Novel mechanisms of Atg16L1 recruitment in non-canonical autophagy.

Katherine Anne Fletcher

Murray Edwards

September 2018

This dissertation is submitted to The University of Cambridge for the degree of Doctor of Philosophy

Under the supervision of:

Dr Oliver Florey

The Babraham Institute

Cambridge

UK
Contents

List of Tables .............................................................................................................................. vii
List of Figures ............................................................................................................................. vii
Declaration .................................................................................................................................. x
Statement of length ..................................................................................................................... x
Acknowledgements .................................................................................................................... xi
Acknowledgements of assistance .............................................................................................. xiii
Summary ..................................................................................................................................... xiv
Papers published relevant to the work in this thesis ................................................................. xv
Abbreviations ............................................................................................................................. xvi

1 Introduction ............................................................................................................................ 1
  1.1 Autophagy ........................................................................................................................... 1
     1.1.1 Molecular mechanisms of autophagy ......................................................................... 1
     1.1.2 Common methods to study autophagy ........................................................................ 8
     1.1.3 Role of Atg8 family proteins in autophagy ................................................................. 11
     1.1.4 Other types of autophagy ........................................................................................... 13
     1.1.5 Non-autophagic roles of autophagy proteins .............................................................. 16
     1.1.6 The role of autophagy proteins in viral infection particularly Influenza infection ...... 17
  1.2 Non-canonical autophagy .................................................................................................... 19
     1.2.1 Definition and discovery ............................................................................................. 19
     1.2.2 How is non-canonical autophagy distinct from autophagy? ........................................ 20
     1.2.3 Pharmacological modulators ..................................................................................... 21
     1.2.4 Toxins ......................................................................................................................... 25
  1.3 Macro-endocytic engulfment events associated with non-canonical autophagy ............. 26
     1.3.1 LC3 associated phagocytosis (LAP) .......................................................................... 26
     1.3.2 LAP in fungal infection .............................................................................................. 31
     1.3.3 Calcium signaling in LAP .......................................................................................... 32
     1.3.4 Assays to study phagocytosis and xenophagy ............................................................ 33
     1.3.5 Macropinocytosis ....................................................................................................... 33
     1.3.6 Entosis ......................................................................................................................... 34
  1.4 Importance of non-canonical autophagy ......................................................................... 37
     1.4.1 LAP maintains homeostasis through clearance of apoptotic debris ......................... 37
     1.4.2 Role of LAP in maintaining the visual cycle ............................................................... 38
     1.4.3 Signalling linked to cell division in c. elgans is controlled by LAP ............................ 39
1.4.4 Antigen presentation .................................................................40
1.4.5 Receptor signaling in B cells .......................................................44
1.4.6 Role of Atg8 lipidation ................................................................44
1.5 Atg16L1 .....................................................................................45
1.5.1 Structure ..................................................................................45
1.5.2 Autophagy protein recruitment ..................................................48
1.5.3 Atg16L2....................................................................................50
1.6 Atg16L1 WD domain .................................................................50
1.6.1 Structure ..................................................................................50
1.6.2 WD domain in canonical autophagy .............................................51
1.6.3 Known binding partners ............................................................52
1.6.4 Crohn’s Disease .........................................................................54
1.7 Aims of this study .........................................................................56
2 Materials and Methods ....................................................................59
2.1 Materials ...................................................................................59
2.2 Cell culture .................................................................................59
2.2.1 HCT116 (human colorectal carcinoma cells) ..................................59
2.2.2 J774.A1 (mouse macrophage cell line), HEK293T (transformed Human Embryonic Kidney cells) and Mouse Embryonic Fibroblasts (MEFs) ........................................59
2.2.3 MCF10A cells (human mammary epithelial cells, female) ...............60
2.2.4 Mouse Embryonic Stem cells (ES cells) ........................................60
2.2.5 Stable cell line creation ...............................................................60
2.2.6 Transient Transfections .............................................................61
2.3 Drugs and antibodies ...................................................................62
2.3.1 Drugs .....................................................................................62
2.3.2 Western Blot Antibodies ............................................................63
2.3.3 Immunofluorescence Antibodies ................................................64
2.4 Molecular Biology .........................................................................64
2.4.1 Polymerase chain reaction (PCR) ...............................................64
2.4.2 Agarose gel electrophoresis ......................................................65
2.4.3 Restriction digests and ligations ..................................................66
2.4.4 Transformation .........................................................................66
2.4.5 DNA extraction and purification from bacterial cultures ..............67
2.4.6 DNA extraction from cells or tissue ..........................................67
2.4.7 Constructs .................................................................................68
Results: The molecular mechanisms of Atg16L1 function during non-canonical autophagy...
3.3.1 Atg16L1 Structure-function Analysis: cell lines and constructs .........................97
3.3.2 Atg16L1 Domain Analysis: canonical autophagy assays ........................................99
3.4 The C-terminal WD domain of Atg16L1 is essential for monensin induced LC3 lipidation in non-canonical autophagy .................................................................102
  3.4.1 Pharmacological assays: Bafilomycin versus Monensin..................................102
  3.4.2 Monensin induced LC3 lipidation: PI3K dependence ....................................103
  3.4.3 Monensin-induced LC3 lipidation: phagocytosis assay .................................103
  3.4.4 Monensin-induced LC3 lipidation: entosis assay ....................................104
3.5 The C-terminal WD domain of Atg16L1 is essential for LC3 lipidation in physiological engulfment events involving non-canonical autophagy .................................................108
  3.5.1 LC3 associated phagocytosis (LAP) assays ..................................................108
  3.5.2 Macropinocytosis assays ..............................................................................108
  3.5.3 Vacuolating toxin A (VacA) assays ...............................................................109
3.6 Atg16L1 ΔWD no longer localises to membranes of the endolysosomal system ....112
3.7 Identification of key residues on the top face of ATG16L1 WD40 C-terminal domain required for non-canonical autophagy .................................................................118
  3.7.1 Atg16L1 WD domain sequence analysis ......................................................118
  3.7.2 Atg16L1 WD domain hotspot analysis ..........................................................123
  3.7.3 Atg16L1 WD domain mutant analysis: F467A and K490A ..............................126
3.8 Recruitment of the WD40 Atg16L1 domain ................................................................138
3.9 Analysis of the T300A Atg16L1 Crohn’s disease mutant in non-canonical autophagy ..140
3.10 Functional implications of Atg16L1 mediated non-canonical LC3 lipidation ............144
3.11 Discussion .............................................................................................................147
4 Results: In vivo functions of non-canonical autophagy: Atg16L1 mutant mouse models. ....152
  4.1 Introduction ........................................................................................................152
    4.1.1 Atg16L1 mouse models ..............................................................................152
    4.1.2 CRISPR technology and in vivo application ..................................................153
  4.2 Characterising Atg16L1 1-230 mouse model from collaborators (Tom Wileman University of East Anglia). .................................................................157
    4.2.1 Atg16L1 1-230 deletion ..............................................................................157
    4.2.2 Atg16L1 1-230: in vitro validation ..............................................................157
  4.3 Using Atg16L1 1-230 to investigate non-canonical autophagy phenotypes ..........160
    4.3.1 Atg16L1 E230 mice: LC3 lipidation and LAP ...........................................160
    4.3.2 Atg16L1 E230 mice: antigen presentation ..................................................160
  4.4 Using CRISPR/Cas9 to generate an Atg16L1 1-336 mouse model .......................164
    4.4.1 CRISPR Cas 9 guides ...............................................................................164
List of Tables

Table 2.1: Details of pharmacological modulators of canonical and non-canonical autophagy ................................................. 62
Table 2.2: Antibodies for detection of specific proteins by western blot ................................................................. 64
Table 2.3: Antibodies for detection of specific proteins by immunofluorescence ....................................................... 64
Table 2.4: PCR reaction reagents and quantities .................................................................................................................. 65
Table 2.5: Thermocycler programme for PCR .................................................................................................................. 65
Table 2.6: Plasmids ......................................................................................................................................................... 69
Table 2.7 Primers to confirm sequence of plasmids. .......................................................................................................... 69
Table 2.8: Primers for site directed mutagenesis of Atg16L1 ........................................................................................ 70
Table 2.9: Guide oligos to clone into a Cas9 vector to form the sgRNA for Atg16L1 ΔWD targeting and synthetic single stranded guide sequence and tracrRNA for Atg16L1 K490A targeting ........................................................................................................................................ 72
Table 2.10: Surveyor assay primers .................................................................................................................................. 74
Table 2.11: Surveyor assay expected size in base pairs of DNA fragments .......................................................................... 75
Table 2.12: Primers in order to make HDR template for creation of Atg16L1 ΔWD ................................................................. 76
Table 2.13: Summary of primers and restriction enzymes for screening and genotyping .................................................... 77
Table 2.14: Volumes of reagents to make poly-acrylamide gels .......................................................................................... 78
Table 2.15: Primers for cloning Atg16L1 K490A from p-BABE to pQCKIN-BirA-Myc vector ....................................... 90

List of Figures

Figure 1.1 Role of Autophagy related proteins in LC3 conjugation ............................................................... 5
Figure 1.2: Molecular mechanisms of autophagy ........................................................................................................... 7
Figure 1.3: Drugs that activate non-canonical autophagy with a detailed mode of action for monensin .................................................................................................................................................. 24
Figure 1.4: Molecular mechanisms of LAP .................................................................................................................... 30
Figure 1.5: Molecular mechanisms of non-canonical autophagy ............................................................................... 36
Figure 1.6: MHC II loading extracellular and intracellular antigens ........................................................................ 43
Figure 1.7: Atg16L1 domain structure of mouse Atg16L1 .......................................................................................... 47
Figure 1.8: Atg16L1 recruitment in autophagy ............................................................................................................. 49
Figure 1.9: Summary of Atg16L1 recruitment and aims of project .................................................................................. 58
Figure 2.1: Antigen presentation assay in vitro ............................................................................................................. 85
Figure 3.1: Atg16L1 recruitment in canonical and non-canonical autophagy .............................................................. 95
Figure 3.2: Upstream signals for LC3 lipidation in non-canonical autophagy are distinct from canonical autophagy ........................................................................................................................................ 96
Figure 3.3: Atg16L1 constructs and expression in various cell lines ................................................................. 98
Figure 3.4: Atg16L1 structure function in canonical autophagy ................................................................................ 100
Figure 3.5: Pharmacological analysis of non-canonical autophagy Atg16L1 in re-complemented cell lines .................................................................................................................................................. 105
Figure 3.6: Using beads and monensin to study non-canonical autophagy in Atg16L1 re-complemented cell lines ........................................................................................................................................ 106
Figure 3.7: Entotic corpse vacuoles treated with monensin to observe non-canonical autophagy in Atg16L1 re-complemented cell lines ........................................................................................................ 107
Figure 3.8: LC3 associated phagocytosis assay in Atg16L1 re-complemented MEFs .................................................... 110
Figure 3.9: Macropinocytosis and VacA assays to observe LC3 lipidation in non-canonical autophagy .................................................................................................................................................. 111
Figure 3.10: Atg16L1 with a C-terminal deletion still forms a functional complex with Atg5-12.

Figure 3.11: Atg16L1 lacking the WD domain no longer localises to bead phagosomes and entotic corpse vacuoles.

Figure 3.12: Membrane fractionation to assess Atg16L1 and Atg5-12 at membranes in cells expressing FL or ΔWD Atg16L1.

Figure 3.13: Alignment of the WD domain of Atg16L1 from human, mouse and rat.

Figure 3.14: Alignment of the WD domain of human Atg16L1 and Atg16L2.

Figure 3.15: Atg16L2 does not support LC3 lipidation in non-canonical autophagy.

Figure 3.16: Atg16L1 WD domain using WDSP predicted amino acids required for protein-protein interactions.

Figure 3.17: Screening Atg16L1 C-terminal WD point mutants for non-canonical autophagy.

Figure 3.18: Atg16L1 point mutants mapped onto the crystal structure of the Atg16L1 WD domain and amino acid conservation.

Figure 3.19: Canonical autophagy response of cells expressing the Atg16L1 WD domain point mutants.

Figure 3.20: Atg16L1 point mutants in response to monensin induced non-canonical autophagy.

Figure 3.21: Generating a double knockout Atg13 and Atg16L1 HEK293 cell line to test if Atg16L1 mutants respond to non-canonical autophagy.

Figure 3.22: Non-canonical autophagy response utilising a Atg13 and Atg16L1 knockout cell line re-constituted with Atg16L1 mutants.

Figure 3.23: Assessing Atg16L1 WD domain point mutants in response to a physiological LAP assay.

Figure 3.24: Atg16L1 point mutants are able to form a complex with Atg5 and Atg12, but no longer recruit to bead containing phagosomes.

Figure 3.25: WD domain of Atg16L1 is not sufficient to recruit to bead containing phagosomes.

Figure 3.26: Atg16L1 T300A variant in non-canonical autophagy.

Figure 3.27: Live entosis assay with WT and T300A Atg16L1GFP-LC3 expressing cells.

Figure 3.28: Influenza A infection is dependent on the viral M2 protein and activates LC3 lipidation through a non-canonical autophagy pathway.

Figure 4.1: Schematic to summarise the use of CRISPR/CAS9 to mutate Atg16L1.

Figure 4.2: Gene editing workflow in mouse ES cells compared to mouse zygotes.

Figure 4.3: In vitro characterisation of Atg16L1 1-230, C-terminal deletion.

Figure 4.4: BMDCs from WT or E230 Atg16L1 mice, expression and LAP.

Figure 4.5: The C-terminal domain of Atg16L1 is essential for exogenous antigen presentation.

Figure 4.6: Surveyor Assay from IDT to test efficiency of guide targeted Cas9 DNA cutting.

Figure 4.7: Validating the guide sequence to target Atg16L1 to produce a WD domain deletion.

Figure 4.8: Original sequence and genotyping strategy for Atg16L1 truncated DNA.

Figure 4.9: Original genotyping of pups from the direct zygote injections for edited Atg16L1 sequence.

Figure 4.10: Re-designed strategy to insert a triple stop codon to form Atg16L1 WD deletion and genotyping strategy.

Figure 4.11: ES cell colonies screened by PCR for Atg16L1 edited DNA and validation PCR.

Figure 4.12: Identified ES cell colonies positive for edited DNA, validation and sequencing.
Figure 4.13: Atg16L1 expression and function in WT and edited ES cells. ........................................... 176
Figure 4.14: Re-design of strategy for Atg16L1 truncation and outline of the screening and genotyping strategy........................................................................................................... 178
Figure 4.15: Screening DNA from ES cell colonies by PCR where one primer is complementary to the predicted edited sequence. ...................................................................................... 179
Figure 4.16: Edited ES cells taken forward for sequencing and validation. ...................................... 180
Figure 4.17: Protein expression and function of Atg16L1 in WT and ΔWD edited ES cells........... 182
Figure 4.18: Generation of chimeric mice after edited ES cell injection and genotyping of subsequent breeding rounds. ........................................................................................................... 184
Figure 4.19: Surveyor assay to test the guide efficiency to produce a double strand break in a specific region of Atg16L1, to produce DNA to encode Atg16L1 K490A........................................... 186
Figure 4.20: Summary of strategy to direct K490A point mutation in the genomic sequence of Atg16L1 ........................................................................................................................................ 187
Figure 4.21: Sequencing of genomic DNA of pups from direct zygote injections and after founder breeding. ........................................................................................................................................ 187
Figure 5.1: Immunoprecipitation using the S-tag Atg16L1 constructs. .............................................. 189
Figure 5.2: Silver stain to assess the total proteins in IP samples from the negative control compared to the Atg16L1 FL sample. ................................................................................................. 197
Figure 5.3: Comparison of IP of Atg16L1 and Atg5-12 when cells were lysed in a triton or CHAPs buffer. ........................................................................................................................................ 200
Figure 5.4: β1-integrin and PLEKH1 do not co-IP with Atg16L1...................................................... 201
Figure 5.5: Summary of BioID method to immunoprecipitate Atg16L1 and proximal interactors. ........................................................................................................................................ 203
Figure 5.6: Expression of Atg16L1 BirA constructs and functional validation.............................. 206
Figure 5.7: Specific biotinylation of proteins from biotin addition and expression of BirA........... 209
Figure 5.8: Western blots to show specific biotinylation and IP of known Atg16L1 interactors. ........................................................................................................................................ 210
Figure 6.1 Summary Diagram....................................................................................................... 221
Declaration

I hereby declare this thesis is my own work and contains nothing that is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements.

Statement of length

This dissertation does not exceed the word limit of 60 000 words (excluding the bibliography and figures) set out by The University of Cambridge Faculty of Biology.
Acknowledgements

I would like to thank everyone involved in making my time at The Babraham Institute enjoyable and everyone who has offered me their support throughout the PhD journey. I would particularly like to thank the past and present members of the signalling department.

I have been at The Babraham Institute for a long time, 5 and a half years! I started out in Michael Wakelam’s lab, where Simon was kind enough to supervise me to get some up to date lab experience. This opportunity was down to a good friend Martin who will take credit for my scientific career to date! Thank you for getting me to Babraham.

I then want to thank Nick and Maria who taught me the ropes of autophagy when I was a research assistant and offered me their time and encouragement. During this time I met some wonderful people. Mary was my screening partner and we built a wonderful friendship, along with the other Italian visitors to the Institute, Arianna, Valentina and Federico. Matt and Maria you have helped me a lot over the years and I loved working with you. Lefteris, Jason, Valerio and Claudio you were also part of our wonderful team.

Of course, I want to thank The Florey lab for being great and offering such a fun atmosphere to be a PhD student. Dr Elise Jacquin, I have no words, your friendship, advice, our teamwork was something special, and I will always fondly remember the conferences we attended together and the time we spent in the lab. Babraham was our playground for boot camp and running and I thank you for getting me motivated to get fit and achieve multiple half marathons (not sure I thanked you after the first!). Jo your life advice, mentoring and eye for detail has taught me a lot and I appreciate all you did to help me with my next career move. Thank you for your support along the way and with the thesis.

Geoff, interviewed me, became my formal assessor at the Institute and has looked out for me throughout my PhD. Dominik taught me so much about molecular biology and was always available to answer my questions and offer encouragement. He provided laughter and positivity.
Of course, this leads me to thank the fellow PhD students of the corridor. I want to thank Katie for being such a supportive PhD student in the lab and for putting up with me in this last year of stress. Chiara you are wonderful and I thank you for the running and your friendship and the same to Beth. Piotr and Izzy you are great, Izzy your outlook on life is a breath of fresh air and Piotr your laugh and the EVDS have been among my highlights! Kate you have been there from the beginning to end and thank you for the mutual stressing whenever it came to doing any presentations or assessments.

Finally, Oli, thank you for teaching me, and looking after me and giving me the chance to learn many skills inside and outside of the lab. Your support and guidance have helped build my confidence and it was so exciting to publish my first paper with you. It has been a privilege to be your first student and to share the beginnings of the Florey lab. I look forward to following the updates from the lab.

I am going to stop waffling in a minute, but finally I want to thank my family. They are my biggest fans and have been celebrating my PhD before I had even started so let’s hope I have done them proud! Dad you are amazing, you have read all of my reports, heard my presentations, and even managed to look interested! Mum and Dad you always know what to do and have encouraged us to be the best we can be. Rach and Nicola I am so lucky to have such inspiritional and supportive sisters and of course thanks to Tom and Rob. Finally, Tom, you have put up with a lot, mainly a lot of science talk and science dreams. Thank you for moving to Cambridge and sharing and supporting me in these years of my PhD, now on to the next adventure...
Acknowledgements of assistance

Dr Oliver Florey was responsible for supervision during this project and teaching in the lab. Dr Joanne Durgan was responsible for mentorship and Dr Geoff Butcher also assisted with my progress and feedback. Dr Elise Jacquin assisted with a few experiments as stated in the text. Some experiments, as stated in the text, were done in collaboration with Dr Rupert Beale’s lab and Dr Tom Wileman’s lab. The *in vivo* work was done under the supervision of Dr Dominik Spensberger and the mice are maintained by staff at the Biological Support Unit. Sanger sequencing was performed by Cogenics Lark and genotyping where stated by Transnetyx. Mass spectrometry experiments were done by Dr David Oxley and Dr Judith Webster, at the proteomics facility at the Babraham Institute. Cells and reagents from other lab groups are acknowledged in the material and methods section.
Summary

Autophagy is a well-studied catabolic process through which cytoplasmic components are targeted for lysosomal degradation by autophagosomes. A key step in this process is the recruitment, processing and lipidation of LC3 to autophagosomes. Recently it has become increasingly apparent that, through the unconventional use of some autophagy related proteins, LC3 can also become lipidated to distinct non-autophagosomal membranes of the endolysosomal system. This process is termed non-canonical autophagy and occurs independently of conventional autophagy initiation signals. Non-canonical autophagy usually occurs after macro-endocytic engulfment events such as macropinocytosis, entosis and LC3 associated phagocytosis (LAP). Certain ionophores and lysosomotropic drugs, such as monensin, can also activate this process and promote LC3 lipidation to lysosomes. This project focuses on Atg16L1, an essential autophagy protein that directs the membrane site where LC3 is lipidated. Atg16L1 is relatively well characterised in autophagy but little is known about the mechanisms underlying its role in non-canonical autophagy. This project used a structure/function approach to assess the importance of different domains of Atg16L1 in the context of autophagy versus non-canonical autophagy. I have demonstrated for the first time, that the Atg16L1 C-terminal WD40 domain (CTD) is dispensable for its role in autophagy but essential for LC3 lipidation during non-canonical autophagy. Furthermore, single point mutants were uncovered in the CTD of Atg16L1 that likewise are dispensable for autophagy but fundamental to LC3 lipidation in non-canonical autophagy. These data provide a novel strategy for dissecting canonical and non-canonical autophagy pathways at a molecular level. This project used an existing mouse model with an Atg16L1 truncation (lacking the CTD and nearby residues) and implicated the lack of non-canonical autophagy to a defect in MHCII antigen presentation. Furthermore this project has generated new refined Atg16L1 mutant models ablating non-canonical autophagy without affecting canonical autophagy. In parallel, proteomic analysis was done to provide mechanistic insights into Atg16L1 binding partners in the context of non-canonical autophagy.
Papers published relevant to the work in this thesis

Fletcher, K, et al., The WD40 domain of ATG16L1 is required for its non-canonical role in lipidation of LC3 at single membranes. EMBO J, 2018. 37 (4) e97840

(with commentary Fracchiolla, D, Martens, S, Sorting out “non-canonical autophagy”. EMBO J, 2018 37 (4) e98895)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM</td>
<td>Atg8 Interacting Motif</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AMDE-1</td>
<td>Autophagy modulator with dual effect 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>5′ adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>Aspergillus fumigatus</td>
</tr>
<tr>
<td>Atgs</td>
<td>Autophagy related gene (also used for autophagy related protein)</td>
</tr>
<tr>
<td>Baf</td>
<td>Bafilomycin</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cells</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophages</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSU</td>
<td>Biological support unit</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CCD</td>
<td>Coiled coiled domain</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestinal phosphatase</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone mediated autophagy</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAPK1</td>
<td>Death-associated protein kinase 1</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>FBD</td>
<td>FIP200 binding domain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fragment crystallisable gamma receptor</td>
</tr>
<tr>
<td>FIP200</td>
<td>FAK-family interacting protein of 200 kDa</td>
</tr>
<tr>
<td>FYCO1</td>
<td>FYVE and coiled coil domain containing 1</td>
</tr>
<tr>
<td>GABARAP</td>
<td>γ-aminobutyric acid receptor-associated protein</td>
</tr>
<tr>
<td>GABARAPL</td>
<td>γ-aminobutyric acid receptor-associated protein like</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>HDR</td>
<td>homologous directed repair</td>
</tr>
<tr>
<td>H. pylori</td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus-1</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immuno-globulin G</td>
</tr>
<tr>
<td>IP</td>
<td>Immuno-precipitation</td>
</tr>
<tr>
<td>KAN</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LAP</td>
<td>LC3 associated phagocytosis</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1A/1B-light chain 3</td>
</tr>
<tr>
<td>LIR</td>
<td>LC3-interacting region</td>
</tr>
<tr>
<td>LLOME</td>
<td>Leu-Leu methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Mon</td>
<td>Monensin</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MVB</td>
<td>Multi-vesicular body</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NOX2</td>
<td>(NADPH) oxidase-2</td>
</tr>
<tr>
<td>Nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCVs</td>
<td>Pathogen containing vacuoles</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methane sulfonyl fluoride</td>
</tr>
<tr>
<td>POS</td>
<td>Photoreceptor outer segments</td>
</tr>
<tr>
<td>PtdSer</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio Immunoprecipitation Assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelial cells</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Rubicon</td>
<td>RUN domain protein as Beclin-1 interacting and cysteine-rich containing</td>
</tr>
<tr>
<td>S</td>
<td>Starvation</td>
</tr>
<tr>
<td>SB</td>
<td>Sample buffer</td>
</tr>
<tr>
<td>SCVs</td>
<td>Salmonella containing vacuoles</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labelling of amino acids in cell culture</td>
</tr>
<tr>
<td>SLE</td>
<td>Systematic lupus erythenatus</td>
</tr>
<tr>
<td>SLRs</td>
<td>Sequestosome like receptors</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth</td>
</tr>
<tr>
<td>Ss</td>
<td>Single stranded</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBK1</td>
<td>Serine/threonine protein kinase 1</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with Tween 20</td>
</tr>
<tr>
<td>TGS</td>
<td>Tris-glycine-SDS</td>
</tr>
<tr>
<td>TIM-4</td>
<td>T cell Ig mucin domain 4</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TMEM</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem mass tag</td>
</tr>
<tr>
<td>TRIM</td>
<td>Tripartite motif containing protein</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51 like autophagy activating kinase 1</td>
</tr>
<tr>
<td>UVRAG</td>
<td>UV radiation resistance-associated gene protein</td>
</tr>
<tr>
<td>VacA</td>
<td>Vacuolating toxin A</td>
</tr>
<tr>
<td>VOD</td>
<td>ATP6 V0d1 (V-ATPase subunit)</td>
</tr>
<tr>
<td>WD domain</td>
<td>WD40 repeat domain forms a β-propeller structure</td>
</tr>
<tr>
<td>WIPI</td>
<td>WD-repeat Protein Interacting with Phosphoinositides</td>
</tr>
<tr>
<td>WM</td>
<td>Wortmannin</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Autophagy

Autophagy is a fundamental catabolic process with important physiological and pathophysiological roles. Autophagy mediates the recycling of cellular material in times of starvation or stress, allowing nutrients to be recovered through lysosomal degradation and retrieval mechanisms. Various related processes facilitate the degradation of other cellular components, such as mitophagy, aggrephagy, lysophagy and xenophagy. Importantly, some key molecular players from the autophagy pathway also play an important role in degradation of material engulfed from outside the cell. This parallel, non-canonical autophagy pathway plays a range of important functions, particularly in the context of immunity and inflammation, and forms the focus of this thesis.

1.1.1 Molecular mechanisms of autophagy.

Autophagy is a cellular recycling process by which cytoplasmic material is sequestered into \textit{de novo} formed, double membrane structures (autophagosomes) to be degraded by the lysosome, where amino acids and other nutrients can be recycled back into the cytoplasm of the cell [1-3]. Autophagy is required by the cell to overcome various stresses such as nutrient depletion, oxidative stress and damage to organelles. Autophagy has complex associations with neurodegeneration, ageing and cancer [4-6].

There are multiple forms of autophagy, including macro-autophagy, micro-autophagy and chaperone mediated autophagy (CMA). Macro-autophagy sequesters cargo for degradation, either in a selective or non-selective manner. Non-selective macro-autophagy will be termed canonical autophagy from this point onwards. Micro-autophagy is where the lysosome directly invaginates and takes in cytoplasmic content to degrade [7], and CMA involves the transport of unfolded or misfolded proteins from the cytosol into the lysosome [8].
Autophagy is a conserved process and was first characterised in yeast, however this project focuses on mammalian autophagy pathways. A series of autophagy related proteins (Atgs) act in a tightly regulated manner to mediate initiation, maturation and degradation of the autophagosome. The overall process of autophagy initiation is summarised in (FIGURE 1.2). Initiation of autophagy depends on the pre-initiation complex made up of a serine threonine kinase ULK1 (Unc-51 like autophagy activating kinase 1), FIP200 (FAK-family interacting protein of 200 kDa), Atg13 and Atg101. All of these components localise to the early autophagosome structure termed the phagophore and are essential for mammalian autophagy [9-12]. How this activated complex is recruited to these membranes has not yet been fully elucidated, however there is evidence that Atg13 may interact with certain lipids found on the autophagy initiation membrane [13].

The ULK1 complex is regulated by the nutrient and energy status of the cell through the action of mammalian target of rapamycin complex 1 (mTORC1), which is a nutrient, energy and stress sensor important in the regulation of growth and many cellular processes [14]. mTORC1 is carefully modulated through the action of the Ragulator complex and, in amino acid rich conditions, mTORC1 is in an active state and interacts with Rheb at the lysosome. In basal, fed conditions mTORC1 hyper phosphorylates Atg13 inhibiting its interaction with ULK1 [15]. But when the cell is starved of amino acids mTORC1 disassociates from the lysosomal membrane and this allows activation of the ULK1 complex [16]. Upon starvation induced mammalian autophagy, or under pharmacological inhibition by Rapamycin, mTORC1 inactivation leads to de-phosphorylation of ULK1 and Atg13[15], as shown in (FIGURE 1.2). In addition, AMPK (5’ adenosine monophosphate-activated protein kinase) is another energy sensing kinase and upon energy depletion phosphorylates ULK1 to aid activation of the pre-initiation complex [17]. This allows for the complex to assemble and concentrate at sites of growing autophagosome formation, permitting self-phosphorylation and further activation of ULK1 complex assembly. Furthermore, the activated ULK1 complex can phosphorylate other substrates, including beclin
that makes up part of the Vps34 complex outlined below. This ULK1 dependent activation of the PI3K complex furthers the initiation of autophagy [18].

The phosphoinositide 3 kinase (PI3K) complex consists of the class III PI3K Vps34, the only mammalian class III PI3K that catalyses the phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P). In canonical autophagy, this acts in conjunction with UVRAG, Beclin1 and Atg14. The activity of the PI3K complex enriches initiation membranes with PI3P, forming an omegasome cradle to act as a platform for the expansion of the double membrane autophagosome, and allowing the recruitment of various PI3P effectors such as WIPI2b [19].

The source of the initiation membrane, also termed the phagophore, has been widely debated. There is now strong evidence to suggest it is the ER, specifically the ER mitochondrial exit sites, that provide the site for nucleation of the phagophore. This is evidenced by mitochondrial markers found on the autophagosome [20, 21] and visualised through live cell imaging of ER mitochondrial exit sites alongside autophagy initiation proteins [13]. However, other membrane sources such as the mitochondria, Golgi and endosomes have also been implicated in forming autophagosomes [22].

The next step involves a small cytosolic protein, LC3 (microtubule-associated protein light chain 3), being recruited and lipidated to the autophagosome to form LC3-phosphatidylethanolamine (LC3-II, LC3-PE) [23]. LC3 is a member of the Atg8 family of proteins and other Atg8 members are lipidated to autophagosomes [24, 25]. This process was first characterised in yeast where Apg8 was shown to relocalise to autophagosomes upon starvation, and Apg8 null yeast had impaired autophagosome formation [26]. There are multiple orthologs of Atg8 in mammals: LC3 [23], GABARAP (γ-aminobutyric acid receptor-associated protein)[27] and GABARAPL (γ-aminobutyric acid receptor-associated protein like) subgroups [28], for the sake of this report LC3 will be the focus and is the most widely studied. The Atg8 proteins will be discussed further in a later section.
The translated LC3 protein is referred to as pro-LC3. In the cytosol most of this pro-LC3 is cleaved by Atg4, a protease, to expose the C-terminal glycine residue, this form of the protein is termed LC3-I.

LC3-I is recruited and lipidated onto the autophagosome via two ubiquitin-like conjugation systems, these are summarised in (FIGURE 1.1). LC3-I can directly bind Atg3 in an intermediate state, assisted by the E1-like activity of Atg7, and is subsequently conjugated to the head group amine of phosphatidylethanolamine (PE) to form LC3-II [29]. This conjugation is reversible as Atg4 can act here to cleave PE from LC3-II [29].

Furthermore, in another ubiquitin-like conjugation reaction, Atg12 covalently attaches to a lysine residue of Atg5, via an isopeptide bond; this reaction is activated by Atg7, and Atg12 forms an intermediate complex with Atg10 before it is then transferred to Atg5. Atg5 and Atg12 then complex with Atg16L1, existing as a homo-oligomer, forming a ~800 kDa complex in eukaryotes [25]. Atg5-12-16L1 complex acts as an E3-like ligase for the transfer of Atg8 from Atg3 onto PE [30], FIGURE 1.1.

It is the Atg16L1 complex that drives the specification of the site for LC3 lipidation [31]. The Atg5-12-16 protein complex is recruited to sites of autophagosome formation via the binding of Atg16L1 to FIP200 [32] and/or WIPI2b [33]. The Atg16L1 complex is recruited to the target membrane and the LC3 Atg3 intermediate binds Atg12, where LC3 is transferred from Atg3 to PE [31]. Atg16L1 itself will be discussed in more detail in a subsequent chapter.
Figure 1.1 Role of Autophagy related proteins in LC3 conjugation.

This shows the two ubiquitin-like conjugation systems that are required for autophagy. LC3 after translation is referred to as pro-LC3 but most LC3 in the cytosol is cleaved by Atg4 a protease that exposes a C-terminal glycine to form LC3-I. This can then be targeted by Atg7 that has an E1 like active site that allows LC3-I to bind Atg3 as an intermediate before it is then conjugated to the phospholipid PE on the autophagosome membrane forming LC3-II. In a similar system, Atg12 is activated by Atg7 and transferred to Atg10 as an intermediate before forming an isopeptide bond with Atg5. This complex of Atg5-12 can then complex with Atg16L1, essential to transfer LC3-I from the Atg3 intermediate onto PE to form LC3-II.
Detection of lipidated LC3 (LC3-II) levels is a common way to experimentally estimate the level of autophagic activity, due to the lipidated LC3 staying attached to the autophagosomal membrane after completion (see section 1.1.2). LC3 also acts itself as an autophagic substrate providing a way to measure autophagic flux [34]. The functional role of LC3 is an area of increasing interest that will be discussed separately (section 1.1.3).

Autophagosomes elongate and close before finally fusing with the lysosome, where their contents are degraded and released back into the cytoplasm of the cell for nutrient recovery to reactivate the status of m-TOR [3]. This fusion process is dependent on the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), syntaxin 17 (Stx17). The structure of Stx17 allows for the specificity of this SNARE to recruit to complete autophagosomes. Stx17 on the autophagosome surface interact with SNARES on the lysosome surface such as VAMP8 [35].

Rab7 [36] and the homotypic fusion and protein-sorting complex (HOPS) [37] have also been implicated in fusion events in autophagy. It has been found that the pleckstrin homology domain containing protein family member 1 (PLEKHM1) interacts with Rab7, HOPs, Stx17 and LC3, assisting autophagosomes fusion with lysosomes [38]. Therefore, there are multiple factors that mediate fusion of autophagosomes to lysosomes and absence of these components can lead to accumulation of autophagosomes, which is then implicated in neurodegeneration and lysosomal storage disease [38].

A major research interest in the Florey lab, and this project, is understanding the processes and molecular mechanisms that are involved in non-canonical autophagy, where Atgs act in a distinct and unconventional manner to recruit and lipidate LC3 to single membranes of the endolysosomal system. This is discussed further in section 1.2 onwards.
When the cell is starved, mTOR is inactive, this can also be achieved by the drug rapamycin, and the phosphorylation status of the ULK1 complex (ULK1, FIP200, Atg13 and Atg101) changes to an active state. This active ULK1 complex can then auto-phosphorylate components and leads to the recruitment of the PI3K complex (Vps34, Beclin1, Vps14 and Vps15). This initiates the growth of a double membrane autophagosome around cytoplasmic substrates. The enzymatic reactions lead to an enrichment of PI3P on the double membrane that then facilitates the action of various autophagy related proteins (Atgs) that allow the LC3-I to become conjugated to PE a lipid on the double membrane structure. The double membrane with the sequestered contents finally seals and fuses to the lysosome where the contents are degraded.

**Figure 1.2: Molecular mechanisms of autophagy**

When the cell is starved, mTOR is inactive, this can also be achieved by the drug rapamycin, and the phosphorylation status of the ULK1 complex (ULK1, FIP200, Atg13 and Atg101) changes to an active state. This active ULK1 complex can then auto-phosphorylate components and leads to the recruitment of the PI3K complex (Vps34, Beclin1, Vps14 and Vps15). This initiates the growth of a double membrane autophagosome around cytoplasmic substrates. The enzymatic reactions lead to an enrichment of PI3P on the double membrane that then facilitates the action of various autophagy related proteins (Atgs) that allow the LC3-I to become conjugated to PE a lipid on the double membrane structure. The double membrane with the sequestered contents finally seals and fuses to the lysosome where the contents are degraded.
1.1.2 Common methods to study autophagy

The tightly controlled process of autophagy, like many signalling pathways, has multiple steps that can be regulated. Therefore, it can be manipulated and studied in various ways to look at the initiation or the flux through the pathway. The flux of the pathway refers to the whole process, from initiation of autophagy through to the degradation and turnover of autophagosomes and their contents. Relevant and common methods are introduced below, for a more comprehensive review of autophagy methods see the following reference [39].

One method to look at autophagosome number is to use GFP-LC3 as a reporter to observe localisation through fluorescence microscopy. When GFP-LC3 is unlipidated (LC3I), it has a diffuse signal in the cytosol. Upon autophagy induction, it gets recruited and lipidated to autophagosomes, forming puncta that correspond to the number of autophagosomes [23]. The isoform of LC3 that is commonly GFP tagged on its N-terminal is LC3B. Other autophagy proteins can be fluorescently tagged to similarly look at their localisation, for instance DFCP1 is an omegasome marker, a very early marker of autophagy initiation [19]. ULK1 and WIPI2b also offer markers for autophagy initiation.

Autophagic function can be monitored by analysing p62, an autophagy cargo protein. p62 is degraded by autophagy and can be studied, via imaging or western blot, to assess the levels of autophagy within a cell. If there is an increase in p62, this is suggestive of defective autophagy and conversely a loss of protein is an indicator of activation of the pathway.

Fixing cells and counting the changes in puncta of various autophagy markers offers a way to quantify the number of autophagosomes at a given time and how this changes depending on different treatments to the cells. This is a powerful way to set up high throughput screens for novel regulators of the autophagy pathway, with automated algorithms to count the puncta per cell. Transmission electron microscopy is another way of looking at the membrane structures forming in a specific layer of the cell at a specific time; this is useful as it can identify
autophagosomes from lysosomes and even autolysosomes. Alternatively, fluorescent reporters can be followed via live cell imaging to look at the dynamics of the signalling pathway. This gives more information than just looking at the number of autophagosomes at a given time, for instance, if the puncta count has increased, this does not give information on whether this is due to increased initiation of autophagy or inhibition of autophagosome degradation.

Western blots are a useful way to look at endogenous proteins involved in the autophagy pathway. The most common approach involves comparing the amounts of unlipidated and lipidated LC3 in cell populations. LC3-I and LC3-II migrate differently on an SDS-PAGE gel, allowing the visualisation of the two forms, where LC3 II migrates faster due to its hydrophobicity. LC3 western blots can be quantified in different ways, either looking at changes in the lipidated LC3 band, or the ratio of LC3II/I (as used in our lab). LC3 blots are sensitive to handling of the cells and therefore the pattern of the change is usually more reproducible than the exact ratios. Other western blot assays for this signalling pathway involve looking at phosphorylation statuses of the upstream initiation components to unravel what parts of the pathway are being regulated. This gives a better idea of what is happening throughout the signalling process.

Additional methods to assess flux through the pathway include the use of a tandem fluorescence tagged LC3. This is an mRFP-GFP-LC3 where only the GFP signal is quenched by the acidic lysosome [40]. Therefore, newly formed autophagosomes can be visualised in yellow, as both tags fluoresce, while the autolysosomes can also be visualised in red, because the mRFP fluorescence is more stable than the GFP [40]. This allows the opportunity, in fixed and live cell imaging, to appreciate if the forming autophagosomes are going onto fuse with the acidic lysosomes.

In addition, lysosomal inhibitors are used to assess flux. Common methods to inhibit lysosomal degradation of autophagosomes exploit drugs, such as bafilomycin A1 and concanomycin, that raise the lysosomal pH. Previously monensin and chloroquine were used for similar functions to block the flux of the pathway, but recent work shows they additionally activate LC3 lipidation to
non-autophagosomal membranes of the endolysosomal system. This will be introduced in more
detail later in the chapter, section 1.2.3, and raises caveats in their use in studying autophagic flux
[41]. Other inhibitors can be used to look at flux, such as leupeptin and pepstatin that inhibit the
lysosomal proteases. Using these drugs, when autophagy is initiated, blocks the flux, resulting in
an increase in autophagosome number. This can be assessed as mentioned previously by imaging
or western blot.

Other methods to study autophagy include flow cytometry to look at unlipidated and lipidated
LC3, a sensitive and reproducible way to quantify changes in LC3 lipidation. This assay uses
detergent, to permeabilise the cell and therefore release cytoplasmic components including
unlipidated LC3, while membrane bound constituents remain, such as LC3-II; this is a way to show
how much lipidated LC3 there is in cells [42]. This method has been used to look at autophagy
levels in B cell activation [43] and the Florey lab also use this as a method to look at Atg8
lipidation. This has potential for screening opportunities [44].

Autophagy is often initiated by modulating mTOR activity where the most common approach is to
starve the cells. When starvation is referred to in this project, the method involves amino acid
and serum starvation. This, as already described, inhibits mTOR and initiates autophagy. Another
way to achieve this is to inhibit mTOR pharmacologically, using rapamycin or PP242. This provides
a way to look at autophagy initiation and, if the turnover of the pathway is functioning, then this
initiation should result in increased LC3II levels in puncta form or by western blot. The opposite
can be done and autophagy initiation can be inhibited, through the use of ULK1 inhibitors or PI3K
inhibitors. This would result in decreased LC3 puncta and LC3II by western blot. Common pan
PI3K inhibitors used are wortmannin, 3-MA and LY294002 [45]. A potent and specific inhibitor of
VPS34 is SAR405 [46]. These pan inhibitors are useful to an extent but they also stop other
signalling processes within the cell and therefore, inhibiting ULK1 more specifically using
MRT67307 and MRT68931 provides a better way to study this process [47].
Finally, further to pharmacological stimulation, a powerful tool to unpick the autophagy pathway in vitro and in vivo is to genetically manipulate the pathway by CRISPR knocking out of specific Atg genes.

1.1.3 Role of Atg8 family proteins in autophagy.

Atg8 exists as a single protein in yeast but has six different isoforms in mammalian cells, which may help explain why the function of this family has become so complex. These proteins are said to be ubiquitin-like, due to their structural similarity [48]. The Atg8s in mammalian cells fall into two families: LC3 (LC3A, B, C) and the GABARAPS (GABARAP, GABARAPL1 and L2). Overall, their expression is tissue wide, although GABARAP L1 and L2 are predominantly found in the brain [49] and LC3C is at its highest in lung tissue [50]. They are all synthesised as cytosolic proteins and then through the autophagy conjugation machinery they are recruited and lipidated to membranes. It has been shown that in autophagy, this conjugation is to PE [51] and it has only been shown in vitro that the Atg8s can also conjugate phosphatidylserine (PS) [52]. There is a study to suggest that LC3 conjugated to PS is not supported at physiological pH and that adding acidic phospholipids to liposomes favours LC3-PE conjugates [53]. This relationship between pH and LC3 lipid conjugation could be interesting when thinking about LC3 lipidation in the context of non-canonical autophagy.

Atg8 proteins in mammalian autophagy have a role as cargo receptors in selective autophagy. Selective autophagy is the degradation of specific cargo, where autophagy receptors link the cargo to the autophagy machinery for degradation. A range of proteins bear an LC3 interacting region (LIR) or Atg8 interacting motif (AIM) that lead to anchorage of selective cargo to autophagosomes.

The first selective autophagy receptor to be identified was p62/SQSTM1 (sequestosome-1), this interacts with Atg8 proteins and also binds ubiquitinylated protein aggregates, targeting these for degradation via autophagy [54, 55]. As previously mentioned, p62 provides a way to study
autophagic flux, when the pathway is inhibited there is an accumulation of p62 and protein aggregates [55]. Other LIR containing proteins have since been identified, sometimes known as sequestosome like receptors (SLRs), and this includes, but is by no means limited to, selective cargo receptors such as NBR1 (Neighbour of BRCA1 gene1)[56], NDP52 (Nuclear dot protein 52 kDa) [57, 58] and optineurin [59]. These are important in selective autophagy processes that will be further covered in the next section.

As well as playing a role in cargo recruitment, Atg8s also control the recruitment of autophagy initiation proteins. For instance, ULK1 has a LIR domain that can interact with Atg8 and this is thought to be a way to concentrate these proteins on the autophagosome membrane [60].

Another process that links into this idea is precision autophagy. This has been termed after discovering more about the role of TRIM proteins in autophagy. These proteins act as cargo receptors and as regulators; there are a large number of TRIMs that have specificity for various cargo. They directly interact with their cargo, without the need for tags such as ubiquitin or galectin, although in some situations TRIM proteins can bind galectins [61]. TRIMs have LIR domains and therefore interact with Atg8s, where there seems to be a preference for GABARAPs and LC3A [62]. As well as these interactions, they act as a platform for autophagy proteins, such as ULK1, beclin1 and Atg16L1, to form complexes that in some settings activate autophagy and in others, like in the case of TRIM17, negatively regulate autophagy [63]. TRIM 20 directly interacts with Atg16L1 to mediate inflammasome signalling [62].

It was long assumed that the Atg8s were essential for autophagy. However, more recent papers suggest that its role is more nuanced. In a paper where they generated CRISPR knockouts of each individual Atg8 family member, they found that when all the Atg8s had been knocked out, initiation and nucleation of autophagosome formation could still occur, although at a slower rate and with smaller autophagosomes [64]. This is supported in another study where knocking out Atg5 and Atg3, other key members of the conjugation machinery, slows down but does not
prevent the elongation and closure of autophagosomes [65]. The knockout of the GABARAP family had a much more profound affect than the knockout of the LC3 family [64]. This may suggest that the Atg8s are required for efficient autophagosome formation while the GABRAPs play an important role in the final stages of autophagosome fusion with the lysosome [64]. There is further evidence that Atg8s are involved in membrane fusion, from in vitro studies that showed lipidated Atg8 led to tethering and fusion of liposomes [66]. Another assay similarly done in a cell free system showed that permanently lipidated LC3B and GABARAPL2/GATE-16 promoted liposome fusion and tethering and the N-terminal part of these Atg8s were also relevant to this function [67].

In this project, we have used LC3 as a reporter. However, it is important to acknowledge that the lipidation of GABARAPs, and other LC3 isoforms, may also be important and we and the field should consider this too. The roles of Atg8 proteins in non-canonical autophagy will be discussed later (see section 1.3.4).

1.1.4 Other types of autophagy

As well as mediating bulk degradation, autophagy also has a role as a selective degradation pathway. Selectivity of cargo, to be degraded by the autophagosomes, is achieved through the action of receptors that bridge the cargo with the autophagy machinery. The cargo receptors often have LC3 interacting motifs (LIR) that can bind members of the Atg8 family. Another common feature is that the targeted cargo is often ubiquitinylated. Mitophagy is a process through which the autophagy machinery selectively degrades old, damaged or dysfunctional mitochondria; further information can be found in the referenced reviews [68, 69]. This project does not analyse mitophagy, but in brief, it is paramount to cellular homeostasis. Dysfunctional and depolarised mitochondria result in the production of ROS and cause stress responses and damage that ultimately lead to apoptosis. Mitochondria have been shown to be targeted to the lysosomes since 1962 [70]. The molecular mechanisms are still being
unravelled, but the process is dependent on the autophagy machinery that is bridged through the action of LIR proteins such as p62, NBR1 and optineurin. The best studied aspect of the process is PINK1/PARKIN dependent ubiquitinylation of depolarised mitochondria, that are then targeted for degradation through selective autophagy [69]. When this process is not functional, there are implications in neurodegeneration and tumorigenesis [69]. There are similar roles and consequences when the process of aggrephagy, to target aggregated proteins, is dysregulated [71]. These are interesting from an ageing perspective and the onset of age related conditions.

Another selective autophagy process is lysophagy, through which damaged lysosomes are targeted for degradation by autophagy. Lysosomes are acidic membrane bound organelles that function themselves in autophagy. When lysosomes are damaged, the contents can be released into the cell, including proteolytic enzymes, calcium and protons, that have cellular implications including the induction of apoptosis [72]. The leftover damaged organelles need to be targeted for degradation to stop abhorrent signalling especially inflammation, this is done via lysophagy [73]. The damaged lysosomal membrane is targeted by the galectin family of proteins, and the released lysosomal proteins are ubiquitinylated, to be targeted via receptors to be degraded by autophagy. Lysosomal damage is thought to increase with age, in neurodegeneration and can be further caused by oxidative stress or by bacterial toxins. It has now been shown that the autophagy receptor and regulator TRIM-16 acts in a ULK-1 dependent fashion to interact with Galectin 3 after membrane damage [61]. TRIM-16 directly regulates beclin1 and Atg16L1 to allow for clearance of damaged membranes, such as after LLOME treatment or Mycobacterium Tuberculosis infection[61].

The selective autophagy process most relevant to this project is xenophagy, which is involved in intracellular pathogen clearance [74]. Some bacteria break out of their bacterial containing vacuoles to invade the cytosol, leaving behind damaged membrane compartments. The broken membrane exposes glycans and other signalling molecules to the cytosol that would not normally
be exposed and this acts to initiate autophagy to eliminate the bacteria. Such signals include the galectin molecules, that act as danger receptors, binding to β-galactosidase containing carbohydrates. For instance, galectin 8 usually monitors the integrity of membrane compartments such as endosomes and lysosomes [57]. In the case of Salmonella enterica serovar Typhimurium this bacteria is restricted by galectin 8 which recruits NDP52, a cargo receptor [57], that also preferentially interacts with LC3C [57, 75]. Furthermore, the bacteria gets ubiquitinylated and this is just as important as galectin 8 for the action of NDP52, without this mechanism the bacteria proliferate [57, 58]. NDP52, p62 and optineurin all restrict Salmonella enterica serovar Typhimurium [57, 58, 76]. The kinase TBK1 has also been shown to play a role in autophagy dependent bacterial clearance, through its recruitment to escaped cytosolic bacteria through multiple signals [58], to enhance autophagy receptor affinity [59]. It is also important as it recruits WIPI2b, linking the downstream autophagy machinery [77]. This process of bacterial clearance is distinct from LC3 associated phagocytosis (LAP) where LC3 is becoming lipidated directly to the intact phagosome membranes, this process will be outlined in later sections (see sections 1.3 and 1.4).
1.1.5 Non-autophagic roles of autophagy proteins.

As more work is done on autophagy related proteins (Atgs), it is increasingly apparent that their roles are not limited to the canonical autophagy system. There are other distinct roles of Atgs [78]. Therefore, careful characterisation is required when studying Atgs, to ensure autophagy is not being wrongly attributed in unconventional settings. As an example, autophagy related proteins control extracellular secretion, completely separate from the lysosomal degradation role of canonical autophagy and bypassing the usual process of ER to Golgi trafficking for secretion [78]. Atgs have also been implicated in IL-β secretion, where inhibiting Atg5 leads to accumulation of IL-β inside the cell [79]. The cytokine was found to sit in between the two autophagosome membranes and somehow is delivered to the plasma membrane for secretion [79].

In addition, Atgs have been shown to control metabolic signalling in response to metabolic stress. Under glucose starvation, LC3 on autophagosomes binds to the Rab GAP TBC1D5, that can also bind components of the retromer complex in an inhibitory fashion [80]. The binding of LC3 releases the retromer complex from TBC1D5, allowing for endosomal transport of the glucose transporter 1 (GLUT1 receptor) through the trans Golgi network to the plasma membrane for increased glucose uptake [80]. This links Atgs to endosomal trafficking in a manner that is distinct from their canonical role in lysosomal degradation.

The reason that these autophagosomes do not go on to fuse with the lysosome in secretory autophagy is thought to be due to the differential functions of SNAREs. For instance, there is evidence that in secretory autophagy the autophagosomes lack SNTX17, required for lysosomal fusion, and therefore these autophagosomes avoid lysosomal degradation [81]. Instead, they have been shown to have the SNARE SEC22b that aids plasma membrane fusion [81]. TRIM16 regulates this process, where it is an essential cargo receptor with binding affinity for LC3, but also Sec22 to mediate the fusion of secretory LC3 positive vesicles with the plasma membrane SNAREs [81].
There is also evidence that Atg proteins promote the secretion of lysosomes in osteoclasts. Osteoclasts release proteolytic enzymes into the extracellular space in order to degrade bone, essential for bone resorption [82]. LC3 has been shown to play a role in this and when Atg5 is inhibited there is much less bone resorption. No autophagosome structures are observed near the ruffled border, therefore, how LC3 controls this is yet to be elucidated [82].

Host defence by autophagy is something that has already been touched upon. Bacterial infection can be cleared by xenophagy, as previously mentioned, but also by LC3 associated phagocytosis (LAP). LAP is a process that uses Atgs in an unconventional way, and will be one of the focuses of this project; it is covered in more detail in later sections 1.3 and 1.4. There is also evidence for other processes that involve Atgs in bacterial tolerance by the cell. For instance, in the case of Toxoplasma Gondii (T. gondii), an intracellular pathogen that attempts to escape immune detection in pathogen containing vacuoles (PCVs) is targeted by the immune system through the action of some of the autophagy machinery. Specifically the PCVs are decorated with GABARAPL2, that recruits interferon (IFN) inducible GTPases to disrupt the membrane and replication of T. gondii (Sasai 2017). However, in other examples, certain Atgs can favour bacterial replication. For instance, in the case of Brucella abortis, the early autophagy machinery, but not the lipidation machinery, is needed to form bacterial containing vacuoles [83].

1.1.6 The role of autophagy proteins in viral infection particularly Influenza infection.

Non-autophagic roles of Atgs have also been uncovered in the context of virology. Autophagy related proteins are often involved in viral infection, depending on the context they can contribute to promotion or inhibition of the viral lifecycle. For instance, viruses can hijack the autophagy machinery and this is common in positive strand RNA viruses such as poliovirus. In this instance, the virus activates autophagy to utilise the forming membranes as scaffolds for viral replication [84]. Depleting autophagy proteins in this case decreases viral yield [84]. Other viruses such as the herpes simplex virus type 1 (HSV-1) inhibit autophagosome initiation, in this
instance achieved via a viral protein binding to beclin1, a key autophagy protein [85]. This inhibition of autophagy is essential for the virulence of HSV-1 [85].

HIV-1 infection also appears to activate an unconventional autophagy pathway. It has been shown that the small transmembrane HIV protein Vpu helps to overcome host restriction factors through interactions with LC3C that, in a manner distinct from autophagy, removes the host restriction factor, BST2, from viral budding sites [86]. These data suggest there maybe a role for a pathway distinct from autophagy that plays a role in virology.

The interplay of viruses and autophagy related proteins is complex and the virus that is most relevant to this project is Influenza A (IAV), which activates LC3 lipidation to membranes. Upon infection, the virion is taken up by endocytosis into host cells. The viral protein M2 (matrix protein 2), an ion channel protein, inserts into the infected cell membrane and through the action of its LIR motif recruits and lipidates LC3 to the plasma membrane [87]. There is also evidence of accumulated LC3 lipidated membranes [88]. This LC3 lipidation is reliant on the Atg16L1-12-5 complex but, interestingly, is independent of FIP200 [87], an autophagy initiation protein, suggesting that these may not be accumulated autophagosomes. Instead, there may be a non-autophagic role of some of the autophagy proteins. The role for LC3 lipidation in this context is thought to contribute to virion budding and stability [87], which is similar to HIV-1 infection where the viral gag protein interacts with LC3 to help promote budding and assembly [89].

Studying IAV infection in more detail may help uncover if non-canonical autophagy, as defined as part of this project, plays a role in this pathology.
1.2 Non-canonical autophagy

1.2.1 Definition and discovery.

For a long time in the autophagy field, levels of cytosolic LC3 and lipidated LC3 were measured solely as a readout for canonical autophagy. However, it has now been accepted that LC3 can also be lipidated in the context of non-autophagosome single membranes through processes known as “non-canonical autophagy” [90, 91].

The term non-canonical autophagy used in this project refers to the promotion of LC3 lipidation to single-membrane endolysosomal compartments occurring through the unconventional use of some autophagy proteins [92]. Although this is not a bonafide autophagic process, many groups have used the term non-canonical autophagy to refer to LC3 lipidation to single non-autophagosomal membranes, for instance in the case of LAP [41, 93-96]. Due to high profile publications using the term non-canonical autophagy, we felt it was important to keep this name for consistency. Other processes have been referred to as non-canonical autophagy, including Atg5 and beclin independent autophagy [97], but these are distinct processes from the classification discussed in this project. In the future, an alternative term for the pathway may be derived to improve clarity.

Non-canonical autophagy, as described here, involves a cell engulfment event such as phagocytosis, entosis or macropinocytosis, where LC3 is recruited and lipidated to phagosomes, entotic vacuoles or macropinosomes respectively, all single membrane compartments of the endolysosomal system. The molecular mechanisms underlying this unconventional LC3 lipidation event are yet to be fully determined and details seem to be context specific, each of these engulfment events will be described in turn later.
1.2.2 How is non-canonical autophagy distinct from autophagy?

Autophagy, as outlined above, is involved in the clearance and turnover of intracellular cytoplasmic material, while non-canonical autophagy is associated with the engulfment and degradation of extracellular material.

Canonical autophagy involves a de novo double membrane forming inside the cell, whereas in an engulfment event, the cytoskeleton is re-modelled to enwrap the cargo in the plasma membrane. This membrane has been shown by electron microscopy (EM) to be a single membrane distinct from double membrane autophagosomes [91].

Evidence suggests that endolysosomal LC3 lipidation shares the common lipidation complex with autophagy. For instance, knocking down Atg5 and Atg7 abolishes LC3 lipidation in both pathways [91]. However, the nature of the LC3 lipidation is somewhat different. While autophagosomes are decorated with lipidated LC3 on both the inner and outer surfaces of the double membranes during their formation, in non-canonical autophagy the vacuole formed following an engulfment event will only be decorated with LC3 on the outer surface of its membrane.

It is the upstream initiation signals that most clearly separate the two LC3 lipidation pathways. For instance, LC3 can become lipidated to non-autophagosome membranes independently of the ULK1 complex (ULK1, FIP200, Atg13) whereas in autophagy this complex is essential for LC3 lipidation to autophagosomes [91]. Knockdown of FIP200, ULK1, Atg13 all confirm that non-canonical autophagy still occurs despite the inhibition of autophagy [41, 91]. The differences in the upstream signaling to LC3 recruitment and lipidation suggest the lipidation machinery could be recruited in a different way to non-autophagosomal membranes.

It is more complex when looking at the involvement of the PI3K complex and the production of PI3P in non-canonical autophagy. The PI3K complex in autophagy is made up of Vps34, UVRAG, Beclin and Atg14 whereas in non-canonical autophagy Rubicon takes the place of Atg14, this will be further described when introducing LAP. In certain cellular processes, Vps34 is required for
endolysosomal LC3 lipidation associated with non-canonical autophagy [91]. However, in experiments involving pharmacological induction of the pathway, such as monensin or chloroquine, LC3 lipidation is insensitive to PI3K inhibitors such as wortmannin and LY29004 [98]. Therefore, it is hypothesized that the production of PI3P is necessary for non-canonical autophagy in the context of vesicle maturation, but it is not required directly for recruitment of the LC3 conjugation machinery to endolysosomal membranes [98].

Finally, non-canonical autophagy acts in a manner distinct from autophagy because p62 and NDP52, common autophagy cargo receptors, are not present at endolysosomal membranes [98]. Also, the membranes targeted by non-canonical autophagy are not damaged, as galectin 3 and 8 are absent [41, 98], therefore making this process distinct from selective autophagy processes such as xenophagy or lysophagy.

1.2.3 Pharmacological modulators

Many high throughput drug screens have been carried out to find modulators of autophagy. The readout depends on where the drug is acting in the autophagy pathway. If the drug is inhibiting early stages of autophagy initiation, the number of autophagosomes decreases, as do lipidated levels of LC3. However, if the drug is blocking the lysosomal degradation of autophagosomes and their contents, flux of the pathway is being inhibited and autophagosome number increases, as do lipidated levels of LC3.

In this study we have used Bafilomycin A1, a potent and specific inhibitor of vacuolar ATPases (V-ATPase) [99], which causes an increase in lysosomal pH, thereby blocking the final degradation step of autophagosomes. This affects autophagic flux and leads to a build-up of autophagosomes [34]. Other drugs such as: monensin, chloroquine, nigericin, hydroxychloroquine, betahistine, procanimide (see publication for further names [41]), have been previously used to look at autophagic flux as they too block the final steps in the signaling pathway. However, the Florey lab discovered that, unlike bafilomycin, these drugs also promote LC3 lipidation to membranes of the
endolysosomal system [41]. This LC3 lipidation is distinct and independent of autophagy but is dependent on V-ATPase activity, which is why bafilomycin does not promote LC3 lipidation to single membranes of the endolysosome [41, 98]. Some of these afore mentioned drugs are shown in (FIGURE 1.3) to summarise their role in LC3 lipidation to endolysosomal compartments dependent on V-ATPase and the Atg16 conjugation machinery. Therefore, these drugs should be used with caution when looking at autophagic flux [98].

AMDE-1 (Autophagy Modulator with Dual Effect-1) is another drug previously identified to increase GFP-LC3 puncta formation through its action on AMPK to then inhibit mTORC1 [100]. It was also shown to block autophagic flux by affecting the lysosomal enzymes [100]. AMDE-1 has now been shown to activate a form of unconventional autophagy that is independent of ULK1, WIPI2b and Beclin, inducing LC3 lipidation to the Golgi [101]. This lipidation of LC3 occurs in a V-ATPase dependent manner, shown using V-ATPase inhibitors such as bafilomycin [101]. This is further evidence that pharmacological modulators assigned to autophagy need to be used with caution.

In this study monensin, an ionophore, was used as shown in (FIGURE 1.3). Monensin exchanges protons with sodium ions, raising the lysosomal pH, which causes water influx to lysosomal compartments [102], and activates endolysosomal LC3 lipidation. Chloroquine was also used, which is a lysosomotropic drug, a basic lipophilic compound that gets protonated and trapped in acidic compartments [103], again raising the lysosomal pH, causing osmotic imbalance and so leading to the influx of water and inducing endolysosomal LC3 lipidation [98].

The induction of endolysosomal LC3 lipidation by these lysosomotropic or ionophores is V-ATPase dependent. For instance, if cells are pretreated with bafilomycin (inhibiting V-ATPase), and then stimulated with monensin, endolysosomal LC3 lipidation is prevented [98]. Indeed, even merely using hypertonic medium is enough to induce endolysosomal LC3 lipidation, which can then be reduced using aquaporin inhibitors (blocking the osmotic change of endolysosomal
compartments) or bafilomycin [98]. How the influx of water activates LC3 recruitment and lipidation at these membranes is yet to be determined.

Monensin and chloroquine thus provide a signal for endolysosomal LC3 lipidation based on osmotic changes to compartments in a V-ATPase dependent manner [41, 98]. These drugs outlined, will be essential tools for examining LC3 lipidation in both canonical and non-canonical autophagy.
Figure 1.3: Drugs that activate non-canonical autophagy with a detailed mode of action for monensin

List of drugs that cause water influx into endolysosomal compartments, in a V-ATPase dependent manner. This results in LC3 recruitment and lipidation dependent on the Atg5-12-16L1 complex. Monensin carries out this activity by acting as an ionophore, exchanging hydrogen H+ ions for sodium ions Na+. This is V-ATPase dependent and results in LC3 lipidation by the Atg5-12-16L1 complex.
1.2.4 Toxins

The drugs outlined above all have properties that cause water influx into endolysosomal compartments, resulting in the activation of the autophagy lipidation machinery in an unconventional manner, leading to the recruitment and lipidation of LC3 to these single membranes. Interestingly, there is evidence that in bacterial pathogenesis certain toxins can insert into membranes and similarly affect the osmotic balance of intracellular compartments. An example for this is *Heliobacter pylori* (*H. pylori*), a bacterium that can infect the digestive tract. This bacteria, following its endocytosis into the cell, has a virulence factor which is a secreted toxin, vacuolating toxin A, VacA [104, 105]. This toxin complexes and inserts into the endosomal membrane to form a selective calcium channel resulting in an influx of calcium ions into the lumenal space [106]. In order to overcome this, the V-ATPase found on the same membrane increases its activity to pump more protons into the lumen [107]. This results in the accumulation of protonated weak bases accumulating in the endosomal lumen, such as ammonium chloride, which in turn creates an osmotic gradient that results in water influx and vacuolation of endolysosomal compartments [108]. As previously mentioned, vacuolation and swelling of endolysosomal compartments after pharmacological stimulation activates LC3 lipidation. Similarly in MEFs treated with VacA toxin, the cells vacuolate and, in an Atg5 and V-ATPase dependent manner, lipidate LC3 to the single membrane endolysosomes [98]. This was shown to be independent of the canonical autophagy proteins such as Atg13 [98]. This toxin will be used to study LC3 lipidation in the context of non-canonical autophagy.
1.3 Macro-endocytic engulfment events associated with non-canonical autophagy.

1.3.1 LC3 associated phagocytosis (LAP)

The best studied non-canonical autophagy pathway where LC3 is recruited and lipidated to non-autophagosomal membranes is LAP. Phagocytosis is defined by engulfment of particles more than 0.5 µm in size. Like autophagy, phagocytosis has a role in maintaining the homeostasis of an organism, through the clearance of both self and foreign particles, and similarly finishes with lysosomal degradation of said particles. Phagocytosis occurs in professional phagocytes in the blood, such as macrophages, neutrophils and dendritic cells, and other specialised cells in the nervous system and liver. Non-professional phagocytes such as epithelial cells can also perform phagocytosis and it is these that play an essential role in clearing debris.

LC3 associated phagocytosis (LAP) occurs when pathogenic material or apoptotic debris are phagocytosed and LC3 is recruited and lipidated directly onto the phagosomal membrane. The phagosomes then mature and fuse with the lysosome for the acidification and degradation of the contents [109].

LAP is not to be confused with xenophagy, a mechanism that targets pathogens and other foreign materials for autophagic processing. Xenophagy has been extensively described and the process relies on LC3 adaptor proteins and membrane damage markers, which are left as evidence when a pathogen tries to escape from an endosome. LAP is distinct, it does not require membrane damage to initiate the process of LC3 lipidation and is arguably a more direct and swift way to clear pathogens or apoptotic debris from the cell [110].

The upstream signals that initiate phagocytosis or LAP involve cell surface receptors recognising and internalising extracellular material, for instance, Toll like Receptors (TLRs) and FcγR found on macrophages, dendritic cells and neutrophils. TLRs recognise surface markers on the pathogen and they signal to the machinery for LC3 lipidation [109]. It has also been shown that cellular
corpses are targeted for degradation by LAP via the action of the phosphatidylserine receptor TIM4 [111]. There is also a role of Dectin-1, recognising fungal antigens [112-114].

Before going onto describe the molecular mechanisms of LAP, it is important to understand the process of phagocytosis. As mentioned above, it is a process mediated by cellular receptors, where often the signalling is activated upon clustering of said receptors. The phagosome cup forms and engulfs particles, reliant on actin cytoskeleton re-arrangements and tightly regulated phosphoinositide signalling. This eventually leads to scission and formation of a nascent phagosome in the cytosol. This nascent phagosome undergoes a series of maturation steps, similar to the steps in endosome maturation. Fusion of early endosomes to the nascent phagosomes results in the accumulation of Rab5, essential to the maturation of the phagosome [115]. Rab5 then has a role in recruiting the Vps34 complex, a PI3K complex, which allows the phagosomal membrane to become enriched with PI3P [115-117]. This in turn allows PI3P effector proteins to be recruited, such as the NADPH oxidase complex. When the phagosome is matured, Rab5 is no longer present at these membranes and Rab7 increases at the phagosome, again this is essential to the maturation of the phagosome [115]. Furthermore, the membrane proteins LAMP1 and LAMP2, found at late phagosomes, endosomes and lysosomes, are thought to be needed to recruit Rab7 [118, 119]. This mature phagosome can then fuse with the lysosome; V-ATPase allows for acidification and the NADPH complex has a role in reactive oxygen species (ROS) production.

Further to the characterisation of phagocytosis, the molecular mechanisms of LAP have begun to be unraveled, a particularly important paper is by Martinez et al [120] and the findings are summarised in (FIGURE 1.4). They show through SILAC (stable isotope labelling of amino acids in cell culture) studies that rubicon is present at the LC3 associated phagosomal membrane, which they term the laposome [120]. Rubicon is a negative regulator of autophagy, when it is knocked down there is more autophagosome formation under autophagy inducing conditions, due to
rubicon inhibiting Vps34 [121]. In non-canonical autophagy, rubicon is essential for LAP, acting in complex with UVRAG, Beclin1 and Vps34 to form a distinct and specific PI3K complex [121], as shown in (FIGURE 1.4), and rubicon is needed for Vps34 activity in this context. The PI3K complex is recruited upon stimulation of cell surface receptors to the laposome and sustains PI3P production at these membranes. Without the engagement of these receptors, for instance if uncoated beads are phagocytosed, no LAP is observed [109, 110, 120]. As noted above, LC3 lipidation is also dependent on V-ATPase activity and inhibiting V-ATPase blocks LC3 lipidation but has no effect on PI3P generation at phagosome membranes, therefore it must act downstream of the PI3K complex and upstream of the lipidation machinery [98].

Rubicon also stabilises the NOX2 complex, which is an NADPH oxidase 2 expressed in phagocytes, which are able to transport electrons across a membrane to produce superoxide and other ROS [122]. NOX2 has previously been shown to activate ROS production in response to cell surface receptor stimulation and results in LC3 lipidation to the phagosome [110]. NOX2, along with p22PHOX, sits at the intracellular side of membranes and in order for it to be able to produce ROS it has to complex with cytosolic p47PHOX, p40PHOX, p67PHOX and Rac1 [122]. Rubicon directly interacts with p22PHOX and stabilises this NOX2 complex [123]. PI3P production acts upstream of NOX2 and both signals are dependent on rubicon and essential for LAP [120]. The PI3P and ROS production leads somehow to the recruitment of the conjugation machinery to lipidate LC3 on the phagosomal membrane to then fuse with the lysosome [110, 120], as shown in (FIGURE 1.4).

Martinez et al. [120] produced a knockout rubicon mouse and analysed bone marrow derived dendritic cells (BMDCs) from these animals, compared with WT cells [120]. The amount of phagocytosis was equal, but in the rubicon knockout cells there was no LC3 lipidated to phagosomes, additionally increased LC3 puncta were observed, due to rubicon no longer having a
negative effect on canonical autophagy [120]. The signals for LAP were independent of WIPI2b and FIP200 [120].

In NOX2 knockout cells, the PI3K complex was recruited to LAPosomes, however, LC3 was not recruited and lipidated to these membranes [120]. Therefore, LAP involves cell surface receptor signaling to activate a distinct PI3K complex where rubicon is essential to also stabilise NOX2 for the production of ROS [120]. This results in the recruitment of the Atg16-5-12 conjugation machinery and LC3 lipidation to the phagosome membrane, how this lipidation machinery is recruited is unclear [120]. Florey et al [98] hypothesise that ROS production has the potential to alter the ionic balance of the phagosome and may therefore introduce osmotic imbalances, that can drive non-canonical LC3 lipidation [124]. Furthermore, solutes are released from degrading cargo in the phagosome lumen that may also cause an osmotic imbalance [125]. An overview of the signals involved in non-canonical autophagy are summarised in (FIGURE 1.5).
Figure 1.4: Molecular mechanisms of LAP

The pathogen or in this case the zymozan particle engages the cell surface receptor to be internalised, NOX2 and the P22 PHOX subunits are present at the membrane. 1. The PI3K complex (Beclin1, Vps34, UVRAG and rubicon) is recruited to the phagosome. 2. The PI3K activity enriches the phagosome with PI3P. 3. PI3P production recruits the cytosolic NOX2 components to the membrane to form a stable NOX2 complex, rubicon stabilises this complex. 4. NOX2 on the phagosome membrane produces ROS. 5. By an unknown mechanism the Atg16L1 conjugation complex is recruited and LC3 is lipidated to the phagosomal membrane. 6. The phagosome then fuses with the lysosome for degradation of the contents.
1.3.2 LAP in fungal infection.

*Aspergillus fumigatus* is an airborne fungus that can lead to invasive aspergillosis. This is common and extremely dangerous in immuno-compromised patients and chronic granulomatous disease (CGD) patients [126]. *A. fumigatus* can be targeted by the immune system and cleared through the action of LAP. This is dependent on the removal of melanin, a component of the fungal cell wall [127], and exposure of β-glucan, to activate Dectin-1 receptor and Syk dependent ROS production, resulting in LC3 lipidation to phagosomes [128].

LAP is inhibited in CGD, an X-linked heritable disease, where the phox subunits of NOX2 are mutated or deleted, resulting in inhibited ROS production in immune cells [129]. CGD murine and human immune cells were used to look at LAP in response to *A.fumigatus* and showed that there was defective LC3 lipidation to phagosomes and increased fungal load, resulting in increased IL-1β, an inflammatory cytokine, in patient cells compared to healthy cells [130]. This was further confirmed by Martinez et al. taking genetically modified animals deficient for LAP and infecting them with *A.fumigatus* resulted in invasive fungal infection and a plethora of inflammatory cytokines [120]. Similarly, patients treated with corticosteroids that inhibit ROS production and therefore LAP see a similar pathogenesis [128]. Therefore, non-canonical autophagy, specifically LAP, is important in the clearance of airborne fungal spores and when LAP is inhibited, because of disease or other factors, the consequences can be fatal.

Interestingly, LAP can be reactivated in certain circumstances. For instance, IFNγ treatment has been used to treat invasive fungal infections [131] and Oikonomou et al. looked at this in the context of LAP after *A.fumigatus* infection [132]. IFNγ activates the expression of death-associated protein kinase 1 (DAPK1), a protein involved in programmed cell death that also plays a role in inflammation [133]. DAPK1 co-localises at the phagosomes with proteins including LC3 and Rubicon following *A.fumigatus* infection [132]. In cells from CGD patients, there was defective IFNγ and DAPK1 expression, furthermore when DAPK1 was silenced in healthy cells LAP was inhibited [132]. Treating the CGD cells with IFNγ resulted in DAPK1 expression and rescued
fungal clearance decreasing inflammation [132]. Interestingly, this mode of action is independent of NOX2 activity, because CGD patients do not have functional NOX2, suggesting DAPK1 may activate an alternate ROS pathway [134]. Therefore, IFNγ treatment offers a therapeutic way to target fungal pathogenesis potentially through LAP.

1.3.3 Calcium signaling in LAP

Recent work implicates calcium signaling, upstream of the rubicon containing PI3K complex, in the regulation of LAP. Calcium is a secondary messenger that underpins many signaling and trafficking processes, including autophagy and phagocytosis. Free cytosolic calcium is an inducer of autophagy [135], while intracellular calcium is linked to the regulation of membrane fusion in phagocytosis, and ROS formation after zymozan engulfment, but this is a largely unstudied area [136, 137].

As mentioned above, melanin protects *A. fumigatus* from LAP through blocking the assembly of the NOX2 complex [127, 138]. Recent work shows that calmodulin, a protein that binds and is activated by calcium, transiently recruits to phagosomes containing melanin deficient *A. fumigatus* conida [139]. Furthermore, global calcium depletion inhibits this calmodulin recruitment to phagosomes and inhibits NOX2 components, ROS production and therefore LAP [139]. This was shown to be specific to intracellular and ER calcium store depletion. Calcium was shown to be released from phagosomes activating calmodulin signalling regulating LAP [139]. Fungal melanin blocks this calcium signalling, by sequestering calcium in the phagosomal lumen to inhibit the recruitment of the key signalling molecules to allow LAP to clear the fungal infection [139]. Interestingly this study went on to link this to human pathogenesis, where a SNP in the calmodulin I gene that decreases calmodulin expression increases risk of invasive fungal infection [139].
1.3.4 Assays to study phagocytosis and xenophagy.

In order to study pathogen clearance in the context of autophagy or non-canonical autophagy, bead experiments in phagocytic and non-phagocytic cells are used in the literature. To model pathogen clearance via autophagy, beads coated with transfection reagent are often used [140]; this mimics the damage the pathogens cause to the endosome/phagosomes via virulence factors such as toxins, causing the initiation of the autophagy machinery [140]. Non-canonical autophagy has also been investigated using beads. Uncoated beads are internalised into the cell but alone will not activate LC3 lipidation to bead containing phagosomes [98, 110, 140]. However, if the beads are ligand-coated, or combined with a drug treatment, they can be used to activate non-canonical autophagy [98]. Using IgG (ligand of FcƳR) or lipopolysaccharide (LPS; ligand of TLR4) coated beads, LC3 recruitment and lipidation onto phagosomes is observed [110], in a manner dependent on NADPH and the production of ROS [110].

1.3.5 Macropinocytosis

In addition to LAP, non-canonical autophagy is also implicated in other engulfment events, including macropinocytosis and entosis (see below). Pinocytosis was originally observed by Lewis in 1931 [141] where extracellular medium was being “drunk” into the cells. It is a form of endocytosis and occurs in a non-selective manner where extracellular macromolecules are taken into the cell via membrane ruffling, dependent on cytoskeletal rearrangements and phosphoinositol signalling [142]. The uncoated vesicles are usually 0.5-10 μm in size but characteristically irregular and it has been reported that they can mature and fuse with lysosomes similar to endosomal and phagosomes maturation [143].

Macropinocytosis has been observed in response to growth factor signaling [144] and implicated in receptor trafficking and antigen presentation [145]. Macropinocytosis is also implicated in pathogenesis due to certain bacteria [146], protozoa [147] and viruses [148, 149] using it as a method of cellular entry. Furthermore, macropinocytosis plays a role in Ras-driven tumours because oncogenic Ras activates macropinocytosis, allowing increased growth factor signalling...
and amino acid delivery to aid tumour proliferation [151]. Therefore, inhibiting macropinocytosis offers a potential therapeutic target [151].

LC3 is often lipidated onto macropinosomes in a manner distinct from autophagy initiation signals and in line with non-canonical autophagy, the summary diagram 1.5, shows that macropinosomes is one of the examples where non-canonical autophagy occurs [91]. The mechanism and function of non-canonical autophagy in this context is unclear. Macropinocytosis will be used in this project to study non-canonical LC3 lipidation in a physiological setting.

1.3.6 Entosis

Entosis is a process where one live epithelial cell pushes into another epithelial cell to form a so-called ‘cell in cell’ structure [152]. This process occurs in epithelial cells following matrix deadhesion, aberrant mitosis [153], or glucose deprivation [154], often in tumours. The internalisation of neighbouring cells depends on the relative deformability of cells, where the stiffer cell pushes into a softer neighbour, in a manner dependent on the formation of an epithelial junction and the force provided by Rho-dependent actin signalling [155, 156].

The internalised entotic cell is housed inside a single membrane vacuole. The internalised cell may occasionally escape back into the cytosol but it is usually killed by lysosomal degradation [152]. Non-canonical autophagy plays a role in entosis as a rapid and transient recruitment and lipidation of LC3 from host cells onto entotic vacuole membranes is apparent, in a manner separate from autophagosomal LC3 lipidation [91]. The LC3 lipidation seems to influence the maturation of the entotic vacuole and subsequent death of the housed cell [91].

Entosis is a “double-edged sword” in the context of cancer. On one hand it has a tumor suppressive role because it eliminates cells that have become detached or undergone aberrant division: *in vitro* assays suggest inhibiting entosis promotes tumor proliferation [152]. On the other hand, entosis promotes ploidy changes, by physically disrupting host cell cytokinesis; this
genetic instability is associated with tumour promotion, encouraging more aggressive cancers and poor patient outcome [157].

The entotic vacuole is targeted for LC3 lipidation via non-canonical autophagy and is a form of engulfment event associated with non-canonical autophagy, therefore it is included in the summary diagram 1.5. Entosis will be used in this project as a system to study non-canonical LC3 lipidation.
Figure 1.5: Molecular mechanisms of non-canonical autophagy.

Multiple engulfment events activate a non-canonical autophagy process dependent on the PI3K complex (Beclin1, Vps34, Vps15, Rubicon) and V-ATPase activity. In LAP NADPH oxidase is needed to produce ROS and the internalised cell surface receptors such as TLRs could be involved in further signalling. These signals all act upstream of the LC3 conjugation machinery, this machinery is common to canonical autophagy. This results in LC3-I becoming lipidated to single endolysosomal membranes LC3-II. These membranes then fuse with the lysosome for the contents to be degraded.
1.4 Importance of non-canonical autophagy.

Although the role for non-canonical autophagy is yet to be elucidated in entosis and macropinocytosis, a number of studies show that LAP is critical in health and disease. LAP is a vital cellular process with important functional consequences, particularly with regards to its role in the immune system. As previously outlined, non-canonical autophagy regulates the degradation of material following macro-endocytic engulfment. LAP is involved in pathogen clearance, including fungal pathogens, impacting on immune responses including antigen presentation. LAP has also been shown to be essential for cellular homeostasis through apoptotic cell clearance, controlling immune responses but also playing a role in maintenance and control of signalling pathways and processes, such as the visual cycle and in cell division.

1.4.1 LAP maintains homeostasis through clearance of apoptotic debris.

The body is challenged not only by foreign invaders but also with the millions of cells that die every second. It is essential that this does not activate an inappropriate innate immune response and this is achieved through engulfment and degradation of apoptotic cells and general cellular debris by phagocytes [158]. The persistence of apoptotic corpses not only causes aberrant inflammation but also could impair parts of phagocytosis itself, for instance it has been seen in *Drosophila* that persistence of corpses could impair phagocyte motility [159].

Dead cells are recognised by phagocytes through “eat me” signals, this is in the form of phosphatidyl serine (PtdSer) which is usually present on the inner leaflet of the plasma membrane but upon apoptosis become exposed [160]. TIM-4 is a receptor on macrophages and dendritic cells that recognise PtdSer and if this receptor is blocked these cells can no longer engulf dead cells [161, 162]. A knockout TIM-4 mouse model was engineered to show that *in vivo* there is inefficient engulfment and consequent clearance of dead cells, resulting in hyperactivity of immune cells and signs of systemic autoimmunity [163]. LAP was shown to be involved in this engulfment event as rapid LC3 lipidation to apoptotic cell containing phagosomes was seen,
peaking at 120-150 min post engulfment, and electron microcopy confirmed it was a single membrane surrounding the apoptotic cell [111].

In macrophages that do not support LAP, for instance Atg7 knockout macrophages, LC3 lipidation to engulfed apoptotic cells was absent and led to decreased acidification and clearance of these compartments [111]. The result of this was the production of inflammatory cytokines IL-1β and IL-6, compared to a dampening anti-inflammatory response evidenced by IL-10 levels in WT cells [111]. This supports what was seen when the autophagy protein, Atg16L1, important for both canonical and non-canonical autophagy, was knocked out, resulting in IL-1β production in mouse macrophages [164].

Mouse models deficient in LAP were also used, both in vitro and in vivo, to look at their response after challenge with apoptotic cells [96]. Mice that supported LAP were able to clear apoptotic cells, independent of FIP200 expression [96]. When LAP was impaired, for instance in knockout rubicon or NOX2 mice, animals were smaller, and after repeated challenge with apoptotic cells had an inflammatory cytokine profile and autoantigens were produced [96]. The mice also suffered kidney damage, all consistent with an autoimmune systematic lupus erythenatasus (SLE) like phenotype [96].

Taken together, this highlights the importance for non-canonical autophagy in homeostasis and the ability to mount an appropriate immune response.

1.4.2 Role of LAP in maintaining the visual cycle.

LAP also plays an important role in maintenance of the visual cycle. In order to maintain the health of the retina and support vision, photoreceptor outer segments (POS) are shed and phagocytosed by the neighbouring retinal pigment epithelial cells (RPE). The mechanism for this phagocytosis is reliant on “eat me” signals on the POS being recognised by receptors and integrins on the RPE surface.
A paper by Kim and colleagues looked at how the autophagy machinery is involved in this maintenance event [94]. They knocked out Atg5 in RPE cells and showed that LC3 was no longer lipidated to phagosomes and there was an accumulation of enlarged phagosomes containing POS leading to a visual defect in mice [94]. They showed further evidence that this was due to a defect in LAP, and not linked to canonical autophagy, by knocking out components such as ULK1, FIP200 and Atg13 and showing no defect in the phagocytosis and clearance of POS by RPE cells [94]. This could be explained by their observations that in the knock out Atg5 RPE cells had decreased lysosomal activity [94]. Transmission electron microscopy showed the membranes surrounding the POS were also single membranes [94]. This paper provides a link for non-canonical autophagy with RPE phagocytosis implicating the process in the visual cycle. This is interesting from an ageing point of view as the age related decline in vision could be linked to the decline in phagocytosis and LAP [94].

1.4.3 Signalling linked to cell division in C. elegans is controlled by LAP.

LAP has also been implicated in controlling signaling linked to cytokinesis through the degradation of the midbody. The midbody is a structure formed when the mitotic spindle meets the contractile ring and controls the final separation of daughter cells. This signaling complex must be tightly regulated to stop aberrant signaling after this process is complete. The fate of the midbody has been disputed. There have been models proposing midbody release, where they can be safely phagocytosed [165-167], or alternatively, suggestions that after cell division the midbody is targeted and degraded by the autophagy machinery [168, 169]. An interesting paper looked at combining both of these theories to assess whether LAP is involved in midbody degradation, where the midbody is released and phagocytosed, then targeted by some of the autophagy machinery in non-canonical autophagy [170]. The study was done using time lapse imaging in Caenorhabditis elegans (C. elegans) embryos. They found that indeed the midbody was released and phagocytosed by the daughter cells. These phagosomes were then lipidated with Atg8
homologs in C. elegans, independent of the canonical autophagy proteins UNC-51 and EPG-8 (homologous to mammalian ULK1 and Atg14) [170].

A similar phenomenon utilizing LAP in C. elegans has been observed in clearance of polar bodies in early embryonic development [171]. Polar bodies are produced after a meiosis event and contain extra genomic information often produced because of asymmetric division, therefore it is essential that these are programmed for apoptosis and then cleared. This process occurs via LAP and shows that undifferentiated pluripotent cells are capable of LAP, knockdown of EPG-8, a homolog of Atg14, was used to show that canonical autophagy did not have a role [171]. Both worm homologs of LC3 (LGG-2) and GABARAP (LGG-1) were shown to localise to phagosome membranes and knocking out Atg7 stopped Atg8 lipidation [171]. The lack of Atg8 lipidation did not affect LMP-1 recruitment (worm homolog of LAMP proteins) but did slow the degradation and breakdown of polar body membrane for degradation [171].

1.4.4 Antigen presentation

In the adaptive immune response pathogen derived antigen fragments are presented on the surface of antigen presenting cells (APCs). They are presented by major histocompatibility complexes (MHC) that come in two classes, MHC-I and MHC-II. Antigens can originate intracellularly or exogenously (extracellularly) to be presented, they can also be cross-presented. A basic overview, as summarised in (FIGURE 1.6), is that extracellular peptides are processed by the lysosome and loaded onto MHC-II complexes at an endosomal MHC loading compartment, while intracellular peptides are processed by the proteasome and loaded onto MHC-I at the ER, to then be presented at the cell surface. This is an oversimplified view and actually, autophagy is another way intracellular peptides are processed and loaded onto MHC-II for presentation. FIGURE 1.6 visualises MHC-II antigen presentation in the context of LAP and autophagy. Once the antigens are presented on the cell surface MHC-I, antigen complexes are recognised and activate CD8+ T cells and the MHC-II antigen complex activates CD4+ T cells.
There have been studies implicating autophagy in MHC-II antigen presentation in vitro, where this has a knock on effect for efficient CD4+ T cell priming. These studies were done by inhibiting autophagy via the PI3K complex [172-174], or by knocking down Atg12 [175]. In vivo studies were done in a mouse model where Atg5 was knocked out in dendritic cells, a form of antigen presenting cell (APC) [176]. This showed a defect in MHC-II antigen presentation in response to HSV and Listeria infections and T cell priming [176]. The defect was not due to functionality of dendritic cell migration, co-stimulatory signalling, MHC-II expression, cytokine secretion, ROS production or the phagocytosis or endocytosis of the antigen itself [112, 176]. What was affected was the delayed phago-lysosome fusion and delivery of cathepsins to the lysosome [176]. Interestingly, there was no impact on MHC-I antigen presentation or cross presentation [176].

However, when canonical autophagy is activated, for instance by starvation, this does not regulate or enhance antigen presentation [176]. Furthermore, when TEM was performed, the structures were single membrane phagosomes. These data suggest the defect in antigen presentation from knocking out Atg5 in vivo may be dependent on non-canonical autophagy [176].

It has also been reported that knocking out LC3B in dendritic cells does not affect their functionality, but there is an impairment in processing and presentation of MHC-II antigen presentation and subsequent T cell priming [112]. The delay in antigen presentation on MHC-II has been suggested to be due to delayed fusion of phagosomes with the lysosome and therefore less processing for antigen presentation [112]. This is the classical view that LC3 lipidation leads to efficient lysosomal fusion and degradation.

Conversely, one lab reports that LAP is actually needed more for the fine-tuning of antigen presentation, and that LC3 on phagosomes maintains antigens for delayed antigen presentation [177]. The kinetics of lysosome phagosome fusion, with and without LC3, showed that in the presence of LC3, fusion took more than 4 h and phagosomes were negative for Rab7 and LAMP2, suggestive of slowed maturation [177]. This maturation process was much faster where LC3 was
absent. This was in human antigen presenting cells, whereas the other studies were all in mouse cells *in vitro* and *in vivo*, therefore, this observation could be cell type specific [177].
Figure 1.6: MHC II loading extracellular and intracellular antigens

A. A peptide is internalised by a cell and the phagosome is targeted for LC3 lipidation. This membrane fuses with an endosomal MHC loading compartment where lysosomal enzymes break down the peptide into fragments and load them onto MHC-II molecules for trafficking to the cell surface for antigen presentation.

B. An intracellular peptide is targeted by the autophagy machinery and the autophagosome fuses with the endosomal MHC loading compartment where lysosomal enzymes break down the peptide into fragments and load them onto MHC-II molecules for trafficking to the cell surface for antigen presentation.

C. Intracellular peptides are also targeted by the proteasome where fragments are loaded onto MHC-I molecules and trafficked to the cell surface for antigen presentation.

Adapted from [178]
1.4.5 Receptor signaling in B cells

B-cells are essential immune cells in the adaptive immune response; they are activated by the binding of antigens to receptors leading to B-cell proliferation and antibody production. TLRs are among the receptors that can contribute to B-cell activation [179]. Some TLRs recognise foreign nucleic acid but can also recognise host derived nucleic acids. In the case of DNA it is TLR9 that can be activated and if inappropriately activated can lead to inappropriate B-cell proliferation and autoimmunity. Integrins have also been implicated in activation of signaling that follows antigen binding to immune receptors [180]. For instance, αv integrins regulate B-cell activation, through promotion of LC3 to TLR containing endosomes, which promotes TLR containing endosome maturation, resulting in decreased receptor mediated B-cell activation [181]. Conversely if the αv integrins, LC3B or Atg5 are knocked out, this leads to autoantibody production as a consequence of uncontrolled B cell proliferation, through the persistence of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling [181]. The maturation of these endosomes is thought to be similar to LAP and is dependent on ROS and Syk [181]. Therefore, this could indicate that non-canonical autophagy has an additional role in controlling immune responses to auto antigens. This notion is further validated by the fact some polymorphisms found in human Atg5 implicate this process and are linked to SLE [182, 183]. Furthermore, potentially linking to this, mice lacking αvβ5 integrin have age related blindness due to less efficient phagocytosis in RPE cells [184].

1.4.6 Role of Atg8 lipidation

In non-canonical autophagy LC3B, as well as GABARAPL1 and GABARAPL2, localise and lipidate to endolysosomal membranes [41, 120]; a more in depth analysis of the Atg8 proteins is currently being carried out within the Florey lab. As previously mentioned, the role of the Atg8 family members have been studied with regard to autophagy, but more needs to be done to elucidate their role in non-canonical autophagy. Similar experiments, such as knocking out the individual
Atg8 proteins [64], could now help elucidate the function of the different Atg8 members in non-canonical autophagy.

As outlined above, in non-canonical autophagy, LC3 lipidation is needed for homeostasis and control of immune responses. Without the LC3 lipidation in LAP, pathogen and apoptotic cell clearance is slower and less efficient, with evidence that this is due to slowed phagosome lysosome fusion [109, 111]. However, more work needs to be done when looking at LAP and the other non-canonical autophagy pathways. This is particularly important as recently there is contradictory evidence that LC3 is not universally required for phagosomal maturation in all systems [185]. This was shown in MEFs and bone marrow derived macrophages that were deficient for Atg5 or Atg7 and therefore could not lipitate LC3. In this system, despite the lack of LC3 lipidation, there was no defect in phagosome maturation [185]. Their conclusions did not exclude LAP, but suggested there is not a universal requirement for LC3 in phagosome maturation, and there must be further details to be uncovered [185]. This idea that the role of Atg8 is more complex is further supported by studies in which LC3 lipidation was shown to slow phagosome maturation to enable prolonged MHC II antigen presentation [177].

Together, published studies have uncovered non-canonical autophagy as an essential, parallel signalling pathway with critical functions in health and disease. The molecular mechanisms that distinguish canonical and non-canonical pathways remain to be fully understood.

1.5 Atg16L1

1.5.1 Structure

This project focuses on the key autophagy protein Atg16. Atg16 was initially found in yeast, designated Apg16 [24], and then further characterised in mice. The human Atg16L1 gene sits on the long arm of chromosome 2 at position 37.1 (2q37.1), the mouse Atg16L1 gene sits on chromosome 1 D. There are two paralogues of Atg16: Atg16L1 and Atg16L2; and Atg16L1 has
three splicing isoforms: α, β, γ [25]. Atg16L1 forms a ~800 kDa complex with Atg5 and Atg12 [25]. The domain structure of the human Atg16L1 protein is outlined in (FIGURE 1.7).

The N-terminal domain (amino acids: 1-79) of Atg16L1 is required for Atg16L1 binding to Atg5. Atg5 and Atg12 form an irreversible complex [29] and are required for LC3 lipidation activity: this was originally shown in Atg5 knockout MEFs [186]. The crystal structure is available for the Atg5-12-16 interaction, but this only includes the first 33 residues of Atg16L1[187]. The E3 like function of Atg12-5-16 [30], and its role in LC3 lipidation, has already been discussed, see figure 1.1.

The middle region of Atg16L1 includes the coiled coil domain (CCD) (amino acids: 79-230), which is required for Atg16L1 homo-dimerisation and therefore functionality [25, 188]. Amino acids 230-265 make up the rest of this middle region, containing the FIP200 binding domain (FBD)(residues 229-242)[32, 189] and WIPI2b binding sites (amino acids 226,230)[33]. Furthermore, Itoh et al.[190] screened for Rab effectors and found that Atg16L1 interacts directly with Golgi resident Rab33B via residues 141-265 in Atg16L1. The interaction is GTP-dependent and is thought to be important in autophagosome biogenesis; if GTPase activity is impaired, more Rab33B and Atg16L1 bind and this increases LC3 lipidation on autophagosomes [190]. The crystal structure is available of the central region of Atg16L1 residues 72-307 [191].

The C-terminal part of the protein contains seven WD-40 repeats. This domain is absent in yeast and its function is yet to be fully determined [25]. The structure has now been solved [192]. This domain will be discussed and introduced in further detail below in section 1.6.
Figure 1.7: Atg16L1 domain structure of mouse Atg16L1.

The N-terminal domain of Atg16L1 amino acids 1-79 is responsible for Atg16L1 interacting with Atg5, essential to the formation and function of the Atg16L1-5-12 complex. The CCD is the coiled coil domain (79-230) and is responsible for the homo-dimerisation of Atg16L1. The 79-265 part of the protein is also responsible for Atg16L1 interacting with proteins such as FIP200 and WIPI2b required for LC3 lipidation to autophagosomes. Finally the WD40 C-terminal domain of Atg16L, where the function is yet to be fully elucidated.
1.5.2 Autophagy protein recruitment

Atg16L1 is indispensable for autophagy and the knockout mouse for this gene shows lethality within one day of delivery due to the inability to survive starvation conditions [1, 164]. In knockout cell lines and mouse models, where Atg16L1 has been knocked out in specific cells of the mice, no LC3 lipidation is observed to autophagosomes [32, 164, 186].

In autophagy, Atg16L1 directly interacts with WIPI2b on the forming autophagosomes [33]. WIPI2b is a PI3P effector and therefore binds the PI3P-enriched autophagosomes. Pull-down experiments with truncated mutants of Atg16L1, and further mutational studies, showed that amino acids at positions 226 and 230 in the coiled coil domain of Atg16L1 are important for WIPI2b binding [33]. Furthermore, this interaction between Atg16L1 and WIPI2b is important to localise the lipidation complex to specify the location of LC3 lipidation. If WIPI2b is forced to the plasma membrane using a CAAX motif, Atg16L1 binds WIPI2b at the plasma membrane and is able to lipidate LC3 to the plasma membrane [33].

Atg16L1 also interacts directly with FIP200 [32, 189] and this interaction is independent of other Atg protein involvement. Residues 229-242 of Atg16L1 are required for FIP200 binding (the FIP200 binding domain, FBD); notably, one of the WIPI2b binding sites also fall within this region. The recruitment of the Atg16L1 complex in canonical autophagy is summarised in (FIGURE 1.8). The E226R E230R mutant Atg16L1, that no longer binds to WIPI2b, still binds FIP200, and in FIP200 knockout MEFs WIPI2b still binds Atg16L1, so this interaction is independent of FIP200 [33].

Interestingly, in ULK1 knockout cells that have undergone glucose starvation, which is a condition distinct from canonical amino acid starvation-induced autophagy, the ΔFBD mutant of Atg16L1 still supports LC3 lipidation [32]. This observation is consistent with the idea of a different mechanism for LC3 lipidation in processes distinct from canonical autophagy, independent of WIPI2, FIP200 binding and ULK1 involvement.
**Figure 1.8: Atg16L1 recruitment in autophagy**

Atg16L1 as a homodimer, each Atg16L1 interacts with Atg12-5 to form a functional complex to lipidate LC3. The middle region domain shown in red represents the domain that contains the FIP200 and WIPI2b binding sites. The double membrane structure is the autophagosome sequestering cytosolic components, where the PI3K complex containing Vps34 builds up PI3P shown in red on the autophagosome. WIPI2b and FIP200 are PI3P effectors that independently recruit Atg16L1 to autophagosomes specifying where the LC3 will become lipidated to the membrane.
1.5.3 Atg16L2

Atg16L2 is a paralogue of Atg16L1 with similar domain structures. Atg16L2 is conserved in mammals [193]. Like Atg16L1 it has splice variants, but only two, α and β Atg16L2, where the β is the more dominant in mouse tissues [193]. The N-terminal domain of Atg16L2 has 32% amino acid identity to Atg16L1; the WD domain has 43% and the middle region has 20.7% amino acid identity [193]. Atg16L2 forms a ~800 kDa complex with Atg12-5, just like Atg16L1 [193]. However, Atg16L2 does not bind WIPI2b [33] or FIP200 [32] and has a much weaker affinity for Rab33b [193]. Atg16L2 is not recruited to the phagophore in canonical autophagy and is unable to activate autophagy when expressed in Atg16L1 knockout cells. When Atg16L2 is knocked down there is no negative effect on the cell’s ability to carry out autophagy [193]. This is interesting because when Atg16L2 is forced to the plasma membrane it can function to lipidate LC3; therefore, the Atg16L2-Atg12-Atg5 complex is functional but is not recruited to autophagosomes [193]. To date, it has not been tested whether Atg16L2 is involved in endolysosomal LC3 lipidation events.

1.6 Atg16L1 WD domain

1.6.1 Structure

In general, the WD40 domain consists of a 6-8 blade, beta propeller structure. The WD40 domain is extremely common and often functions as a platform for protein-protein interactions to regulate signalling. Although the domain is common, there is low sequence conservation between WD domain containing proteins [192, 194-196]. It is the variable regions, not important for the folding, that are most likely to be responsible for specific protein-protein interactions. Before the crystal structure was solved for the WD domain of Atg16L1, an online database was available to predict the structures of WD domains based on their primary sequence. This offered predictions for potential amino acid ‘hotspots’ that would be important in protein-protein interactions, including the WD domain of Atg16L1 [195]. The crystal structure of the WD domain
of human Atg16L1 (1.55 Å) was solved part way through this project and offered further insight into which sites in the domain could be strong candidates for protein binding and thus good mutagenesis targets [192].

The crystal structure is useful because it shows the properties of different regions of the domain. For instance, the top face is largely acidic and has a lot of hydrophobic residues, whereas the bottom face is basic with negatively charged residues [192]. Furthermore, there is a positively charged cavity, accessible from the bottom and the side of the beta propeller, between blades 3 and 4 [192]. The WD domain has important residues that allow for the dimerization of two WD domains [192]. The published structure of the WD domain of Atg16L1 will be a good tool to help define the role of this domain in canonical and non-canonical autophagy.

1.6.2 WD domain in canonical autophagy

It has been shown that the WD domain of Atg16L1 is dispensable for canonical autophagy. Fujita et al. expressed a ∆WD mutant of ATG16L1 (1-249) in Atg16L1-deficient MEFs [197] and showed that the C-terminal deleted Atg16L1 still formed the complex with Atg12-5 and the dimerization of the complex was not impaired [197], which supports previous literature [25]. Furthermore, the WD domain was dispensable for Atg16L1 recruitment to punctate structures, upon starvation induced canonical autophagy, and subsequent LC3 recruitment and lipidation at these membranes [197].

The effect of deleting the WD domain on xenophagy was also studied, where the re-complemented cells were challenged with *S. typhimurium* and the lipidation of LC3 was measured, as well as the replicative ability of the bacteria [197]. There was no difference in the LC3 lipidation to the bacteria and the replicative ability of the bacteria was the same in cells with the FL Atg16L1 or the ∆WD mutant of ATG16L1 (1-249) [197].
The conserved nature of the WD domain of Atg16L1 in eukaryotes is suggestive of an important function, but this is yet to be determined, and is distinct from the role of Atg16L1 in canonical autophagy.

1.6.3 Known binding partners

A range of proteins have been found to bind to the WD domain of Atg16L1. There is some literature to suggest that WD proteins can bind Ubiquitin (Ub) [198], this was shown for the WD domain of Atg16L1, playing a role in selective and starvation induced autophagy in conjunction with other interactors such as FIP200 [199].

TMEM 166 (transmembrane protein 166) is another protein that interacts with the WD domain of Atg16L1. Knocking down TMEM 166 reduces autophagy and Atg16L1 localisation to autophagosomes [200]. This is a beclin independent initiation of autophagy.

Another protein that interacts with a large subset of autophagy related proteins is interferon induced TRIM 20 (tripartite motif containing protein). These interactions include binding to the WD domain of ATG16L1 [201]. TRIM20 acts as an autophagy receptor and a platform to assemble autophagy components thought precision autophagy and is implicated in inflammasome signalling [201]. So far these examples are not relevant to non-canonical autophagy.

There are other known interactors of the Atg16L1 C-terminal WD domain that implicate this domain in bacterial clearance. TMEM59, a glycosylated transmembrane protein found predominantly on late endosomes and lysosomes, binds Atg16L1 and results in LC3 lipidation to endosomal and lysosomal membranes [202]. This process seems to be distinct from autophagy, as the signalling is independent of starvation and p62 levels are unchanged, furthermore electron microscopy confirms that LC3 is lipidated to non-autophagosome membranes [202]. TMEM59 co-localises with LAMP2, CD63 and transiently with EEA1, suggesting a conventional endocytosis pattern of events. TMEM59 is responsible for LC3 lipidation directly onto endocytic compartments, this is an example of an unconventional autophagy pathway [202]. Furthermore,
TMEM59 was shown to directly interact with the WD domain of Atg16L1, and using site directed mutagenesis, the important residues for this interaction were uncovered. This interaction occurs between a specific binding motif on the intracellular domain of TMEM59 \([YW]\)⁻\(X_3\)⁻\([ED]\)⁻\(X_4\)⁻\([YWF]\)⁻\(X_2\)⁻L and the WD domain of Atg16L1 [202]. Using this novel motif, a flexible prosite search was carried out to identify other proteins with this Atg16L1 binding motif. The flexible input was \([(YW)]\(-X_{(2,6)}\)⁻\([ED]\)⁻\(X_{(2,6)}\)⁻\([YWF]\)⁻\(X_2\)⁻L), where the square brackets are OR, and the round brackets represent the number of amino acids in between defined residues [202]. This search identified CARD1, a domain of NOD2, interestingly NOD1 lacked this motif. TLR2 also had this motif. These interactions were confirmed by co-IP. In line with the idea the WD domain of Atg16L1 could mediate bacterial clearance, when TMEM59 was knocked down, LC3 lipidation to *Staphylococcus aureus* containing vacuoles was decreased [202]. Other interactors that have not been previously implicated in autophagy included T3JAM and DEDD2 [202].

The Atg16L1 WD domain also binds NOD like receptor proteins. These are cytosolic proteins that bind pattern recognition receptors of invading bacteria [203, 204]. A signalling cascade is activated in response to this recognition. NODs have caspase activation and recruitment domains (CARD) and it is via this domain of NOD that the interaction with Atg16L1 occurs. Interestingly, NOD2 contains the same \([YW]\)⁻\(X_3\)⁻\([ED]\)⁻\(X_4\)⁻\([YWF]\)⁻\(X_2\)⁻L motif found in TMEM59, and also TLR2, indicating a conserved binding mechanism shared by several interactors [47, 22]. The role of NOD is to recruit Atg16L1 to the site of bacterial entry; for instance, this occurs in the case of *Shigella flexneri* [205]. However, NODs’ involvement in bacterial clearance is linked to selective autophagy, not LAP [205].

Whether TMEM59 mediated LC3 lipidation is due to non-canonical autophagy and a LAP-like phenomenon is unclear, but evidence suggests it is bafilomycin independent and PI3K independent, suggesting it may be another form of unconventional autophagy [202]. Therefore, a more extensive search needs to be done for novel Atg16L1 binding partners in the context of
endolysosomal LC3 lipidation in non-canonical autophagy. This could provide the mechanistic answers for the recruitment of Atg16L1 in non-canonical autophagy.

### 1.6.4 Crohn’s Disease

Atg16L1 has been extensively studied in the context of Crohn’s disease after a single nucleotide polymorphism (SNP), close to the WD domain of Atg16L1, was picked up in a genome wide screen of SNPs associated with the disease [206]. The nucleotide change from adenine to guanine causes a change in amino acid sequence, threonine to alanine at position 300 in Atg16L1 (T300A) in humans, T316A in murine models. There is a strong association between people with this Atg16L1 variant and their risk of developing Crohn’s disease, but the variant itself is not sufficient to cause the disease. There are other identified SNPs, including other variants of Atg16L1, Atg16L2 [207] and NOD2 [208] to name a few, therefore the mechanisms in which these variants contribute to Crohn’s disease are unclear. A variant of NOD2 is associated with early onset and severity of Crohn’s disease, NOD2 is involved in targeting Atg16L1 to sites of bacterial entry [208]. But the focus here is on the T300A Atg16L1 variant.

Crohn’s disease causes abnormal gut homeostasis and chronic inflammation and is one of the most common types of inflammatory bowel disease. The role of the Atg16L1 T300A variant is debated. In cells derived from a knock in mouse model of Atg16L1 T300A, selective autophagy is decreased and therefore anti-bacterial defence is compromised, increasing inflammatory cytokines such as IL-β [209], much like what is observed in Atg16L1 knockout cells [164]. *Shigella Flexneri* was used in this study due to its role in xenophagy, and infection with this bacteria in T300A Atg16L1 expressing MEFs has increased replication and IL-β production [209]. Furthermore, in this study there were morphological defects in Paneth and goblet cells in cells expressing the T300A variant suggestive of a role of Atg16L1 in gut homeostasis [209]. Furthermore, decreased bacterial handling has been observed in cells expressing the ATG16L1 T300A variant with consequent implications on the innate immune response, for instance lowered T cell response due to reduced antigen presentation [210] [211] [212]. This has been
observed in the case of *salmonella* [212] and uptake and processing of *E.coli* in Crohn’s disease patient dendritic cells [211]. How the Atg16L1 T300A variant mediates these pathways is unclear.

Recent evidence attributes the decline in selective autophagy to loss of functional Atg16L1, due to the T300A variant preceding a caspase 3 cleavage site (DxxD) on Atg16L1, amino acids 296-299 [209, 213]. The variant of Atg16L1 promotes caspase cleavage in response to stress signals such as metabolic stress, tumour necrosis factor alpha (TNFα) signalling or gut infection pathogens such as *Yersinia enterocollica* [213]. The cleavage *in vitro* was studied using TNFα or staurosporine induced apoptosis, that activates caspase 3 cleavage, and the T300A variant of Atg16L1 was significantly more susceptible to cleavage following these treatments; uncleaved Atg16L1 and 34 kDa and 36 kDa cleavage products could be observed by SDS PAGE. Increased cleavage of Atg16L1 T300A, results in less functional protein and lower levels of autophagy [209, 213]. Challenging the Atg16L1 T316A mouse model with *Y.enteroclica*, a physiologically relevant bacterial infection to study, results in an increase in inflammatory cytokine production compared to WT mice [213].

The observations of increased caspase cleavage of Atg16L1 T300A were followed up when looking at TMEM59 mediated autophagy [214]. As outlined previously TMEM59 can interact with the WD domain of Atg16L1 [202]. Therefore, stress mediated caspase-3 cleavage would result in less full length Atg16L1 to bind TMEM59, as the N-terminal region (1-299) gets uncoupled from the C-terminal region (300-607) [214]. In cells expressing the cleavage products, no TMEM59 mediated autophagy was observed [214]. Canonical autophagy, in basal and rapamycin induced conditions, was not affected by the uncoupling of the two domains [214], providing further evidence that the WD domain is not involved in canonical autophagy. Furthermore, this study showed that TMEM59 interacts less efficiently with Atg16L1 T300A compared to WT Atg16L1 in basal conditions, where no caspase 3 has been activated, potentially due to structural changes of the WD domain of Atg16L1 resulting from the polymorphism [214]. Therefore, the T300A variant disrupts TMEM59 mediated autophagy through probable structural changes, changing the
binding affinity of this protein to Atg16L1. The stress induced caspase cleavage, that is more prominent in Atg16L1 T300A, results in uncoupling the C-terminal domain of Atg16L1 and therefore WD domain related functions of Atg16L1 such as xenophagy cannot be carried out [214].

However, the story is not this simple, as reports also suggest that Atg16L1 T300A variant has protective functions against bacterial invasion, in the case of *Salmonella* [215], and Atg16 knockout mice are protected against *Citrobacter rodentium* infection [216].

It is to be noted that the crystal structure of the Atg16L1 WD domain does not characterise the T300A variant as being in the WD domain of Atg16L1 [192], however, it may still affect the function of the C-terminal contribution of Atg16L1.

Atg16L1 T300A has not been looked at extensively in the context of endolysosomal LC3 lipidation events. A paper recently came out suggesting that the Atg16L1 T316A murine variant for Crohn’s disease had negligible impact on LAP in mouse BMDC upon the addition of fluorescent zymozan [120]. This project also includes investigation into whether Atg16L1 T300A affects LC3 lipidation in non-canonical autophagy.

1.7 Aims of this study

Currently there is very little known about the mechanisms regulating non-canonical autophagy. This project aimed to increase our understanding of the signalling underpinning endolysosomal lipidation and the important physiological processes in which it occurs [111]. At present distinguishing autophagy from non-canonical autophagy at a genetic level is difficult due to the dependence on the same autophagy related proteins.
Therefore, this project aimed to determine which domains of Atg16L1 were required for LC3 lipidation in canonical versus non-canonical autophagy. The work quickly identified the WD domain of Atg16L1 as being dispensable for canonical autophagy but critical to non-canonical autophagy.

Therefore, the next aim of the project was to develop an in vivo mouse model of Atg16L1 ΔWD, a model that genetically can separate canonical and non-canonical autophagy. Where the long-term aim is to use this model to look at phenotypic consequences of specifically inhibiting LC3 lipidation to endolysosomal membranes.

Alongside the generation of the in vivo model, site directed mutagenesis studies aimed to further refine our model, and pin point key residues in the C-terminal WD domain of Atg16L1 that were important for LC3 lipidation to endolysosomal membranes. This then directed the generation of another in vivo model of Atg16L1 K490, that in the cellular system phenocopied the WD deletion of Atg16L1.

Whilst generating our own mouse models, we aimed to use an existing model of an Atg16L1 WD deletion, E230 from a collaborator, to show a functional consequence of the lack of LC3 lipidation to endolysosomal membranes.

The final aim was to use the characterised cellular system from this project, to do subtractive proteomics to look for binding partners of Atg16L1 in non-canonical autophagy.
**Figure 1.9: Summary of Atg16L1 recruitment and aims of project.**

On the left this depicts a growing double membrane autophagosome sequestering cytoplasmic material. The pre-initiation complex made up of Ulk1, Atg13, FIP200 alongside the PI3K complex made up of Beclin1, Vps34, Vps15 and Atg14 allow autophagy to be initiated and PI3P to enrich the autophagosome. This recruits PI3P effectors such as WIPI2b and the middle domain shown in red of Atg16L1 interacts directly with WIPI2b and FIP200. This position Atg16L1 in complex with Atg5 and Atg12 to help conjugate LC3 to the autophagosome membrane. The right hand picture shows that at single membrane endolysosome compartments following an engulfment event Atg16L1 in complex with Atg5 and Atg12 lipidates LC3 onto these membranes. The recruitment of Atg16L1 in this context is unknown and this is the main aim of this project.
2 Materials and Methods

2.1 Materials

All materials were purchased from Sigma, Corning or Thermo Fisher Scientific unless otherwise stated.

2.2 Cell culture

All cells were maintained in an incubator at 37°C with 5% CO\textsubscript{2}, in medium containing penicillin and streptomycin (10,000 Units/ml and 10,000 µg/µl, respectively; Gibco Life Technologies).

2.2.1 HCT116 (human colorectal carcinoma cells)

Cells were cultured in McCoy’s 5A with L-Glutamine (Lonza BE12-688F) medium with 10% Fetal Bovine Serum (FBS, Gibco Life Technologies F9665 lot 034M3398). Several derivatives of this line were used all from Dr David Boone: HCT116 WT GFP-LC3, HCT116 KO Atg16L1 GFP-LC3 and HCT116 Atg16L1 T300A GFP-LC3 cells [215]. Other clonal HCT116 GFP-LC3 cells were used, deficient for Atg16L1 and were engineered by Dr Rupert Beale’s lab using CRISPR/Cas9. These cells are expressing GFP-LC3B.

2.2.2 J774.A1 (mouse macrophage cell line), HEK293T (transformed Human Embryonic Kidney cells) and Mouse Embryonic Fibroblasts (MEFs)

Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco Life Technologies, 41966-029) containing 10% Fetal Bovine Serum (FBS). The following MEF derivatives were used: MEF KO Atg16L1 GFP-LC3, a cell line produced by traditional methods based on homologous recombination [197] and re-constituted with Atg16L1 constructs kindly provided by Dr Noor Gammoh [32] to give MEF FL ATG16L1 GFP-LC3, MEF ΔWD Atg16L1 GFP-LC3 and MEF ΔFBD Atg16L1 GFP-LC3. Atg16L1. These cells are expressing GFP-LC3B.
2.2.3 MCF10A cells (human mammary epithelial cells, female)

MCF10A were cultured in DMEM F12 (Gibco, 11320-074) containing 5% horse serum (Gibco, 16050-122) EGF (20 ng/ml, Peprotech), Hydrocortisone (0.5 mg/ml), cholera toxin (100 ng/ml) and insulin (10 µg/ml). These cells are expressing GFP-LC3A.

2.2.4 Mouse Embryonic Stem cells (ES cells)

Media, gelatin and trypsin were all kindly made and donated by Dr Dominik Spensberger and the cells were frozen and stored by the Babraham Institute Gene Targeting facility. The media consisted of knockout Dulbecco’s Modified Eagle’s Medium (KO-DMEM) (GIBCO, 10829-018), GlutaMAX/Glutamate 2mM (Invitogen, 35050061), 1x 2-mercaptoethanol (Sigma, M7522), 3 ml Penicillin/Streptomycin final 50 U/ml (Invitrogen, 15140-122), 300 µl LIF – from prepared stock 2000U/ml (Miltenyi Biotech), FBS final 15 %. Gelatin was made with 0.1 % w/v gelatin (Sigma 9391) in water or 1X PBS. Autoclaved to dissolve the gelatin and stored at 4 °C.

2.2.5 Stable cell line creation

Briefly, Atg16L1 constructs were used to virally transduce various knockout Atg16L1 cell lines. Atg16L1 constructs, with N-terminal Flag-S tags in the retroviral pBABE expression vector, were kindly donated by Dr Noor Gammoh (see tab 2.6 for information of DNA constructs).

Virus production and cell infections were performed by Oliver Florey in a CatII lab, as follows.

Retroviral preparation: HEK293T cells were seeded onto Poly-L-lysine coated plates. Triple transfections were performed in a tissue culture hood, using Lipofectamine 2000 (Invitrogen) and OptiMEM (Gibco) with the following amounts of DNA: 0.4 µg of expression retroviral vector, 0.2 µg VSV-G packaging proteins and 0.25 µg packaging Gag/Pol commercial retroviral enzymes. The method involved preparing two tubes, one with the DNA and 100 µl of OptiMEM and another with 100 µl of OptiMEM and 2.5 µl of Lipofectamine 2000. These tubes were incubated at room temperature for 5 min and then combined and mixed and incubated for a further 20 min. The cells were then washed with OptiMEM and 800 µl was added to each well before dropping on the
transfection mix. Cells were incubated for 6-16 h with the transfection mix and then the medium was replaced with growth medium for the collection of viral supernatant. Cell debris was removed by centrifugation x300 g for 5 min and virus was aliquotted and stored at -80 °C.

Cells to be virally infected were plated at 70 000 cells per well of a 6-well plate. Cells were incubated with viral supernatant and 8 µg/ml of polybrene, using a plate spinner to aid infection of the cells. The plates were spun at 22 °C at 1400 rpm for 45 min.

After 24-48 h, cells were selected with puromycin for the pBABE plasmid (HCT116 0.8 µg /ml, MEF 1.5 µg /ml, MCF10A 2.5 µg /ml) for 2-5 days. Protein expression was checked via western blot.

2.2.6 Transient Transfections

HCT116 cells were seeded at 200 000 cells per well onto a 6-well plate for 48 h. When cells were 70-90% confluent they were transfected in a tissue culture hood with 1 µg of DNA using Lipofectamine 2000 (Thermo Fisher Scientific) and OptiMEM (Gibco). For each well of a transfection, tubes were prepared where one tube contained 200 µl of OptiMEM and 1 µg of DNA to be combined with a tube of 190 µl of OptiMEM and 10 µl of Lipofectamine 2000 for 20 min at room temperature. Fresh media was put on the cells, 1.6 ml per well, and the transfection mix was added dropwise. 24 h later cells were re-seeded onto glass coverslips in a 12-well plate for imaging or onto a 6-well plate for western blotting.
2.3 Drugs and antibodies

2.3.1 Drugs

The following drugs were used in this project in order to study Atg16L1 in the context of canonical and non-canonical autophagy.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Function</th>
<th>Working concentration</th>
<th>Company</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>Ionophore, exchanging sodium and hydrogen ions. Blocks autophagic flux and induces endolysosomal LC3 lipidation.</td>
<td>100 µM (stock solution in methanol)</td>
<td>Sigma (M5273)</td>
<td>1 h</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>Inhibits V-ATPase activity. Blocks autophagic flux.</td>
<td>100 nM (stock solution in DMSO)</td>
<td>R&amp;D systems (1334)</td>
<td>1 h</td>
</tr>
<tr>
<td>PP242</td>
<td>Potent and selective inhibitor of ATP domain of mTOR. Activates autophagy.</td>
<td>1 µM (stock solution in DMSO)</td>
<td>Sigma (P0037)</td>
<td>1 h</td>
</tr>
<tr>
<td>Hank's Balanced Salt Solution HBSS</td>
<td>Serum and amino acid starvation.</td>
<td>Commercial</td>
<td>Sigma (H3934)</td>
<td>1 h</td>
</tr>
<tr>
<td>Starvation media from Dr Nicholas Ktistakis.</td>
<td>Serum and amino acid starvation.</td>
<td>140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 20 mM Hepes pH 7.4 + 1% BSA</td>
<td>[19]</td>
<td>1 h-2 h (depends on cell type).</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Potent and specific inhibitor of PI3K.</td>
<td>67 µM (DMSO)</td>
<td>Sigma (W1628)</td>
<td>1 h</td>
</tr>
</tbody>
</table>

Table 2.1: Details of pharmacological modulators of canonical and non-canonical autophagy.
### 2.3.2 Western Blot Antibodies

<table>
<thead>
<tr>
<th>Primary Antibody (animal, size, company, storage)</th>
<th>Dilution in Blocking buffer (5% BSA TBS-T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-LC3 A/B (rabbit, 14, 16 kDa, Cell Signalling #4108, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-GAPDH (rabbit, 37 kDa, Santa Cruz #25778, 4 °C)</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-ATG16L1 (rabbit, 68 kDa, Cell Signalling #8089, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-ATG5 recognises Atg5-12 complex (rabbit, 55 kDa, Cell Signalling #2630, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-ATG16L2 (rabbit, 68 kDa, Abcam #136093, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-Atg13 (rabbit, 72 kDa, Cell Signalling #13273, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-s tag (mouse, Novagen #71549-3, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-WIPI2 2A2 (mouse, 54 kDa, Biorad #MCA5780GA, 4 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-RB1CC1 (FIP200) (Rabbit, 200 kDa, Protein Tech #10043-2-AP, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>ATP6 V0d1 (V-ATPase subunit) (mouse, 37 kDa, Abcam #ab56441, 4 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-cop (From Dr Nickolas Ktistakis, mouse, dilution at) 4 °C</td>
<td>1:1000 + azide</td>
</tr>
<tr>
<td>Lamp1 (mouse, 110 kDa, BD pharmingen #611042, -20 °C)</td>
<td>1:500</td>
</tr>
<tr>
<td>PLEKHF1 (rabbit polyclonal, 31 kDa, Protein tech, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td><strong>Primary Antibody (animal, company, storage)</strong></td>
<td><strong>Dilution in TBS-TWEEN 20 with 3-5% milk (block and secondary also in TBS-TWEEN 20 with 3-5% milk)</strong></td>
</tr>
<tr>
<td>Anti-FLAG (mouse, Sigma #f1804, -20 °C)</td>
<td>1:1000 (3%)</td>
</tr>
<tr>
<td>Anti-cleaved caspase 3 (rabbit, 17, 19 kDa, #9661S Cell Signalling, -20 °C)</td>
<td>1:1000 (5%)</td>
</tr>
<tr>
<td>B-1 Integrin (rabbit, 115,135 kDa, Giancotti lab MSKCC Cyto SM158)</td>
<td>1:2500 (5%)</td>
</tr>
</tbody>
</table>
### Table 2.2: Antibodies for detection of specific proteins by western blot.

<table>
<thead>
<tr>
<th>Secondary Antibody (animal, company, storage)</th>
<th>Dilution in Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish Peroxidase (HRP)-conjugated anti-mouse or anti rabbit-antibodies (Cell Signalling #7076 #7074, -20 °C)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-streptavidin HRP (Cell Signalling #3999, -20 °C)</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

2.3.3 Immunofluorescence Antibodies

<table>
<thead>
<tr>
<th>Primary Antibody (animal, company, storage)</th>
<th>Fixation and dilution in Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ATG16L1 (rabbit, Cell Signalling #8089, -20 °C)</td>
<td>3.7% PFA, 1:100</td>
</tr>
<tr>
<td>Anti-ATG16L1 (rabbit, MBL #PM040, -20 °C) preferable</td>
<td>3.7% PFA 1:100</td>
</tr>
<tr>
<td>LAMP1 (human CD107a) (Mouse, BD Pharmingen #555798, 4 °C)</td>
<td>MetOH, 1:100</td>
</tr>
<tr>
<td>LAMP1 (mouse CD107a) (Rat, BD Pharmingen #555792, 4 °C)</td>
<td>MetOH, 1:100</td>
</tr>
<tr>
<td>Anti-LC3 A/B (rabbit, Cell Signalling #4108, -20 °C)</td>
<td>MetOH, 1:100</td>
</tr>
<tr>
<td>Anti-s tag (mouse, Novagen #71549-3, -20 °C)</td>
<td>3.7% PFA, 1:200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibody (animal, company, storage)</th>
<th>Dilution in Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa fluor 568 (goat anti mouse, Life Technologies, 4 °C)</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa fluor 568 (goat anti rabbit, Life Technologies, 4 °C)</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa fluor 568 (goat anti-rat, Life Technologies, 4 °C)</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa fluor 488 (goat anti rabbit, Life Technologies, 4 °C)</td>
<td>1:500</td>
</tr>
</tbody>
</table>

### Table 2.3: Antibodies for detection of specific proteins by immunofluorescence.

2.4 Molecular Biology

2.4.1 Polymerase chain reaction (PCR)

Different polymerases were used depending on the PCR. For genotyping and diagnostics MyTaq red mix (Bioline) was used. For generating plasmids or fragments, to be sequenced and used for future work, a higher fidelity DNA polymerase was used, Phusion (Thermo Fisher Scientific).

PCR was also performed from bacterial colonies for diagnostic work, to directly check for the presence of specific DNA. In order to do PCR from bacterial colonies, the colony was picked with an inoculating loop and re-streaked onto a new agar plate to store for future work. The remaining bacteria on the loop was put into 50 µl of water and boiled for 5 min to lyse the bacterial cells. A
PCR was set up with 2 µl of this acting as the DNA template. PCR was done using specific primers, detailed in subsequent methods, to amplify specific DNA, where a negative control of just water was used and a positive control was used where possible. PCR products were purified as per the manufacturer’s instructions with the GeneJET kit from Thermo Fisher Scientific, eluting in 20 µl of water. TABLE 2.4 summarises the different reaction set ups used for PCR and TABLE 2.5 summarises the reaction conditions, where the temperature cycles were achieved using an Eppendorf MasterCycler.

<table>
<thead>
<tr>
<th>Phusion High Fidelity (HF) PCR reaction</th>
<th>Volumes for 1x reaction</th>
<th>MyTaq Red Mix PCR</th>
<th>Volumes for 1x reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Phusion HF buffer</td>
<td>4 µl</td>
<td>MyTaq red mix: loading dye, buffer, dNTPs, polymerase</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>10 mM dNTPs (Invitrogen)</td>
<td>0.4 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 µM Forward primer</td>
<td>1 µl</td>
<td>10 µM Forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 µM Reverse primer</td>
<td>1 µl</td>
<td>10 µM Reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Phusion Taq Polymerase</td>
<td>0.2 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Template DNA 100 ng</td>
<td>100 ng</td>
<td>Template DNA 100 ng</td>
<td>100 ng</td>
</tr>
<tr>
<td>H₂O</td>
<td>Make up to volume</td>
<td>H₂O</td>
<td>Make up to volume</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Table 2.4: PCR reaction reagents and quantities.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>X35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>94 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denature</td>
<td>94 °C</td>
<td>20 s</td>
</tr>
<tr>
<td>Anneal</td>
<td>58 °C</td>
<td>20 s</td>
</tr>
<tr>
<td>Extend</td>
<td>72 °C</td>
<td>20 s</td>
</tr>
<tr>
<td>Extend</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>Infinity</td>
</tr>
</tbody>
</table>

Table 2.5: Thermocycler programme for PCR.

2.4.2 Agarose gel electrophoresis

In order to separate DNA by size agarose gels were used. 1-2% agarose was prepared in 1xTBE buffer, depending on the size of the DNA fragments/vectors. This mixture was boiled, then cooled for 10 min, before adding ethidium bromide at 0.5 µg/ml (Bio-Rad) or 1:10 000 SYBR safe (Invitrogen), both allow for visualisation of the DNA. The DNA samples were then prepared with sample buffer (Thermo Scientific R0611) and either a 100 bp or 1 kbp (GenRuler Plus, Thermo
Scientific) ladder. The agarose gel was run at 75 V for varying times and then the DNA was visualised under UV light using a BioRad gel dock.

2.4.3 Restriction digests and ligations

Restriction digests were set up as per the manufacturer’s instructions (NEB) and following the online tools, NEBcutter, to help choose appropriate restriction enzymes, and NEBcloner, for protocol information. Various restriction enzymes were used in this project; specifics are detailed where appropriate, with digests incubated for at least 30 min at 37°C. Linearised plasmids were treated with a calf intestinal phosphatase (CIP) if the restriction enzyme left compatible ends, to de-phosphorylate the 5’ ends to stop the vector re-circularising. This was done by heat inactivating the restriction enzyme at 65 °C for 20 min, adding the CIP at 1 Unit per pmol of DNA ends for 1 h at 37°C and then inactivating the enzyme at 65°C for 20 min. Restriction digests were run on agarose gels to check the expected DNA band pattern, and then gel purified (gel extraction kit from Qiagen as per manufacturer’s instructions). DNA was eluted in 20 µl of water and quantified to set up ligation reactions. Ligations were done using the manufacturer’s instructions using T4 DNA ligase (NEB) and the NEBiocalculator. 100 ng of vector was used with various ratios of insert for standard ligations, 1:1, 1:3 and 1:10.

2.4.4 Transformation

Plasmid DNA was transformed into chemically competent commercial bacteria using heat shock. Either One Shot Stbl3 Chemically Competent *E.coli* (ThermoFisher Scientific), or DH5α *E.coli* High efficiency (NEB #C29871), were used. 20 µl of Stbl3 or 50 µl of the DH5α were thawed on ice and added to pre-chilled 40 ml round bottom falcons (BD). 1 µl- 2 µl of (~2 ng vector) was added to the bacteria and left on ice for 30 min. Heat shock was performed in a water bath at 42 °C for 30-40 s. The tubes were then added to ice for 2 min and then 1 ml of room temperature super optimal broth SOC (NEB) or lysogeny broth (LB) medium was added and the tubes were shaken (Infors HT multitron standard) at 37 °C for 1 h at 225 rpm. The bacteria were then spun down in a table top centrifuge at 2000 rpm for 2 min and 800 µl of supernatant was removed and the
pelleted bacteria were re-suspended in the remaining media and plated onto 100 µg/ml ampicillin or kanamycin (Melford #A0104) agar plates and incubated for 16 h at 37 °C. Controls included a positive control (2 ng/µl of an existing plasmid), and re-ligated vector with and without phosphatase treatment.

2.4.5 DNA extraction and purification from bacterial cultures

Bacterial colonies were picked and re-streaked onto antibiotic agar plates in order to save for future use. In parallel, the picked colony was grown up in 3 ml of LB, containing the relevant antibiotic, at 37°C shaking for 16 h. The DNA was then extracted using a Qiagen miniprep kit as per the manufacturer’s instructions, eluting the DNA in 50 µl of water. The extracted DNA was then either tested via restriction digest for successful plasmid production or sent for Sanger sequencing using Cogenics Lark. Bacterial colonies that had the desired plasmid were then grown up in 100 ml of LB (volume for high copy plasmids) containing the relevant antibiotic at 37°C shaking for 16 h. The DNA was extracted using a Qiagen maxiprep kit as per the manufacturer’s instructions, eluting the DNA in 250 µl of water. All plasmids were sequenced using Sanger sequencing (Cogenics Lark) and the DNA quality and quantity was check using a Nanodrop 2000 (Thermo Fisher Scientific).

2.4.6 DNA extraction from cells or tissue

ES cells in a 96 well format were taken to extract the DNA. The plate was spun at 2000 rpm for 10 min at RT. The media was flicked out and 30 µl of water was added to each well and then incubated for 95°C for 10 min. 10 µl of proteinase K (final concentration 100 µg/ml) was added to each well and incubated for 1 h at 56°C, then 95°C for 10 min. This plate was stored at 4°C.

For genotyping, ear clips were taken from mice at 10 days old, this was done by the Biological Support Unit (BSU) at the Babraham Institute. DNA was extracted from this tissue using 200 µl of lysis buffer: 100 mM TRIS-HCL (pH=8.0), 5 mM EDTA, 0.2% SDS, 200 mM NaCl with the addition of 100 µg/ml proteinase K before use. This was incubated at 55°C shaking for 1 h at 1000 rpm. 200 µl of isopropanol was added and inverted, to precipitate the DNA, and pelleted by centrifugation for
5 min at 13000 rpm. The aqueous phase was removed and the pellet was washed with 70% ethanol, then air dried and finally re-suspended in 50 µl of water. This could then be used as template DNA for PCR.

### Constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Antibiotic resistance</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBABE F-S-Atg16L1</td>
<td>AMP</td>
<td>Mouse Atg16L1 aa 1-623 (Dr Noor Gammoh) Flag-S tag N-terminus</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 ΔFBD</td>
<td>AMP</td>
<td>Mouse Atg16L1 aa Δ229-242 (Dr Noor Gammoh) Flag-S tag N-terminus</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 ΔWD</td>
<td>AMP</td>
<td>Mouse Atg16L1 aa 1-335 (Dr Noor Gammoh) Flag-S tag N-terminus</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 ΔN</td>
<td>AMP</td>
<td>Mouse Atg16L1 aa 80-623, made as part of this project. Atg16L1 ΔN: Subcloned by using a) restriction sites XhoI and Sall to cut pBABE F-S-Atg16L1 vector and b) PCR to generate the Atg16L1 ΔN sequence, introducing Sall restriction sites, this gives compatible ends with XhoI cut sites. The primers used to do this were: Fwd: 5′-3′ GCAGCAGTCGACATGAGTCAACTACAAGAAATGGCCCAG and Rev 5′-3′ CTTAAGTCGACTCAAGGCTGTGCCCACAGCAC</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 Just the WD domain</td>
<td>AMP</td>
<td>Mouse Atg16L1 aa 336-623 (Dr Noor Gammoh) Flag-S tag N-terminus.</td>
</tr>
<tr>
<td>pcDNA3.1 Atg16L1 1-230 ΔWD</td>
<td>AMP</td>
<td>Dr Sharon Tooze</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 E324A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 N326A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 M342A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 N386A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 K410A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 L412A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 H428A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 N453A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 F467A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 K490A</td>
<td>AMP</td>
<td>Site directed mutagenesis from pBABE F-S-Atg16L1</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 D536A</td>
<td>AMP Site directed mutagenesis from pBABE F-S-Atg16L1.</td>
<td></td>
</tr>
<tr>
<td>pBABE F-S-Atg16L1 N581A</td>
<td>AMP Site directed mutagenesis from pBABE F-S-Atg16L1.</td>
<td></td>
</tr>
<tr>
<td>pEGFP-C1 CRISPR repair complex ΔWD Atg16L1</td>
<td>KAN See further information in text.</td>
<td></td>
</tr>
<tr>
<td>pSpCas9(BB)-2A-GFP Guide 2 Atg16L1 for Atg16L1 ΔWD</td>
<td>AMP Made as part of this project to target mouse Atg16L1 to produce Atg16L1 ΔWD. See details in text.</td>
<td></td>
</tr>
<tr>
<td>pQCXIN-BirA-Myc Atg16L1 FL</td>
<td>AMP Mouse Atg16L1 aa 1-623 (Dr Noor Gammoh) BirA-Myc C-terminal</td>
<td></td>
</tr>
<tr>
<td>pQCXIN-BirA-Myc Atg16L1 ΔWD</td>
<td>AMP Mouse Atg16L1 aa 1-335, BirA-Myc C-terminal, made as part of this project</td>
<td></td>
</tr>
<tr>
<td>pQCXIN-BirA-Myc Atg16L1 ΔWD Atg16L1 K490A</td>
<td>AMP Mouse Atg16L1 K490A, BirA-Myc C-terminal, made as part of this project</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6: Plasmids

2.4.8 Sequencing Primers

<table>
<thead>
<tr>
<th>Constructs/Plasmids</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>BirA Atg16L1 constructs</td>
<td>5’-3’ NotI Fwd GCACGGGAGCCGCATGTCGTCGGGCCTGCGCGC</td>
</tr>
<tr>
<td>SgRNA plasmid pSpCas9(BB)-2A-GFP</td>
<td>U6 fwd primer, bottom strand of ordered sgRNA used as a reverse primer.</td>
</tr>
<tr>
<td>pcDNA3.1 Plasmids</td>
<td>CMV fwd CGCAAATGGGCGGTAGGCGTG and BGH rev TAGAAGGCACAGTCGAGG</td>
</tr>
<tr>
<td>pBABE plasmids</td>
<td>pBABE forward and reverse supplied primers by Cogenics Lark.</td>
</tr>
<tr>
<td>pEGFP-C1 CRISPR repair complex ΔWD Atg16L1</td>
<td>EGFP-c fwd (Cogenics Lark supplied) CATGTTCTGCTGGAGTTCGTG and Genotyping ΔWD fwd: TGGAGGTTCTAGAAAAGACAAC</td>
</tr>
</tbody>
</table>

Table 2.7 Primers to confirm sequence of plasmids.

2.5 Site-directed mutagenesis

Primers were designed to introduce single amino acid changes to alanine for different residues of Atg16L1. The primers were re-constituted in water to 100 ng/µl, see TABLE 2.8 for the list of primers. The method for the mutagenesis was based on the QuikChange Site-directed Mutagenesis Kit (Stratagene). This involved a 50 µl reaction consisting of: 1 x Pf u UltraII buffer (Agilent Technologies), 0.2 mM dNTPs (Invitrogen), 10 % DMSO (Sigma), 1 µl Pf uUltra II (Agilent...
Technologies), 250 ng of forward primer and 250 ng of reverse primer, 50 ng of Flag-S Atg16L1 p-BABE DNA template made up to the total volume with water. A negative control consisted of a reaction without the primers, that resulted in just the template DNA. A thermocycler was used to create the mutation and amplify the DNA. The thermocycler programme was set at 95 °C for 2 min, 95 °C 30 s, 55°C for 1 min, 68 °C for 10 min and this was cycled 16 times.

<table>
<thead>
<tr>
<th>Position of Atg16L1 for alanine substitution</th>
<th>Primers 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>E324A For</td>
<td>CGCATGACGGAGCGGTCAACGCAGTG</td>
</tr>
<tr>
<td>E324A Rev</td>
<td>CACTGCGGTGACCGCTCGTCATGCG</td>
</tr>
<tr>
<td>N326A For</td>
<td>CATGACGGAGAGGTCGGCGCAATGACTGCA</td>
</tr>
<tr>
<td>N326A Rev</td>
<td>CTGAACCTGCACGCGCGACCTTCCGTATG</td>
</tr>
<tr>
<td>M342A For</td>
<td>GCCACCGAGCGGCAGCCAGGGGTG</td>
</tr>
<tr>
<td>M342A Rev</td>
<td>CACCCCTCGGTCCGCGTCATGAC</td>
</tr>
<tr>
<td>N386A For</td>
<td>CTTACCTATAGCAGCTCAGCTGATTTTGCAAGCCGGAATC</td>
</tr>
<tr>
<td>N386A Rev</td>
<td>GATTCGGGCTGACAAATCAGGTAGGCTAATAGGTAAG</td>
</tr>
<tr>
<td>K410A For</td>
<td>GGCCACACGCAGCGCGACCTCCCTCTG</td>
</tr>
<tr>
<td>K410A Rev</td>
<td>GGCAGGAGAGGCGCCCTCCTCCTG</td>
</tr>
<tr>
<td>L412A For</td>
<td>CACAGCGGGAAGCGGCTCGTCAGCCAGAG</td>
</tr>
<tr>
<td>L412A Rev</td>
<td>GGAACCTGACGAGCGGACTTCCCTCCG</td>
</tr>
<tr>
<td>H428A For</td>
<td>GATTTGCTCAGGAGAGGTCCGACGGACCTC</td>
</tr>
<tr>
<td>H428A Rev</td>
<td>CTTGTTAGGTTGCTCGGACGGCAACTTCTAG</td>
</tr>
<tr>
<td>N453A For</td>
<td>GCAGTATACGCTGCCTGACATGTTGTAAC</td>
</tr>
<tr>
<td>N453A Rev</td>
<td>GTGCAAAACTAGTGCAAGCGAGCTGAGT</td>
</tr>
<tr>
<td>F467A For</td>
<td>GTGTAATGAGTGGACATGCTGACAAGAAATCTCCTG</td>
</tr>
<tr>
<td>F467A Rev</td>
<td>CAGAAACGTATTTTCTTTGTCACATGTCATCCTCATAC</td>
</tr>
<tr>
<td>K490A For</td>
<td>GATGAACGTATGAGGCGGAGTACGCTGCAG</td>
</tr>
<tr>
<td>K490A Rev</td>
<td>GTTCCAGAGCAGTAGTGACCGGCCTACGCTCAT</td>
</tr>
<tr>
<td>D536A For</td>
<td>CAAATGCCGCTCTGAGCAGCCCGGTTG</td>
</tr>
<tr>
<td>D536A Rev</td>
<td>CACCCCGGATCCAGGGAGCGGCGCTTACG</td>
</tr>
<tr>
<td>N581A For</td>
<td>CAGCTCTCTATCGCTCAGTGGGCTGGG</td>
</tr>
<tr>
<td>N581A Rev</td>
<td>CCCAGGCCACGAGAGGAGAGGAGC</td>
</tr>
</tbody>
</table>

*Table 2.8: Primers for site directed mutagenesis of Atg16L1*
The product was digested using 1 µl DpnI 10 U/ul (Promega) 1 h at 37 °C. This digested methylated DNA and therefore destroyed the starting template DNA, leaving just the new mutated plasmid. The negative control, where no primers were added, acted as a control for the DpnI digestion; it should have digested all of the template DNA and no colonies should grow on antibiotic agar plates. 2 µl of DpnI treated DNA was transformed into high efficiency DH5α E.coli and the DNA was extracted and sequenced to confirm the mutation.

2.6 CRISPR

2.6.1 CRISPR to generate a cell line.

A HEK 293 knock out Atg13 GFP-LC3 cell line was created by Dr Elise Jacquin, a previous member of the Florey lab. This cell line was used to produce a double knockout line by also editing Atg16L1. An existing sgRNA plasmid to target Atg16L1 for knock out was used as follows; this was produced and validated by Celine Judon and Dr Elise Jacquin: 5’ CACCGGTCAGATCTTCATTCAGTGT 3’ pSpCas9 (BB)-2A-GFP plasmid. HEK ATG13 KO cells were plated on a 6-well plate and co-transfected with the guide Atg16L1 plasmid and a puromycin plasmid in order to select for the transfected cells. The transfection used Lipofectamine 2000 as previously described (Transient transfections), using 1 µg in total of DNA (where the plasmids were at a 50:50 ratio). 24 h post transfection fresh media was added containing 2 µg/ml of puromycin, a control of untransfected cells was also used. Selection with puromycin was achieved in 48 h and a proportion of cells were frozen down. The transfected population of cells was checked by western blot for knockdown of Atg16L1 in comparison to WT cells. A limiting dilution of the transfected population was performed in order to get one cell per well of a 96 well plate. Four 96 well plates were prepared, with a more concentrated cell dilution in one well to help focus the microscope. After ~ 10 days, single cells started grow in well of the 96 well plate, cells were grown and propagated until clonal populations could be tested for Atg16L1 knockout via western blot. Some were tested by imaging the GFP-LC3 response to chloroquine. Among the successful double knock out cells, clone 3C11
was used subsequently to produce stable HEK Atg13 KO Atg16 KO cells re-expressing the different Atg16L1 constructs.

2.6.2 In vivo CRISPR reagents

Chapter 4 outlines the theory and method for how CRISPR/Cas9 technology was used to generate two mouse models please refer to this section for more information.

2.6.2.1 Guide sequences: design and cloning

Two sgRNA sequences to target mouse Atg16L1 were designed using the online software DNA 2.0. The sgRNA were resuspended at 100 µM in water. These guide sequences were cloned into an expression plasmid with Cas9. This is important to direct the Cas9 to the desired part of the genome to produce a double strand break.

<table>
<thead>
<tr>
<th>sgRNA Top oligo 5’-3’</th>
<th>sgRNA bottom oligo 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence 1 (Atg16L1 ΔWD):</td>
<td>Sequence 1 (Atg16L1 ΔWD):</td>
</tr>
<tr>
<td>CACCGATGGACACTCATCCTCTTC</td>
<td>AAACGAAGCAGGATGAGTGTCCATC</td>
</tr>
<tr>
<td>Sequence 2 (Atg16L1 ΔWD):</td>
<td>Sequence 2 (Atg16L1 ΔWD):</td>
</tr>
<tr>
<td>CACCGAGACATACGAGGCAGTAGTT</td>
<td>AAACAACTACTGCTCGTATGTCTC</td>
</tr>
<tr>
<td>Ordered synthetic guide sequence for K490A</td>
<td>Ordered synthetic guide sequence for K490A</td>
</tr>
<tr>
<td>GUUAGGGGCCAUCACGGCUCGUUUUAGAGCUAUGCUGUUUUG (20 nmol)</td>
<td>GUUAGGGGCCAUCACGGCUCGUUUUAGAGCUAUGCUGUUUUG (20 nmol)</td>
</tr>
</tbody>
</table>

**Table 2.9:** Guide oligos to clone into a Cas9 vector to form the sgRNA for Atg16L1 ΔWD targeting and synthetic single stranded guide sequence and tracrRNA for Atg16L1 K490A targeting.

The oligos were annealed together by setting up the following reaction: SgRNA top and bottom 1 µl each, T4 DNA ligation buffer 10x 1 µl (NEB), T4 Polynucleotide kinase (PNK) 1 µl (NEB), this catalyses the transfer and exchange of a phosphate from the gamma position of ATP onto the 5’ hydroxyl terminus of the nucleotide and also removes the 3’ phosphate. The volume was made up to 10 µl with water. This reaction was then put in a thermocycler set to 37°C for 30 min, 95°C for 5 min, and the temperature was ramped down 5°C per min to 25°C. This reaction was then diluted 1 in 200 with water.
A single reaction was set up to digest 100 ng of plasmid pSpCas9(BB)-2A-GFP (PX458 from Dr Dominik Spensberger) and 2 µl of the diluted sgRNA were ligated into this linearised vector. The reaction also had 2 µl of 10 x Tango Buffer (Thermo Fisher Scientific), 1 µl of 10 mM DTT (Melford Biolaboratories), 1 µL 10Mm ATP (NEB), 0.5 µL T4 DNA ligase (NEB), 1 µL BbsI high fidelity (aka Bpil Thermo Fisher Scientific), made up to a total volume of 20 µl with water. A thermocycler was used to do six cycles of 37°C for 5 min and 21°C for 5 min.

The resulting product, the Cas9 plasmid also containing the guide sequence, was transformed into Stbl3 E.coli and the bacterial colonies were screened by PCR, using the U6 forward primer (that will bind to the promoter region just before the sgRNA) and the sgRNA bottom oligo as a reverse primer. The PCR product was checked on an agarose gel. The successful bacterial colonies were grown up and the DNA was extracted and sequenced.

2.6.2.2 Surveyor assay: to test the guide efficiency

A surveyor assay is a way to assess how efficiently and accurately the guide sequence navigates the Cas9 to the DNA region of interest, to then form a double strand break. This is then repaired by the error prone method of NHEJ in cells and leads to the production of mismatches. A specific nuclease can be used to cleave the sites where a mismatch has occurred. This can be visualised by size separation of DNA fragments on an agarose gel to visualise the untargeted DNA versus the targeted Cas9 cleaved DNA. FIGURE 4.6 and Chapter 4 have more information.

Primers were designed to amplify regions of Atg16L1 around the site that was targeted for editing. It was important that the primers gave a clean single PCR product, and that when targeted by Cas9 the cleaved products were not of equal size. The following primers were designed and tested for this assay.
### Table 2.10: Surveyor assay primers.

<table>
<thead>
<tr>
<th>Primer Forward 5′-3′</th>
<th>Primer Reverse 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplify region for Atg16L1 ΔWD edit TGAGACTGCTTCAGTGCTGG</td>
<td>Amplify region for Atg16L1 ΔWD edit TGCTCCAGAGGATGTCCCTA</td>
</tr>
<tr>
<td>Amplify region for Atg16L1 K490A edit TGCTTAGTTCCCCAAGAACA</td>
<td>Amplify region for Atg16L1 K490A edit GTTAGCAGCCGCTACAGTG</td>
</tr>
</tbody>
</table>

The efficiency of the guides to target Cas9 to the specific site on the DNA for cleavage was tested by transfecting the sgRNA into ES cells. In the case of the Atg16L1 ΔWD targeting, 2 µg of sgRNA plasmid was added to 50 µl of serum free media. Another tube with 8 µl of Lipofectamine 2000 in 50 µl of serum free media was prepared and left for 5 min. The tubes were then combined and incubated at RT for 20 min. During this time a 6-well plate was coated with gelatin and full ES media was pre-equilibrated. One million ES cells were re-suspended in 100 µl of full media and were added to the transfection mixture dropwise over two wells. Cells were fed 24 h later and 48 h after transfection. The cells were sorted for the expression of GFP by flow cytometry. Trypsinised and filtered cells were taken on ice to the Flow Cytometry facility at the Babraham Institute and 100 000 GFP positive, transfected cells were saved to extract DNA with the Blood extraction kit (Qiagen) as per the manufacturer’s instructions.

For the Atg16L1 K490A targeting, Dr Dominik Spensberger transfected ES cells with crRNA for ATG16L1 K490A, tracrRNA and Cas9. Cells were lysed and DNA was extracted. From this point on the samples were treated the same.

A PCR was performed, to amplify the region around the edit site, and the PCR product was then purified. A hybridisation was then set up with 300 ng of DNA in total, where 150 ng WT PCR product was hybridised with 150 ng of Atg16L1 ΔWD PCR product or K490A. This reaction was made up to 20 µl in water with 2 µl of NEB buffer 2. The DNA was left to hybridise in a thermocycler set to 37°C for 30 min, 95°C for 5 min, and the temperature was ramped down 5°C per min until it reached 25°C. Then, 1 µl of T7 endonuclease (NEB), the nuclease that cleaves
mismatched DNA, was added to this reaction and left at 37 °C for 20 min. The product was run on a 1.5% agarose gel to detect indel formation. Expected DNA band sizes are summarised in the table below.

<table>
<thead>
<tr>
<th></th>
<th>Atg16L1 ΔWD surveyor</th>
<th>Atg16L1 K490A surveyor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR:</td>
<td>549 bp</td>
<td>608 bp</td>
</tr>
<tr>
<td>Cleaved DNA Cas9</td>
<td>334 bp, 215 bp</td>
<td>431 bp, 177 bp</td>
</tr>
</tbody>
</table>

Table 2.11: Surveyor assay expected size in base pairs of DNA fragments.

2.6.2.3 Homologous directed repair templates

Once the efficiency and precision of the guide sequence had been tested, a repair template needed to be designed to allow for homology directed repair (HDR). This allowed for the double strand break at the targeted region of DNA to be repaired in a controlled manner to introduce an ‘edit’.

The repair complexes were ordered as PAGE purified ultramer ssDNA. See Chapter 4 for details of the repair template sequences.

The final Atg16L1 ΔWD repair template was made in a slightly different way, in order to add an SV40 termination sequence to enhance stability of the edited mRNA. A PCR was used to generate two flanking homology sequences complementary to the targeted DNA. Then an additional PCR was done to amplify an SV40 termination sequence. Please refer to TABLE 2.12, for details of the primers. Primers were designed using NEBuilder Assembly tool v1.12.12. The PCR products were purified and a pEGFP-C1 vector was linearised with EcoR1 (NEB) and treated with CIP phosphatase (NEB). These components were then taken to do a complex ligation using Gibson Assembly mix (NEB), as per the manufacturer’s instructions using a linearised vector to PCR product ratio of 1:2:2:2. The negative control was linearised vector without inserts. These reactions were transformed using DH5α high efficiency *E.coli* and colonies were screened via PCR, using the genotyping primers for Atg16L1 ΔWD, (TABLE 2.13), where a positive colony gave a PCR
product of 586 bp and a negative colony gave no PCR product. Colonies were grown, and DNA was extracted and quantified and confirmed by Sanger sequencing (See table of sequencing primers, TABLE 2.7).

<table>
<thead>
<tr>
<th>Primer name and description</th>
<th>Primer sequence 5’-3’</th>
<th>Table 2.12: Primers in order to make HDR template for creation of Atg16L1 ΔWD.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fwd 1 Left Homology arm Atg16L1</td>
<td>ACTCAGATCTCGAGCTCAAGCTCGTCTTGATGCTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>Rev 1 Left Homology arm Atg16L1</td>
<td>CAGTTATCTAGATCTACTAGAGCAATACGAGGAGTTGG</td>
<td></td>
</tr>
<tr>
<td>Fwd 2 SV40 termination sequence</td>
<td>TGCTCTCTAGATCTACTAGATACTGATGCTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>Rev 2 SV40 termination sequence</td>
<td>AAGGCTACTTACTAAAGATACATAGATGCTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>Fwd 3 Right homology arm Atg16L1</td>
<td>TCACTCTATGATGCTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>Rev 3 Right homology arm Atg16L1</td>
<td>GGGCGCGGTACCAGTCCGACTGAGGCAGGAAGTATGTC</td>
<td></td>
</tr>
</tbody>
</table>

2.6.3 Screening and genotyping CRISPR targeted cells

See chapter 4 for full details on screening ES cell colonies for edited DNA, and for genotyping strategies. DNA was extracted from cells or ear clips from mice as previously described. PCR was performed with MyTaq red polymerase with the previously described method. TABLE 2.13 shows the primers used in this part of the project with a summary of their use and the expected PCR product sizes. Where relevant, restriction digests were also used to see if the CRISPR Cas9 targeting had worked, Bfa1 (NEB) and Bcl1 (NEB) were used as advised by the manufacturer and details are summarised in TABLE 2.13. Genotyping part way through the project, after the embryo transfers, was outsourced to Transnetyx.

<table>
<thead>
<tr>
<th>Primer name/description or Restriction enzyme</th>
<th>Sequence 5’-3- or restriction site</th>
<th>Expected PCR product sizes, base pairs (bp) and restriction digest where relevant.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotyping primers Atg16L1 Δ WD common to all protocol design.</td>
<td>Fwd TGGAGGGTCTAGAAAGACAAC Rev AGTCAGCAATGGAAAGCCTAG</td>
<td>WT template 344 bp Edited DNA template 362 bp, Edited DNA template SV40 method: 590 bp</td>
</tr>
<tr>
<td>Bfa1 digest of genotyping PCR product (first Atg16L1 ΔWD strategy, polyA repair)</td>
<td>TCTA</td>
<td>WT: Bfa1 product 315 bp Edited DNA: Bfa1 232 bp, 101 bp</td>
</tr>
<tr>
<td>Screening primers Atg16L1 ΔWD for triple stop codon repair.</td>
<td>Fwd TGGAGGGTCTAGAAAGACAAC Rev CAAAGCATACTAATTCATTATA</td>
<td>WT template: no PCR product Edited Template: 255 bp</td>
</tr>
<tr>
<td>Bcl1 digest of genotyping PCR product (Atg16L1 ΔWD strategy, triple stop codon repair)</td>
<td>TGATCA</td>
<td>WT: 344 bp (no restriction site) Edited DNA: 239 bp and 105 bp</td>
</tr>
<tr>
<td>Screening primers Atg16L1 ΔWD for SV40 repair.</td>
<td>Fwd TGGAGGGTCTAGAAAGACAAC Rev AAATTTGTGATGCTATTGC</td>
<td>WT template: no PCR product Edited Template: 801 bp</td>
</tr>
<tr>
<td>Genotyping primers for Atg16L1 K490A</td>
<td>Fwd TGCTTAGTTCCCCAAGAACA Rev GTAGCAGCGGCGTACAGTG</td>
<td>WT template: 608 bp Edited template: 608 bp</td>
</tr>
</tbody>
</table>

**Table 2.13: Summary of primers and restriction enzymes for screening and genotyping.**

2.7 Western blotting

2.7.1 Sample preparation and protein quantification

HCT116 cells were seeded at 150,000 cells per well of a 6-well plate. 48 h later cells were subjected to experimental treatment and lysed. MEF or MCF10A cells were seeded at 150,000 cells per well of a 6-well plate for 24 h and then treated and lysed. Cells were lysed on ice, using Radio Immunoprecipitation Assay (RIPA) buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % sodium deoxycholine, 1 % Triton, 0.1 % SDS, protease/phosphatase inhibitor cocktails were added 1:200, 1:100 respectively (ready to use protease and phosphatase inhibitors Sigma P8340 and P0044). Protein quantification assays were performed using a spectrophotometric BCA assay, (ThermoFisher Scientific) as per manufacturer’s instructions. Lysates containing 15 µg of protein were mixed with 1 x sample buffer diluted in RIPA buffer (5 x Sample Buffer: 0.2 M Tris-HCl pH 6.8, 10 % SDS w/v, 20 % glycerol v/v, 10 mM β-mercaptoethanol, 0.05 % Bromophenol Blue w/v, H₂O) and boiled at 100 ºC for 5 min.

2.7.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Prepared samples were loaded onto hand-cast acrylamide gels (Biorad system). Different percentage resolving gels were needed depending on the size of the proteins being separated for detection, TABLE 2.14 shows the most common gels used for this project. The stacking gel is the same for all gels.
<table>
<thead>
<tr>
<th>Resolving/Running Gel</th>
<th>10%</th>
<th>15%</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of gels</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1.5M TrisHCl pH 8.3+0.4% SDS</td>
<td>2.8 ml</td>
<td>2.8 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>30% Acrylamide 0.8% Methylene bis acrylamide (BioRad)</td>
<td>3.72 ml</td>
<td>5.56 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.92 ml</td>
<td>2.52 ml</td>
<td>6.4 ml</td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS)</td>
<td>40 µl</td>
<td>40 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED (BioRad)</td>
<td>9.2 µl</td>
<td>9.2 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 2.14: Volumes of reagents to make poly-acrylamide gels.

Gels were run at 125 V for ~90 min in 1x running buffer diluted in water from a 10 x stock: 25 mM Tris, 192 mM Glycine, 0.1% SDS pH 8.3 (10x TGS Biorad) and a pre-stained molecular weight marker was run alongside samples (Fisher BioReagents EZ-Run™ Prestained Rec Protein Ladder).

2.7.3 Transfer of proteins onto a membrane

Wet transfers were performed using PVDF membranes (Immobilon Millipore). The following 10 x transfer buffer (250 mM Tris (30.2 g), 1920 mM Glycine (144.1 g) 1 L H₂O) was diluted in water giving a final concentration of 20 % methanol and 1 x transfer buffer. Transfers were carried out at 100 V for 1 h, an ice pack was used to keep the transfer cold.

2.7.4 Detection of specific proteins

Membranes were blocked with 1 x Tris buffered saline TBS- 0.1 % TWEEN20 diluted in water from 10 x TBS (200 mM Tris (24.22 g) 1370 mM NaCl (80.06 g) 1 L H₂O Adjust pH 7.6 with 37% HCl ~14 ml) with the addition of 5 % BSA or 3 % milk (Marvel powdered milk, Harts Office Supplies), on a shaker at room temperature for 1 h. Primary antibodies were prepared in blocking buffer, and incubated overnight at 4 °C. Membranes were washed for 3 x 5 min with TBS-TWEEN20, shaking vigorously at room temperature. The secondary antibody was used at a dilution of 1:1000 in blocking buffer, shaking for 1 h at room temperature; Horseradish Peroxidase (HRP)-conjugated
anti-mouse or anti rabbit-antibodies (Cell Signalling) were used. The TBS-T washes were then repeated.

Blots were developed using enhanced chemiluminescent reagent (Invitrogen) or a more sensitive ECL reagent (Millipore Immobilon Western #WBKLS0500), film (Scientific Laboratory Supplies Ltd (Sls)) and an automated developer (Varispeed X150 developer, Xograph Imaging Systems Ltd.). ImageJ software was used to quantify the detected band, taking into account the loading control. LC3 quantification was expressed as a ratio of LC3II/LC3I.

2.8 Cell based assays and imaging

2.8.1 Immunofluorescence

Cells were plated onto 12-well plates containing autoclaved 18 mm coverslips (SLS Scientific laboratory supplies). Cells were fixed for immunofluorescence by two different methods, refer to the methods below and see TABLE 2.3 for the antibodies. The coverslips were protected from the light throughout.

The first method was methanol fixation, where cells were washed twice with PBS and then fixed with ice cold methanol for 5 min at -20˚C. The cells were then washed at room temperature for 5 min with PBS three times. Cells were left in PBS at 4˚C until they were ready to block at room temperature with PBS 5 % BSA for 1 h. Coverslips were placed face down on parafilm with 80 µl of the primary antibody dilution per coverslip, see table 2.3 of antibodies for dilution. This was then placed in a humidified container and left overnight at 4˚C. The coverslips were then put face up into a 12-well plate and washed at room temperature for 5 min with PBS three times. 500 µl of the secondary antibody dilution was added to each coverslip at room temperature for 1 h; see the antibody table 2.3 for dilution in blocking buffer. The PBS washes were repeated and then DAPI was used to stain the nuclei, at a final concentration of 1 µg/ml, protected from the light and added for 10 min at room temperature. The cells were then mounted onto 76x26mm
microscope slides (ThermoFisher Scientific) using Prolong gold anti-fade reagent (Life Technologies).

The alternative method of fixation was used for some antibodies, see antibody TABLE 2.3, and was adapted from a protocol used by Dr Nicholas Ktistakis, Babraham Institute. The cells were washed twice with room temperature 3.7 % paraformaldehyde (PFA) and left at room temperature for 20 min. The PFA was quenched using DMEM with 10 mM HEPES, the cells were washed twice with the HEPES solution and left at room temperature for 10 min. The cells were then washed with NETgel (NaCl 150 mM, EDTA pH 8.5 mM, Tris-HCl pH 7.4 50 mM, NP40 aka igeple 0.05 %, gelatin (type 4 bloom 6650) 0.25 %, Sodium Azide 0.02.%), gently shaking for 5 min. The cells were then permeabilised using 0.25% NP40 (Nonidet nonionic, non-denaturing detergent now purchased as IGEPAL CA-630) for 15 min, gently shaking at room temperature. Washes with NETgel were performed 3 x 5 min, gently shaking, and the plate was left at 4°C overnight. The primary antibody dilution was added, as explained previously, but only for 30 min at room temperature and diluted in NETgel. The coverslips were then washed in NETgel for 3 x 5 min with gentle shaking. The secondary antibody was diluted and added, as described previously, for 30 min at room temperature. The following steps of washing and mounting the coverslips are the same as the methanol fixation method.

2.8.2 Microscopes

Fixed cells were viewed using an oil emersion 63 x lens objective on the Zeiss 780 confocal microscope (Carl Zeiss Ltd.).

Live cell imaging was carried out using a Zeiss 780 confocal or a wide field Nikon live cell imager (Nikon), where both had an incubation chamber heated to 37 °C and 5 % CO₂ supply.

2.8.3 Latex bead assay

3 µm uncoated polystyrene beads (Polysciences) were washed and re-suspended in PBS. The beads in PBS were vortexed and added to culture cell media at a 1:200 dilution. The cells were...
incubated in media containing the beads for 4 h, to allow time for engulfment, before they were treated with 100 µM monensin for 1 h, to stimulate non-canonical autophagy. The cells were then washed and fixed for immunofluorescence.

2.8.4 Entotic corpse assay

MCF10A cells were plated on glass coverslips in a 12-well plate, 80 000 cells per well. The cells were incubated for 24 h to allow cell-in-cell structures to form and corpses to be generated. The cells were then treated with 100 µM monensin for 1 h, to stimulate non-canonical autophagy. The cells were then washed and fixed for immunofluorescence.

2.8.5 Entosis cell fate assay

HCT116 cells were seeded onto glass bottomed 6-well plates (Matek Corporation) at 150 000 cells per well and incubated for 48 h. The Nikon Live cell imager was set up to image at 8 min intervals for 20 h with the 40 x oil lens. NIS Elements software was used to analyse the videos for LC3 positive entotic deaths.

2.8.6 Macropinocytosis assay

MEF cells were plated on 35 mm glass bottomed dishes (MatTek corporation) at 100 000 cells per dish for 24 h. The cells were then serum starved overnight (DEMEM + 1% penicillin and streptomycin), and stimulated with 0.1 µg/ml PDGF (Peprotech (100-14B)) in the presence of 0.1 mg/ml Tetramethylrhodamine conjugated 10 000 MW Dextran (fluoro-Ruby) (D-1817 Life Technologies). Cells were imaged immediately via confocal microscopy and representative images were captured.

2.8.7 Apoptotic corpse LC3 associated phagocytosis (LAP) assay

These assays were performed using MEF cells (engulfing hosts) and Hct116 cells (apoptotic corpses). First, MEF cells were plated on 35 mm glass bottomed dishes (MatTek corporation), 150 000 cells per dish for 24 h. HCT116 cells were prepared by adding cell tracker red CMTPX (Invitrogen #C34552) at a final concentration of 10 µM to a 10 cm dish of cells for 30 min at 37°C.
To induce apoptosis, the Hct116 cells were washed and 16 J/m² of energy was applied, using a UV stratalinker 2400 (Stratagene), in full media with the lid was off the dish; the cells were then returned to the incubator for 5 h. The dying/dead HCT116 cells were washed and added to the plated MEFs at a ratio of 5 apoptotic cells to 1 plated cell. The cells were then left for 14 h in the incubator and imaged using the Zeiss 780 confocal with the 63 x oil lens. Engulfed apoptotic cell corpses were marked in red and twenty corpses were counted, scoring whether they were negative or positive for LC3. This was repeated three times and plotted on a graph showing the standard error of the mean (SEM). An unpaired Student’s t-test was performed to look at statistical significance, where \( p \leq 0.5 \) is significant.

2.8.8 VacA toxin assay

MEFs cells were plated on 35 mm glass bottomed dishes (MatTek corporation) at 150 000 cells per dish for 24 h. Purified vacuolating toxin A, VacA, was kindly provided by Dr Tim Cover as previously acknowledged [98]) and was stored at 4 °C in PBS, 1mM EDTA, 0.2% sodium azide pH7.5. In order to disassemble the oligomeric structure, the pH was adjusted to 2-3. This was achieved by the addition of 60 µl of 200 mM HCL to 50 µl of toxin and left at room temperature for 30 min. This was then made up to a final concentration of 10 µM, with 5 mM ammonium chloride in full culture media. The cells were incubated with this solution for 4 h and then imaged via live Zeiss 780 confocal microscopy with the 63 x oil lens, where representative images were captured from two experiments.

2.9 Primary cell assays

2.9.1 Primary bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC) isolation and culture

In order to culture BMDCs and BMDMs, complete RPMI media was used: RPMI 1640 (Gibco), 10 % FBS, 1 % Pen/Strep, 50 µM 2-Mercaptoethanol (Life Technologies). For differentiation: BMDC differentiation medium: Complete RPMI supplemented with 20 ng/ml murine GM-CSF (Peprotech), 10 ng/ml IL-4 (Peprotech) and 50 ng/ml (Peprotech) and Fungizone (Amphotericin B,
Gibco); BMDM differentiation medium: Complete RPMI supplemented with 30 ng/ml murine M-CSF (Peprotech) and Fungizone (Amphotericin B, Gibco).

BMDCs or BMDMs were isolated by sacrificing two 13-15 week male mice per condition. Their legs were dissected to leave the tibias and femurs. The following steps were all done with cold reagents including culture media. Bones were kept in sterile 1x PBS + 2 % FBS on ice and incubated for approximately 15 min to soften the tissues. In a tissue culture hood the bones were cleaned of all flesh and tendons on a sterile bacterial culture plate using a sterile scalpel and tweezers. The extremities of the bones were cut with the scalpel to allow the bone marrow to be flushed through. Then, using a 16 gauge sterile needle (Camlab limited), the bottom of a sterile 0.5 ml Eppendorf was pierced and the lid was cut off, it was important to ensure there was minimal touching of the sides of the tube to ensure sterility. The 0.5 ml tube was placed in a 1.5 ml sterile Eppendorf containing 100 µl of 1x PBS + 2 % FBS. The tubes were spun at 13 000 rpm for 20-30 s. The bone marrow was flushed through into the solution and a red pellet in the 1.5 ml Eppendorf was visible. The pellet was re-suspended in the media that had been flushed through, transferred to a 15 ml tube containing 14 ml of complete RPMI media, and then spun for 5 min at 1800 rpm to wash the cells. The cell pellet was re-suspended in 1 ml of 1x red blood cell lysis buffer diluted in water (10x stock (100 ml) : 8.99 g NH₄Cl, 1g KHCO₃, 37 mg Na₂ EDTA, pH to 7.3 in H₂O) and incubated at room temperature for 1 to 2 min with occasional agitation of the tube. The red blood cell lysis was stopped by the addition of 15 ml of complete RPMI. Cells were centrifuged for 5 min at 1800 rpm and the supernatant was discarded. The cells were re-suspended in 50 ml of differentiation media, pooling two mice into a T175 flask. On day three the majority of the media was removed and cells were fed with 50 ml of fresh differentiation media and fed with an additional 50 ml of differentiation media on day six. Cells were plated on day eight by gently scraping the cells from the flask.
2.9.2 Antigen presentation assay by flow cytometry

An antigen presentation assay was used in vitro to look at MHC II antigen presentation in bone marrow derived dendritic cells (FIGURE 2.1). This assay involved the addition of a fluorescent antigen to the cells in culture. The cells were cultured for 24 h with the antigen to allow uptake. The cells were then processed and analysed by flow cytometry where CD11b and CD11c transmembrane proteins were used to gate for the BMDC population. An antibody that recognises the antigen in complex with MHC II was used to assess antigen presentation. There is also a theory that the fluorescence of the antigen is affected by presentation and processing therefore the GFP fluorescence was measured.
Figure 2.1: Antigen presentation assay in vitro

An outline of the method, where BMDCs were plated for 24 h with the EaGFP fluorescent antigen. The cells were then re-suspended and transferred to flow cytometry tubes. The cells were then blocked to stop non-specific binding of antibodies. Antibodies were used to detect CD11c and CD11b in order to gate for the BMDCs to then analyse the amount of antibody detecting the MHC II receptors in complex with the antigen, Yae. Furthermore, the GFP fluorescence was also measured.
2.9.2.1 Plating, blocking and staining cells

BMDCs were plated on a 24-well plate at 250,000 cells per well and mixed with 0 µg/ml, 25 µg/ml, 50 µg/ml or 100 µg/ml of Ea-GFP antigen (kindly donated from Dr Michelle Linterman), then incubated for 24 h. Cells were gently scraped from the plate and transferred to cold flow cytometry tubes. 4 ml of PBS 2% FBS was added per tube and cells were spun at 1800 rpm, 4°C for 5 min. The supernatant was removed and the cells were re-suspended in 50 µL of FC Block (FC Block: Anti-Mouse CD16/CD32 Clone 93 eBioscience #14-0161-82 (100 µg) (stored at 4°C, working dilution 1:200). The tubes were vortexed briefly to homogenise and incubated for 10 min at 4°C to block FC receptors and reduce non-specific signal. The cells were then washed by adding 4 ml PBS 2% FBS per tube and spinning cells at 1800 rpm, 4°C for 5 min. The cells were then stained with biotin-label Y-Ae antibody (eBioscience, 13-5741-85), which recognises the Ea-MHCII complex. The tube was vortexed briefly to homogenise and incubated for 1 h at 4°C. The cells were then washed and incubated with PE-labelled anti-CD11b (clone M1/70, BD Horizon, 562287), APC-eFluor780-labelled anti-CD11c (clone N418, eBioscience, 47-0114-82), Alexa Fluor 700-labelled anti-MHC class II (clone M5/114.15.2, eBioscience, 56-5321-82) and PE-labelled streptavidin (eBioscience, 12-4317-87) for 1 h at 4°C. The cells were then washed and re-suspended in 150 µL of PBS 2% FBS.

2.9.2.2 Preparing for flow cytometry compensations

Tubes for flow cytometry compensations were prepared by distributing a drop of beads (One Comp eBeads Compensation Beads Invitrogen (Thermo Fisher Scientific) #01-1111-42) into flow cytometry tubes. There was one tube of single stained beads for each antibody used to stain the cells. 1 µL of each of the antibodies used above was added to each tube to single-stain the beads. An additional tube was set up using a FITC-conjugated antibody for E-GFP compensation. The beads were incubated for 1 h at 4°C. The beads were not washed but 150 µL of PBS without FBS was added per tube.
2.9.2.3 Flow cytometry analysis

Flow cytometry analysis was performed with assistance from Dr Elise Jacquin and the Flow cytometry facility at The Babraham Institute, on the Fortessa A machine (Beckton Dickinson). The voltage for the cells was set and the compensation was done on the single-stained beads. Cells were gated on the single cell population, to remove debris from the analysis, and gated on CD11b+/CD11c+ cells, assumed to be the BMDC cellular population. The data was analysed by Dr Elise Jacquin using FlowJo software. Graphs were produced to show the cell gating and FSC-A was plotted against SSC-A to determine where the single cell population was. Then CD11b expression was plotted over CD11c expression to show the gating for the double positive cell population assumed to be the dendritic cells. Then the mean fluorescence intensities (MFI) for the Eα GFP alone, the Eα peptide in complex with MHCII and MHCII alone, were analysed. Graphs detailing the MFI at different antigen concentrations were plotted and error bars represent standard deviation. 4 technical repeats were plotted on graphs and the trend was compared across three independently repeated experiments. Graphs presented are representative where the error bars represent the standard deviation and a Student’s t-test was the statistical test used.

2.9.3 LAP in primary cells.

Autoclaved glass coverslips were added to a 12-well plate and coated with 1 mL of Poly-L-lysine (0.1 mg/mL in TC-grade H2O) per well and incubated at 37°C for 10 min. The Poly-L-lysine was kept to be re-used and the coverslips were washed 2 or 3 times with 1 mL of tissue culture grade H2O and one more time with complete culture medium. BMDCs or BMDMs were collected and seeded at 100 000 cells per well. 24 h-48 h later 5 µl of zymosan at a final concentration of 25µg/ml, was added to each well. This gave a ratio of five zymosan particles to one plated cell. This zymosan stock suspension was made up in PBS to a concentration of 5 mg/ml, 10x10⁷ particles/ml and vortexed to homogenise. The zymosan particles were incubated with the cells for 15-30 min. Cells were then fixed with methanol and immunofluorescence was done to visualise the localisation of LC3 and LAMP1.
2.10 Biochemical Assays

2.10.1 Subcellular Fractionation

HCT116 cells were seeded 2.5 million cells per condition on a 15 cm dish and cultured for 48 h. Cells were treated with and without 100 μM monensin for 1 h. Cytosolic and membrane proteins were fractionated using the Mem-Per Plus Membrane Protein Extraction Kit (89842, Thermofisher Scientific) following the manufacturer’s guidelines. The protein concentration from fractions was measured by BCA assay, where equal amounts of proteins were then separated by SDS-PAGE. Western blots were done to look at levels of specific proteins in the membrane or cytosol using control proteins to account for purity of fractions and loading (membrane proteins such as LAMP1 and cytosolic proteins such as tubulin). Quantification of the western blots was done using ImageJ, to quantify the protein levels in relation to the relevant control protein. This was repeated three times and the data was plotted on a graph to show the standard error of the mean (SEM). An unpaired Student’s t-test was performed to look at statistical significance, where \( p \leq 0.5 \) is significant.

2.10.2 Immuno-precipitation (IP) using FLAG-S tagged Atg16L1 constructs

MEF Atg16L1 -/- GFP-LC3 cells stably re-expressing Flag-S tagged Atg16L1 constructs were seeded onto four 144 x 20mm tissue culture dishes at 1.5 million cells per dish for 48 h. MEF wild type cells were used as a negative control, that are not expressing Flag-S tagged proteins. Cells were unstimulated or treated with monensin, 100 μM, for 1 h. The treatments were staggered so only four dishes were being lysed at any one time. The dishes were washed twice with ice cold PBS before being lysed in 2 ml of lysis buffer either 1% Triton buffer (50 mM Tris HCL, 15 mM NaCl, 1 mM EDTA, 1% Triton X-100) or 0.3% CHAPS buffer (0.3% CHAPS (Melford), 50 mM TrisHCl, 150 mM NaCl, 1 mM EDTA) where protease/phosphatase inhibitor cocktails were added 1:200, 1:100 respectively (ready to use protease and phosphatase inhibitors Sigma P8340 and P0044) and 1 mM PMSF and 10 mM Sodium orthovanodate on the day. The dishes were scraped and the
lysates were left in iced 15 ml falcon tubes before spinning at 4 °C 4000 rpm for 15 min. The Triton or CHAPs soluble material was transferred to a new iced 15 ml falcon tube and the pellet of insoluble material discarded. A proportion of the lysates were saved here. The lysates were pre-cleared using 160 µl of Immunoglobulin G (IgG) conjugated 4% agarose bead slurry (Sigma, 4°C ); these had been pre-washed three times with the full lysis buffer described above with a 10-20 s 1500 rpm centrifugation step after each wash in order to pellet the beads. The lysates were left to slowly rotate at 4°C with the IgG agarose beads for 1 h. The tubes were centrifuged for 5 min 1500 rpm in order to pellet the IgG beads and anything that was now bound to the beads. The supernatant was transferred to a fresh 15 ml tube and added to 160 µl of an S-protein agarose bead slurry (Novagen) designed to bind S-tagged proteins. The beads had been washed in preparation in the same way as the previous beads. The lysates were left to slowly spin at 4°C with the S-beads for 6 h. The tubes were centrifuged for 5 min 1500 rpm in order to pellet the S-beads and anything that was now bound to the beads, a proportion of the supernatant was saved here to analyse the depleted sample. The bead pellets were washed three times in full lysis buffer and then replaced with 50 µl of 1x SB, the samples were then boiled at 100 °C for 5 min to elute the bound proteins and centrifuged in order to remove the beads. The samples were analysed via western blotting for the presence of tagged protein in the total lysate (input sample), in the depleted sample and in the IP sample. A silver staining kit (SilverQuest Invitrogen) was used to sensitively visualise proteins in the IP samples. This was done by running pre-cast acrylamide gradient gels (Mini-protean TGX gels Bio-Rad) for sterility and optimal separation range, and then the silver staining was performed as per the manufacturer’s instructions.

Once optimised the samples were sent for mass spectrometry analysis (Core facility at The Babraham Institute headed by Dr David Oxley). At the core facility, the samples were run on an SDS-PAGE gel ~5 mm into the gel and then the proteins were stained with Coomassie. The stained proteins were excised and put into individual tubes to be de-stained, reduced and alkylated before being digested with trypsin [217]. The digested products were then labelled with Thermo
Fisher Scientific TMT10plex label regent set prior to mixing. [218]. The principal of mass spectrometry is to identify molecules based on their mass to charge ratio. The TMT labelling offers a robust way to allow relative abundance of peptides to be compared.

2.10.3 BioID

This method was adapted from the referenced paper [219], with advice from Dr Noor Gammoh. Chapter 5 has further information on the theory of the method. In short, this method utilises a promiscuous biotin ligase that is fused to a protein of interest. Upon the addition of biotin, the promiscuous biotin ligase on the protein of interest biotinylates proteins that are in close proximity. After this process had occurred, cellular lysates were generated and immunoprecipitation of biotinylated proteins were prepared for analysis.

The pQCXIN-BirA-Myc plasmid with Atg16L1 FL expressing the BirA (promiscuous biotin ligase) and myc at the C-terminal was kindly donated by Dr Noor Gammoh. This was then taken to produce pQCXIN-BirA-Myc vectors expressing Atg16L1 K490A and Atg16L1 ΔWD. This was done using PCR to introduce Not1 and Pac1 restriction sites flanking either the genetic sequence for Atg16L1 K490A or the ΔWD using the primers in TABLE 2.15. The pQCXIN-BirA-Myc plasmid and the PCR products were then digested with Pac1 and Not1. The PCR digested products were then ligated into the BirA plasmid and grown in DH5α E.coli where the colonies were screened and DNA sequenced. See molecular biology techniques for more detail.

<table>
<thead>
<tr>
<th>Primer sequence to introduce <strong>Not1</strong> to the start of Atg16L1 sequence. (Common to cloning for Atg16L1 K490A and ΔWD). 5′-3′.</th>
<th>Primer sequence to introduce <strong>Pac1</strong> to the end of the sequence of Atg16L1 or the end of Atg16L1 ΔWD. 5′-3′.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCAGCAGCGGCCGCATgtcgtgcggctgctgcgcgc</td>
<td>GCAGCAGCTTAAATTAAaggctgtgcccacagcag</td>
</tr>
<tr>
<td>GCAGCAGCTTAAATTAAcaacgcatcacacagcagtcctgcggctgctgcgcgc</td>
<td>GCAGCATTCATTTAagggcctcccacagcagcag</td>
</tr>
</tbody>
</table>

Table 2.15: Primers for cloning Atg16L1 K490A from p-BABE to pQCXIN-BirA-Myc vector.

MEF Atg16L1 -/- GFP-LC3 cells stably re-expressing C-terminally tagged BirA Atg16L1 constructs were plated at a density of 1.5 million cells per 15 cm dish per condition for 48 h. The cells were
treated with 50 μM biotin (Sigma B4639) alongside 1 μM PP242 to modulate canonical autophagy or 100 μM monensin to modulate non-canonical autophagy. Cells were then washed three times with ice cold PBS and lysed in 1 ml of RIPA buffer with phosphatase and protease inhibitor cocktails per 15 cm dish. Cells were scraped and lysed on ice and lysates were pipetted up and down and left on ice for 10 min. Lysates were spun at 13000 rpm for 15 min at 4 °C. 10% of the supernatant was kept here as the total lysate (input) sample. The protein concentration was determined through a BCA assay, see western blot method. Equal amounts of protein were added to pre-washed streptavidin conjugated Dynabeads (MyOne Streptavidin C1; Invitrogen). Washing of the beads involved three washes with lysis buffer, pelleting the beads with a magnetic rack (Cell Signalling). 30 μl of bead slurry per condition was used and this will give 15 μl of beads per condition where the binding capacity for this amount of beads is ~12 mg of protein. The total amount of beads for all conditions were washed together and then separated into the respective tubes. It is important to ensure pipette tips are cut at the end (<1000 μl) to preserve the integrity of the beads. The lysates were left rotating at 9 rpm at 4 °C overnight. The beads were pelleted using the magnet and the supernatant was kept as the depleted sample. The beads were then washed four times in RIPA lysis buffer with phosphatase and protease inhibitors. To elute the bound proteins 30 μl of 2x SB was added to each tube and boiled at 95 °C for 10 min, the beads were then removed to leave the IP sample. The samples were analysed by western blot where 15 μg of total lysate and 10 μl of IP sample were separated via SDS-PAGE and specific protein levels were detected by western blot. For mass spectrometry, the samples were handed over to the Babraham Institute mass spectrometry facility and the samples were prepared and TMT labelled as previously described.

2.11 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism software. Two-tailed unpaired Student’s t-test was the common statistical analysis used.
3 Results: The molecular mechanisms of Atg16L1 function during non-canonical autophagy.

3.1 Introduction: non-canonical autophagy and Atg16L1.

It is now accepted that the lipidation of LC3 cannot be purely used as a readout of canonical autophagy because LC3 can be lipidated to single membrane endolysosomal compartments, through a so-called ‘non-canonical’ autophagy pathway [91, 98, 109, 111]. This single membrane lipidation is dependent on the canonical autophagy lipidation machinery, where Atg16L1 plays a key role, acting in complex with Atg12 and Atg5 to direct the site for LC3 lipidation [25, 31, 91]. However, non-canonical LC3 lipidation is independent of some upstream signals involved in autophagy, such as the ULK1 complex [91, 120], with the underlying molecular mechanisms largely unknown.

To investigate non-canonical autophagy, this project utilises the ionophore monensin, which exchanges hydrogen ions for sodium ions in the lysosome, raising the lysosomal pH but also changing the osmotic balance of endolysosomal compartments [220, 221]. This alteration of endolysosomal osmotic properties has been shown to induce LC3 lipidation to these single membranes [98]. Monensin induced LC3 lipidation to single membranes is independent of the PI3K complex needed for canonical autophagy [41, 98]. While Atg16L1 is recruited in canonical autophagy via direct interaction with FIP200 and the PI3P effector protein WIPI2b [32, 33, 189], these signals are not important for non-canonical LC3 lipidation to membranes of the endolysosome.

The aim of this chapter was to explore the mechanisms controlling Atg16L1 recruitment in non-canonical autophagy. Using a structure function approach, existing Atg16L1 mutants [32] were expressed in Atg16L1 knockout cells, to uncover which domains are important for Atg16L1 localisation and LC3 lipidation in the context of non-canonical autophagy. Further refinement of the role of Atg16L1 was achieved using site directed mutagenesis. Biochemical assays alongside
imaging were the methods used to analyse LC3 lipidation in non-canonical autophagy, in pharmacologically stimulated or physiological settings. The results from the structure function approach led to a collaboration with Dr Rupert Beale looking at the effect of Influenza A on LC3 lipidation to begin to assess the functional consequences of the developed cellular system based on Atg16L1 manipulation. The Atg16L1 T300A variant associated with Crohn’s disease was also assessed to see if it had a role in non-canonical autophagy.

3.2 Atg16L1 recruitment and LC3 lipidation during non-canonical autophagy is independent of canonical autophagy signals.

Atg16L1 acts in complex with Atg5 and Atg12 to lipidate LC3 to PE and has been well studied in the context of canonical autophagy. Atg16L1 co-localises with LC3 puncta corresponding to autophagosomes upon nutrient starvation of cells [31]. Consistent with this, GFP-LC3 puncta formed and co-localised with Atg16L1 in HCT116 cells, following starvation, as shown in (Figure 3.1A). LC3 can also be lipidated to single membrane endolysosomal compartments, dependent on the Atg16L1-Atg5-Atg12 complex. Therefore, the hypothesis was that LC3 would also co-localise with Atg16L1 at these membranes. Indeed, Atg16L1 was detected at LC3 positive, single-membrane phagosomes containing zymozan in a macrophage cell line J7741 (FIGURE 3.1B). To further study this, the ionophore monensin was used to promote robust LC3 lipidation to acidic endolysosomal compartments. Examples of these endolysosomal membranes include the entotic vacuoles that form after cell cannibalism events or bead containing phagosomes that form following the phagocytosis of uncoated latex beads. In both of these cases, monensin stimulated LC3 lipidation to endolysosomal membranes as well as Atg16L1 recruitment; FIGURE 3.1C shows this on the entotic vacuole and FIGURE 3.1D on the bead containing phagosome. These data confirm that Atg16L1 recruits to autophagosomes, and importantly, show that it also recruits to single membranes during non-canonical autophagy.

Published data from the Florey lab, showed that monensin induces LC3 lipidation to single membranes during non-canonical autophagy independently of VPS34 activity, a PI3K involved in
canonical autophagy [98]. In canonical autophagy, growing autophagosomes are enriched with PI3P, leading to the recruitment of the PI3P effector WIPI2b that subsequently interacts with Atg16L1. In agreement with this, cells treated with the PI3 Kinase inhibitor wortmannin did not form autophagosomes following starvation, as assessed by microscopy and GFP-LC3 puncta formation (FIGURE 3.2A). The same result was also seen analysing LC3 lipidation via western blot, wortmannin significantly reduced LC3 lipidation in response to starvation (FIGURE 3.2C and D). However, in keeping with previously published data [98], pre-treatment of cells with wortmannin, followed by activation of non-canonical autophagy with monensin treatment, did not affect LC3 lipidation to entotic corpse vacuoles (FIGURE 3.2B). This again was supported by western blots to show that lipidated LC3 in response to monensin was not affected by the inhibition of PI3K (FIGURE 3.2 C AND D).

In order to further support this conclusion, the localisation of the PI3P effector, WIPI2b, was looked at in the context of canonical and non-canonical autophagy. Following starvation induced canonical autophagy, WIPI2b puncta co-localised with GFP-LC3 puncta corresponding to autophagosome structures (FIGURE 3.2E). However, when cells were treated with monensin to drive LC3 lipidation to single endolysosomal membranes WIPI2b was absent from these membranes, supporting the dispensability of VPS34 and PI3P in monensin-induced non-canonical autophagy (FIGURE 3.2F).

Together these data show that Atg16L1 is recruited to single-membrane endolysosomal compartments during non-canonical autophagy and that this is independent of known mechanisms that recruit Atg16L1 to autophagosome membranes. This suggests the existence of a novel molecular mechanism of Atg16L1 function during non-canonical autophagy.
Figure 3.1: Atg16L1 recruitment in canonical and non-canonical autophagy.

A. HCT116 GFP-LC3 cells +/- starvation 1 h and fixed and stained for Atg16L1. Images were captured by confocal microscopy. The arrow heads show puncta positive for Atg16L1 and LC3. Scale bar is 10 µm.

B. J774A GFP-LC3 cells with engulfed zymosan, cells were fixed 30 min after zymosan addition and stained Atg16L1. Images were captured by confocal microscopy. The arrows indicate LC3 and Atg16L1 positive zymosan containing phagosomes. The scale bar is 5 µm.

C. MCF10A GFP-LC3 cells +/- 100 µM monensin for 1 h and then fixed and stained for Atg16L1. Images were captured by confocal microscopy, arrows indicate an LC3 and Atg16L1 positive entotic vacuole where the asterisk marks the entotic corpse. The scale bar is 10 µm.

D. HCT116 GFP-LC3 cells with engulfed latex beads +/- 100 µM monensin for 1 h and then fixed and stained for Atg16L1. Images were captured by confocal microscopy, arrows indicate LC3 and Atg16L1 positive bead containing phagosome. The scale bar is 5 µm.
Figure 3.2: Upstream signals for LC3 lipidation in non-canonical autophagy are distinct from canonical autophagy.

A. MCF10A GFP-LC3 cells were starved for 1 h or pre-treated for 15 min with 67 µM wortmannin (WM) followed by 1 h starvation still in the presence of WM. Cells were then fixed and the DNA was stained with DAPI and images were captured by confocal microscopy. The scale bar is 10 µm.

B. MCF10A GFP-LC3 cells were treated with 100 µM monensin for 1 h or pre-treated for 15 min with 67 µM wortmannin (WM) followed by 1 h monensin treatment still in the presence of WM. Cells were then fixed and the DNA was stained with DAPI and images were captured by confocal microscopy. The scale bar is 10 µm.

C. HEK293 cells were starved or treated with 100 µM monensin for 1 h or pre-treated for 15 min with 67 µM wortmannin (WM) followed by these treatments. A western blot was used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control.

D. Quantification of the ratio of LC3II/I. n=3 where the error bars represent the standard error of the mean (SEM) *P<0.04 (unpaired Student’s t-test).

E. HCT116 GFP-LC3 cells were starved for 1 h and fixed and stained for WIPI2b. Images were captured by confocal microscopy. Arrows indicate puncta positive for LC3 and WIPI2b. The scale bar is 10 µm.

F. MCF10A GFP-LC3 cells -/+ 100 µM monensin for 1 h. Fixed and stained for WIPI2b. Images were captured by confocal microscopy. The scale bar is 10 µm.

*This work was produced in collaboration with Dr Elise Jacquin.
3.3 The C-terminal WD domain of Atg16L1 is dispensable for canonical autophagy.

3.3.1 Atg16L1 Structure-function Analysis: cell lines and constructs

To begin studying Atg16L1 in the context of both canonical and non-canonical autophagy a structure function approach was used. This exploited existing pBabe retroviral Atg16L1 constructs: full length Atg16L1 (FL) (1-623), Atg16L1 lacking amino acids 219-242, which house the FIP200 and WIPI2b binding sites (ΔFBD), and Atg16L1 with a C-terminal deletion of the WD40 domain (ΔWD) (1-335), where they all have N-terminal Flag and S tags (FIGURE 3.3A).

These constructs were re-expressed in a variety of different Atg16L1 deficient cell lines: i) Clonal HCT116 GFP-LC3 cells, deficient for Atg16L1, were engineered by Dr Rupert Beale’s lab using CRISPR/Cas9. ii) Previous members of the lab, Dr Elise Jacquin and Celine Judon, used CRISPR/Cas9 technology to produce MCF10A Atg16L1 deficient cells expressing GFP-LC3, iii) this project also utilised an existing MEF Atg16L1 deficient GFP-LC3 cell line produced by traditional methods based on homologous recombination [197]. The cells deficient for Atg16L1 were complemented with ATG16L1 constructs, where re-expression was achieved by using viral transduction to stably express the constructs at equal levels, as shown in FIGURE 3.3B-D.
Figure 3.3: Atg16L1 constructs and expression in various cell lines.

A. Schematic representation of the different Atg16L1 Flag S (F-S) tagged constructs. Full length Atg16L1 (FL), Atg16L1 with a deletion of the FIP200 binding domain (∆FBD), Atg16L1 with a deletion of the C-terminal WD domain (∆WD).

B-D. Western blot to detect Atg16L1 in various knockout Atg16L1 cell lines stably re-expressing Atg16L1 constructs to a similar level. The arrows show the specific Atg16L1 bands. B: HCT116 GFP-LC3 Atg16L1 +/- C. MEF GFP-LC3 Atg16L1 +/- D. MCF10A GFP-LC3 Atg16L1 +/-.
3.3.2 Atg16L1 Domain Analysis: canonical autophagy assays

Canonical autophagy assays were performed with the Atg16L1 re-complemented cell lines to see which domains of Atg16L1 control LC3 lipidation to autophagosomes. The first experiment was to test their response to nutrient starvation by observing and quantifying the GFP-LC3 puncta per cell through confocal imaging. In agreement with published work [32, 189], FIGURE 3.4 A-D shows that deletion of the FBD domain of Atg16L1, in MEF and HCT116 cells, impairs GFP-LC3 puncta formation, a measure of LC3 localisation and lipidation to autophagosomes. However, the deletion of the C-terminal WD domain of Atg16L1 had no significant effect on GFP-LC3 puncta formation in response to starvation [197]. Using PP242, an m-TOR inhibitor, as an alternative treatment to activate canonical autophagy, HCT116 cells re-expressing the Atg16L1 constructs were used to look at LC3 lipidation levels via western blotting (Figure 3.4 E-F). These data support the immunofluorescence results, in that the FBD domain of Atg16L1 is required for efficient canonical autophagy while the WD domain is dispensable.

To reinforce these observations, the localisation of WIPI2b under starvation was analysed. WIPI2b localises to autophagosomes upstream of Atg16L1 recruitment. Therefore, as expected, there was no significant difference in WIPI2b recruitment to autophagosomes in cells expressing the different Atg16L1 constructs (FIGURE 3.4 G AND H). These results demonstrate that signalling upstream of LC3 lipidation was functional in all cell lines.
**Figure 3.4: Atg16L1 structure function in canonical autophagy.**

A. Fixed confocal images of MEF GFP-LC3 Atg16L1 -/- cells complemented with full length (FL), ∆FBD or ∆WD Atg16L1 -/+ starvation for 1 h. The scale bar is 10 µm.

B. Quantification of GFP-LC3 puncta from A, 100 cells were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM) ***P<0.0001, **P<0.001 (unpaired Student’s t-test).

C. Fixed confocal images of HCT116 GFP-LC3 Atg16L1 -/- cells complemented with full length (FL), ∆FBD or ∆WD Atg16L1 -/+ starvation for 1 h. The scale bar is 10 µm.

D. Quantification of GFP-LC3 puncta from C, 100 cells were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM) ***P<0.0004, **P<0.002, (unpaired Student’s t-test).

E. Lysates from HCT116 cells re-complemented with Atg16L1 constructs treated -/+ 1 µM m-TOR inhibitor PP242 for 1h.

F. Quantification of the ratio of LC3II/I. n=3 where the error bars represent the standard error of the mean (SEM) *P<0.02 (unpaired Student’s t-test).
Figure 3.4 continued: Atg16L1 structure function in canonical autophagy.

G. Fixed confocal images of HCT116 GFP-LC3 Atg16L1 -/- cells complemented with full length (FL), ΔFBD or ΔWD Atg16L1 -/+ starvation for 1 h. The cells were fixed and stained for WIPI2b. The scale bar is 10 µm.

H. Quantification of WIPI2b puncta from G, 100 cells were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM) ***P<0.0006, **P<0.005 (unpaired Student’s t-test).
3.4 The C-terminal WD domain of Atg16L1 is essential for monensin induced LC3 lipidation in non-canonical autophagy.

Having established the domain requirements of Atg16L1 in canonical autophagy, the same cell lines were next used to assess Atg16L1 structure-function with regards to non-canonical autophagy. To do this, a series of experiments were performed, using pharmacological stimulations and a range of assays for non-canonical autophagy.

3.4.1 Pharmacological assays: Bafilomycin versus Monensin

Comparative analysis of the drugs Bafilomycin A and monensin can be performed to assay for canonical versus non-canonical autophagy. As outlined in the introduction, Bafilomycin A1 is a V-ATPase inhibitor that blocks proton pump activity and raises lysosomal pH, leading to a block in autophagic flux and an accumulation of lipidated LC3, specifically on autophagosomes (LC3-II).

Monensin also raises lysosome pH, but by exchanging protons for sodium ions, resulting in a block in autophagic flux and a similar increase in canonical LC3 lipidation. Importantly, however, monensin also stimulates parallel LC3 lipidation to single-membrane compartments through osmotic regulation of non-canonical autophagy [41, 98], in a manner that can be inhibited by Bafilomycin A1. As such, while both drugs increase LC3 lipidation at autophagosomes, they have opposing effects on non-canonical autophagy – monensin induces, while Bafilomycin A1 inhibits. These pharmacological differences provide a useful means by which to compare canonical versus non-canonical autophagy.

Bafilomycin A1 and monensin were used to treat the Atg16L1 re-complemented cells and the LC3 lipidation levels were assessed by western blot (FIGURE 3.5A). This assay enabled visualisation of the extra LC3 lipidation that monensin causes, beyond the blockage of autophagic flux. In HCT116 cells re-expressing FL Atg16L1 or ∆FBD ATG16L1, there was more LC3 lipidation occurring when the cells were treated with monensin in comparison with bafilomycin (FIGURE 3.5A). Therefore, FL and ∆FBD Atg16L1 support monensin induced LC3 lipidation to endolysosomes. Strikingly, in cells where the Atg16L1 is lacking the C-terminal WD domain, there was little difference in the
lipidated LC3 levels in cells treated with bafilomycin or monensin (FIGURE 3.5A). This suggests that deleting the WD domain of Atg16L1 has stopped the additional LC3 lipidation to single membranes that is caused by monensin. These data provide the first evidence that the WD domain of Atg16L1 is required for LC3 lipidation in non-canonical autophagy.

3.4.2 Monensin induced LC3 lipidation: PI3K dependence

To complement the above work, another drug was used to help distinguish LC3 lipidation from canonical and non-canonical autophagy pathways. Wortmannin, a PI3K inhibitor, inhibits canonical autophagy but not monensin induced non-canonical LC3 lipidation (Figure 3.2A-D). It was evident in cells expressing FL Atg16L1 and ∆FBD Atg16L1 that there was wortmannin insensitive LC3 lipidation, which can be attributed to LC3 lipidation to single membranes in non-canonical autophagy (FIGURE 3.6B). However, in the cells expressing the ∆WD Atg16L1, wortmannin significantly inhibited monensin driven LC3 lipidation. As such, when the C-terminal domain of Atg16L1 is deleted, LC3 lipidation induced by monensin can mostly be attributed to canonical autophagy and the blockage of flux, suggesting non-canonical autophagy is not active.

3.4.3 Monensin-induced LC3 lipidation: phagocytosis assay

In order to visualise LC3 lipidation to membranes of the endolysosomal system in non-canonical autophagy, an assay was developed using uncoated latex beads. Following bead engulfment, cells were treated with monensin, which induced LC3 lipidation to the bead containing phagosomes. Samples were fixed and stained for LAMP1 to identify phagosomes and GFP-LC3 localisation was quantified. The Florey lab have previously shown that this method detects LC3 lipidation independent of canonical autophagy [98]. The lab have also shown that monensin induced LC3 lipidation to single membranes is not due to endolysosomal damage, as no galectin 3 or 8 were seen on endolysosomal membranes following monensin treatment [41]. This assay detected monensin induced LC3 lipidation to bead containing phagosomes in cells expressing the Atg16L1 FL and ∆FBD, where ~35% of LAMP1 bead containing phagosomes were GFP-LC3 positive at a
fixed timepoint (FIGURE 3.6 A-B). Strikingly, in cells lacking the WD domain of Atg16L1 there was no incidence of GFP-LC3 localisation to bead containing phagosomes (FIGURE 3.6 A-B). LC3 lipidation in this context was thus dependent on the C-terminal WD domain of Atg16L1, in a monensin dependent fashion.

3.4.4 Monensin-induced LC3 lipidation: entosis assay

To support these findings, another cell-based assay was utilised with MCF10A cells. These cells, following matrix deadhesion, have a high frequency of entotic live cell engulfment. During entosis, the internalised cell dies, while housed in an entotic corpse vacuole, an acidic, lysosome like compartment. It has previously been shown that monensin will promote the lipidation of LC3 to entotic corpse vacuoles through activation of non-canonical autophagy [41, 98].

Re-complemented Atg16L1 MCF10A cells were matrix detached, to induce entosis, then reseeded overnight, fixed and stained for LAMP1 to mark the resulting entotic corpse vacuoles; the GFP-LC3 localisation was quantified (FIGURE 3.7A-B). The cells expressing either the FL or ∆FBD Atg16L1 supported monensin induced LC3 lipidation to entotic vacuoles, +80% of vacuoles captured were LC3 positive (FIGURE 3.7A-B). Again, there was no incidence of LC3 lipidation to entotic vacuoles when the cells lacked the C- terminal domain of Atg16L1 (FIGURE 3A-B).

Taken together, these data show for the first time that the WD40 C-terminal domain of ATG16L1 is essential in drug induced non-canonical autophagy. Next, this observation was extended to analyse physiological examples of non-canonical autophagy.
### Figure 3.5: Pharmacological analysis of non-canonical autophagy Atg16L1 in re-complemented cell lines.

**A.** Lysates from HCT116 cells re-complemented with Atg16L1 constructs treated +/- 100 nM bafilomycin or 100 µM monensin for 1 h. A western blot was used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control. Quantification of the ratio of LC3II/I. n=3 where the error bars represent the standard error of the mean (SEM) *P<0.02, ***P<0.001, not significant (NS) (unpaired Student’s t-test).

**B.** Lysates from HCT116 cells re-complemented with Atg16L1 mutants were treated with +/- 100 µM monensin or 67 µM wortmannin (WM) for 1 h or pre-treated for 15 min with 67 µM WM followed by a 1 h monensin treatment. A western blot was used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control. Quantification of the ratio of LC3II/I. n=3 where the error bars represent the standard error of the mean (SEM) **P<0.0002 and not significant (NS) (unpaired Student’s t-test).
Figure 3.6: Using beads and monensin to study non-canonical autophagy in Atg16L1 re-complemented cell lines.

A.  HCT116 GFP-LC3 Atg16L1 -/- cells re-complemented Atg16L1 constructs, where with 3 µm latex beads were added for 4 h -/+ 100 µM monensin for 1 h. The cells were fixed and stained for LAMP1 and images were captured by confocal microscopy. Cropped images show bead phagosomes. The scale bar is 5 µm.

B.  Quantification of GFP-LC3 positive bead containing phagosomes from A, 100 LAMP1 positive bead containing phagosomes were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM).
Figure 3.7: Entotic corpse vacuoles treated with monensin to observe non-canonical autophagy in Atg16L1 re-complemented cell lines

A. MCF10A GFP-LC3 Atg16L1 -/- cells re-complemented with Atg16L1 constructs and treated +/- 100 µM monensin for 1 h. The cells were fixed and stained for LAMP1 and images were captured by confocal microscopy. The cropped images represent entotic corpse containing vacuoles. The scale bar is 5 µm.

B. Quantification of GFP-LC3 positive corpse containing entotic vacuoles from A, 100 LAMP1 positive entotic corpse vacuoles were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM).
3.5 The C-terminal WD domain of Atg16L1 is essential for LC3 lipidation in physiological engulfment events involving non-canonical autophagy.

3.5.1 LC3 associated phagocytosis (LAP) assays

LC3 associated phagocytosis (LAP) is involved in the phagocytic engulfment of pathogens, but also of apoptotic cells, and is a physiological example of non-canonical autophagy. MEF cells are able to engulf apoptotic cells [222] and have previously been shown to be competent for LAP [223]. Therefore, using Atg16L1 knockout MEF cells, re-complemented with the Atg16L1 constructs, LAP was analysed following phagocytosis of apoptotic HCT116 cells.

Cell tracker red labelled HCT116 cells were UV treated to induce apoptosis and added to MEF monolayers. Live cell imaging was performed to look at GFP-LC3 recruitment and lipidation to apoptotic corpse containing phagosomes. This assay showed that in FL and ∆FBD cells ~50% of apoptotic cell phagosomes were positive for GFP-LC3 (FIGURE 3.8 A and B). Strikingly, LAP was not supported in cells lacking the C-terminal WD domain of Atg16L1, as apoptotic cell phagosomes were negative for GFP-LC3 (FIGURE 3.8). These data support the conclusions from the monensin induced model and show that the WD domain of Atg16L1 is essential during LAP and non-canonical autophagy.

3.5.2 Macropinocytosis assays

It has been previously shown that LC3 can lipidate to macropinosomes [91]. This allowed another strategy to observe LC3 lipidation during non-canonical autophagy. MEF cells were serum starved overnight and stimulated with PDGF to activate macropinocytosis. A red dextran tracer was used as a fluid phase marker to observe the red macropinosomes and the localisation of GFP-LC3 via live cell imaging. It was found that GFP-LC3 was recruited to macropinosomes in cells expressing FL Atg16L1 or ∆FBD Atg16L1 but not in ∆WD Atg16L1 expressing cells (FIGURE 3.9A).
3.5.3 Vacuolating toxin A (VacA) assays

Another system to study GFP-LC3 lipidation in non-canonical autophagy involves treatment of cells with Vacuolating toxin A (VacA) derived from *Helicobacter pylori*. It has previously been shown that VacA induces vacuolation of endolysosomal compartments, which are then targeted by LC3 lipidation [98]. In MEF cells, the FBD domain of Atg16L1 was dispensable for VacA induced LC3 recruitment to vacuoles. However, the WD C-terminal domain of Atg16L1 was essential for LC3 lipidation in this context (FIGURE 3.9B).

Together, these data from three distinct cellular models show for the first time that the WD domain of Atg16L1 is essential for lipidation of LC3 during non-canonical autophagy and associated processes such as LAP.
Figure 3.8: LC3 associated phagocytosis assay in Atg16L1 re-complemented MEFs.

A. Atg16L1 complemented GFP-LC3 MEF cells that have phagocytosed red-labelled apoptotic cells for 14 h and then live confocal microscopy captured representative images. The cropped image is of an apoptotic corpse phagosome. The scale bar is 5 µm.

B. Quantification of GFP-LC3 positive apoptotic corpse containing phagosomes from A, 20 phagosomes were counted per experiment, n=3. The error bars represent the standard error of the mean (SEM), ****P<0.0001 (Unpaired Student’s t-test).
Figure 3.9: Macropinocytosis and VacA assays to observe LC3 lipidation in non-canonical autophagy.

A. Atg16L1 complemented GFP-LC3 MEF cells were serum starved overnight and then stimulated with 0.1 µg/ml PDGF in media containing tetramethylrhodamine conjugated dextran to visualise macropinocytosis. Live confocal microscopy was used to capture representative images and the DIC channel was an additional way to visualise macropinocytosis. Images were cropped to show a single macropinosome. The scale bar is 5 µm.

B. Atg16L1 complemented GFP-LC3 MEF cells treated with vacuolating toxin A (VacA) at 10 µM for 4 h. The cells were imaged by live confocal microscopy and representative images were captured. The DIC channel helped to visualise the vacuolation. The scale bar is 5 µm.
3.6 Atg16L1 ∆WD no longer localises to membranes of the endolysosomal system.

Next the possible mechanism underlying the function of the WD domain of Atg16L1 during non-canonical autophagy was explored. It has been assumed that the ∆WD Atg16L1 still has the ability to form a functional complex with Atg5 and Atg12 to support lipidation of LC3. This assumption is based on published work [209] and the fact that cells expressing ∆WD Atg16L1 still have the ability to lipidate LC3 to autophagosome membranes in canonical autophagy (FIGURE 3.4). It was confirmed that the ∆WD Atg16L1 does successfully form a complex with Atg5 and Atg12 to support LC3 lipidation. Following immunoprecipitation of FL Atg16L1 or ∆WD Atg16L1, both constructs can pull down the ATG5/12 conjugates (FIGURE 3.10A). ATG5 and Atg12 were also identified at similar levels in pull downs from both Atg16L1 constructs by mass spectrometry (FIGURE 3.10B). Thus, it can be concluded that Atg16L1 lacking the WD domain can form a functional complex with Atg5 and Atg12, and the lack of LC3 lipidation during non-canonical autophagy must be through a different mechanism.
Figure 3.10: Atg16L1 with a C-terminal deletion still forms a functional complex with Atg5-12.

A. Lysates from MEF Atg16L1−/− cells re-complemented with Flag-5 tagged FL or ΔWD Atg16L1 that had been treated for 1 h with 100 μM monensin were used to perform immunoprecipitation (IP) with anti-s-tag beads. A western blot was used to detect Atg16L1 or Atg5-12 in the total lysate/input (IN) and IP sample. The negative (−/−) comes from WT MEFs.

B. The graph represents data from n=2 mass spectrometry analysis for Atg5 and Atg12 levels pulled down in samples from cells expressing FL or ΔWD Atg16L1.
Next the recruitment of Atg16L1 to single-membrane compartments was tested to see whether it was affected by the WD domain. As shown earlier, Atg16L1 localises to bead containing phagosomes upon cellular treatment with monensin (FIGURE 3.1D and 3.11A). It was further found that, when the C-terminal domain of Atg16L1 is absent, it is no longer able to recruit to bead containing phagosomes upon monensin treatment (FIGURE 3.11A). This observation was extended by looking at Atg16L1 recruitment to entotic corpse vacuoles after monensin treatment. In MCF10A cells re-complemented with the FL Atg16L1 or the ΔFBD, it was evident that there was Atg16L1 localisation to corpse containing vacuoles in a monensin dependent fashion (FIGURE 3.11B). However, in the cells re-complemented with ΔWD Atg16L1, this version of Atg16L1 no longer recruited to corpse containing vacuoles (FIGURE 3.11B). Therefore, the WD domain of Atg16L1 is required for its correct localisation to single-membranes in non-canonical autophagy and the subsequent LC3 lipidation to these compartments.

In support of this conclusion from the imaging based assays, a membrane fractionation protocol was optimised in re-complemented HCT116 cells, followed by western blotting, to look at Atg16L1 and Atg5-12 levels in the membrane fraction following monensin treatment (FIGURE 3.12). Membrane proteins LAMP1 and V-ATPase subunit V0D1 were chosen as controls, to ensure purity of the fractionation and act as a loading control. Cytosolic protein alpha tubulin was also used to ensure that cytosolic proteins were not contaminating the membrane fraction (data not shown). It was evident that FL Atg16L1 was found at slightly increased levels at the membrane after monensin treatment, along with Atg16L1 lipidation complex partners Atg5-12 (FIGURE 3.12 A, C AND D). These changes are subtle, perhaps due to the fact that this is data from total membrane fractions rather than specifically endolysosomal membranes, but nevertheless reproducible. However, such an increase after monensin treatment was not observed in ΔWD Atg16L1 levels at the membrane or in the levels of its Atg5-12 binding partners (FIGURE 3.12 B-D). These findings, alongside the imaging data, show that Atg16L1 lacking the C-terminal domain is
failing to recruit to membranes of the endolysosomal system, leading to a lack of LC3 lipidation at these membranes.
Figure 3.11: Atg16L1 lacking the WD domain no longer localises to bead phagosomes and entotic corpse vacuoles.

A. HCT116 GFP-LC3 Atg16L1-/- cells re-complemented with FL or ΔWD Atg16L1 where 3 µm latex beads were added for 4 h followed by treatment +/- 100 µM monensin for 1 h. The cells were fixed and stained for Atg16L1 and images were captured by confocal microscopy. Cropped images show bead containing phagosomes. The scale bar is 3 µm. Line profile analysis of Atg16L1 and GFP-LC3 fluorescence intensity is shown for representative phagosomes and this was performed in ImageJ.

B. MCF10A GFP-LC3 Atg16L1-/- cells re-complemented with FL or ΔWD Atg16L1 +/- 100 µM monensin for 1 h and the cells were fixed and stained for Atg16L1. Images were captured by confocal microscopy and cropped images show representative corpse containing vacuoles. The scale bar is 10 µm.
Figure 3.12: Membrane fractionation to assess Atg16L1 and Atg5-12 at membranes in cells expressing FL or ΔWD Atg16L1.

A. HCT116 GFP-LC3 Atg16L1 +/- cells re-complemented with FL Atg16L1 +/- 100 μM monensin for 1 h. Cells were then lysed and fractionated, using a kit, into membrane and cytosolic fractions. Western blot analysis was done to detect Atg16L1 and Atg5-12 in the membrane fraction where LAMP1 and V-ATPase subunit D1 (VOD1) were used as known membrane proteins for loading purity control.

B. At the same time as A the experiment was done in HCT116 GFP-LC3 Atg16L1 +/- cells re-complemented with ΔWD Atg16L1.

C. Quantification of A and B to show the amount of Atg16L1 and ΔWD Atg16L1 at the membrane with and without monensin stimulation. Western blots were quantified in ImageJ where numbers were normalised to the untreated sample, and corrected for loading and purity using LAMP1. These data were from n=3 and the error bars are the standard error of the mean (SEM). *P<0.02 (Unpaired student t-test).

D. Quantification of A and B to show the amount of Atg5-12 at the membrane in respective samples with and without monensin stimulation. Western blots were quantified in ImageJ where numbers were normalised to the untreated sample, and corrected for loading and purity using VOD1. These data were from n=3 and the error bars are the standard error of the mean (SEM). *P<0.02 (Unpaired student t-test).
3.7 Identification of key residues on the top face of ATG16L1 WD40 C-terminal domain required for non-canonical autophagy

3.7.1 Atg16L1 WD domain sequence analysis

To gain more insight into the molecular details of the WD domain, bio-informatic alignments were performed to look at the conservation of the WD domain of Atg16L1 in species that support non-canonical autophagy. The alignments showed that the WD domain is evolutionarily conserved in species known to support non-canonical autophagy and cemented the idea that the domain must be carrying out an important function. FIGURE 3.13 shows the amino acid homology of Atg16L1 WD domains in rat, mouse and human. Further alignments were done as part of FIGURE 3.18C.

Atg16L2, a paralog of Atg16L1, which has a similar domain structure, was used to uncover important information on how specific the WD domain of Atg16L1 is in its function in non-canonical autophagy. A sequence alignment of the WD domains from Atg16L1 and Atg16L2 was performed to look at sequence homology (FIGURE 3.14), there was 43% homolog. It has been previously shown that although Atg16L2 can act in complex with Atg12-Atg5, it is not sufficient for canonical autophagy [193]. Therefore, it was next interesting to see if Atg16L2 was able to support LC3 lipidation in non-canonical autophagy. This was done in HCT116 GFP-LC3 Atg16L1 KO cells, transiently transfected with Atg16L1 or Atg16L2. By western blot, re-expression of Atg16L1 in HCT116 Atg16L1 KO cells supported LC3 lipidation in response to bafilomycin and monensin (FIGURE 3.15 A AND B). However, when the cells are just expressing Atg16L2, minimal LC3 lipidation is observed in response to canonical or non-canonical autophagy stimulations (FIGURE 3.15B). These conclusions were consistent when looking at GFP-LC3 via confocal microscopy, cells just expressing Atg16L2 no longer support monensin induced GFP-LC3 structure formation (FIGURE 3.15C AND D). These data suggest that Atg16L2 cannot support LC3 lipidation to endolysosomal membranes. The differences in the WD domain of these two Atg16 paralogs may
provide a clue to which residues may be essential for the function of the WD domain of Atg16L1 in non-canonical autophagy.
Figure 3.13: Alignment of the WD domain of Atg16L1 from human, mouse and rat.

Amino acid sequence alignment of the WD domain of human, mouse and rat Atg16L1. The single amino acids are listed and the graph shows the degree of similarity across the alignment. This was produced with the CLC sequence viewer 7 software.
Figure 3.14: Alignment of the WD domain of human Atg16L1 and Atg16L2.
Amino acid sequence alignment of the WD domain of human Atg16L1 and Atg16L2. This depicts amino acid similarity in a graphical form and numerically there was 43% sequence homology. This was generated using CLC sequence viewer 7.
Figure 3.15: Atg16L2 does not support LC3 lipidation in non-canonical autophagy.

A. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected with FL Atg16L1 or Atg16L2, cells were lysed and a western blot was performed to analyse the expression of Atg16L1 and Atg16L2. GAPDH was used as a loading control.

B. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected with FL Atg16L1 or Atg16L2 -/-+ 100 nM bafilomycin or 100 µM monensin for 1 h. Cells were lysed and a western blot was done to analyse unlipidated and lipidated LC3 (LC3I and LC3II). GAPDH was used as a loading control.

C. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected with FL Atg16L1 or Atg16L2 -/-+ 100 µM monensin. Cells were fixed and representative images were captured by confocal microscopy.

D. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected with FL Atg16L1 or Atg16L2 and 3 µm latex beads were added for 4h and then the cells were treated -/-+ 100 µM monensin for 1 h. Cells were fixed and stained for LAMP1. Representative images were captured by confocal microscopy and the cropped images show the bead containing phagosome.
3.7.2 Atg16L1 WD domain hotspot analysis

Next, a recently described algorithm was used that predicts “hotspot” residues potentially involved in protein-protein interactions in different WD domain containing proteins [195]. Based on the mouse Atg16L1 protein, 12 potential “hotspots” were identified, all located on the top face of the predicted β-barrel structure of the WD40 domain (FIGURE 3.16).

Site directed mutagenesis was done on these 12 candidate residues in the WD domain of Atg16L1, mutating each residue separately to alanine. This was done via an adaptation of the QuikChange II agilent technologies method and the results were sent off for Sanger sequencing. 12 point mutant Atg16L1 constructs were successfully produced in the pBabe retroviral plasmid. The Atg16L1 C-terminal domain point mutants were virally transduced to re-constitute Atg16L1 KO HCT116 GFP-LC3 cells in order to produce stable cell lines. The mutant Atg16L1 proteins were expressed at similar levels (FIGURE 3.17A). These cells were then used to assess whether the point mutation in the WD domain of Atg16L1 had changed the response of cells to non-canonical autophagy.

The initial assay used to screen these mutant cell lines was western blotting analysis of LC3 lipidation following monensin and/or wortmannin treatment, as previously used in (FIGURE 3.5B). It was evident that when canonical autophagy was inhibited by wortmannin, monensin was still able to stimulate non-canonical autophagy in some cells re-constituted with Atg16L1 point mutations (e.g. N326A, N342A) (FIGURE 3.17B). This suggests that these point mutations in the C-terminal of Atg16L1 have not affected LC3 lipidation in non-canonical autophagy. However, there were five point mutations (E342A, N386A, N453A, F467A, K490A) in Atg16L1 that seemed to impair monensin induced LC3 lipidation (FIGURE 3.17B). This result was promising as it suggested that Atg16L1 point mutants could phenocopy the C-terminal WD deletion of Atg16L1.
Figure 3.16: Atg16L1 WD domain using WDSP predicted amino acids required for protein-protein interactions.

Using the WDSP database as referenced in the text, using the amino acid sequence of Atg16L1, this is the predicted structure and potential residues important for protein-protein interactions. They are all shown on the top face of the beta barrel. The table shows the alanine substitutions made at various residues in the WD domain of Atg16L1 by site directed mutagenesis.
Figure 3.17: Screening Atg16L1 C-terminal WD point mutants for non-canonical autophagy.

A. HCT116 Atg16L1 -/- GFP-LC3 cells re-complemented with Atg16L1 single point mutants. Cells were lysed and western blots were performed to detect Atg16L1 expression. GAPDH was used as a loading control.

B. Lysates from HCT116 Atg16L1 -/- cells re-complemented with Atg16L1 point mutants that had been treated with +/- 100 µM monensin or 67 µM wortmannin (WM) for 1 h or pre-treated for 15 min with 67 µM WM followed by a 1 h monensin treatment. Western blots were used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control. Quantification of the ratio of LC3II/I was done using ImageJ. n=3 where the error bars represent the standard error of the mean (SEM). The red asterisk marks those that are not conserved in Atg16L1.
3.7.3 Atg16L1 WD domain mutant analysis: F467A and K490A

Two C-terminal Atg16L1 point mutants were taken forward for further validation: Atg16L1 F467A and K490A. These residues were mapped onto the recently reported crystal structure of the WD domain of Atg16L1 [192]. Atg16L1 F467, K490, and another positive hit N453, were all in close proximity to one another and generated a pocket on the top face of the WD40 C-terminal domain (FIGURE 3.18A AND B). Importantly, these particular residues were conserved across species (FIGURE 3.18C). Furthermore, F467 and K490 are not conserved in Atg16L2, which has a WD domain that does not support non-canonical autophagy, further validating that these residues could be essential in the WD domain of Atg16L1 that does support LC3 lipidation to single membranes (FIGURE 3.18D).

The point mutants were next analysed for their ability to support canonical and non-canonical autophagy using the experiments described earlier. Firstly we showed that each point mutant has no effect on starvation induced canonical autophagy, as assessed by GFP-LC3 puncta formation (FIGURE 3.19 B AND C). Similarly, WIPI2b puncta formation was not affected by the point mutants (FIGURE 3.19 B AND D). Western blots supported the immuno-fluorescence data; all of the Atg16L1 mutants supported an induction in LC3 lipidation when the m-TOR inhibitor PP242 was used to induce autophagy (FIGURE 3.19E AND F). Therefore, the alanine substitutions at positions F467 and K490 in the C-terminal of Atg16L1 have no effect on LC3 lipidation in canonical autophagy.

The initial screen by western blot identified the F467A and K490A Atg16L1 point mutants to impair monensin induced LC3 lipidation to endolysosomes in non-canonical autophagy (FIGURE 3.17). In order to further investigate this inhibition of LC3 lipidation to single membranes, cell lines were used to look at LC3 lipidation to bead containing phagosomes after monensin treatment. Cells stably re-expressing the Atg16L1 point mutants were significantly impaired for LC3 lipidation to bead containing phagosomes after stimulation with monensin (FIGURE 3.20).
These data further confirm that the residues F467 and K490 in the WD of Atg16L1 are essential for its function in LC3 lipidation in non-canonical autophagy.
Figure 3.18: Atg16L1 point mutants mapped onto the crystal structure of the Atg16L1 WD domain and amino acid conservation.

A. Ribbon model of the top face of Atg16L1 WD domain, with critical residues in ball and stick. Structural image generated in NGL viewer using Protein Database.

B. Surface of Atg16L1 WD domain coloured to electrostatic potential (blue positive 2, red negative 2). Cropped and zoomed area of critical residues. Image generated in Swiss-Protein database Viewer.

C. Annotated alignment of Atg16L1 sequences amino acids (477-496) from different species. Asterisks shows conserved critical residues.

D. Annotated alignment of a region of the WD domain of Atg16L1 in comparison to the WD domain of Atg16L2. Asterisks show un-conserved critical residues.
Figure 3.19: Canonical autophagy response of cells expressing the Atg16L1 WD domain point mutants.

A. Lysates taken from HCT116 GFP-LC3 Atg16L1 -/- cells re-complemented with Atg16L1 mutants. Western blot to detect expression levels of Atg16L1. GAPDH was used as a loading control.
B. Confocal images of cells from A starved for 1 h and fixed and stained for WIPI2b.
C. Quantification of GFP-LC3 puncta per cell +/- 1 h starvation. 100 cells were counted per experiment and an average was taken. n=3 where the error bars represent the standard error of the mean. *P<0.03 **P<0.003 ***P<0.0001 (Unpaired Student’s t-test).
D. Quantification of WIPI2b puncta per cell +/- 1 h starvation. 100 cells were counted per experiment and an average was taken. n=3 where the error bars represent the standard error of the mean. **P<0.005 (Unpaired Student’s t-test).
E. Lysates from re-complemented HCT116 cells treated +/- 1 µM m-TOR inhibitor PP242 for 1h. Western blot to detect unlipidated and lipidated LC3 (LC3I and LC3II) where GAPDH was used as a loading control.

F. Quantification of the ratio of LC3II/I using ImageJ. n=3 where the error bars represent the standard error of the mean (SEM) NS= not significant (unpaired Student’s t-test).
Figure 3.20: Atg16L1 point mutants in response to monensin induced non-canonical autophagy.

A. HCT116 GFP-LC3 Atg16L1 +/- cells re-complemented with Atg16L1 mutants where 3 µm latex beads were added for 4 h +/- 100 µM monensin for 1 h. The cells were fixed and stained for LAMP1 and images were captured by confocal microscopy. Cropped images show bead phagosomes. The scale bar is 5 µm.

B. Quantification of GFP-LC3 positive bead containing phagosomes from A, 100 LAMP1 positive bead containing phagosomes were counted per experiment and this represents n=3. Error bars represent the standard error of the mean (SEM).
To extend our studies of these key residues identified in the WD domain of Atg16L1, a new cell line was developed. This involved the generation of an Atg13 and Atg16L1 double knock out, by taking an existing Atg13 KO cell line (FIGURE 3.21A), which is deficient for canonical autophagy [41], and using CRISPR/Cas9 to knock out Atg16L1. Single cell clones were screened through western blotting for LC3 and Atg16L1 (FIGURE 3.21A AND B). The double knockout would render the cell deficient for LC3 lipidation associated with either canonical or non-canonical autophagy (FIGURE 3.21B). Indeed there was no evidence of GFP-LC3 re-localisation in these cells following monensin treatment (FIGURE 3.21C).

The double knockout cell line was useful to study which Atg16L1 constructs specifically rescued non-canonical autophagy when stably re-expressed in these cells at similar levels (FIGURE 3.22A). This was a clean system as the KO of Atg13 allowed LC3 lipidation in these cells to be attributed solely to non-canonical autophagy. These cells were then tested for LC3 lipidation in response to bafilomycin and monensin treatment (FIGURE 3.22B). These data show that only cells re-expressing the FL Atg16L1 rescued LC3 lipidation, while ΔWD Atg16L1 and selected point mutants were unable to support any LC3 lipidation in this background (FIGURE 3.22B). This validates our conclusions thus far that the WD domain of Atg16L1 and residues F467 and K490 are essential for Atg16L1 to function in non-canonical autophagy.

The role of the Atg16L1 point mutants in a physiological LAP assay was tested next using Atg16L1 KO GFP-LC3 MEFs re-constituted with FL Atg16L1, ΔWD Atg16L1, Atg16L1 F467A or K490A. As expected, the ΔWD Atg16L1 expressing cells did not support LAP (FIGURE 3.8). Furthermore, the point mutant Atg16L1 expressing cells also had a significant impairment for LC3 lipidation to apoptotic corpse phagosomes (FIGURE 3.23). These data show for the first time that mutating F467 or K490 in the WD domain of Atg16L1 does not affect canonical autophagy but impairs LC3 lipidation to membranes of the endolysosomal system, including LAP.
Figure 3.21: Generating a double knockout Atg13 and Atg16L1 HEK293 cell line to test if Atg16L1 mutants respond to non-canonical autophagy.

A. HEK293 GFP-LC3 Atg13/- cells generated by Dr Elise Jacquin were used to further knockout Atg16L1. Western blots to show Atg13 and Atg16L1 expression.

B. HEK293 GFP-LC3 Atg13/- cells generated by Dr Elise Jacquin targeted by CRISPR/Cas9 to knockout Atg16L1. Colonies from single cell dilutions were tested for successful Atg16L1 knockout. Western blots to show Atg16L1 expression from different populations of cells, unlipidated and lipidated (LC3I and LC3II) was also probed where GAPDH is a loading control.

C. HEK293 GFP-LC3 Atg13/- cells or HEK293 GFP-LC3 Atg13/- Atg16L1/- cells +/- 100 µM monensin for 30 min. Cells were fixed and representative confocal images were taken. The scale bar is 5 µm.
Figure 3.22: Non-canonical autophagy response utilising a Atg13 and Atg16L1 knockout cell line re-constituted with Atg16L1 mutants.

A. HEK293 GFP-LC3 Atg13 and Atg16L1 knockout cells re-complemented with Atg16L1 mutants. Cells were lysed and a western blot was performed to detect Atg16L1 protein expression levels.

B. Lysates from HEK293 GFP-LC3 Atg13 and Atg16L1 knockout cells re-complemented with Atg16L1 mutants treated -/+ 100 nM bafilomycin or 100 µM monensin for 1 h. A western blot was used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control.
Figure 3.23: Assessing Atg16L1 WD domain point mutants in response to a physiological LAP assay.

A. Atg16L1 complemented GFP-LC3 MEF cells that have phagocytosed red-labelled apoptotic cells for 14 h and then live confocal microscopy was used to capture representative images. The cropped images are of apoptotic corpse phagosomes.

B. Quantification of GFP-LC3 positive apoptotic corpse containing phagosomes from A, 20 phagosomes were counted per experiment, n=3. The error bars represent the standard error of the mean (SEM), **P<0.002 ***P<0.0002 (Unpaired Student’s t-test).
Finally, similar to the WD domain truncation, the point mutants appear to be competent for binding Atg5-12, but defective in recruitment to single membranes. As shown in FIGURE 3.24A, mutating residues F467 and K490 in the C-terminal of Atg16L1 did not affect the formation of the conjugation complex with Atg5 and Atg12. However, Atg16L1 is no longer recruited to bead containing phagosomes when F467 or K490 have been mutated to alanine (FIGURE 3.24 B AND C).

Together, these data reveal that F467A and K490A mutations in the WD domain of Atg16L1 phenocopy its deletion, as they fail to recruit to single membranes in non-canonical autophagy and therefore no longer support LC3 lipidation at these membranes.
Figure 3.24: Atg16L1 point mutants are able to form a complex with Atg5 and Atg12, but no longer recruit to bead containing phagosomes.

A. Immunoprecipitation (IP) using anti s-tag beads from MEF Atg16L1 -/- cells re-complemented with Flag-S tagged FL or Atg16L1 F467A or K490A point mutants. Cells were treated for 1 h with 100 µM monensin and lysed (total lysate = IN) and from this an IP was performed. A western blot was used to detect Atg16L1 or Atg5-12 in the total lysate/input (IN) and IP sample. The WT cells offer a negative control.

B. HCT116 GFP-LC3 Atg16L1 -/- cells re-complemented with Atg16L1 mutants where 3 µm latex beads were added for 4 h/-/+ 100 µM monensin for 1 h. The cells were fixed and stained for Atg16L1 and images were captured by confocal microscopy. Cropped images show bead phagosomes. The scale bar is 5 µm.

C. Quantification of B where cells had been treated with monensin. 100 bead containing phagosomes were counted per experiment, and of this, the number that were Atg16L1 and GFP-LC3 positive were recorded. n=3 the error bars represent the standard error of the mean (SEM), ***P<0.0003 (Unpaired Student’s t-test).
3.8 Recruitment of the WD40 Atg16L1 domain.

The data so far show the WD domain, and specific residues within the C-terminal domain, to be essential for Atg16L1 recruitment to endolysosomal membranes (FIGURE 3.11 AND 3.24). Therefore, it was interesting to look at whether the C-terminal domain alone was sufficient to localise to bead containing phagosomes. Using cells stably expressing a Flag-S-tag C-terminal WD domain of Atg16L1 (FIGURE 3.25 A), we were unable to detect any recruitment to bead containing phagosomes following monensin treatment (FIGURE 3.25 B) This leads to the conclusion that the C-terminal domain of Atg16L1 is necessary for recruitment in non-canonical autophagy, but is not sufficient.
Figure 3.25: WD domain of Atg16L1 is not sufficient to recruit to bead containing phagosomes.

A. HCT116 GFP-LC3 -/- Atg16L1 cells re-complemented with Flag-S tagged FL Atg16L1 or Atg16L1 WD C-terminal domain (CTD). Cells were lysed and a western blot was used to detect the S-tag on Atg16L1.

B. HCT116 GFP-LC3 Atg16L1 -/- cells re-complemented with Flag-S tagged FL Atg16L1 or Atg16L1 WD C-terminal domain (CTD) where 3 µm latex beads were added for 4 h +/- 100 µM monensin for 1 h. The cells were fixed and stained for the S-tag and images were captured by confocal microscopy. Cropped images show bead phagosomes. The scale bar is 5 µm.
3.9 Analysis of the T300A Atg16L1 Crohn’s disease mutant in non-canonical autophagy

The Atg16L1 T300A variant is associated with increased risk of Crohn’s disease [206], which falls close to the start of the WD domain (FIGURE 3.26A). The molecular mechanisms of how this variant is associated with pathogenesis remains unclear, and considering our data demonstrating the importance of this domain in non-canonical autophagy, it was assessed whether the T300A variant impacted the pathway.

HCT116 GFP-LC3 cells stably expressing the Atg16L1 T300A variant were used to compare with WT GFP-LC3 cells. Firstly, LC3 lipidation in response to starvation, bafilomycin and monensin was analysed by western blotting (FIGURE 3.26 B AND C). As described previously, starvation inactivates m-TOR and activates autophagy, bafilomycin blocks autophagic flux and monensin both blocks autophagic flux and induces single membrane LC3 lipidation. In WT cells and cells expressing the Atg16L1 T300A variant, there is a canonical autophagy response shown by the increase in LC3 lipidation upon starvation and upon blocking of the flux (FIGURE 3.26 B and C). When the cells were treated with monensin there was robust LC3 lipidation in both cell lines, beyond the LC3 lipidation detected from bafilomycin treatment, suggesting that both the WT T300 Atg16L1 and the T300A variant support LC3 lipidation to single membranes in non-canonical autophagy (FIGURE 3.26 B AND C). In order to visualise LC3 lipidation to non-canonical autophagy membranes, the cells were set up to engulf latex beads and then treated with monensin to provide the signal for LC3 lipidation to bead containing phagosomes. Both WT HCT116 cells and Atg16L1 T300A expressing cells support monensin induced LC3 lipidation to bead containing phagosomes to a similar extent (FIGURE 3.26 D AND E). In untreated cells there was a small difference in LC3 lipidation to bead containing phagosomes, however, the difference is not significant based on statistical unpaired Student’s t-test (FIGURE 3.26E). Both WT Atg16L1 and the T300A variant localised to bead phagosomes upon monensin treatment (FIGURE 3.26F).

Therefore, the T300A variant in Atg16L1, that is associated with Crohn’s disease, does not affect
localisation of Atg16L1 to single membrane endolysosomes or its ability to lipidate LC3 to these membranes upon monensin stimulation.

To complete these studies, the same cells were taken to assess whether LC3 lipidation in a physiological non-canonical autophagy setting might be affected by the Atg16L1 T300A variant. For this we used an entosis assay, where we image cells following a live cell engulfment [153]. Figure 3.27A shows an example of fluorescent time-lapse imaging of LC3 recruitment to the entotic vacuole housing a live engulfed cell. The number of entotic cell deaths were counted in wild type and T300A cells and the presence of LC3 re-localisation to the entotic vacuoles was quantified (FIGURE 3.27B). The T300A variant in Atg16L1 does not affect LC3 lipidation in entosis and the supports the conclusion that the T300A variant in Atg16L1 is not implicated in non-canonical autophagy.
Figure 3.26: Atg16L1 T300A variant in non-canonical autophagy.

A. Cartoon of Atg16L1 where the asterisk shows where the T300A mutant falls.
B. HCT116 GFP-LC3 cells expressing WT Atg16L1 or the T300A variant were starved or treated with 100 nM bafilomycin or 100 µM monensin for 1 h. Cells were lysed and a western blot was used to detect unlipidated and lipidated LC3 (LC3I or LC3II) and GAPDH was used as a loading control.
C. Quantification of the ratio of LC3II/I using ImageJ from B. This was done from n=2 and the treated samples were plotted as an increase from the untreated LC3II/I ratio.
D. HCT116 GFP-LC3 cells expressing WT Atg16L1 or the T300A variant where 3 µm latex beads were added for 4 h +/- 100 µM monensin for 1 h. The cells were fixed and stained for LAMP1 and images were captured by confocal microscopy. Asterisks mark the bead containing phagosomes and the arrows indicate GFP-LC3 positive bead containing phagosomes.
E. Quantification of D, 100 LAMP1 positive bead containing phagosomes were counted per experiment and the number of GFP-LC3 positive beads was recorded. n=3 the error bars represent the standard error of the mean (SEM), NS= not significant (Unpaired Student’s t-test).
F. HCT116 GFP-LC3 cells expressing WT Atg16L1 or the T300A variant where 3 µm latex beads were added for 4 h +/- 100 µM monensin for 1 h. The cells were fixed and stained for Atg16L1 and images were captured by confocal microscopy. Asterisks mark the bead containing phagosomes that have been cropped and zoomed. The arrow head indicates GFP-LC3 and Atg16L1 positive phagosomes.
Figure 3.27: Live entosis assay with WT and T300A Atg16L1GFP-LC3 expressing cells.

A. Representative stills from live cell imaging of entosis looking at GFP-LC3 fluorescence and DIC in HCT116 GFP-LC3 cells with WT Atg16L1 or stable expression of the T300A Atg16L1 variant. The asterisk marks the inner cell and the arrow head shows the GFP-LC3 on the entotic vacuole. The first panel shows the cell in cell that goes onto the middle panel where GFP-LC3 gets transiently recruited and in the final panel, after the LC3 has left, the inner cell dies.

B. Quantification of A from n=1 where videos were analysed and counted for entotic events and the percentage of these events that had transient LC3 recruitment was plotted on a graph.
3.10 Functional implications of Atg16L1 mediated non-canonical LC3 lipidation

The cellular system characterised so far defines a novel strategy to separate canonical autophagy from non-canonical autophagy through the dependence of the WD domain of Atg6L1 in LC3 lipidation to endolysosomal membranes. Therefore, this provides a tool to find novel non-canonical autophagy processes. This rationale led to a collaboration with Dr Rupert Beale’s group (University of Cambridge), who previously reported that Influenza A (IAV) infection leads to LC3 lipidation at the plasma membrane and perinuclear region, dependent on the viral proton channel protein M2 [87]. Interestingly, this LC3 lipidation is independent of FIP200, suggestive of an involvement of non-canonical autophagy [87]. Thus, using the cellular systems outlined so far it was tested whether Influenza activates non-canonical autophagy.

Our lab have previously implicated the importance of proton gradients in LC3 lipidation to single membranes induced by monensin and the VacA toxin [98]. Accordingly, one hypothesis is that the M2 proton channel offers a related mechanism that may induce non-canonical autophagy. However, there have been conflicting reports as to whether this LC3 lipidation depends on the proton channel activity of M2 [88, 224]. Therefore, the first experiments performed in Rupert Beale’s lab were to uncover further evidence for the involvement of M2 in Influenza A induced LC3 lipidation. This used the antiviral drug amantadine, which is a selective blocker of the M2 proton channel in sensitive Influenza strains such as Udorn (Mud), but not in a laboratory-adapted Influenza A virus (IAV) PR8 strain (amantadine resistant). Consistent with a role for the M2 protein, Beale and colleagues found that LC3 re-localisation in response to PR8 infection was not affected by amantadine, while it was blocked when using the sensitive Mud strain (FIGURE 3.28A).

Next HCT116 cells expressing various Atg16L1 mutants were tested for the effect of IAV infection on GFL-LC3 localisation. Interestingly, it was found that IAV induced LC3 re-localisation dependent on the WD domain of Atg16L1 and the key residue K490 (FIGURE 3.28B).
Therefore, the cellular system to separate canonical and non-canonical autophagy has uncovered that indeed IAV infection induces LC3 lipidation associated with non-canonical autophagy. This was further supported by western blot data. Cellular lysates were taken from the re-complemented HCT116 Atg16L1/- cells by Dr Rupert Beale, and the lysates and western blots were directly run as part of this project. The cells with the Atg16L1 C-terminal deletion induce less LC3 lipidation in response to IAV infection (FIGURE 3.28C). These data suggests there must be a role for non-canonical autophagy in the cellular response to IAV infection; this collaboration is ongoing to gain further insights into the mechanism and implications of these data.
Figure 3.28: Influenza A infection is dependent on the viral M2 protein and activates LC3 lipidation through a non-canonical autophagy pathway.

A. Confocal images of HCT116 GFP-LC3 cells infected at MOI 1 with IAV strains PR8 (amantadine resistant) or MUD (amantadine sensitive). Amantadine was added 3 h post infection. Samples were fixed at 16 h post infection and stained for M2 (red) and DAPI. Scale bar is 10 µm.

B. Confocal images of GFP-LC3 transduced WT or Atg16L1−/− HCT116 cells re-complemented with the indicated Atg16L1 mutants. Samples were fixed at 16 h post infection with IAV PR8 at MOI of 5 and stained for M2 (red). Scale bar is 20 µm.

C. Influenza-infected GFP-LC3 transduced WT or Atg16L1−/− HCT116 cells re-complemented with the indicated Atg16L1 mutants. Lysates were taken 16 h post infection with PR8 IAV and western blotting was used to detect unlipidated and lipidated LC3 (LC3I or LC3II). GAPDH was used as a loading control.

* A and B generated by Dr Rupert Beale et al and C was generated in collaboration.
3.11 Discussion

This chapter has outlined a novel mechanism where Atg16L1 recruits to single endolysosomal membranes to lipidate LC3 in non-canonical autophagy, following engulfment processes such as LAP, or driven by lysosomotropic drugs. This project shows that the C-terminal WD domain of Atg16L1 is necessary, but not sufficient, for targeting Atg16L1 to single membrane endolysosomes, where it forms the complex with Atg5-Atg12 to lipidate LC3. This chapter has further identified novel sites within the top face of the WD C-terminal domain required for the non-canonical activity of Atg16L1.

This chapter supports published data showing that the WD domain of Atg16L1 is not required for canonical autophagy [197] and the lack of the WD domain does not affect Atg16L1 ability to form the functional LC3 lipidation complex [209]. Interestingly, the WD domain of Atg16L1 is absent in yeast [25] and therefore it could be that higher eukaryotes have evolved to carry out non-canonical autophagy.

Data in this chapter also support published work on the FBD domain of Atg16L1, housing the FIP200 binding sites, that showed if this domain is deleted there is inefficient autophagy [32, 189]. Furthermore, this work has proven the FBD domain to be dispensable for LC3 lipidation in non-canonical autophagy, strengthening the existence of a distinct mechanism for Atg16L1 recruitment during non-canonical autophagy. WIPI2b, a fundamental regulator of Atg16L1 recruitment in canonical autophagy [33], was also shown to be absent from endolysosomal membranes. This is further supported by experiments that show that, unlike canonical autophagy, PI3P generation and VPS34 activity are not required for LC3 lipidation in non-canonical autophagy. These findings could appear contradictory to recent work showing that LAP is dependent on Rubicon-mediated VPS34 activity and PI3P generation on the phagosome membrane [120]. However, it is possible that in the context of phagocytosis, VPS34 and PI3P are required at an upstream step to mature the phagosome to a state competent for LC3 lipidation, without being directly involved in Atg16L1 recruitment. This idea is supported by the experiments.
using the ionophore monensin that show LC3 lipidation to be independent of VPS34 activity. This could be because the drug bypasses the PI3P dependent maturation of the single membrane, as monensin targets already mature endolysosome compartments for LC3 lipidation. Therefore, there may be a distinct signalling pathway to PI3P generation that is required more directly in Atg16L1 recruitment to endolysosomal membranes.

It is now accepted that there are non-autophagy roles for many autophagy proteins and that LC3 lipidation is involved in more than just autophagy. Furthermore, due to overlap of some autophagy proteins in both canonical and non-canonical autophagy pathways it is possible that some processes historically attributed to canonical autophagy may actually have more relevance to non-canonical autophagy. Therefore, the work in this chapter has led to a clear genetic system that can separate canonical from non-canonical autophagy that will help to uncover novel pathways implicated in non-canonical autophagy. This rationale has already uncovered that Influenza A activates LC3 lipidation through non-canonical autophagy, rather than activating LC3 lipidation to autophagosome.

This clear separation of autophagy from non-canonical autophagy using Atg16L1 is the first clean system to study one pathway without the other. Previous methods focused on comparing cells deficient in canonical autophagy (knockouts for ULK1 complex proteins) to cells deficient for both pathways (knockouts for Atg proteins in involved in LC3 lipidation) and inferring a role for non-canonical autophagy [94, 96]. Alternatively, in the context of phagocytosis, Rubicon has been used to separate canonical autophagy and LAP. This method focused on the different roles of Rubicon in the two distinct pathways: Rubicon is a negative regulator of autophagy but an essential component for LAP [120]. Thus, while knocking out Rubicon does provide a model deficient for LAP, it is not totally specific, as loss of Rubicon may have additional effects on endosomal maturation and canonical autophagy. In addition, Rubicon is linked to PI3P signalling and, as touched on before, this signalling is specific to LAP. The work in this project suggests there
must be distinct signals that are more directly involved in Atg16L1 recruitment to endolysosomal membranes and can be applied to all non-canonical autophagy pathways.

Therefore, this project offers an improved system where the C-terminal WD truncation or point mutations in ATG16L1 cause the cells to be deficient in non-canonical autophagy while remaining competent for canonical autophagy. Additional benefits are that Atg16L1 is a well-characterised autophagy protein and the C-terminal deletion of Atg16L1 affects all types of non-canonical autophagy processes including pharmacological and physiological processes.

The WD domain of Atg16L1 which is essential for LC3 lipidation in non-canonical autophagy, would be expected to be involved in protein-protein interactions, although between different WD domains there is not a huge amount of homology. The published structure and directed mutagenesis studies [192, 195] could help to unravel how the WD domain of Atg16L1 is acting in the recruitment of Atg16L1 to single endolysosomal membranes. Further work looking at Atg16L1 binding partners using the Atg16L1 truncation and point mutation is described in chapter 5.

It will be intriguing to uncover if there is one mechanism of recruitment of Atg16L1 through the WD domain or whether there are process-specific mechanisms. For instance could it be that the mechanism for Atg16L1 recruitment to phagosomes is different to its recruitment to entotic vacuoles. Currently, these data show that the sites identified in Atg16L1 are important in all tested examples of non-canonical autophagy suggestive of a common mechanism and the domain could act as a molecular hub for non-canonical autophagy processes.

It is interesting to note that the WD domain of Atg16L1 alone is not sufficient for its recruitment. This may not be surprising however, as Atg16L1 normally acts as a dimer and the WD domain alone lacks the dimerization motif. Experiments could be done in the future to force dimerization of the WD domain, through the use of a GST recombinant version of the protein, to test whether this enables the domain to recruit to single-membrane compartments. Further, the WD domain alone is unable to bind with ATG5, which has been shown to play a role in recruitment of the
Atg16L1 complex to autophagosomes [25]. Consistent with this, our preliminary work, using an Atg16L1 mutant that lacks the N-terminal domain and can no longer complex with Atg5-12, hints that the N-terminal domain of Atg16L1 is also essential for its recruitment to single-membrane compartments during non-canonical autophagy (data not shown). There also may be other essential N-terminal Atg16L1 binding motifs that assist Atg16L1 recruitment in non-canonical autophagy and therefore this needs to be considered in future work.

The WD domain of Atg16L1 has previously been implicated in the activation of an unconventional autophagy pathway through its interaction with TMEM59 and TMEM166/EVA1 [200, 202]. Both TMEM proteins have the same binding motif that interacts with the WD domain of Atg16L1 [202]. However, as yet, it is not clear whether these represent true non-canonical autophagy pathways that fit in with the data presented here. The TMEM59 dependent autophagy is implicated in the role of Atg16L1 in Crohn’s disease [214]. The Crohn’s disease-associated point mutation, T300A, is located near the C-terminal domain of ATG16L1 and has been implicated in affecting autophagy processes. One mechanism is that, under stress conditions, the T300A Atg16L1 variant is more susceptible to caspase 3 cleavage and therefore the cell has less functional Atg16L1 [209, 214]. The cleaved Atg16L1 product, that lacked the WD domain, decreased TMEM59 dependent autophagy that is reliant on the WD domain of Atg16L1 [214]. However, as part of this project replication of apoptosis induced caspase cleavage of Atg16L1 was performed in accordance to the methods in the paper by Lassen et al [209]. The controls showed that apoptosis had been induced and caspase 3 had been cleaved however, there was no detectable Atg16L1 cleavage (data not shown). Furthermore, this project showed that the Atg16L1 T300A variant had no significant effect on LC3 lipidation in non-canonical autophagy. These results support previously reported data where the T300A mutation was shown not to affect LAP [120].

The significance of LC3 lipidation, or lack of LC3 lipidation, in non-canonical autophagy has previously been looked at with regard to impaired degradation of phagosome content and the
homeostatic consequences of this [225]. This chapter further shows Influenza A infection to be involved in non-canonical autophagy, but how this signalling pathway is involved in the Influenza infection model or host defence is yet to be elucidated; the collaboration with Dr Rupert Beale is ongoing. The function of LC3 lipidation in non-canonical autophagy is explored further in following chapters and is the remit of future work.

This project has uncovered a powerful method to study the function of non-canonical autophagy without affecting canonical autophagy.
4 Results: *In vivo* functions of non-canonical autophagy: Atg16L1 mutant mouse models.

4.1 Introduction

4.1.1 Atg16L1 mouse models

The data presented so far show that the Atg16L1 WD domain, and specific sites within it, are indispensable for LC3 lipidation to endolysosomal membranes, but not required for canonical autophagy. As such, we have identified a genetic manipulation of Atg16L1 to separate LC3 lipidation in canonical and non-canonical autophagy processes.

In this chapter, this system is exploited to investigate the phenotypic consequences of inhibiting LC3 lipidation during non-canonical autophagy through *in vivo* studies, guided by the previous results. While the full extent of non-canonical autophagy function in cells is not completely understood, it has been implicated in a number of immune-related processes including pathogen clearance [109, 112] and presentation of exogenous antigens [112, 176]. Commonly this has been achieved by inhibiting the LC3 conjugation machinery, for instance knocking out Atg5 or Atg7. To begin to look at the functional consequence of impairing non-canonical autophagy more specifically, we have now developed studies of Atg16L1 mutant mice instead.

Firstly, in collaboration with Dr Tom Wileman’s lab, from the University of East Anglia, a mouse model expressing the Atg16L1 1-230 truncation (E230), in essence a WD domain deletion, has been produced and analysed. The Atg16L1 1-230 construct was tested *in vitro* and in primary cells from WT or E230 mice, to look at the role of non-canonical autophagy in MHC II antigen presentation.

Alongside this, using CRISPR/Cas9 technology, this project aimed to create new mouse models of Atg16L1 ΔWD and the Atg16L1 K490A variant, to complement the *in vitro* data shown in the previous chapter.
4.1.2 CRISPR technology and in vivo application

A huge advance in the field of genomics has come with the CRISPR Cas system, a highly efficient and precise technique, to target and edit mammalian genomic DNA, which has revolutionised the field of in vivo modelling, as editing the genome is now relatively fast and cheap. This technology came from the microbial adaptive immune response system that uses CRISPR to target and cleave foreign DNA.

The type II system is the best characterised and uses the Cas9 nuclease from *Streptococcus pyogenes*, this is the system used in this project. The Nature protocols publication by Ran et al was used to direct the design of this part of the project [226]. The Cas9 enzyme is targeted to DNA via a short approximately 20 nucleotide guide sequence (crRNA), and requires a repetitive scaffolding sequence called the trans-activating tracrRNA, that positions the Cas9 nuclease to the targeted region of DNA to be cleaved. These components, if altogether on a plasmid, are known collectively as the sgRNA. The individual components can also be added separately as an alternative method. The Cas9 nuclease cleavage occurs three nucleotides upstream of a protospacer adjacent motif (PAM) sequence, the PAM for Cas9 is 5’ –NGG. The DNA is left with a double strand break (DSB) and the cell’s default would be to repair the DSB via non-homologous end joining (NHEJ) however, this is error prone and undirected. Therefore, usually homology directed repair is utilised, this concept is summarised in the simplified cartoon (FIGURE 4.1). This is where a repair template is provided that has two homology arms either side of the DSB and then a sequence in between these arms that will direct the editing of the DNA.

This process of genome editing can be done in mouse embryonic stem cells to produce transgenic mice (FIGURE 4.2). The CRISPR/Cas9 reagents are transfected into the ES cells and these cells are then diluted to single cells, where the colonies are eventually screened for edited DNA. The selected ES cells are then injected into mice blastocysts resulting in a mix of cells with WT DNA and cells with the edited DNA. This gives rise to chimeric pups where hopefully the germline cells have edited DNA and therefore can breed to produce heterozygote mice (FIGURE 4.2). This
process involves a long screening step and success of germline transmission at the chimera stage can require multiple attempts [227]. It is estimated 12 months to produce a transgenic mouse using ES cell editing [227]. An alternative and quicker method, estimated closer to 7 months, is taking the CRISPR/Cas9 reagents and injecting them into the mouse zygote, this can directly produce offspring with edited DNA (FIGURE 4.2) [227]. Both methods were used as part of this project and this work was done with close supervision of Dr Dominik Spensberger (who at the time was Head of the Gene Targeting facility at The Babraham Institute).
Figure 4.1: Schematic to summarise the use of CRISPR/CAS9 to mutate Atg16L1.

Cartoon to show how the nuclease Cas9 was targeted to Atg16L1 to introduce a double strand break (DSB). The targeting was achieved through a specific guide sequence that targets just upstream of the protospacer adjacent motif (PAM). The DSB is then repaired using donor mediated homologous recombination. In this diagram, the red box is depicting the added DNA sequence that in this case is a termination sequence, when the sequence is transcribed and translated, a truncated Atg16L1 protein will be produced. This same principal was used for producing the Atg16L1 with a single point mutation.
Figure 4.2: Gene editing workflow in mouse ES cells compared to mouse zygotes.

Adapted from [227]: Two methods for producing transgenic mice using CRISPR/Cas9. This can be done in ES cells where the sgRNA is transfected into the mouse ES cells and the transfected cells are selected. The selected cells are then diluted into single cells and the colonies are expanded and screened for edited DNA. Edited ES cells are then injected into a blastocyst to get a mix of WT and edited DNA. The blastocyst is then transferred into a pseudo pregnant mouse from this chimeric mice are born, this is the founder animal. The chimeras will have varying degrees of cells with edited DNA and the edited DNA needs to be present in the germline to be passed on. The chimeras are bred and if there is germline transmission heterozygote mice are produced and these can be bred to get homozygote mice. Alternatively CRISPR reagents can be injected into the mouse zygote and the zygote can be transferred to a pseudo pregnant mouse and from this a heterozygote or homozygous pup for the DNA could be born. There is still a chance that not all of the cells in the offspring will have the edited DNA this is what mosaic means. Therefore, founder mice must be bred to get heterozygotes and then bred again to get homozygotes.
4.2 Characterising Atg16L1 1-230 mouse model from collaborators (Tom Wileman University of East Anglia).

4.2.1 Atg16L1 1-230 deletion

Firstly, it is important to note, that the Atg16L1 1-230 (E230) construct from Dr Tom Wileman is a larger C-terminal deletion than the Atg16L1 1-336 ΔWD that has featured in the project so far. The cartoon domain structure of Atg16L1 depicts how much of Atg16L1 has been deleted in the 1-230 construct compared to the FL Atg16L1 (FIGURE 4.3A), where the ΔWD Atg16L1 mutation is 1-336 that we have characterised so far. The larger Atg16L1 E230 C-terminal deletion removes the middle region of Atg16L1, which could therefore also interfere with normal autophagy responses in cells. Nevertheless, whilst waiting for the generation of mouse models for Atg16L1 1-336 and Atg16L1 K490A that are more refined, this was a good model to test whether the E230 mice were deficient for LC3 lipidation associated with non-canonical autophagy and to develop assays.

4.2.2 Atg16L1 1-230: in vitro validation

The Atg16L1 E230 construct was transiently transfected into HCT116 GFP-LC3 Atg16L1 -/- cells in order to test the function of this truncated Atg16L1 in vitro. FIGURE 4.3B shows the successful transfection of Atg16L1 FL or 1-230 protein at similar levels. The transfected HCT116 cells were treated with bafilomycin, to inhibit autophagic flux, and with monensin, to inhibit autophagic flux but also lipidate LC3 to swollen lysosomal compartments. Western blot analysis from three independent experiments showed that the cells expressing the Atg16L1 1-230 construct support LC3 lipidation in response to bafilomycin but there is no additional LC3 lipidation after treatment with monensin (FIGURE 3.4C), indicating effective canonical autophagy but defective non-canonical autophagy.

Transiently transfected cells were also analysed by microscopy to look at the LC3 lipidation response as result of monensin treatment. The cells expressing the FL Atg16L1 have GFP-LC3 localisation to swollen lysosomal compartments (FIGURE 4.3D). However, in the cells expressing the Atg16L1 E230 construct the cells were only able to form GFP-LC3 puncta, suggesting they still
support some canonical autophagy but the GFP-LC3 was not forming larger ring like structures indicative of LC3 lipidation to swollen endolysosomal compartments (FIGURE 4.3D). Further analysis of primary cells from E230 animals (FIGURE 4.4B), including work done in Dr T. Wileman’s lab, are consistent with these data that the Atg16L1 230 still supports canonical autophagy.

Further evidence supporting the lack of non-canonical autophagy in E230 Atg16L1 expressing cells is shown using the latex bead assay and monensin to stimulate LC3 lipidation to bead containing phagosomes (FIGURE 4.3E). The cells expressing the FL of Atg16L1 supported monensin induced LC3 lipidation however, there was no monensin induced LC3 lipidation to beads in cells re-expressing E230 Atg16L1 (FIGURE 4.3E). Therefore, just like the Atg16L1 ΔWD characterised in the previous chapter, the Atg16L1 1-230 truncation does not support non-canonical autophagy. The 1-230 Atg16L1 truncation also seems to support some canonical autophagy, which is supported by results from the collaborating lab (data not shown).
Figure 4.3: In vitro characterisation of Atg16L1 1-230, C-terminal deletion.

A. Cartoon of Atg16L1 domain structure showing the full length (FL) structure compared to the Atg16L1 1-230 truncation.

B. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected using Lipofectamine to express FL Atg16L1 or Atg16L1 1-230 C-terminal deletion, cells were lysed and a western blot was performed to analyse the expression of Atg16L1.

C. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected using Lipofectamine to express FL Atg16L1 or Atg16L1 1-230 C-terminal deletion were treated -/+ 100 nM bafilomycin or 100 µM monensin for 1 h. Cells were lysed and a western blot was done to analyse unlipidated and lipidated LC3 (LC3I and LC3II). GAPDH was used as a loading control.

D. HCT116 GFP-LC3 Atg16L1 -/- cells transiently transfected using Lipofectamine to express FL Atg16L1 or Atg16L1 1-230 C-terminal deletion -/+ 100 µM monensin for 1 h and then fixed and imaged via confocal microscopy to look at GFP-LC3. Zoomed images represent the GFP-LC3 localisation upon monensin treatment. The scale bar is 5 µm.

E. Transient transfection of HCT116 GFP-LC3 Atg16L1 -/- cells with the FL Atg16L1 or the 1-230 construct. Cells engulfed 3 µm beads for 4 h -/+ 100 µM monensin for 1 h and then the cells were fixed and stained for LAMP1. Cells were imaged via confocal microscopy to look at GFP-LC3 and LAMP1.

*Atg16L1 1-230 constructs were provided by Dr Tom Wileman.
4.3 Using Atg16L1 1-230 to investigate non-canonical autophagy phenotypes.

4.3.1 Atg16L1 E230 mice: LC3 lipidation and LAP

The Wileman lab produced homozygote Atg16L1 E230 mice, which were viable and had no apparent phenotype in terms of their appearance compared to the WT mice. To explore non-canonical autophagy in these mice, LC3 lipidation and LAP were examined in bone marrow derived dendritic cells (BMDCs) (FIGURE 4.4C). Western blotting confirmed the expression of the truncated form of Atg16L1 in cells from the E230 mice (FIGURE 4.4A). The BMDCs were first tested for their response to canonical autophagy stimulation, by using PP242, or for non-canonical autophagy, by monensin treatment (FIGURE 4.4B). BMDCs from both WT and E230 mice respond to canonical autophagy, PP242 has induced LC3 lipidation (FIGURE 4.4B). However, in the E230 BMDCs there is no longer a robust response of LC3 lipidation after monensin treatment (FIGURE 4.4B), consistent with a defect in non-canonical autophagy. The BMDCs were further tested by immunofluorescence to look at LC3 lipidation to zymozan containing phagosomes (FIGURE 4.4C). It was found that BMDCs from the WT mice supported LAP, evidenced by LC3 positive zymozan containing phagosomes, however, the BMDCs from the E230 mice were deficient for LAP (FIGURE 4.4C). This is an important observation in which a novel mouse model is described which is specifically deficient in non-canonical autophagy.

4.3.2 Atg16L1 E230 mice: antigen presentation

BMDCs are professional antigen presenting cells. Autophagy proteins have previously been implicated in antigen presentation but the specific role of non-canonical autophagy is unclear. The E230 Atg16L1 in vivo system offered a way to address this question. Isolated mouse BMDCs were used to look at the presentation of exogenous antigen on MHC class II. This was done with guidance and materials from Dr Michelle Linterman’s lab, and experiments and analysis were done together with Dr Elise Jacquin, a former Post Doc from the lab. BMDCs were incubated with the GFP-\(\alpha\) peptide, a fluorescent antigen (kindly donated by Dr Michelle Linterman), for 24 h. In
this time antigen is taken up by cells and processed for loading on MHC II for subsequent
presentation at the cell surface. Cells were then prepared for analysis by flow cytometry. Single
cell dendritic populations were gated by CD11c and CD11b positivity (FIGURE 4.5A). An antibody
that detects the Eα peptide in complex with MHC II, YAe, was used to measure presentation
within this population (FIGURE 4.5B) [228]. Strikingly, while WT BMDCs showed a dose dependent
increase in antigen presentation, there was a significant defect in E230 BMDCs (FIGURE 4.5B).
This defect was not due to a failure in uptake of the Eα peptide, as both WT and E230 cells
displayed similar GFP signal (FIGURE 4.5C). Indeed, E230 BMDCs displayed increased GFP signal
compared to WT cells, which could be explained by the fact the antigens are not being processed
as efficiently in these cells and therefore have higher retention of the GFP signal (FIGURE 4.5C).
These data demonstrate a functional consequence of inhibiting non-canonical autophagy through
the targeting of the ATG16L1 WD C-terminal domain. It verifies a role for non-canonical
autophagy in antigen presentation and provides a clean system with which to study this process.
In the future these experiments will be repeated with the other refined mouse models expressing
Atg16L1 1-336 ΔWD or the Atg16L1 point mutant K490A.
Figure 4.4: BMDCs from WT or E230 Atg16L1 mice, expression and LAP.

A. Cell lysates were taken from WT and E230 Atg16L1 Bone marrow derived dendritic cells (BMDCs) and western blots were performed to detect Atg16L1. Arrows indicate Atg16L1, and asterisks mark non-specific band.

B. Cell lysates were taken from WT and E230 Atg16L1 BMDCs +/- 1 µM m-TOR inhibitor PP242 or 100 µM monensin for 1 h. Western blots were done to analyse unlipidated and lipidated LC3 (LC3I and LC3II). GAPDH was used as a loading control, n=2.

C. WT or E230 Atg16L1 BMDCs were left to engulf zymozan for 15 min and then the cells were fixed and stained for LC3 and DAPI. Confocal images were captured and the zoomed insert is a zymozan containing phagosome. Scale bar: 20 µm.
**Figure 4.5: The C-terminal domain of Atg16L1 is essential for exogenous antigen presentation.**

A. Gating strategy for WT and E230 Atg16L1 bone marrow derived dendritic cells (BMDCs). The first plot is the forward and side scatter in order to gate on the cellular population, this excludes any debris. Then the side scatter and width of particles was plotted in order to gate just to obtain data from single cells. Finally gating was done to select the double positive population expressing CD11b and CD11c to carry out the exogenous antigen presentation analysis just on the BMDC population.

B. Mean fluorescent intensity (MFI) flow cytometry analysis of Y-Ae an antibody that recognises MHC II in complex with the Ea-GFP antigen in wild-type (open circles) and E230 (filled circles) BMDCs exposed to different concentrations of Ea-GFP. Data are presented as mean ± SD from four replicates. Data are representative of three independent experiments **P < 0.002.

C. Mean fluorescent intensity (MFI) flow cytometry analysis of Ea-GFP in wild-type (open circles) and E230 (filled circles) BMDCs exposed to different concentrations of Ea-GFP. Data are presented as mean ± SD from four replicates. Data are representative of three independent experiments ***P < 0.0001 (Student’s t-test).

*Reagents and guidance from Dr Michelle Linterman. Experiments done together with Dr Elise Jacquin. Analysis performed by Dr Elise Jacquin.
4.4 Using CRISPR/Cas9 to generate an Atg16L1 1-336 mouse model.

CRISPR/CAS9 technology was exploited to generate a mouse model of the Atg16L1 1-336 C-terminal WD domain deletion that matches the characterised Atg16L1 in vitro system described in the previous chapter. The diagram in the introduction to this chapter has a cartoon overview of how the CRISPR/Cas9 system was utilised (FIGURE 4.1).

4.4.1 CRISPR Cas9 guides

First of all, using the mouse sequence of Atg16L1, two guide sequences were designed using the online tool DNA 2.0 (now ATUM). These guide DNA sequences were cloned into a GFP Cas9 vector, PX458, that encodes the Cas9 and the sequence that allows the guide and enzyme to complex to target to the DNA. The plasmid was transfected into mouse ES cells, the cells were then sorted by flow cytometry (with assistance from the Flow Cytometry facility at The Babraham Institute) based on their GFP expression.

4.4.2 Surveyor assay

DNA was isolated from the sorted population of cells to check the efficiency of the targeting and cutting of Cas9 by the chosen guides. This was done using a surveyor assay. FIGURE 4.6 describes the principle of this assay.

Both guide sequences specifically target Cas9 to nick the DNA just after exon 10 of Atg16L1 (FIGURE 4.2 and 4.7). The DNA cleavage products were separated at the expected size on an agarose gel showing both guides targeted the Cas9 to the precise location on the DNA to cause a DSB (FIGURE 4.7). There was roughly 40% cutting efficiency, where the intensity of the uncleaved WT band was compared to the intensity of the bands of the cleaved products (analysis not shown from FIGURE 4.7). Guide sequence two was selected, as it was the better sequence to design the donor template to repair the DNA double strand break.
Figure 4.6: Surveyor Assay from IDT to test efficiency of guide targeted Cas9 DNA cutting.

Adapted from https://thegenetherapynotes.wordpress.com/2016/02/15/surveyor-nuclease/, a schematic to explain how the surveyor assay works. Where cells transfected with the Cas9 and guide sequence will have a mixed population of WT DNA and DNA that has been cleaved by Cas9. Then the cell will have randomly repaired the double strand break to form an indel. DNA was extracted from the cells and PCR amplified the region of interest. The DNA was de-natured and re-hybridised to get a mixture of WT DNA and mismatched DNA, the mismatched DNA was cleaved by the surveyor nuclease and the DNA could then be analysed on an agarose gel to work out how efficiently and precisely the guide was targeting the Cas9 enzyme.
Figure 4.7: Validating the guide sequence to target Atg16L1 to produce a WD domain deletion.

This figure outlines the two guide sequences highlighted in light blue and the red is the proto spacer motif (PAM) –NGG, this should direct the Cas9 to form a DSB in the DNA, 3 nucleotides from the PAM. The guides were tested using a surveyor assay as described in the previous figure and in the methods. The arrows indicate the nuclease cleaved DNA that correspond to the expected band sizes outlined to the side of the agarose gel picture.
4.4.3 Repair

The repair donor sequence was designed based on the theory of homology directed repair, where the Cas9 induced double strand break is repaired through homologous recombination. The donor repair sequence has homology to the sequence either side of the edit site, but it also has additional nucleotides for the addition of stop codons and a PolyA tail (FIGURE 4.8). This should result in a genome edit that should encode Atg16L1 1-336 ΔWD.

4.4.4 Zygote injection

The donor repair sequence was ordered as PAGE purified single stranded (ss) DNA. The guide sequence was also ordered in ssRNA format alongside a TracrRNA sequence that is responsible for forming the secondary structure needed for the guide sequence and Cas9 to form a complex to target the DNA. Dr Dominik Spensberger injected these reagents alongside recombinant Cas9 protein into mouse zygotes to directly edit mouse genomic DNA in the zygote (FIGURE 4.2). 33 pups were born following these injections.

4.4.5 Genotyping

Ear clips were taken by staff at the BSU (Biological support unit, Babraham Institute) and then the DNA was isolated to genotype the pups. PCR was done to amplify the region of the Atg16L1 gene that was targeted for editing. FIGURE 4.8 shows the primers that were designed and the potentially inserted sequence that would be present in genome edited pups. The edited DNA sequence should have inserted an extra Bfa1 restriction site, therefore the amplified DNA was taken and a digest was done using Bfa1 (FIGURE 4.8 AND 4.9). Through this method all pups looked WT, this was confirmed by sequencing (FIGURE 4.9). The mouse zygote injections using CRISPR/Cas9 was a relatively new technology to the Institute at this time and therefore it was advised by Dr Dominik Spensberger to switch to using the CRISPR technology to edit mouse ES cells (FIGURE 4.2).
Figure 4.8: Original sequence and genotyping strategy for Atg16L1 truncated DNA.

Nucleotide sequence of the Atg16L1 targeted region, showing the forward and reverse primers used to amplify the region of interest. The sequence in red is the “edited” inserted stop codons and poly A. The blue italics shows where the Bfa1 restriction enzyme will cut, where the edited sequence gives an extra cut site. Below is a schematic of the sequence where the red box is the inserted stop sequence and the table describes the size of the products expected for genotyping.
Figure 4.9: Original genotyping of pups from the direct zygote injections for edited Atg16L1 sequence.

A. DNA was isolated from 33 ear clips and PCR was used to amplify the DNA around the targeted site of Atg16L1 where WT DNA would give a product of 344 base pairs (bp) and edited DNA would give a product of 362 bp. The first lane is a negative control (PCR without DNA) and WT is DNA from WT mouse genomic DNA. 16-23 are representative of all PCR product results when run on a 2% agarose gel.

B. The PCR product from A was PCR purified and a DNA digest was set up with or without the restriction enzyme Bfa1. The WT PCR product should be cut to give a 315bp product and 2x < 15 bp fragments. The edited Atg16L1 PCR product should give products at 232 bp and 101 bp.
4.4.6 Alternative strategy: injection of mouse ES cells

The project switched to using CRISPR Cas9 technology in mouse ES cells to be able to screen for “edited” cells to then implant into a mouse blastocyst. The design for the edited sequence was also slightly different, switching to inserting a triple stop codon and creating a new restriction site for Bcl1, specific to the edited DNA sequence (FIGURE 4.10).

Dr Dominik Spensberger transfected mouse ES cells with a CD4 expressing plasmid also containing the same guide sequence and scaffolding sequence, as previously, alongside the newly designed repair donor DNA. The transfected population was selected for using CD4 as a marker for positively transfected ES cells. He then took the transfected population and did a single cell dilution and expanded these colonies on a 96 well format, with 2 x 96 well plates of ES cells originating from single cells. The cells were returned as part of this project to be screened for edited DNA.

The DNA was extracted and PCR was used to screen the DNA, where a PCR product was only produced if the genomic DNA from the ES cell colony had the edited Atg16L1 ΔWD, by designing the reverse primer to bind within the edited sequence (FIGURE 4.10). A reverse primer downstream of the edited region was also used to verify that the sequence downstream of the edit was unchanged (FIGURE 4.10). FIGURE 4.11 shows that one 96-well plate was screened in this way and had multiple positive ES cell colonies for the DNA edit. Therefore, the second 96 well plate was not screened. Furthermore, at random some of these were validated using a reverse primer that binds outside of the edited region of DNA (figure 4.11B). The colonies with the edited genomic DNA were selected based on experience of how healthy the cells looked, the asterisks mark the ES cell colonies taken forward (FIGURE 4.11A).

The selected clones were taken and a PCR was performed to check the DNA downstream of the edit (FIGURE 4.12A). Also, a Bcl1 digestion was done, where the PCR product with edited DNA will be cleaved but the WT DNA will not have this restriction site and will be uncut (FIGURE 4.12B).
Therefore, on an agarose gel it was possible to make a comment on whether the cells were heterozygous or homozygous for the edit. WT DNA is uncut and there is only one band (FIGURE 4.12B). DNA from ES cell colonies C7 and D6, look homozygous for the edit because the gel shows two bands, indicative of the PCR product being cleaved (FIGURE 4.12B). There is a faint shadow of uncleaved DNA which could be due to incomplete cutting of the PCR product by Bcl1. PCR amplified DNA from ES cell colonies C9, D3, D5 upon Bcl1 addition give three bands on a gel suggesting the genetic edit has only occurred in one allele (FIGURE 4.12B).

The DNA from these selected ES cell colonies was sequenced to confirm that random mutations had not occurred and to give a clearer answer on whether the edits were homozygous or heterozygous (FIGURE 4.12C). The results matched the restriction digest results, except for D3; this could be explained by inefficient cutting of the PCR product in this tube. The sequencing confirmed that there were ES cell clones with the Atg16L1 ΔWD genetic sequence to take forward for implantation.
Figure 4.10: Re-designed strategy to insert a triple stop codon to form Atg16L1 WD deletion and genotyping strategy.

Nucleotide sequence of Atg16L1 targeted region, showing the forward and reverse primers. Two reverse primers were designed, one for screening where only edited DNA will give a PCR product, and another for validating the edit to check nothing has changed downstream of the edit. The sequence in red is the “edited” inserted triple stop codon. The blue italics shows where the Bcl1 restriction site has been introduced if the DNA is edited. Below is a schematic where the red box is the inserted triple stop codon and the table describes the outcome of genotyping PCRs.
Figure 4.11: ES cell colonies screened by PCR for Atg16L1 edited DNA and validation PCR.

A. DNA was isolated from 96 ES cell colonies originating from single cells. The PCR was performed where the reverse primer had been designed to anneal if a stop codon had been inserted into the specific site of the Atg16L1 gene. Therefore, a PCR product was detected on an agarose gel at 255 base pairs (bp) only if the DNA from the ES cell colony had been edited. The red asterisk marks the ES cell colonies.

B. The screening PCR results shown above, were validated by performing another PCR on some of the DNA isolated from ES cell colonies D3-12. This used primers shown in the previous figure that amplify DNA around the edited site, both WT and edited DNA will give a DNA product, only differing a few base pairs in length.
Figure 4.12: Identified ES cell colonies positive for edited DNA, validation and sequencing.

A. PCR to show that selected ES cell colonies still give a PCR product at the expected length with a forward and reverse primer either side of the edited region. Where – was a PCR reaction without DNA.

B. The PCR product from A was PCR purified and a restriction digest was performed with the enzyme Bcl1. The DNA was then run on an agarose gel for analysis by size separation. The table shows the expected outcomes.

C. The PCR products from A were sent off for Sanger sequencing and the table summarises whether the amplified DNA is representative of the WT Atg16L1 sequence or homozygous or heterozygous for the edit.
4.4.7 Alternative strategy: functional validation of edited ES cells

Before, the ES cell colonies were implanted into pseudo pregnant mice, some of the ES cells were taken to verify Atg16L1 protein expression and function (FIGURE 4.13). Firstly, a western blot was performed to look at Atg16L1 protein expression. The Atg16L1 antibody has non-specific bands as indicated (FIGURE 4.13A), but it was apparent that there was no WT or ΔWD Atg16L1 being expressed in ES cell colonies, despite them having the genetic sequence to encode ΔWD Atg16L1 (FIGURE 4.13A). This unexpected result was further confirmed by a western blot looking at LC3 lipidation in response to starvation, LC3 lipidation has been induced by starvation in WT ES cells, however, this is not evident in the cells with the DNA edit (FIGURE 4.13B).

Taken together, these data suggest that Atg16L1 has been knocked out, due to the lack of protein detected by western blot and therefore the function of LC3 lipidation is not observed. Immunofluorescence experiments looking at endogenous LC3 and LAMP1 in response to starvation or monensin show that the WT ES cells respond to both canonical and non-canonical autophagy (FIGURE 4.13C). However, edited ES cells have no LC3 puncta in response to any of these signals, reminiscent of Atg16L1 knockout cells (FIGURE 4.13C). The unintended knockout of Atg16L1 in cells targeted for ‘ΔWD’ was assumed to be because of nonsense-mediated decay, where the m-RNA from the edited genomic DNA was recognised by the cell as an error due to an introduction of a premature stop codon.
Figure 4.13: Atg16L1 expression and function in WT and edited ES cells.

A. WT mouse embryonic stem (ES) cells or ES cells positive for a ΔWD Atg16L1 DNA sequence (C7-D6) were lysed and analysed by western blot to look at the expression of Atg16L1. The black asterisk are non-specific bands and the white asterisk marks the only specific Atg16L1 band.

B. WT mouse embryonic stem (ES) cells or ES cells homozygous for the ΔWD Atg16L1 DNA sequence -/+ starvation 1 h. The cells were then lysed and a western blot was run to detect the levels of un-lipidated and lipidated LC3 (LC3I or LC3II). GAPDH was used as a loading control.

C. WT mouse embryonic stem (ES) cells or ES cells homozygous for the ΔWD Atg16L1 DNA sequence -/+ starvation or 100 µM monensin for 1 h. Cells were fixed and stained for LC3 and LAMP1 and images were captured by confocal microscopy. The scale bar is 5 µm.
4.4.8  Troubleshooting of ES cell editing: revised repair template

Re-design of the repair template was carried out in order to introduce a larger, more physiological SV40 termination sequence that the cell should not recognise as a mistake (FIGURE 4.14). The re-designed donor repair DNA, with the SV40 stop poly A sequence, had two homology arms homologous to Atg16L1 either side of the targeted site. This was made by doing three PCRs, getting the homologous sequences using mouse ES cell genomic DNA as a template and the third PCR was using c1-eGFP plasmid as a template to amplify the SV40 termination sequence.

With the new repair template, and the same reagents as previously, the ES cell transfection was repeated by Dr Dominik Spensberger with the CRISPR reagents. The single cell dilution was done to give two 96 well plates of colonies where a similar screening approach was taken. A reverse primer for PCR was designed complementary to the DNA edit (FIGURE 4.14), this screening was again successful at a reasonable efficiency for DNA editing (FIGURE 4.15). PCR was also done with genotyping primers as outlined in (FIGURE 4.16A) to determine if the edit was heterozygote or homozygote. The figure shows the outcome of this, there was a mixture of homozygote and heterozygote edits and this was confirmed by sequencing (FIGURE 4.16). Sequencing confirmed edited DNA where there has been successful insertion of the SV40 sequence at the correct site to encode Atg16L1 ΔWD.
Figure 4.14: Re-design of strategy for Atg16L1 truncation and outline of the screening and genotyping strategy.

Nucleotide sequence of Atg16L1 targeted region, showing two sets of primers, where the screening primers will be used to where only edited DNA will give a PCR product, and another for genotyping to look at whether the edit has occurred in both alleles. The sequence in red is the “edited” inserted SV40 termination sequence. Below is a schematic of a region of the Atg16L1 gene where the yellow box is the inserted SV40 sequence and the table describes the expected size of the PCR products with the various primers.
Figure 4.15: Screening DNA from ES cell colonies by PCR where one primer is complementary to the predicted edited sequence.

DNA was isolated from 192 ES cell colonies originating from single cells. The PCR was performed where the reverse primer had been designed to anneal if a stop codon had been inserted into the specific site of the Atg16L1 gene. Therefore, a PCR product was detected on an agarose gel at 801 base pairs (bp) only if the DNA from the ES cell colony had been edited. The red asterisk marks the ES cell colonies taken forward for further analysis, where H12 has been selected as a WT.
Figure 4.16: Edited ES cells taken forward for sequencing and validation.

A. PCR was performed using DNA from the selected ES cell colonies and using the genotyping primers. The – sample is where a PCR was set up without DNA and H12 was used as a WT control. The table shows the expected PCR product size, PCR products were run on an agarose gel for size separation where a 100 bp ladder was used as a reference.

B. PCR products were also sent for Sanger sequencing where the table summarises these results.

C. Example of sequencing trace to show successful edited DNA sequence introducing the SV40 termination sequence compared with the WT sequence.
The selected ES cells were taken to look at Atg16L1 protein expression and functional analysis of LC3 lipidation by western blot and imaging (FIGURE 4.17). This time it was evident that there was Atg16L1ΔWD protein expression as well as some full length Atg16L1 protein expression (FIGURE 4.17A). This was confirmed by immunofluorescence where the WT ES cells responded to starvation through an increase in LC3 puncta and monensin through colocalisation of LC3 with LAMP1 endolysosomal compartments (FIGURE 4.17B). However, an edited ES cell colony that was homozygote for the DNA sequence, that encodes Atg16L1 ΔWD, responded to starvation but no longer supported non-canonical autophagy (FIGURE 4.17B). This is the first time genome editing to produce ΔWD Atg6L1 in mouse ES cells confirms the in vitro system from Chapter 3, where the WD domain is dispensable for canonical autophagy but essential for LC3 lipidation to endolysosomal membranes.
Figure 4.17: Protein expression and function of Atg16L1 in WT and ΔWD edited ES cells.

A. WT mouse embryonic stem (ES) cells or ES cells positive for a ΔWD Atg16L1 DNA sequence were lysed and analysed by western blot to look at the expression of Atg16L1. The black asterisk mark non-specific bands and the arrow indicates specific Atg16L1 bands.

B. WT mouse embryonic stem (ES) cells or ES cells homozygous for the ΔWD Atg16L1 DNA sequence -/+ starvation or 100 µM monensin for 1 h. Cells were fixed and stained for LC3 and LAMP1 and images were captured by confocal microscopy.
4.4.9 Injection of optimised mouse ES cells

Dominik Spensberger injected edited ES cell colony plate 2 H8 into mice blastocysts and chimeric mice were born and genotyped (FIGURE 4.18A). These male chimeras were then bred with WT female mice. Unfortunately, this was unsuccessful as no germline transmission was evident and all pups were WT (FIGURE 4.18B).

Another edited ES cell colony was selected, plate 2 D4, and injected into mice blastocysts and the rate and degree of chimerism was much stronger than previously (FIGURE 4.18C). Chimeric males were bred with females, with a successful germline transmission. Heterozygous pups were confirmed by Transnetyx genotyping (FIGURE 4.18D) and further breeding rounds led to confirmed homozygote mice for the WD C-terminal deletion of Atg16L1 (FIGURE 4.18E).

Therefore, this project has successfully developed an in vivo mouse model for Atg16L1 ΔWD that will be an invaluable tool to observe the effects of specifically inhibiting non-canonical autophagy in a living system.
Figure 4.18: Generation of chimeric mice after edited ES cell injection and genotyping of subsequent breeding rounds.

A. PCR using DNA extracted from ear clips from pups born from H8 ES cell injection. The table summarises the expected PCR product sizes and the PCR products were run on an agarose gel for size separation. Pups were chimeric if there was evidence of both WT PCR product and PCR products from the edited DNA sequence. A negative control was set up, where no DNA was present in the PCR reaction and a positive control was using edited ES cell DNA from the injected colony.

B. A series of pups were born from breeding WT mice with the identified male chimeras from A. Ear clips from these offspring were used to isolate DNA and PCR was performed using the genotyping primers to assess whether there had been a germline transmission and production of heterozygote pups, the expected PCR sizes are the same as in A and were separated on an agarose gel by size. A negative control was set up, where no DNA was present in the PCR reaction and a positive control was using edited ES cell DNA from the injected colony. This is a representative image of multiple negative genotyping results.

C. PCR using DNA extracted from ear clips from pups born from D4 ES cell injection where the genotyping experiment was the same as A.

D. A series of pups were born from breeding WT mice with the identified male chimeras from C. Ear clips from these offspring were sent off to Transnetyx an external genotyping company. They do reverse PCR based on given information where they have designed two probes ones that will give a product with WT DNA and another that will give a product for edited DNA. The red asterisk marks examples of heterozygote pups.

E. A series of pups were born from breeding heterozygote mice. Ear clips from these offspring were sent off to Transnetyx, the same as D. The red asterisk marks examples of homozygote pups that have the C-terminal WD deletion in Atg16L1.
4.5 Using CRISPR/Cas9 to generate an Atg16L1 K490A mouse model.

The technology and capability within the Institute to do direct injection of mice zygotes with CRISPR reagents improved during the course of this project. Therefore, this method was used to generate a refined mouse model of Atg16L1 with a point mutation in the WD C-terminal domain, Atg16L1 K490A. This was identified in the previous chapter to act in the same way as deleting the WD domain of Atg16L1. Reagents were designed to produce transgenic mice expressing Atg16L1 K490A a single point mutation in the WD domain.

A guide sequence was designed and tested for the precision and cutting efficiency of the Cas9, via a similar method as previously (FIGURE 4.6). FIGURE 4.19 shows that the guide cuts efficiently. Therefore a repair donor template was designed to introduce the point mutation and mutate the PAM sequence to stop re-targeting of the Cas9 complex to already edited DNA (FIGURE 4.20).
Figure 4.19: Surveyor assay to test the guide efficiency to produce a double strand break in a specific region of Atg16L1, to produce DNA to encode Atg16L1 K490A.

This figure shows the results of a surveyor assay (see methods and Figure 4.6) for a guide sequence designed to target the Atg16L1 gene to cause a double strand break that will be targeted to repair to produce the sequence to encode Atg16L1 K490A. The arrows indicate the nuclease cleaved DNA that correspond to the expected band sizes outlined to the side of the agarose gel picture.
**Figure 4.20: Summary of strategy to direct K490A point mutation in the genomic sequence of Atg16L1**

This figure shows the nucleotide sequence of a region of the Atg16L1 gene that is targeted for editing. The repair donor is introducing nucleotide changes that will cause an amino acid change from lysine (K) to alanine (A). There are two silent mutations that will stop the CAS9 from re-targeting to edited DNA, shown just upstream of the protospacer adjacent motif PAM sequence where the Cas9 will target to the DNA to cause a double strand break ~3 nucleotides away from the PAM sequence.
Dr Dominik Spensberger injected the CRISPR/Cas9 reagents into mouse zygotes. Pups born from these injections were genotyped by extracting DNA from ear clips and using PCR to amplify the region around the potential point mutation. The PCR products were sent for Sanger sequencing, as the PCR products from WT DNA and edited DNA had the same number of base pairs and could not be genotyped by running an agarose gel, and there were no restriction sites introduced as part of the edit. The results came back with four heterozygote animals, two male and two female animals had successful introduction of the point mutation in one allele. FIGURE 4.21A shows the heterozygote sequencing profile for one of these mice, where there is evidence of the point mutation encoding an amino acid change from lysine (K) to alanine (A) and with silent mutations as expected, which do not encode a different amino acid (FIGURE 4.21A).

These pups were taken forward as founder animals and bred with WT mice to get pure heterozygote animals to then breed to produce homozygote animals. Genotyping after the founder animals was done via Transnetyx to confirm heterozygote animals (FIGURE 21.B). Further breeding rounds led to confirmed homozygote mice for the Atg16L1 K490A (FIGURE 4.21C).

Therefore, this project has successfully developed a second, even more refined in vivo mouse model for Atg16L1 K490A that will be an invaluable tool to observe the effects of specifically inhibiting non-canonical autophagy in a living system.
Figure 4.21: Sequencing of genomic DNA of pups from direct zygote injections and after founder breeding.

A. Genomic DNA was extracted from pups born from direct zygote injections. Here is one representative example of a Sanger sequencing trace to show the nucleotide sequence in the edited region of Atg16L1 where the pup is heterozygote for the edited nucleotide sequence that should encode an amino acid change from lysine to alanine and the silent mutations are also evident. This is an example of a founder heterozygote Atg16L1 K490A mouse.

B. Founder heterozygote mice were bred with WT mice and ear clips from these offspring were sent off to Transnetyx an external genotyping company. They do reverse PCR based on given information where they have designed a probe that will recognise the mutated DNA in the specific region of Atg16L1. The red asterisk marks examples of heterozygote pups, these are representative of other such heterozygote pups.

C. Heterozygote mice such as those from B were bred and ear clips from these offspring were sent off to Transnetyx an external genotyping company. They do reverse PCR based on given information where they have designed a probe that will recognise the mutated DNA in the specific region of Atg16L1. The red asterisk marks examples of homozygous pups successfully expressing Atg16L1 K490A.
4.6 Discussion

The mouse model of E230 Atg16L1 from collaborators offered a new way to study the consequences of deleting the C-terminal domain of Atg16L1. The mouse is viable, which is surprising because the E230 Atg16L1 deletion houses the FIP200 binding domain (229-242), sites that directly recruit Atg16L1 in autophagy [32, 189]. A knockout Atg16L1 mouse model would not survive birth [164]. The mouse model seems to still support some autophagy and data from the collaborating lab confirms this. This Atg16L1 mutant still has both WIPI2b binding sites at positions 226 and 230 [33] suggesting WIPI2b binding is enough to support canonical autophagy [33]. This has previously been shown in cells expressing Atg16L1 that cannot bind WIPI2b, FIP200 is not enough to rescue autophagy [33]. Furthermore, the lack of FIP200 in cells does not affect WIPI2b binding and the autophagy response [33]. These data support the literature suggesting that WIPI2b is more important than FIP200 in the recruitment of Atg16L1 to support canonical autophagy.

In the context of non-canonical autophagy, primary cells from mice with the C-terminal deletion of Atg16L1 were deficient for LAP and monensin stimulated non-canonical autophagy. Furthermore, in dendritic cells from these mice, exogenous antigen presentation via MHC II was impaired; this is hypothesised to be linked to the impairment of LAP. These conclusions support published work that show a dependence on autophagy proteins in antigen presentation, specifically MHC II antigen presentation [112, 176, 178]. This includes work where in the absence of LC3, fungal antigen presentation is impaired in mouse dendritic cells [112]. Furthermore, Lee et al showed a defect in in vivo antigen presentation in dendritic cells lacking Atg5. This was not due to differences in phagocytosis but in the absence of Atg5 there is inefficient fusion of the phagosome and lysosome and therefore acidification affecting the MHC II processing [176].
Non-canonical autophagy could be playing a similar role in antigen processing to its role in TLR signalling, where LC3 lipidation to TLR containing endosomes regulates B cell signalling through increased fusion of lysosomes to acidify and degrade the contents of the TLR containing endosomes [93]. In autophagy it has been shown that ~50% of autophagosomes fuse with MHC II loading compartments and this is dependent on the Atg16-12-5 complex and LC3 lipidation, this delivery is essential to efficient MHC II antigen presentation [229]. Therefore, LC3 positive phagosomes could have a similar role in delivering contents through the fusion to late endosomal compartments such as the MHC II loading compartment and this could explain why when lipidation of LC3 is inhibited there is a decrease in efficient MHC II antigen presentation.

Vesicle trafficking has also been shown to be regulated by LC3, where LC3 on the outer membrane of autophagosomes can interact with FYCO1 (FYVE and coiled coiled domain containing 1) [230]. FYCO1 is a protein that has a LIR domain and therefore can interact with LC3 to mediate microtubule transport [230]. FYCO1 also has a role in LAP and similarly interacts with LC3 in this non-canonical autophagy context to aid vesicle transport for increased maturation and acidification of phagosomes [113], this could be linked to the role of LAP in antigen presentation. These potential mechanisms for the role of LAP in antigen presentation will be looked at in future work from the lab with primary cells from the refined mouse models produced in this chapter.

There is however contradictory literature, where one paper shows that Atg5 and Atg7 have no role in phagosome maturation [185]. Another paper even shows the opposite, that the presence of LC3 in human dendritic cells delays phagosome maturation. This leads to prolonged antigen presentation and it is the lack of LC3 that leads to rapid turnover of phagosomes [177]. Therefore, further analysis is needed to unpick the molecular mechanisms of how non-canonical autophagy is acting in phagosome maturation implicating antigen presentation.

The complexity and role of the Atg8 family is relevant when thinking about the function of non-canonical autophagy and its function in phagosome maturation and antigen presentation. This
study has only looked at LC3 lipidation which is inhibited in the model of the Atg16L1 WD domain deletion but the GABARAPs have not been looked at. Therefore a more complete analysis of the Atg8 members in non-canonical autophagy needs to be done as it is beginning to emerge that the GABARAP proteins may be more significant in the role of lysosome fusion than LC3 [64]. Therefore, further downstream analysis from the Atg8 lipidation step needs to be done.

The in vivo models generated as part of this project are a major step in the study of non-canonical autophagy. By targeting a bona fide autophagy protein we are able to inhibit non-canonical autophagy while leaving canonical autophagy unaffected. This is unlike the previously reported Rubicon knockout mouse, which also shows defects in LAP [96]. However, Rubicon is not a specific autophagy protein and has major roles in the endocytic system. It is likely that loss of Rubicon alters the PI3P and ROS production at phagosomes, irrelevant as to whether LC3 would be recruited or not. Indeed, LC3 lipidation to phagosomes from Rubicon knockout macrophage can be induced by stimulation with monensin (data not shown). This suggests that Rubicon plays a role in generating the signal to activate non-canonical autophagy specifically during phagocytosis, and may not play a role in other examples of the pathway, such as macropinocytosis and entosis. Also, Rubicon is a negative regulator of autophagy, so its loss can lead to increased levels of canonical autophagy which may impact the interpretation of results.

Thus, we believe our Atg16L1 model can be used to explore the functions of non-canonical autophagy. Future work includes experiments that challenge the mice with Influenza infection, in vitro and in vivo antigen presentation assays, clearance and immune consequences of fungal infection or addition of apoptotic corpses.
5 Results: Proteomic analysis of Atg16L1 complexes during non-canonical autophagy.

5.1 Introduction

The work in Chapter 3 implicates the WD domain of Atg16L1, and specific residues within this domain, in recruitment to, and subsequent LC3 lipidation at, membranes of the endolysosomal system. WD domains are relatively common and found in many proteins. They do not have catalytic activity, but instead are classified as a scaffolding domain [231], implicated in protein-protein interactions. They are considered to be promiscuous domains because they can interact with a range of proteins, and interactome studies suggest there is not a clear pattern to the types of proteins that they generally bind [231]. Nevertheless, individual WD40 domains do interact with specific partners, in a manner that is often controlled by key ‘hot-spot’ residues [195]. This concept was already utilised in Chapter 3 by using a published algorithm to predict Atg16L1 WD domain residues, on the top face of the beta barrel structure, that could be important in its protein-protein interactions [195]. Building on this, the WD domain of Atg16L1 became the focus of the project to search for protein interactors.

Proteomic analysis of Atg16L1 has previously been carried out in the context of canonical autophagy. Through various methods, direct interactors of Atg16L1 have been characterised such as FIP200, WIP1b [32, 33, 189]. Furthermore, the WD domain of Atg16L1 has been implicated in the binding of various proteins during unconventional autophagy processes, such as its interaction with TMEM59 [202] and TRIM 20 [201]. There is some evidence that there may be some common features among Atg16L1 WD domain protein interactors, as a specific motif [YW]-X$_1$-[ED]-X$_4$-[YWF]-X$_2$-L was found on multiple proteins that interact with the WD domain of Atg16L1 [202].

This chapter aimed to identify Atg16L1 interactors via proteomics in the context of non-canonical autophagy. The cellular system produced in Chapter 3 uses different mutants of Atg16L1, re-
expressed in an Atg16L1 null background, to separate canonical and non-canonical autophagy, providing an ideal system to study this.

In order to carry out proteomic analysis of Atg16L1 interactors in the context of non-canonical autophagy, endolysosomal LC3 lipidation was stimulated using the ionophore monensin, which exchanges hydrogen ions for sodium ions in the lysosome, raising the lysosomal pH but also changing the osmotic balance of endolysosomal compartments\[220, 221\]. This alteration of the osmotic properties of the endolysosomal compartment has been shown to induce LC3 lipidation to these single membranes, whilst also inhibiting autophagy flux and therefore increasing LC3 lipidation from accumulated autophagosomes (in WT cells) \[98\]. After cellular stimulation, the cells were lysed and the various Atg16L1 mutants were immunoprecipitated (IP) to look for proteins that had been pulled out of the lysate with Atg16L1.

Two proteomic methods are outlined in the following sections and summarised in FIGURE 5.1 and 5.5. Various biochemical techniques were used to optimize methods and validate experiments before ultimately using mass spectrometry to compare samples from cells expressing the FL Atg16L1, competent for both canonical and non-canonical autophagy, in comparison with the C-terminal WD deletion or point mutant K490A Atg16L1, where cells are deficient for non-canonical autophagy. Our hypothesis was that the WD domain may be required to bind key partners in non-canonical autophagy, which may be revealed by comparative proteomics.

Samples sent as part of this project used a semi-quantitative mass spectrometry approach where all the samples could be run at the same time and compared to one another. The Mass Spectrometry Facility at The Babraham Institute, took the IP samples and ran them ~5 mm into an SDS-PAGE gel, the proteins were stained and excised from the gel. They were then de-stained and trypsin digested before being TMT labelled, a robust way to allow relative abundance of peptides to be compared. The samples were then analysed by mass spectrometry, to identify the molecules based on their mass to charge ratio.
5.2 IP using Flag-S tagged Atg16L1 constructs.

5.2.1 Introduction to IP methodology using Flag-S tagged Atg16L1 constructs.

The first method used to look at Atg16L1 binding partners in the context of non-canonical autophagy utilised the Flag-S tag on the Atg16L1 constructs. Figure 5.1 shows a schematic of the IP strategy and subtractive nature of the experiment.

**Figure 5.1: Immunoprecipitation using the S-tag Atg16L1 constructs.**

1. The cell lysate is pre-cleared where uncoated agarose beads are rotated with the lysate and this removes proteins that just stick randomly to the beads.
2. The pre-cleared lysate is then rotated with agarose beads that are conjugated to an antibody that recognises the S-tag present on the Atg16L1 constructs. The unbound proteins are removed and the beads are washed.
3. The beads with the bound Atg16L1 and its binding partners were then boiled to elute the proteins from the beads.
4. The IP sample was then run ~5 mm into an SDS-PAGE gel, the proteins were stained and excised from the gel. Where they were then de-stained and trypsin digested before being TMT labelled for mass spectrometry analysis. A subtractive analysis was used where anything bound to the Atg16L1 with the WD deletion was not important but proteins found in the FL Atg16L1 sample but not the ΔWD were the interesting hits, visualised by the shapes with asterisks.
5.2.2 IP of Atg16L1 and known binding partners

Atg16L1−/− MEF GFP-LC3 cells were re-complemented with Flag-S tagged FL Atg16L1 and the ΔWD, as shown previously in FIGURE 3.3C. MEF cells were treated with monensin, to stimulate non-canonical autophagy, and then cells were harvested with lysis buffer containing 1% triton. After pre-clearing, magnetic Flag beads were initially used to IP tagged Atg16L1 FL or ΔWD. Western blots were carried out and Atg16L1 was successfully immunoprecipitated. However, known complex partners, such as Atg5 and Atg12, were never detected with this method (data not shown). Furthermore, by silver stain, the magnetic Flag beads showed high background levels of protein binding in control samples, which has commonly been reported with this reagent (data not shown).

As an alternative, beads that would bind the S-tag on Atg16L1 were used instead to IP Atg16L1. FIGURE 3.10 showed there was successful IP of Atg16L1, and co-IP of Atg5-12 as internal controls. The pull down of these proteins was specific to the cells expressing the tagged Atg16L1, WT MEF cells were used as a negative control (FIGURE 3.10). Furthermore, a silver stain was done to crudely look at the total proteins in the IP samples and to get an idea of the background proteins in the negative control (FIGURE 5.2). There are extra bands in the IP sample of tagged Atg16L1 compared with the negative control, indicating specific interacting partners have been recovered (FIGURE 5.2).
**Figure 5.2: Silver stain to assess the total proteins in IP samples from the negative control compared to the Atg16L1 FL sample.**

IP samples were prepared by using beads specific to the s-tag to pull out Atg16L1 from cell lysates. A negative control was WT MEF cells that do not have tagged Atg16L1. IP samples were run on a pre-cast gradient gel to separate proteins by size. The proteins were then silver stained and the arrows indicate obvious bands present in the Atg16L1 FL IP sample compared to the negative control which shows background levels of protein in the IP sample.
5.2.3 Mass spectrometry analysis of IP samples

Now that the method had been optimised to immunoprecipitate Atg16L1, and known interactors Atg5-12 and the background had been checked, samples were prepared for mass spectrometry analysis. WT MEF cells were used as a negative control, as there is no tagged protein, then MEF cells reconstituted with FL Atg16L1 or with the C-terminal WD deletion of Atg16L1 were all treated with monensin to activate LC3 lipidation to endolysosomal membranes. It is to be noted that this treatment would also have led to the accumulation of autophagosomes. However, binding partners specific to Atg16L1 in non-canonical autophagy were to be assessed by using subtractive analysis. For instance, proteins identified in the IP sample of the negative control would be the background proteins. Proteins identified in the Atg16L1 FL IP sample that were not in the Atg16L1 ΔWD IP sample were the proteins of interest as these would technically be the interactors to the WD domain of Atg16L1. FIGURE 5.1 visualises the subtractive nature of this experiment.

Therefore, these samples were prepared for mass spectrometry analysis, where Dr David Oxley at the mass spectrometry facility at The Babraham Institute, ran the samples into an SDS-PAGE gel for trypsin digestion and TMT labelling. The labelled samples were then run to get a semi-quantitative list of proteins for each sample. The results came back with background proteins in the negative control but reassuringly in the IP samples from the cells expressing the tagged Atg16L1 known Atg16L1 interactors: Atg16L1, Atg5 and Atg12 were present in the initial list.

Unfortunately, there were no proteins present in the IP sample from the cells expressing FL Atg16L1 that were not in the sample from the ΔWD Atg16L1. Therefore, no candidates were identified that specifically bind the WD domain of Atg16L1 to mediate non-canonical autophagy. There was also not many membrane bound proteins or lysosome associated proteins as would be expected, suggesting the method of lysis could be too harsh to preserve membrane protein interactors.
5.2.4 Optimisation of cell lysis for IP

The next round of experiments were done with an alternative lysis buffer containing 0.3% CHAPs, a gentler detergent, to keep protein complexes intact, more commonly used when looking for membrane proteins. The same experiment was performed but with the different buffer. Successful IP of Atg16L1 and co-IP ATG5-12 was achieved whether using 1% triton or 0.3% CHAPs buffer to lyse the cells (FIGURE 5.3).

5.2.5 Optimised IP samples for mass spectrometry analysis

The same samples as 5.2.3 were prepared for mass spectrometry analysis. The data came back with much less background and again identified known interactors Atg16L1, Atg5 and Atg12 and Atg16L2. Nevertheless, again, the subtractive approach of looking for specific binding partners to the WD domain of Atg16L1 was not successful; there were no proteins that were present in the FL Atg16L1 sample but not the ΔWD Atg16L1 sample.

Two proteins that bound both full length and WD Atg16L1 looked interesting as novel general Atg16L1 interactors: β-integrin and PlekHF1. Therefore, IP experiments were repeated and western blots were performed to see if indeed these proteins were interacting with Atg16L1, this would be a way to validate mass spectrometry hits. FIGURE 5.4 shows that by western blot, β-integrin and PlekHF1 could not be detected in the IP sample of cells expressing Atg16L1 FL or Δ WD. Therefore, these proteins were not followed up any further.

We next moved to using the BioID technology in the hope of picking up transient or weaker interactors of Atg16L1 in the context of non-canonical autophagy. In addition, at this stage of the project the Atg16L1 K490A mutant had been identified which could be used as a more refined comparison for proteomic analysis.
Figure 5.3: Comparison of IP of Atg16L1 and Atg5-12 when cells were lysed in a triton or CHAPs buffer.

Cells were lysed with either 1% triton or 0.3% CHAPs and IP samples were prepared by using beads specific to the s-tag to pull out Atg16L1 from cell lysates. A negative control (-) was WT MEF cells that do not have tagged Atg16L1. IP samples were run on acrylamide gels to separate proteins by size. A western blot was performed to detect Atg16L1 levels or Atg5-12 levels as the Atg5 antibody recognises this complex. LE=Low exposure and HE=High exposure.
Figure 5.4: β1-integrin and PLEKH1 do not co-IP with Atg16L1.

Cells were treated for 1 h with monensin and lysed with 0.3% CHAPs buffer. IP samples were prepared by using beads specific to the s-tag to pull out Atg16L1 from cell lysates. A negative control (-) was WT MEF cells that do not have tagged Atg16L1. IP samples were run on acrylamide gels to separate proteins by size. A western blot was performed to detect β1-integrin and PLEKH1 in the total lysate and in the IP sample.
5.3 Using the BioID system to look for Atg16L1 binding partners.

5.3.1 Introduction into the BioID methodology

The BioID system involves a 321 amino acid, ~35 kDa, bacterial biotin ligase (Bir-A) from *E.coli* being fused to a bait protein of interest, in this case Atg16L1. The BirA used in this project has been engineered to have the point mutation R118G, and this allows the biotin ligase to have a promiscuous activity [219]. The promiscuous BirA can biotinylate proteins in close proximity (~10 nm range) of the bait protein, upon the addition of exogenous biotin, providing a means of identifying proximal proteins.

BirA works by catalysing the synthesis of activated biotin, 5′-AMP biotin, in its reactive centre. In the promiscuous version of BirA, this intermediate, active biotin, is turned over quickly, resulting in a “cloud” of active biotin. This active biotin can form amide bonds with amino groups of lysine residues on proximal proteins, allowing biotin to label proteins in a living cell [232]. This method was developed as a way to identify more transient and dynamic interactors and identify protein complexes. It is superior to previous proteomic methods due to the labelling of protein complexes *in vivo*, there are less concerns about lysis buffers and washes destroying the protein-protein complexes. One limitation is that biotinylation can also occur endogenously [233]. For instance, biotin exists endogenously within the cell and is used as a co-factor by a variety of carboxylases, for instance for the biotinylation of histones [234]. But this is reported as a rare modification and the background biotinylation in theory can be easily corrected for [233].

FIGURE 5.5 summarises the methodology used for this proteomic approach. In order to assess Atg16L1 interactors in the context of non-canonical autophagy a subtractive approach was used.
1. Cells in culture where Atg16L1 is expressing a promiscuous biotin ligase (BirA). Then biotin was added to the cells alongside monensin to stimulate non-canonical autophagy. This will lead to any proximal proteins to Atg16L1 becoming biotinylated. The cells were lysed.

2. The cell lysate was then rotated with streptavidin beads that bound biotinylated proteins.

3. Boil the beads to release the specifically bound proteins.

4. Run IP sample on SDS-PAGE gel, digest, TMT label, mass spectrometry.

Figure 5.5: Summary of BioID method to immunoprecipitate Atg16L1 and proximal interactors.

1. Cells in culture where Atg16L1 is expressing a promiscuous biotin ligase (BirA). Then biotin was added to the cells alongside monensin to stimulate non-canonical autophagy. This will lead to any proximal proteins to Atg16L1 becoming biotinylated. The cells were lysed.

2. The cell lysate was then rotated with streptavidin beads that bound anything that was biotinylated. The unbound proteins were removed and the beads were washed.

3. The beads with the bound biotinylated proteins were then boiled to elute the proteins from the beads.

4. The IP sample was then run ~5 mm into an SDS-PAGE gel, the proteins were stained and excised from the gel. Where they were then de-stained and trypsin digested before being TMT labelled for mass spectrometry analysis. A subtractive analysis was used where proteins found in the FL Atg16L1 sample but not the Atg16L1 K490A sample were the interesting hits, visualised by the shapes with asterisks.
5.3.2 Creating BirA Atg16L1 constructs and cell lines

The use of the BioID system first of all required some cloning. The BirA C-terminally tagged Atg16L1 construct was kindly donated by Dr Noor Gammoh. Their lab had validated that the C-terminal BirA tag did not affect Atg16L1’s function in canonical autophagy in MEF cells re-expressing this tagged version. From this plasmid, the Atg16L1 ΔWD and Atg16L1 K490A mutants were cloned into the BirA vector and confirmed by Sanger sequencing.

FIGURE 5.6A shows the re-expression of the BirA-Atg16L1 constructs in Atg16/- MEFs. Proteomic studies from this point onwards used comparisons of BirA-Atg16L1 FL to BirA-Atg16L1 K490A. This was because expression of the BirA-Atg16L1 ΔWD was not detected by western blot, although functional assays suggest that the cells expressing the BirA-Atg16L1 ΔWD still function to lipidate LC3 suggestive of protein expression (data not shown). Furthermore, the Atg16L1 K490A mutant offered a better comparison, as the BirA was in the same place on Atg16L1, and therefore this controlled for functional difference that may have come about due to positioning of the enzyme.

The re-complemented cells were then tested to check that the C-terminal BirA tag had not affected the function of Atg16L1 to lipidate LC3 in non-canonical autophagy. This is shown in FIGURE 5.6B, using western blotting to detect LC3 lipidation following monensin and/or wortmannin treatment, to distinguish LC3 lipidation from canonical or non-canonical autophagy, as previously used in FIGURE 3.5B. Wortmannin, a PI3K inhibitor, inhibits canonical autophagy, but not monensin induced non-canonical LC3 lipidation. As previously shown, cells expressing FL Atg16L1 have wortmannin insensitive LC3 lipidation, which can be attributed to LC3 lipidation to single membranes in non-canonical autophagy (FIGURE 3.5B) and this function is not affected upon the addition of the C-terminal BirA tag (FIGURE 5.6B). However, as expected, wortmannin significantly inhibited monensin driven LC3 lipidation in cells expressing the K490A mutation in the WD domain.
of Atg16L1 (FIGURE 3.17B) and this is still observed upon the addition of the C-terminal BirA tag (FIGURE 5.6B).
Figure 5.6: Expression of Atg16L1 BirA constructs and functional validation.

A. WT MEF cells or Atg16L1 -/- cells re-complimented with Atg16L1 FL, ΔWD,F467A or K490A all tagged with BirA. Cells were lysed and a western blot was performed to detect Atg16L1 expression where GPADH was used as a loading control.

B. Western blot to detect un-lipidated (LC3I) and lipidated LC3 (LC3II) in lysates from MEF Atg16L1 -/- cells re-complimented with Atg16L1 FL or K490A tagged with BirA, that were untreated, treated with 67 µM wortmannin (WM) or 100 µM monensin or both. GAPDH was used as a loading control.
5.3.3 Biotin addition and BirA dependent biotinylation of proteins.

The next step was to assess whether, upon the addition of biotin, there was successful biotinylation of proteins. Further to this, it was assessed whether the BirA on the Atg16L1 mutants was causing additional biotinylation above the background levels. This was done by adding biotin to the re-complemented MEF cells in culture, where WT MEFs were used as a negative control. The cells were lysed, the total lysate was run on an SDS-PAGE gel and a western blot was performed using a streptavidin HRP antibody to detect total biotinylated proteins.

FIGURE 5.7A shows that in cells where no biotin has been added we can detect endogenous biotinylated proteins. Addition of biotin increases the intensity of these background biotinylated proteins. Moreover, it was apparent that in cells expressing the BirA- FL Atg16L1, biotin addition has led to the biotinylation of proteins beyond the background (FIGURE 5.7A). Biotinylation of proteins is occurring upon the addition of biotin and the expression of the BirA tagged Atg16L1 is increasing biotin dependent biotinylation in the total lysate.

Next, a similar treatment was done using the addition of biotin and then lysing the cells. These lysates were then processed with streptavidin beads to pull out the biotinylated proteins from the lysate, leaving an IP sample that should only contain the biotinylated proteins. Total lysates and IP samples were analysed via western blot using a streptavidin HRP antibody to detect biotinylated proteins (FIGURE 5.7B). These data show that the IP using the streptavidin beads has enriched for biotinylated proteins and indeed there are extra biotinylated proteins in the IP sample from cells expressing BirA-FL Atg16L1 compared to those in the negative control (FIGURE 5.7B).

Experiments were optimised at this stage for timings of the addition of biotin, and the timing for the rotation of samples with the streptavidin beads. Suboptimal levels of biotinylated proteins were produced after two hours of biotin addition and therefore 6 h were tested and chosen to be the time point used. This fits in with published data where optimal biotinylation had been shown around 6 h [219] (data not shown).
5.3.4  Biotinylation and IP of known Atg16L1 binding partners.

The system had been shown to function as expected in terms of biotinylation and enrichment of biotinylated proteins using streptavidin beads. The system was further validated for known Atg16L1 protein interactors using the drug PP242, an m-TOR inhibitor, which activates canonical autophagy. This allowed for a proof of principle to show that expected binding partners of Atg16L1, in the context of canonical autophagy, were detected only upon the addition of biotin and expression of BirA. The WT MEFs were used as a negative control and the Atg16L1 KO MEFs, reconstituted for BirA-Atg16L1 FL, were treated with and without biotin and PP242 for 6 h. The cells were lysed and the total lysate was kept as an input sample and quantified so equal protein amounts were loaded onto the streptavidin beads for the IP of biotinylated proteins. FIGURE 5.8A shows a biotin and BirA specific biotinylation and IP of known binding partners, such as FIP200 and WIPI2b, as well as Atg16L1 and Atg5-12, further validating the system. Notably, there also looked to be a potential drug dependent increase in Atg16L1 binding to FIP200. This was not completely reproducible, seen in 2 out of 3 repeats, so would need to be tested more thoroughly for clarification.

Finally, the conditions for samples to be sent for mass spectrometry analysis were also validated by western blot. The control samples were: i) WT MEFs, treated with biotin, to assay for endogenously biotinylated proteins and ii) MEF cells re-complemented with the BirA-FL Atg16L1, where no biotin had been added, again to assess background biotinylation caused by the expression of the BirA. The samples to be analysed were MEF cells, re-complemented with the BirA-FL Atg16L1 or the BirA–K490A Atg16L1, treated with biotin alone, biotin and PP242 or biotin with monensin for 6 h. Streptavidin blots were performed and specific interactors were also blotted for (FIGURE 5.8B). Again, there was a biotin and BirA specific IP of expected proteins, Atg16L1 and Atg5-12, in the case of FIGURE 5.8B. Furthermore, the streptavidin blot identified proteins additional to background biotinylation (FIGURE 5.8B).
Figure 5.7: Specific biotinylation of proteins from biotin addition and expression of BirA.

A. Cells were treated for 6 h with −/+ 50 µM biotin and lysed with RIPA buffer giving the total lysate, INPUT sample. 15 µg of protein was loaded onto an SDS-PAGE gel to separate proteins by size. A western blot was performed to visualise biotinylated proteins using a streptavidin HRP antibody. The numbers indicate the protein size in kDa. Where the – sample is the negative control, WT MEFs with no BirA expression.

B. Cells were treated for 6 h with −/+ 50 µM biotin and lysed with RIPA buffer giving the total lysate, INPUT sample. Equal protein amount was loaded onto magnetic streptavidin beads to pull out biotinylated proteins and this is the IP sample. A negative control (−) was WT MEF cells that do not have BirA tagged Atg16L1. INPUT and IP samples were run on acrylamide gels to separate proteins by size. A western blot was performed to detect biotinylated proteins using a streptavidin HRP antibody. The numbers indicate the protein size in kDa.
Figure 5.8: Western blots to show specific biotinylation and IP of known Atg16L1 interactors.

A. Cells were treated for 6 h with 1 µM PP242 and -/+ 50 µM biotin and lysed with RIPA buffer giving the INPUT sample. Equal protein concentration was loaded onto magnetic streptavidin beads to pull out biotinylated proteins and this is the IP sample. A negative control (-) was WT MEF cells that do not have BirA tagged Atg16L1. INPUT and IP samples were run on acrylamide gels to separate proteins by size. A western blot was performed to detect Atg16L1, FIP200, Atg5 and WIPI2b in the total lysate and in the IP sample.

B. Cells were treated for 6 h -/+ 50 µM biotin, with -/+ 1 µM PP242 and with -/+ 100 µM monensin and lysed with RIPA buffer giving the INPUT sample. Equal protein concentration was loaded onto magnetic streptavidin beads to pull out biotinylated proteins and this is the IP sample. A negative control (-) was WT MEF cells that do not have BirA tagged Atg16L1. INPUT and IP samples were run on acrylamide gels to separate proteins by size. A western blot was performed to detect Atg16L1, Atg5 and streptavidin HRP antibody detected biotinylated proteins in the total lysate and in the IP sample.
Given that the protocol had been thoroughly validated, the same samples as verified in 5.3.4 were prepared for mass spectrometry. The control samples were: i) WT MEFs, treated with biotin, to assay for endogenously biotinylated proteins and ii) MEF cells re-complemented with the BirA-FL Atg16L1, where no biotin had been added, again to assess background biotinylation caused by BirA. The experimental samples to be analysed were MEF cells, re-complemented with the BirA-FL Atg16L1 or the BirA–K490A Atg16L1, treated with biotin alone, biotin and PP242 or biotin with monensin for 6 h. The entire IP sample was submitted to the Mass Spectrometry Facility at The Babraham Institute, to maximise the amount of each candidate binding protein for detection. They ran the samples into an SDS-PAGE gel, digested the proteins and tagged the peptides with TMT, then analysed by mass spectrometry.

A list of ~3500 proteins came back, disappointingly, the results of this analysis showed no significant difference between the peptides in the experimental samples compared with any of the control samples. Atg16L1, which would certainly be expected to biotinylate itself, was not a prominent hit. Similarly, known Atg16L1 binders, which had been detected by western blot using the BioID system, were also absent. Even proteins that are known to undergo endogenous biotinylation, such as the carboxylases, were not detected in the control samples. These important omissions suggest that there was a major problem in sample preparation, handling or analysis, which confounded our ability to identify novel binding proteins.

Unfortunately, as the whole IP had been digested and TMT labelled, to maximise the amount sent for analysis, no sample was available with which to go back and assess whether the experiment had worked as before. Therefore, it cannot be known whether the problem lies in the preparation of this particular batch of samples, or somewhere during the mass spectrometry procedure. This will be discussed further below.
Due to time constraints, the mass spectrometry analysis could not be repeated in full for this thesis. However, the samples were re-prepared, this time withholding a small proportion of these samples, which were validated by western blot; similar results to FIGURE 5.9B were obtained and ensured the system was working as expected in the exact samples sent for mass spectrometry analysis. These have been stored for future mass spectrometry analysis.

5.4 Discussion

Atg16L1 binding partners in the context of canonical autophagy have been previously studied, with most interactors found using cross linking to stabilize weak interactions, using GST-tagged proteins as bait. This suggests that Atg16L1 is not an easy protein to work with when uncovering binding partners. For instance, chemical cross-linking was required to identify the interaction between Atg16L1 and WIPI2b, suggesting stabilisation of this complex was required due to the potential transient nature of this interaction [33]. Chemical crosslinking was also required in the case when the FIP200 and Atg16L1 interaction was discovered [189]. Similarly, an alternative method, using a system that stabilised Atg16L1 at membranes, in an Atg3-/- background, was needed to observe the same interaction of Atg16L1 with FIP200 [32]. In this case, the IP was done from a membrane fraction to further enrich for context specific binding partners. Therefore, this may explain the lack of success we had using traditional IP methods, with the S-tagged Atg16L1 constructs, without any enrichment or cross-linking strategies to stabilise Atg16L1’s interactions. As such, future work with this method could be to try cross-linking or membrane fractionation prior to the IP, both with the aim to stabilise or enrich interactors of Atg16L1.

Encouragingly, the BioID proteomics approach was sensitive enough to detect interactors such as FIP200 and WIPI2b, that had previously required cross-linking, through proximity to Atg16L1. Therefore, this suggests BioID as a method that has the potential to pick up a more global view of Atg16L1 proximal interactors and is more sensitive than previous methods used to look at Atg16L1 interactors.
Further tentative evidence for BioID being a more sensitive approach, comes from the suggestion that FIP200 may interact more with Atg16L1 in autophagy induced conditions when using BioID proteomics. Previously it was shown that the interaction between FIP200 and Atg16L1 was independent of starvation [32, 189], which is perhaps surprising as it might be expected to be a regulated interaction. It is possible that this inducible binding was missed due to a limitation in the traditional IP methods. However, further work would be needed to confirm that ours is a true result, as starvation induced FIP200 binding was only seen on two of three occasions.

In spite of its advantages, BioID does have some drawbacks because of its sensitivity. It can label many proximal proteins, some that may just be in the vicinity due to random chance, therefore repeats of the BioID preparation and mass spectrometry would be essential. Unfortunately, due to time constraints, sample preparation and mass spectrometry analysis for a specific set of conditions was not repeated.

As part of this project a BioID method has been successfully optimised and validated by western blot to be a useful tool for proteomic analysis of Atg16L1. Unfortunately, mass spectrometry results from the BioID proteomics were so far unsuccessful, because no known protein interactors were pulled out from any samples, and our controls suggested a general problem with that batch of samples. The reason for this is hard to determine, as the specific samples prepared were not validated before being sent to mass spectrometry, it was assumed that the system was working as optimised. In future work, it will be important to validate a small fraction of the exact samples to be sent, prior to mass spectrometry as a quality control step.

There could be some possible explanations for the failure of this experiment. The results may suggest that either the biotin addition or biotinylation did not occur at an optimal level, or a combination of both, which could explain why no endogenously biotinylated proteins were enriched for in the control sample and why no known interactors were present. Alternatively, it could be the case that the IP failed, and the proteins were being biotinylated however, the
streptavidin IP did not work or the biotinylated proteins were not eluted from the beads properly before handing over the samples for mass spectrometry. This would explain why even the endogenously biotinylated proteins were not detected by mass spectrometry and would further explain why known interactors were not identified. Seeking advice from the mass spectrometry facility, they suggest it is unlikely that the gel running, TMT labelling or mass spectrometry could have contributed to the failure of this experiment, as previous samples have been TMT labelled in the same way and there was detection of known interactors. Furthermore, when repeating this experiment to re-send validated samples for mass spectrometry analysis, the first attempt showed that biotinylated proteins were not enriched for by IP, suggesting that something within the IP method is temperamental, indeed adding a confirmation that the problem came before the mass spectrometry analysis. It is evident that validation of IP samples should always be done before sending and waiting for mass spectrometry analysis. Therefore, new samples have now been prepared and validated to be re-submitted for mass spectrometry analysis, experiments have shown successful biotinylation within the system and enrichment of these biotinylated proteins after IP and Atg16L1 and Atg5-12 have been specifically detected in relevant samples and not in the controls. This will form the basis of future work within the lab.

Depending on the results from this experiment, which will be beyond the scope of this thesis, maybe an additional control sample in the future could be added. Looking at cells that express BirA alone, with biotin addition, to then subtract the background biotinylation. This seems a common method [235] and was something that was not used in this project when submitting samples for mass spectrometry analysis. Although other ways to correct for background biotinylation were used such as cells with no BirA tagged protein and cells with the tagged BirA Atg16L1 but with no biotin addition. More stringent washes could be used to optimise the protocol in future, such as higher concentrations of SDS, due to the strong nature of the biotin and streptavidin interaction [219, 236]. The washes used as part of this project were more reminiscent of normal IP washes where much more care needed to be taken.
If the subtractive approach to identify Atg16L1 interactors specific to non-canonical autophagy does not work, the BirA FL Atg16L1 construct could be expressed in the Atg13 −/- Atg16L1 −/- HEKs developed in chapter 3. This cell line would be deficient for canonical autophagy and therefore all interactors identified are candidates for binding partners involved in non-canonical autophagy.

Furthermore, future work could involve even more refined proteomic approaches that are now available including an alternative system such as the APEX system. This is based on an engineered form of ascorbate peroxidase that in the presence of biotin-phenol and the addition of hydrogen peroxidase rapidly biotinylates proximal proteins in a matter of minutes rather than hours [237]. This cuts out having to add biotin for long periods of time and doing unnecessary long drug treatments, where the downstream methodology is the same using streptavidin beads. The shorter addition of biotin may reduce background biotinylation. However, literature detailing the APEX system has largely been done in targeted cellular compartments such as the inner mitochondrial space [237] although it has been done in non-membrane enclosed organelles [238].

The other method that can be used as an advancement of the BioID method in this project is BioID2 [236]. It is based on the BioID method outlined but involves a much smaller promiscuous biotin ligase that allows for a better identification of proximal interactors due to it being smaller and less disruptive to its fusion bait protein. A flexible linker can also be added to improve the range of the enzyme. It also requires significantly less biotin addition to cells and this may affect the background biotinylation levels. With the mouse models developed in Chapter 4, the BioID technology has the potential to be used in vivo to identify protein interactions directly in a living system.

Finally, this work has focused on proteomic approaches to investigate the role of the WD domain of Atg16L1 in non-canonical autophagy, the other possibility is that lipid interactions may regulate the recruitment of Atg16L1. Lipidomic strategies will also be considered as part of future work.
6 General discussion and Impact of thesis

Autophagy is a fundamental and widely studied pathway, that has roles in both physiological and pathophysiological processes. The importance of autophagy is evidenced by the award of the Nobel Prize in Physiology or Medicine in 2016 to Yoshinori Oshumi for his contributions to the field. Therefore, autophagy is an exciting and rapidly developing field to be working in. Since the recent discoveries that LC3 can be recruited and lipidated to membranes distinct from autophagosomes, it was exciting to contribute to the novel non-canonical autophagy field.

Non-canonical autophagy refers to the unconventional lipidation of LC3 to single endolysosomal membranes, which often follows cell engulfment events such as LAP, macropinocytosis or entotic cell cannibalism. The term ‘non-canonical autophagy’ can be thought of as misleading by some, because ‘auto’ implies self-eating, and this pathway targets material ingested from the outside. However, as the field continues to grow this term has begun to be used by many groups, in high profile publications [41, 93-96], and as such, it was used for this project. We do not feel that LAP can be substituted, as it does not encompass the whole pathway, but in the future, a better name may emerge. One suggestion could be LC3 Lipidation to Endolysosomal Membranes (LLEM); this offers a broader name and detaches the pathway from autophagy.

This project set out to uncover the role of Atg16L1 in the context of non-canonical autophagy. Firstly, Atg16L1 was shown to localise to single membranes of the endolysosome and function with Atg5-12 to lipidate LC3 to these membranes. Previous work suggested that LC3 lipidation to single membrane endolysosomes was independent of some canonical autophagy proteins such as the ULK1 complex [91]. This project further validated this and showed that Atg16L1 lacking the FIP200 binding domain still supported LC3 lipidation in non-canonical autophagy. This project also confirmed that the WD domain of Atg16L1 was indeed dispensable for LC3 lipidation to autophagosomes [197].
Using a structure function approach, this project then went on to demonstrate that the C-terminal WD domain of Atg16L1 is essential for LC3 lipiddation in non-canonical autophagy, under both drug induced and physiological settings such as LC3-associated phagocytosis (LAP). A more refined model was also determined through site directed mutagenesis; single amino acid residues were identified within the WD domain of Atg16L1 that are essential to the role of Atg16L1 in non-canonical autophagy. Specifically, the WD domain of Atg16L1, and key residues, were shown to be essential for the recruitment of Atg16L1 to single membrane endolysosomes upon non-canonical autophagy stimulation.

This novel observation provided a genetic means to specifically dissect canonical and non-canonical autophagy pathways, allowing the impact of inhibiting non-canonical autophagy to be assessed without affecting autophagy. The use of truncated or mutated Atg16L1 offers some advantages to existing models. For instance, knock out of the Atg8-conjugation machinery, such as Atg5, will affect both pathways so lacks specificity. Knockout of rubicon, a component of the class 3 PI3 kinase Vps34, has been used to model inhibition of LAP[96], but rubicon has other functions that will impact phagocytosis separately from any role it has in non-canonical autophagy. As such it is unclear whether results from using rubicon knockout cells, stems from inhibition of LC3 recruitment or some other defect in phagocytosis. Furthermore, not all non-canonical autophagy associated processes may depend on rubicon. Thus, we feel that our model, that targets a bona fide autophagy protein, will more specifically inhibit LC3 lipidation associated with all non-canonical autophagy processes. Nevertheless, it has to be acknowledged that Atg16L1 may have functions outside of its role in lipidating LC3. Indeed, there is evidence that Atg16L1 may function in regulating inflammatory responses to pathogens independently of its conjugation function [239] or IFNγ responses to murine norovirus [240]. Whether these processes depend on the WD domain is not known and so some caution should still be considered in what effect deletion of this domain will have.
This study has focused on Atg16L1 recruitment and LC3 lipidation, however, the function of LC3 at these single membrane endolysosomes, particularly in entosis and macropinocytosis, remains elusive. LC3 lipidation in LAP is reported to speed up the clearance of apoptotic debris or pathogens [109, 111], however, this is not a universal view [185] and these functional outcomes of LC3 lipidation were not studied as part of this project. However, existing mice models that have inferred the role of non-canonical autophagy, from the comparison of various autophagy related protein knockouts, have interesting phenotypes. For instance non-canonical autophagy has been implicated in the visual cycle of mice [94], controlling autoimmune signalling in B cell activation [181] and controlling autoimmune pathologies such as SLE [96]. Further work will need to be done to look at the functional implications of LC3 lipidation using the in vivo mouse models developed as part of this project. They are one of the first mammalian systems that will lack non-canonical autophagy without affecting canonical autophagy. A model of both Atg16L1 ΔWD and Atg16L1 K490A were made using CRISPR Cas9 technology. While there appears to be no obvious problems or phenotype with the mice, as they are maintained in a clean mouse facility, it is predicted that phenotypes may emerge as they are challenged with pathogens, as there is consensus that non-canonical autophagy plays important roles in the immune system.

This project has already utilised an Atg16L1 E230 mouse model from Dr Tom Wileman’s lab, a C-terminal Atg16L1 deletion, which also appears to inhibit specifically non-canonical autophagy. Using this model we uncovered an involvement for LC3 lipidation to endolysosomal membranes in MHC II antigen presentation. This is something that will be interesting to study in more detail and repeat with the mice produced through this project. Understanding at what point in the antigen presentation process non-canonical autophagy impacts will provide molecular insight into the pathway. It was also shown as part of this project, in collaboration with Dr Rupert Beale, that Influenza A infection, through the action of the viral protein M2, activates non-canonical autophagy. The possible functions of this lipidation are currently being investigated. The hope is that non-canonical autophagy may be a pathway to target therapeutically. Activating the pathway
could possibly increase the efficiency of antigen presentation and vaccination. Dampening the pathway could help protect from autoimmune responses. The data generated in this project on the critical residues of Atg16L1 could help guide new drugs to modulate non-canonical autophagy.

The Atg8 protein LC3 has been a major focus in this project. For western blot analysis, LC3 A/B have been detected, and cells have been expressing GFP-LC3A or GFP-LC3B. However, a recent publication discusses the role of LC3 and GABARAPs in autophagy, where GABARAPs seem to be more important than LC3 family members in the efficient formation and fusion of autophagosomes [64]. Therefore, to build on this project, it would be worthwhile to look at the GABARAPs in non-canonical autophagy. Furthermore, published work shows that GABARAPs, as well as LC3, go to non-canonical autophagy membranes [41, 120, 171] and are lipidated in the same way as LC3, by Atg16L1 in complex with Atg5. Therefore, the conclusions of this project could be relevant to Atg8 proteins more broadly.

To begin to understand the molecular details of how the WD domain of Atg16L1 targets it to single-membrane compartments, proteomic analysis was used to identify binding partners of Atg16L1 during non-canonical autophagy. Optimised conditions were achieved using the BioID system to biotinylate proteins in proximity to Atg16L1 during non-canonical autophagy activation. Pilot studies were performed, conditions were optimised, building important preliminary data for a successful BBSRC grant application in our lab. However, the comprehensive analysis of WD-domain interactors, induced upon activation of non-canonical autophagy, was not fully concluded in the period of this project. Optimised samples have been prepared for analysis, but the identification and follow up on candidate interacting proteins will now form the basis of future work in the lab. The critical residues identified on the top face of the WD domain were proposed to be important in protein-protein interaction, which is a common feature of WD domain containing proteins [231]. Nevertheless, it is possible that the WD domain could interact with a
lipid. So future work could also analyse lipid interactions and their possible role in recruiting Atg16L1 to membranes.

Currently, these data show that the sites identified in Atg16L1 are important in all tested examples of non-canonical autophagy: LAP, macropinocytosis, Influenza induced non-canonical autophagy and entosis. This is suggestive of a common mechanism with the WD domain of Atg16L1 acting as a molecular hub for non-canonical autophagy processes. It will be intriguing to uncover if there is one mechanism of recruitment of Atg16L1 through the WD domain or whether there are process-specific mechanisms. For instance, could it be that the mechanism for Atg16L1 recruitment to phagosomes is distinct to its recruitment to entotic vacuoles, possibly involving different WD domain binding proteins.

In conclusion, this project offers an important new way to separate two related pathways, autophagy where LC3 gets lipidated to autophagosomes and non-canonical autophagy where LC3 can become lipidated to single membranes of the endolysosome. This provides an important strategy to study non-canonical autophagy and further understand the physiological relevance of the signalling pathway. Future work will build directly on these findings, identifying binding partners to reveal molecular mechanisms, and exploring mouse model phenotypes, to uncover the physiological roles of non-canonical autophagy.
Atg16L1 complexes with Atg5 and Atg12 to form the lipidation complex that localises to both
autophagosomes and single membranes during non-canonical autophagy. These single endolysosomal
membranes are formed after phagocytosis, macropinocytosis, and entosis and can be targeted in a
manner distinct from canonical autophagy for LC3 recruitment and lipidation. Endolysosomal membranes
in influenza infection and after the use of lysosomotropic drugs show activation of this non-canonical LC3
lipidation. Atg16L1 recruitment in non-canonical autophagy is due to the C-terminal WD40 domain and
specific residues within this domain that are dispensable for canonical autophagy. This offers a novel
strategy to distinguish between canonical and non-canonical autophagy processes and utilising this
approach, in vivo mouse models have been generated. Initial studies implicate the C-terminal of Atg16L1
in MHC class II antigen presentation in dendritic cells. There is still a question mark about what signals
are involved in the recruitment of Atg16L1 to these single membrane compartments.

**Figure 6.1 Summary Diagram**

Atg16L1 complexes with Atg5 and Atg12 to form the lipidation complex that localises to both
autophagosomes and single membranes during non-canonical autophagy. These single endolysosomal
membranes are formed after phagocytosis, macropinocytosis, and entosis and can be targeted in a
manner distinct from canonical autophagy for LC3 recruitment and lipidation. Endolysosomal membranes
in influenza infection and after the use of lysosomotropic drugs show activation of this non-canonical LC3
lipidation. Atg16L1 recruitment in non-canonical autophagy is due to the C-terminal WD40 domain and
specific residues within this domain that are dispensable for canonical autophagy. This offers a novel
strategy to distinguish between canonical and non-canonical autophagy processes and utilising this
approach, in vivo mouse models have been generated. Initial studies implicate the C-terminal of Atg16L1
in MHC class II antigen presentation in dendritic cells. There is still a question mark about what signals
are involved in the recruitment of Atg16L1 to these single membrane compartments.
7 Bibliography


