Effect of Autophagy Induction via TRPML1 Activation on Hepatic Steatosis

by

Claudia Tersigni

A thesis submitted in conformity with the requirements
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Institute of Medical Science
University of Toronto

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2019

Discrepancies between the number of liver transplant candidates and available organs has led to an increased use of marginal grafts, including steatotic liver grafts. Steatotic grafts are frequently discarded as their use has been associated with poor transplant outcomes; further exacerbating the organ shortage. Recent studies have demonstrated that autophagy regulates lipid metabolism in hepatocytes, leading to the idea that autophagic function might regulate the development of steatotic liver diseases. A lysosomal calcium channel, TRPML1, has been implicated in the regulation of autophagy through downstream TFEB activation and successive upregulation of autophagic and lysosomal gene expression. The objective of the present study is to explore the link between the induction of autophagy via TRPML1 activation and hepatic steatosis. If an interplay between these factors is elucidated, this may delineate a mechanism by which steatotic livers may be improved during the \emph{ex vivo} preservation period prior to transplantation.
Acknowledgments

“The race is long, and in the end, it is with yourself.” Before graduating from eighth grade, our teacher showed us a motivational YouTube video from which this quote comes from. It is something that has always ‘stuck’ with me, and I think about it often. However, I have never found it to be more applicable in life than during this degree. Each day for the past two years, I’ve had to constantly motivate myself, teach myself, and show up for myself. This degree has been a mass of learning experiences and tribulations, sprinkled with some triumphs and successes; it is the latter that served as the fuel to my fire and as the force that propelled me to keep moving forward. However, this was not enough, and I owe the completion of this thesis to many individuals, whom I would like to thank here.

I would like to begin by deeply thanking my supervisor, Dr. Nicola Jones, for her support, guidance, and overt optimism over the past two years. You are truly an inspiration to myself and all your students, and I am so grateful for the time that I got to spend in your tight-knit lab. Thank you for not only being an amazing supervisor, but also a role model, advocate and mentor. I also need to thank the members of my program advisory committee, Dr. Peter Kim, Dr. Nazia Selzner, and Dr. Sonya MacParland for taking the time out of your busy schedules and offering your very welcomed input on this project. I would like to specifically thank Dr. MacParland for her lab’s collaboration and contributions to this project.

I would have never been able to complete this journey if it weren’t for the amazing Jones lab members. To Mariana – thank you for being ‘mom’, for your amazing hugs and optimistic outlook on life, and for imparting so much knowledge to me, both in life and in science. To Laurel – thank you for being twinnie, for the constant laughs and outrageous stories, and for your immense support, guidance and friendship. To Akriti – thank you for being the realist of the group, for the limitless supply of snacks, and for inspiring me every day to push a little harder. To Sunny – thank you for your words of advice, insightful questions, and for dragging me to the gym! And to the previous members of the lab, especially Dana, Majd and Ana, thank you for your kindness, mentorship and radiating positivity.

I would also like to thank all the members of the Cell Biology Department. We have made so many memories together over the years, and I am thankful I got the chance to share this chapter in my life with you all. To my lab/floor mates Zuhra, Vritika, Gaby, Kelvin, Dustin, Kate, Joel, Fernando, Vikki, Paris, Farooz, Siv, Nick, Victoria, Kyla, Bushra, Diana, Frank, Vikrant – and so many more, thank you for your amazing friendship, vision, and collaboration.

To my loving parents, Tony and Mary – you have always been my biggest supporters and number one fans, always pushing me and encouraging me to do my best and pursue my dreams. To my sisters – you have always been there for me, thanks for being a constant outlet and source of entertainment. To my amazing friends, Andrea, Collin and Emerson and to all the friends I have made over the past two years, thank you for all your support. I love you all!
To my love, Gareth – you have been there for me every single day of this degree; I cannot thank you enough for your unwavering support, motivation and love. Thank you for the countless homemade dinners, rides home, hugs, and putting me back on my feet every time I fell. Thank you for sincerely trying to understand what I was studying so that you knew what I was talking about half the time, and thanks for being so understanding of my bizarre and crazy schedule. I am so grateful to have you in my life, and truly cannot wait to see where this next chapter takes us.

Finally, I would like to dedicate this thesis to my late grandfather, Luigi Panacci, who unfortunately passed during my studies here. It only seems fitting that with the culmination of this degree, your house was sold and emptied. Thank you for braving a move across the world to cultivate a better life for our family. Truly without you, none of this would be possible.
Statement of Contributions

All experiments, analysis, and results presented in this thesis were performed by the author. Primary hepatocytes were isolated by the MacParland Lab by Damra Camat and Max Ma.
# Table of Contents

Acknowledgments .................................................................................................................. iii

Statement of Contributions ................................................................................................... v

Table of Contents ................................................................................................................... vi

List of Abbreviations ................................................................................................................. x

List of Tables ........................................................................................................................... xii

List of Figures .......................................................................................................................... xiii

1 Chapter 1: Introduction .......................................................................................................... 1

1.1 Liver transplantation and hepatic steatosis ........................................................................ 1

1.1.1 Liver transplantation ................................................................................................. 1

1.1.2 Hepatic steatosis and NAFLD .................................................................................. 3

1.1.3 The pathogenesis of hepatic steatosis ..................................................................... 3

1.1.4 Hepatic steatosis in the transplant setting ............................................................... 6

1.1.5 Defatting steatotic livers using *ex vivo* transplantation systems ......................... 6

1.2 Autophagy .......................................................................................................................... 10

1.2.1 Autophagy Overview ............................................................................................. 10

1.2.2 The Autophagy Pathway ......................................................................................... 10

1.3 Autophagy in the liver ....................................................................................................... 15

1.3.1 Lipid droplets .......................................................................................................... 15

1.3.2 The liver, lipid droplets and autophagy .................................................................. 15

1.3.3 Autophagy regulates intrahepatocellular LDs ......................................................... 16

1.3.4 Intracellular LDs regulate autophagy ..................................................................... 18

1.3.5 Autophagy and the pathogenesis of hepatic steatosis .......................................... 18
1.3.6 Activating autophagy to decrease hepatic steatosis in the transplant setting......19

1.4 Transcription factor EB as the master regulator of autophagy and lysosomal biogenesis.................................................................19
1.4.1 TFEB as a master regulator of lysosomal biogenesis..........................19
1.4.2 TFEB as a master regulator of autophagy........................................20
1.4.3 Regulation of TFEB activity............................................................22
1.4.4 Regulation of TFEB activity via lysosomal calcium release..............24

1.5 TRPML1: a lysosomal calcium channel............................................26
1.5.1 The transient receptor potential family..........................................26
1.5.2 The TRPML1 channel...................................................................26
1.5.3 TRPML1 and lysosomal function....................................................27
1.5.4 TRPML1 and the induction of autophagy........................................28
1.5.5 TRPML1 agonism via synthetic agonist MLSA1..............................29

2 Chapter 2: Research Aims and Hypothesis.............................................31
2.1 Hypothesis.........................................................................................31
2.2 Research Aims Summary.................................................................31
2.3 Research Aim 1..............................................................................31
2.3.1 Research Aim 1: Rationale and Significance.................................31
2.4 Research Aim 2..............................................................................32
2.4.1 Research Aim 2: Rationale and Significance.................................32
2.5 Research Aim 3..............................................................................32
2.5.1 Research Aim 3: Rationale and Significance.................................32

3 Chapter 3: Methods.............................................................................34
3.1 Cell Cultures....................................................................................34
3.1.1 Hep3B cells................................................................................34
3.1.2 Primary hepatocytes ........................................................................................................34

3.2 Bacterial Cultures and Growth Conditions ....................................................................35

3.2.1 Preparation of conditioned culture media supernatants ...........................................35

3.3 Fat-overloading induction in cells ..................................................................................35

3.3.1 Preparation of FFA stock solutions .........................................................................35

3.3.2 Preparation of FFA-BSA conjugates ......................................................................35

3.3.3 Inducing lipid overloading in cells ..........................................................................36

3.3.4 Pharmacological Treatments ....................................................................................36

3.4 Antibodies .......................................................................................................................37

3.5 Immunoblotting ..............................................................................................................37

3.6 Densitometry Analysis ..................................................................................................38

3.7 Immunofluorescence ....................................................................................................40

3.8 Oil Red O Staining ..........................................................................................................42

3.9 Statistical Analysis ........................................................................................................42

4 Chapter 4: Results ...........................................................................................................43

4.1 TRPML1 is functional in Hep3B cells and primary hepatocytes and MLSA1 rescue is effective ........................................................................................................................................43

4.1.1 TRPML1 immunoblot analysis ...............................................................................43

4.1.2 TRPML1 functional assay: disrupting autophagy by \( H. pylori \) ..........................45

4.1.3 Activation of TRPML1 via MLSA1 reverses accumulation of VacA-induced LAMP-1-positive vacuoles .................................................................45

4.1.4 MLSA1-mediated TRPML1 activation does not reverse autophagy defect following VacA treatment .......................................................................................51

4.2 Developing an in vitro hepatic steatosis model .............................................................56

4.2.1 FFA treatment induces lipid droplet accumulation .................................................56

4.2.2 FFA treatment does not impair autophagic flux .....................................................67
4.3 MLSA1 induces LC3 accumulation in Hep3B cells..............................................................72
4.4 MLSA1 addition does not rescue FFA-induced lipid droplet accumulation ...............77

5 Chapter 5: Discussion ........................................................................................................89
  5.1 TRPML1 functionality in Hep3B cells and in primary hepatocytes..........................89
  5.2 Effect of FFA supplementation on LD accumulation...............................................92
  5.3 Effect of FFA supplementation on autophagic flux...............................................93
  5.4 Effect of MLSA1 on autophagy following FFA supplementation .........................94
  5.5 Effect of MLSA1 addition following FFA supplementation on LD accumulation ....95
  5.6 Translational relevance .........................................................................................96
  5.7 Conclusions.............................................................................................................97

6 Chapter 6: Future Directions............................................................................................98
  6.1 Can we further characterize TRPML1 channels? .....................................................98
  6.2 Would more potent TRPML1 agonists be able to reduce LD accumulation? ..........98
  6.3 Is TRPML1-mediated autophagy induction the mechanism behind LD breakdown?.....101
       6.3.1 Does TRPML1 activation mediate changes in fat catabolism? ......................102
       6.3.2 Does a lack of TRPML1 expression increase LD accumulation in the liver? .....102
  6.4 Would it be advantageous to target multiple routes simultaneously to defat livers? ....103
  6.5 What is the effect of TRPML1 activation in more physiologically relevant models?..104
       6.5.1 Development of macrosteatotic culture systems ..............................................104
       6.5.2 Liver organoids ...............................................................................................105
  6.6 The use of MLSA1 in the transplant setting ...............................................................106

References........................................................................................................................107
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMEM</td>
<td>Advanced Minimum Essential Medium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of the variance</td>
</tr>
<tr>
<td>ATG</td>
<td>autophagy-related</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>autophagy-related 16 like 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAF</td>
<td>bafilomycin</td>
</tr>
<tr>
<td>BODIPY</td>
<td>4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCMS</td>
<td>conditioned culture media supernatant</td>
</tr>
<tr>
<td>CLEAR</td>
<td>coordinated lysosomal expression and regulation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHSB</td>
<td>developmental studies hybridoma bank</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>HFD</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>lysosome-associated membrane protein 1</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated light chain 3</td>
</tr>
<tr>
<td>LD</td>
<td>lipid droplet</td>
</tr>
<tr>
<td>LDLT</td>
<td>living-donor liver transplantation</td>
</tr>
<tr>
<td>LELs</td>
<td>late endosomes and lysosomes</td>
</tr>
<tr>
<td>LT</td>
<td>long-term</td>
</tr>
<tr>
<td>MCOLN</td>
<td>mucolipin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MLIV</td>
<td>mucolipidosis type IV</td>
</tr>
<tr>
<td>MLSA1</td>
<td>mucolipin synthetic agonist 1</td>
</tr>
<tr>
<td>MLSA3</td>
<td>mucolipin synthetic agonist 3</td>
</tr>
<tr>
<td>MLSI3</td>
<td>mucolipin synthetic inhibitor 3</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NMP</td>
<td>normothermic machine perfusion</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORO</td>
<td>oil red O</td>
</tr>
<tr>
<td>PA</td>
<td>palmitic acid</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PGC1α</td>
<td>peroxisome proliferator-activated receptor-γ coactivator 1α</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SCS</td>
<td>static cold storage</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>ST</td>
<td>short-term</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline - Tween 20</td>
</tr>
<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPML1</td>
<td>transient receptor potential membrane channel mucolipin 1</td>
</tr>
<tr>
<td>VacA</td>
<td>vacuolating cytotoxin A</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
</tr>
</tbody>
</table>
List of Tables

Table 1. Antibodies used for immunoblotting. ................................................................. 39

Table 2. Antibodies used for immunofluorescence. ........................................................ 41
List of Figures

Figure 1.1. Number of patients/mortalities on the liver transplant waitlist in Canada. .................. 2
Figure 1.2. Pathogenesis of hepatic steatosis. ............................................................................ 5
Figure 1.3. *Ex vivo* liver perfusion system ............................................................................. 8
Figure 1.4. Regulation of autophagy via mTORC1 .................................................................... 12
Figure 1.5. An overview of the autophagy pathway. ................................................................. 14
Figure 1.6. Process of LD breakdown by lipophagy. ................................................................. 17
Figure 1.7. TFEB regulates lysosomal biogenesis and autophagy. .......................................... 21
Figure 1.8. Mechanism of TFEB regulation via mTORC1 ....................................................... 23
Figure 1.9. Mechanism of TFEB regulation via lysosomal calcium release. .......................... 25
Figure 1.10. TRPML1 is activated by MLSA1 .......................................................................... 30
Figure 4.1. TRPML1 immunoblot analysis did not validate TRPML1 expression .................. 44
Figure 4.2. TRPML1 activation reverses the accumulation of VacA-mediated LAMP-1-positive vacuoles in Hep3B cells .................................................................................. 48
Figure 4.3. TRPML1 activation also reverses the accumulation of VacA-mediated LAMP-1-positive vacuoles in primary hepatocytes. ................................................................. 50
Figure 4.4. MLSA1 does not rescue VacA-induced autophagy disruption in Hep3B cells. ...... 53
Figure 4.5. MLSA1 does not rescue VacA-induced autophagy disruption in primary hepatocytes. ........................................................................................................................................ 55
Figure 4.6. ST FFA incubation induces LD accumulation in Hep3B cells. ......................... 59
Figure 4.7. LT FFA incubation induces LD accumulation in Hep3B cells. ......................... 61
Figure 4.8. ST FFA incubation induces LD accumulation in primary hepatocytes. ................. 64

Figure 4.9. LT FFA incubation induces LD accumulation in primary hepatocytes. ............... 66

Figure 4.10. Autophagic flux assays with bafilomycin reveal that ST FFA supplementation does not disrupt autophagy. ......................................................................................................................... 69

Figure 4.11. Autophagic flux assays with bafilomycin reveal that LT FFA supplementation does not disrupt autophagy. ......................................................................................................................... 71

Figure 4.12. MLSA1 addition following ST FFA supplementation results in an increase in LC3-II. ........................................................................................................................................ 74

Figure 4.13. MLSA1 addition following LT FFA supplementation results in an increase in LC3-II. ........................................................................................................................................ 76

Figure 4.14. MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in Hep3B cells. ........................................................................................................................... 79

Figure 4.15. MLSA1 treatment does not decrease LD accumulation following 24 h FFA incubation in Hep3B cells. ........................................................................................................................... 81

Figure 4.16. MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in primary hepatocytes. ........................................................................................................................... 84

Figure 4.17. Longer MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in primary hepatocytes. ........................................................................................................................... 86

Figure 4.18. MLSA1 treatment does not decrease LD accumulation following 16 h FFA incubation in primary hepatocytes. ........................................................................................................................... 88

Figure 6.1. MLSA1 and MLSA3 possess very different chemical structures..........................100
Chapter 1: Introduction

1.1 Liver transplantation and hepatic steatosis

1.1.1 Liver transplantation

For many patients with end-stage liver disease, liver transplantation is the only therapeutic option. Livers can be donated from a living donor or from individuals who have been declared brain dead. In Canada, over 400 liver transplants are performed per year\(^1\). Despite the number of successful liver transplants and the advances made in this field in the recent decades, there still exists long liver transplant wait lists, high waitlist mortality, and an overall organ shortage\(^2\) (Figure 1.1). Therefore, transplant centers often resort to utilizing suboptimal grafts, such as steatotic livers for transplantation.
Figure 1.1. Number of patients/mortalities on the liver transplant waitlist in Canada. These data show that around 400 individuals are still on the wait list each year, while 50-100 mortalities occur.

Data obtained from the last available Canadian Organ Replacement Register (CORR) Annual Statistics, 2017: Liver Transplants, 2006 to 2015.
1.1.2 Hepatic steatosis and NAFLD

Liver steatosis (fatty liver) is the earliest stage in the spectrum of conditions that characterize nonalcoholic fatty liver disease (NAFLD) and is described as the deposition of lipid droplets in the cytoplasm of hepatocytes\(^3\). The prevalence of NAFLD in the general population of the United States has been reported at 46\(^{\%}\)\(^4\) and occurs in more than 65\(^{\%}\) of individuals who are obese\(^5\). In addition to obesity, other factors associated with the development of NAFLD include dyslipidemia, type 2 diabetes mellitus, and metabolic syndrome\(^6\). NAFLD has been described as the “hepatic manifestation” of metabolic syndrome, which incorporates insulin resistance, hypertension, hyperlipidemia, hyperglycemia, and obesity\(^7\).

Steatosis in the liver can be defined both quantitatively and qualitatively. Quantitative assessment is based on the percentage of fat within the hepatocytes. These assessments are graded as mild (<30\(^{\%}\)), moderate (30-60\(^{\%}\)), and severe (>60\(^{\%}\))\(^8\). Qualitative assessment further separates fatty livers in two categories, macrosteatosis and microsteatosis. Macrosteatosis is characterized by a single fat vacuole in hepatocytes, which displaces the nucleus to the edge of the cell. Macrosteatosis is more commonly associated with obesity and diabetes. Microsteatosis is characterized by tiny lipid vesicles in the cytoplasms of hepatocytes, without nuclear displacement.

1.1.3 The pathogenesis of hepatic steatosis

In normal conditions, the liver does not store triglycerides (TGs). Free fatty acids (FFAs) derived from the diet, adipose tissue lipolysis, or de novo lipogenesis are normally balanced with the clearance of FFAs. FFAs can be cleared via β-oxidation, whereby FFAs are oxidized and esterified into TGs, or via secretion by very-low-density lipoprotein (VLDL). In stressed conditions, such as in obesity or increased carbohydrate/fat intake, abnormal lipid metabolism occurs, causing hepatic lipid accumulation\(^9\). In addition, genetic defects in genes involved in FFA uptake or clearance are associated with the development of hepatic steatosis\(^10\) (Figure 1.2).

Hepatic fat has been associated with metabolic dysfunctions\(^11\). In fact, intrahepatic fat, and not visceral fat, is correlated with insulin resistance, potentially leading to the dyslipidemia associated with hepatic steatosis\(^12,13\). Approximately 60\% of patients with fatty liver present with dyslipidemia and hyperglycemia\(^14\). Hepatic steatosis is also linked to mitochondrial
dysfunction\textsuperscript{15,16}. The mitochondria is the cellular location wherein energy in the form of adenosine triphosphate (ATP) is generated via fatty acid oxidation. Heterozygous mutations in the mitochondrial trifunctional protein, responsible for catalyzing steps involved in β-oxidation, lead to insulin resistance and hepatic steatosis in mice fed a low-fat chow diet\textsuperscript{17}. Moreover, an excess of FFAs in the liver perturbs mitochondrial homeostasis and causes mitochondrial dysfunction, resulting in the production of reactive oxygen species (ROS) from an accumulation of incompletely oxidized substrates\textsuperscript{18,19}. In addition to increased mitochondrial stress, lipid accumulation in the liver also increases endoplasmic reticulum stress; both of these stresses result in lipotoxic effects seen in fatty liver\textsuperscript{20}. In sum, increased hepatic TG could lead to the development of metabolic dysfunction resulting in insulin resistance, dyslipidemia, and subsequent progression to inflammation and liver damage – termed non-alcoholic hepatic steatosis (NASH). Complications related to NASH include fibrosis, cirrhosis and hepatocellular carcinoma\textsuperscript{20}. 
Figure 1.2. Pathogenesis of hepatic steatosis.

(A) Under physiologic conditions, FFAs derived from the diet, adipose tissue lipolysis, or *de novo* lipogenesis are esterified into TGs and are cleared via β-oxidation in the mitochondria or secreted by VLDL. (B) When a significant increase in the FFA pool occurs, abnormal lipid metabolism causes hepatic lipid accumulation, ER stress, and mitochondrial dysfunction with subsequent ROS production. An increase in hepatic TGs leads to fatty liver and subsequent metabolic dysfunctions.
1.1.4 Hepatic steatosis in the transplant setting

Hepatic steatosis is commonly encountered during hepatic organ procurement with rates as high as 30% in cadaveric and 20% in potential living donors. Given the steady increase in the prevalence of metabolic syndrome and obesity, it is expected that rates of steatosis will become increasingly prevalent in potential donors.

Transplant outcomes are not greatly affected by hepatic microsteatosis or mild macrosteatosis. However, liver allografts with severe macrosteatosis have a significant risk of graft failure and are frequently discarded. This is largely due to the fact that use of steatotic livers for transplantation is associated with increased incidence of primary graft non-function and delayed graft function. Furthermore, patients who receive livers with more than 30% of macrosteatosis have poorer outcomes, such as longer hospital and ICU stays, compared to patients who receive a liver with 5% or less steatosis.

Due to poorer recipient outcomes, livers with significant steatosis are discarded, further contributing to the organ shortage problem. Therefore, in vivo or ex vivo manipulations that reduce hepatic steatosis prior to liver transplantation could increase the number of suitable organs and drastically reduce current waitlists.

1.1.5 Defatting steatotic livers using ex vivo transplantation systems

The concept of defatting livers prior to transplantation has been attempted in live donor liver transplants (LDLT) in humans with steatosis. In one study, a combination of a protein-rich diet, exercise, and administration of a drug that reduces triglyceride levels by stimulating fatty acid β-oxidation, bezafibrate, for 2-8 weeks led to a threefold reduction in macrosteatosis in 11 LDLT candidates with steatosis. In another study, three living donors who had biopsy-proven macrosteatosis (>30%) consumed omega-3 fatty acids for 1 month to attempt to decrease hepatic macrosteatosis. In both studies, the defatting strategies used led to decreased hepatic macrosteatosis, normalized donor liver tests including total bilirubin and alanine aminotransferase, normalized lipid metabolism, thereby allowing for successful graft procurement. Despite these results, these studies use defatting strategies would only be
relevant in the case of LDLT. In procured liver grafts from deceased donors, the kinetics of defatting must occur in a matter of hours to fit into the logistics of liver transplantation (i.e. procurement to transplant in a maximum of 12 h)\textsuperscript{30,31}.

Novel preservation techniques, such as machine perfusion systems, offer a platform for the delivery of defatting agents prior to transplant. This organ preservation system consists of two perfusion circuits for the hepatic artery and portal vein which include a pump, flow meter, and pressure sensor\textsuperscript{32} (Figure 1.3). An oxygenator is placed in the arterial circuit, and a computer controls flow conditions and temperatures of the preservation solution. Machine perfusion techniques are categorized by the temperature of the perfusate and include hypothermic oxygenated machine perfusion (10ºC), sub-normothermic machine perfusion (25ºC) and normothermic machine perfusion (NMP; 37ºC)\textsuperscript{33}. Machine perfusion has been shown to be superior to static cold storage (SCS) by decreasing preservation injury and improving organ viability in lean, ischemic and steatotic livers\textsuperscript{34}. Perfusion technology offers a potential platform to preserve, assess and repair marginal donor organs, including steatotic livers\textsuperscript{35}. 

Figure 1.3. *Ex vivo* liver perfusion system.

This organ preservation system consists of two perfusion circuits for the hepatic artery (HA) and portal vein (PV) which include a pump, flow meter, and pressure monitors. An oxygenator is placed in the arterial circuit, and a computer controls flow conditions and temperatures of the preservation solution; temperatures can be altered based on the perfusion technique chosen.
Of these techniques, NMP may be the most promising in improving the quality of steatotic grafts as the liver is still metabolically active. This setting provides the opportunity to perform a thorough evaluation of hepatic function and suitability for transplantation in comparison with SCS or cold perfusion\textsuperscript{34}. The overall goal of liver defatting is to rapidly decrease the proportion of macrosteatotic hepatocytes, while maintaining high viability and functionality of the organ. To achieve this, a shift needs to occur in lipid metabolism from TG synthesis to breakdown\textsuperscript{36}. Working within the realm of NMP may allow for the modulation of lipid metabolism in a more physiologically relevant system.

Experimental studies have previously reported macrosteatotic liver defatting in animal models using \textit{ex vivo} perfusion. First, Bessems \textit{et al.} procured macrosteatotic livers from choline-methionine deficient diet rats [a common model of NASH] and performed a 1 h NMP on them. Following this, cell death markers were reduced while liver function markers were significantly higher compared to SCS livers\textsuperscript{37}. Moreover, prolonged NMP (48 h) in a porcine fatty liver model resulted in decreased hepatic fat content from 30% to 15% during perfusion, as assessed by histology\textsuperscript{38}. In 2009, Nagrath \textit{et al.} went on to assess the combination of pharmacological interventions with NMP in reducing liver steatosis within the clinically relevant time scale of several hours\textsuperscript{36}. The authors developed a defatting cocktail that was supplemented with pharmacological agents used to target the pathways involved in triglyceride secretion and β-oxidation (i.e. peroxisome proliferator-activated receptor (PPAR)-α and PPAR-δ agonists, as well as mimetics of insulin and glucagon). Macrosteatotic livers procured from obese Zucker rats were perfused \textit{ex vivo} using NMP with this cocktail of defatting agents. Remarkably, after only 3 h of perfusion, there was a reduction in lipid droplet size and the intracellular lipid content was reduced by greater than 50\%\textsuperscript{36}. In sum, the development of new innovative preservation techniques, coupled with pharmacological agents, is of great interest to improve organ viability within the \textit{ex vivo}, pre-transplant setting. Such innovative therapies would allow for the increased utilization of fatty livers for transplantation.

In 2018, the first randomized clinical trial with NMP in patients was published. Results from this trial revealed a significant decrease in the peak of aspartate transaminase, less early allograft dysfunction and lower discard rate in the NMP compared to SCS group\textsuperscript{39}. There are no published
clinical trials that explore the effects of NMP on fatty livers, however these results show promise in using NMP to improve function of marginal grafts, such as those with steatosis. One potential mechanism to enhance the removal of fat in steatotic grafts using NMP is through enhancing autophagy.

1.2 Autophagy

1.2.1 Autophagy Overview

Autophagy is a highly conserved cellular degradation and recycling pathway; essential for both maintaining cellular energy homeostasis in times of nutrient deprivation, and for removing aged or damaged cellular components\textsuperscript{40,41}. Autophagy is activated in response to cellular stressors, such as starvation or via specific signaling pathways, or environmental stressors, such as nutrient and oxygen deprivation, pathogen presence, or amino acid starvation\textsuperscript{42,43}. There are three different types of autophagy, classified by how substrates are trafficked to the lysosomes for degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy. The major type of autophagy, macroautophagy, sequesters and transports a heterogeneous set of cargo in vesicles, called autophagosomes, to the lysosome. In the lysosome, cargo is broken down by lysosomal hydrolases, and the resulting macromolecules are recycled. Autophagy dysregulation has been linked to various pathological consequences, including neurodegeneration, NAFLD, and tumor progression\textsuperscript{44–46}.

1.2.2 The Autophagy Pathway

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase which acts as a sensor for growth factors, nutrient signals, and energy status. mTOR exists in a complex, mTORC1 (mammalian target of rapamycin complex 1), which regulates several cellular processes including autophagy, cell growth, proliferation, and protein synthesis\textsuperscript{47}. Interestingly, autophagy induction requires nutrient or growth factor deprivation whereas low cellular energy levels inhibit mTORC1 activity, demonstrating an inverse relationship between autophagy induction and mTORC1 activation\textsuperscript{48}.

Initiation of autophagy requires inhibition of mTORC1. During starvation, mTORC1 is inhibited, resulting in increased autophagy-initiating ULK1/2 kinase activity\textsuperscript{49}. ULK1/2 phosphorylate other components of the ULK complex, including ATG13, FIP200, and ULK1/2 itself. In
addition, mTORC1 also regulates the VPS34 complex, a class III phosphoinositide 3-kinase, whose activity is crucial for promoting autophagosome formation\textsuperscript{50}. Together, these kinase complexes promote autophagy initiation and drive the formation of the phagophore, which sequesters cytoplasmic cargo targeted for degradation\textsuperscript{48,51} (Figure 1.4).
Figure 1.4. Regulation of autophagy via mTORC1.
Under conditions of sufficient nutrients and growth factors, autophagy is inhibited via mTORC1 activation. On the other hand, during times of nutrient deprivation, mTORC1 is inhibited, thereby resulting in the downstream activation of the ULK and Vps34 complexes, which are essential for the early steps of autophagosome formation. When mTORC1 is inhibited, these suppressed complexes become activated, and in concert promote autophagy initiation and phagophore formation.

*Figures created using templates from Biorender.*
The molecular mechanism of autophagy includes several stages following autophagy induction: phagophore initiation, elongation, maturation, and cargo degradation\textsuperscript{52}. After autophagy induction, two ubiquitin-like conjugation systems facilitate the expansion of the phagophore and subsequent shaping and sealing of the autophagosome. The first ubiquitin-conjugation system requires autophagy-related (ATG) proteins, ATG12-ATG5-ATG16L (autophagy-related 16 like 1) and is associated with the elongation of the outer autophagosomal membrane. This protein complex permits the second conjugation reaction to occur\textsuperscript{53}. The second ubiquitin-like conjugation system involves the lipidation of microtubule-associated light chain 3 (LC3); an essential process for the formation of mature autophagosomes. The ATG12-ATG5-ATG16L protein complex serves as a E3 ligase which facilitates the addition of the phospholipid, phosphatidylethanolamine (PE), to LC3, forming LC3-II\textsuperscript{54}. Lipidated LC3-II associates with newly forming autophagosome membranes and remains on mature autophagosomes until fusion with lysosomes. Following the maturation and closure of the autophagosome, fusion with lysosomes occurs to form autolysosomes. These soluble N-ethylmaleimide-sensitive factor-attachment protein receptor-mediated fusion events result in the acidification of the lumen and degradation of the sequestered cargo through lysosomal hydrolases (Figure 1.5).

The sum of these molecular events constitutes autophagic flux: from autophagic induction and autophagosome formation to autolysosomal degradation and recycling. LC3-I to LC3-II conversion and LC3-II to actin ratios are well-known markers of autophagy induction as the amount of LC3-II can be indicative of autophagosomal number\textsuperscript{55,56,57}. However, because LC3-II accumulation can also be the result of decreased autophagosome degradation and thus impaired autophagic flux, this marker alone is not sufficient to assess flux. A marker of autophagic flux is the ubiquitin-associated protein p62, which binds to LC3\textsuperscript{58} and physically links autophagic cargo to the autophagic membrane\textsuperscript{59}. Since both proteins are degraded through the autophagy process, they are often used as markers of autophagic activity.
Figure 1.5. An overview of the autophagy pathway.

Autophagy is activated during times of cellular environmental stress, such as nutrient deprivation. The goal of autophagy is to maintain cellular homeostasis and quality of cellular components. The overall pathway includes several stages: phagophore initiation, elongation, autophagosome maturation, and cargo degradation upon lysosomal fusion. Macromolecules are then recycled back into the cytosol. The ATG12-ATG5-ATG16L complex facilitates the addition of PE to LC3, forming LC3-II, which localizes to both the inner and outer autophagosome membrane. LC3-II is only degraded once the autophagosome is fused with the lysosome.
1.3 Autophagy in the liver

1.3.1 Lipid droplets

In cells, lipids are stored within organelles called lipid droplets (LDs), which vary in size and appearance between organisms and cell types. Despite this variation, the structure and organization of LDs is highly conserved. LDs consist of a neutral hydrophobic lipid core, mainly consisting of TGs and cholesteryl esters, surrounded by a monolayer of phospholipids decorated by several type of proteins including perilipins.

LDs play an important role in scavenging FFAs, which would otherwise be toxic to the cell by increasing endoplasmic reticulum stress, insulin resistance, mitochondrial dysfunction, and apoptosis; a phenomenon described as lipotoxicity. LDs store these potentially toxic intracellular FFAs as TGs. Lipid droplets are highly dynamic organelles and alternate between periods of growth and consumption depending on the cell’s energy requirements. In times of nutrient deprivation, TGs can be broken down to their component FFAs by cytosolic lipases in a process known as lipolysis. Lipolysis uses FFAs to supply the cell with ATP through mitochondrial \( \beta \)-oxidation. When nutrients are abundant, lipolysis re-esterifies FFAs back into TGs.

Aside from cytosolic lipases, lipases have also been found in the lysosome (named lysosomal acid lipases), which play a role in hydrolyzing parts of TG-rich lipoproteins. Studies using lysosomal inhibitors (i.e. ammonium chloride/chloroquine) demonstrated that lysosomal enzymes also contribute to the lipolysis of intracellular lipids. It is now widely accepted that the lysosomal pathway of autophagy degrades LD-stored TGs.

1.3.2 The liver, lipid droplets and autophagy

The liver plays an important role in the packaging, redistribution, and processing of fatty acids. In the liver, autophagy-mediated degradation of LDs is crucial for lipid turnover. Recent evidence suggests that the catabolism of glycogen, lipid droplets and proteins through autophagy affects basic hepatic metabolic functions including glycogenolysis, gluconeogenesis, and \( \beta \)-oxidation. Dysregulation of the mechanisms by which the liver mobilizes stored lipid for energy production can promote liver disease or dysfunction – namely, hepatic steatosis.
1.3.3 Autophagy regulates intrahepatocellular LDs

Numerous studies have identified a selective form of autophagy that specifically targets LDs. In 2009, Singh et al. demonstrated that lipid droplet delivery to the lysosomes occurs through the sequestration of LDs by autophagosomes followed by fusion with lysosomes, leading to degradation of LDs by lysosomal acid hydrolases\textsuperscript{44,67}. Through the autophagic breakdown of LDs into their essential component FFAs, the rate of mitochondrial B-oxidation is enhanced and ATP production increases (Figure 1.6).
Figure 1.6. Process of LD breakdown by lipophagy.

Entire small LDs or portions of larger ones are first sequestered by autophagosomes. Then, autophagosomes fuse with lysosomes to form autolysosomes in which the substrates of the autophagosome are degraded by lysosomal hydrolytic enzymes. Lipid breakdown leads to degradation products such as FFAs which are then released to the cytoplasm. FFAs sustain rates of mitochondrial β-oxidation to maintain cellular energy homeostasis.
1.3.4 Intracellular LDs regulate autophagy

Under normal conditions, lipophagy regulates hepatocellular lipid accumulation following acute lipogenic stimuli\(^\text{68}\). Sustained lipogenic or acute elevated abnormal lipid levels compromise lipophagic function. Singh et al. demonstrated that in more chronic model systems of hepatic steatosis [such as in mice fed a high-fat diet (HFD) for prolonged periods], there is a paucity in the clearance of LDs through lipophagy\(^\text{68}\). In this study, electron microscopy revealed evidence of decreased LC3-positive membranes on lipid droplets and overall reduced degradation of LDs following chronic lipogenic stimuli.

A follow-up study by Koga et al. demonstrated that following chronic lipogenic stimuli, there is defective autophagy for all forms of autophagic cargo, not just lipids, suggesting that the primary defect lies in the autophagic machinery itself\(^\text{69}\). The researchers dissected each of the individual steps of autophagy following HFD feeding and concluded that the defect occurs at the level of autophagosome—lysosome fusion\(^\text{69}\). They went on to suggest that changes in membrane lipid composition following HFD feeding could possibly lead to decreased autophagosome—lysosome fusion. The Western diet may lead to similar autophagic defects in humans. Prolonged exposure to diets rich in fat may similarly impair lipid breakdown through defects in autophagosome—lysosome fusion, leading to a vicious cycle of hepatic fat accumulation.

1.3.5 Autophagy and the pathogenesis of hepatic steatosis

The role of autophagy in the pathogenesis of hepatic steatosis was first suggested by the finding that autophagy mediates the breakdown of intracellular lipids in hepatocytes\(^\text{68}\). Studies by Singh et al. in cultured hepatocytes and in mice showed that inhibition of autophagy led to an increase in LD accumulation in response to lipid challenges such as FFA supplementation. Researchers also found support for the autophagic regulation of intracellular lipids via the co-localization of LDs with autophagic components such as lysosome-associated membrane protein 1 (LAMP-1) and LC3 during nutrient deprivation. This novel finding illustrated that autophagy can regulate the intracellular level of lipids by removing lipid droplets; a process they coined lipophagy\(^\text{68}\).

Further studies also illustrated that the inhibition or induction of autophagy increases or decreases lipid accumulation in the liver, respectively. Liver-specific knockdown of autophagy genes, Atg14 or Atg7, increased hepatic TG content and cholesterol levels, whereas their
overexpression improved hepatic steatosis in mouse models of obesity (ob/ob mice and HFD-fed mice)\textsuperscript{70,71}. In addition, pharmacological promotion of autophagy in vivo with autophagy enhancers, carbamazepine or rapamycin, was protective in reducing steatosis in murine models of non-alcoholic and alcoholic fatty liver disease\textsuperscript{72,73}. Similarly, knockout of transcription factor EB, a transcriptional regulator of autophagy and lysosome biogenesis, resulted in accumulation of hepatocellular LDs\textsuperscript{74}. Conversely, overexpression of TFEB ameliorated steatosis and improved glucose homeostasis in the liver of obese mice\textsuperscript{74} and improved liver injury and inflammation in a murine model of alcoholic fatty liver disease\textsuperscript{75}. The ability of autophagy to regulate LD accumulation in hepatocytes further highlights the fact that autophagy in the liver may regulate the development of steatotic liver diseases, such as non-alcoholic and alcoholic steatohepatitis\textsuperscript{44,76,77}.

### 1.3.6 Activating autophagy to decrease hepatic steatosis in the transplant setting

It is important to note that some autophagy enhancers may not be suitable, effective, or safe to use in the transplant setting. For instance, rapamycin, an mTORC1 inhibitor, has been shown to be protective in reducing steatosis in studies using a variety of fatty liver model systems\textsuperscript{72}. However, rapamycin cannot be use in the peri-transplant period as it has been linked to an increased risk of hepatic artery thrombosis\textsuperscript{78}. The ideal method of autophagy induction in the regulation of lipid metabolism should rapidly defat the liver without adversely affecting the viability of the organ, its functions, or the patient. This demands the use of a more specific agent to upregulate autophagy and decrease steatosis in the transplant setting. A potential candidate includes the activation of transcription factor EB to upregulate autophagy and lysosomal biogenesis.

### 1.4 Transcription factor EB as the master regulator of autophagy and lysosomal biogenesis

#### 1.4.1 TFEB as a master regulator of lysosomal biogenesis

Lysosomes are membrane-bound organelles present in all cell types. Most notably, they are responsible for degrading cargoes at the final step of many cellular pathways such as autophagy, phagocytosis, and endocytosis\textsuperscript{79}. The lysosome is also responsible for integrating the cell’s response to nutrient status, growth factors, and hormones\textsuperscript{80}. As such, they are key regulators in
catabolic processes of the cell, and their correct function is essential for maintaining cell homeostasis.

Promoter analysis of lysosomal genes revealed that they share the coordinated lysosomal expression and regulation (CLEAR) motif; a 10-base E-box-like palindromic sequence. Transcription factor EB (TFEB), a member of the bHLH leucine-zipper family of transcription factors, binds directly to CLEAR elements. Accordingly, TFEB promotes the expression of the entire network of genes that contains the CLEAR regulatory motif in their promoter. TFEB overexpression enhances lysosomal catabolic activity by increasing both lysosome number and levels of lysosomal enzymes. Thus, lysosomal biogenesis and function are controlled by transcriptional regulation via TFEB.

1.4.2 TFEB as a master regulator of autophagy

TFEB controls the expression of a broad number of genes involved in lysosome-associated processes, including autophagy. TFEB binds to the promoter regions of numerous autophagy genes, such as BECN1 and GAPARAP, which play a role in the regulation of autophagy. Moreover, TFEB induces autophagosome biogenesis and autophagosome-lysosome fusion. TFEB overexpression modulates organelle-specific autophagy; for example, by enhancing the clearance of lipid droplets (Figure 1.7).

TFEB modulates lysosomal biogenesis and autophagy by orchestrating a transcriptional program. Although TFEB is essential for enhancing its targets’ transcriptional levels in response to environmental changes, it does not regulate the basal transcription of its targets. Transcriptional regulation in response to environmental cues allows TFEB to maintain cellular degradative pathways and promotes intracellular clearance.
Figure 1.7. TFEB regulates lysosomal biogenesis and autophagy.
TFEB has been shown to bind to the promoter regions of numerous autophagy and lysosomal genes, upregulating their expression and function. Figure illustrates some of the downstream effects of TFEB activation.
1.4.3 Regulation of TFEB activity

During nutrient-rich conditions, TFEB is largely cytosolic and inactive, however upon starvation or lysosomal dysfunction TFEB translocates to the nucleus, where it can activate the transcription of its target genes\(^{81,83}\). The activity of TFEB is strictly regulated by its phosphorylation status, which dictates its cellular localization and activity (Figure 1.8). Two serine residues in the TFEB protein are responsible for regulating its subcellular localization, Ser142 and Ser 211\(^{83–85}\). When both serine residues are phosphorylated, TFEB is kept inactive in the cytosol.

The main protein kinases known to phosphorylate TFEB are the extracellular signal-regulated kinase 2 and mTORC1, both master regulators of cell growth\(^{84,85}\). In normal, nutrient-rich conditions, these kinases phosphorylate TFEB, localizing it to the cytoplasm. When mTORC1 is inhibited, such as during starvation, mTORC1 is released from the lysosomal membrane and becomes inactive. This results in TFEB dephosphorylation and subsequent translocation to the nucleus, where it is active and functions as a master regulator of lysosome biogenesis and autophagy. Interestingly, the lysosomal membrane is the site of mTORC1-mediated TFEB phosphorylation\(^{86}\).
Figure 1.8. Mechanism of TFEB regulation via mTORC1.
The lysosomal membrane serves as the site of mTORC1-mediated TFEB phosphorylation. Under nutrient-rich conditions, mTORC1 phosphorylates TFEB, sequestering it in the cytoplasm.
1.4.4 Regulation of TFEB activity via lysosomal calcium release

The signaling role of lysosomes in the regulation of TFEB activity is not limited to the mTORC1 pathway. Lysosomes also act as an intracellular calcium (Ca$^{2+}$) store. The release of lysosomal calcium stores can have indirect effects on autophagy and lysosome biogenesis through TFEB activation\textsuperscript{87}. Nutrient deprivation also results in the release of lysosomal Ca$^{2+}$ through the transient receptor potential membrane channel mucolipin 1 (TRPML1; also known as MCOLN1 or ML1) located on the lysosomal membrane. Lysosomal calcium release activates the serine/threonine phosphatase calcineurin. Calcineurin dephosphorylates TFEB residues Ser211 and Ser142, promoting its nuclear translocation\textsuperscript{88} (Figure 1.9). Complete depletion of this Ca$^{2+}$ channel inhibits subsequent TFEB activation and halts autophagy induction in times of nutrient deprivation\textsuperscript{88}. In addition, in calcineurin-silenced cells the effect of mTORC1 inhibition on nuclear TFEB translocation is significantly suppressed\textsuperscript{88}. This suggests that the control of TFEB subcellular localization by calcineurin is independent from mTORC1 activity, and that calcineurin acts downstream of mTORC1 in TFEB regulation.

The signaling cascades described above further outline the lysosome’s role as an integrative center; essential for sensing nutrient availability and coordinating the activation of a transcriptional program in response to the changing metabolic demands of the cell. Lysosomal-driven adaptation to cellular environmental cues is a self-sustaining response that is tightly regulated by feedback loops. Many factors that regulate TFEB activity (i.e. the Ca$^{2+}$ channel TRPML1) are themselves transcriptionally regulated by TFEB\textsuperscript{74}. Moreover, TFEB activation promotes its own transcription, further highlighting the role of feedback loops in sustaining lysosomal signaling and function.
Figure 1.9. Mechanism of TFEB regulation via lysosomal calcium release.
The lysosome also serves as a store for intracellular Ca\textsuperscript{2+}. Under starvation conditions, lysosomal calcium is released through TRPML1, activating the phosphatase calcineurin. Calcineurin dephosphorylates TFEB and promotes its nuclear localization, where it transcriptionally activates the lysosomal/autophagic pathway.
1.5 TRPML1: a lysosomal calcium channel

1.5.1 The transient receptor potential family

The transient receptor potential (TRP) family consists of seven subfamilies of integral membrane proteins that function as ion channels with diverse physiological roles, localized to many different cell and tissue types. Most TRPs are non-selective cation channels, though few are highly selective for calcium (Ca$^{2+}$). When TRP channels become activated, the cellular membrane becomes depolarized which activates voltage-dependent ion channels, changing the intracellular Ca$^{2+}$ concentration. Because of their role in regulating Ca$^{2+}$ release, TRP channels are important for proper cellular organelle functioning, such as in endosomes and lysosomes. Mutations in TRP genes have been implicated in various pathological states such as neurodegenerative diseases, polycystic kidney disease, and skeletal dysplasia. The mucolipin (MCOLN) gene encodes the seven subfamilies of the TRP family. One of these subfamilies is the transient receptor potential mucolipin (TRPML) family, which has 3 known members TRPML1, TRPML2, and TRPML3.

1.5.2 The TRPML1 channel

The first member of the mucolipin subfamily, TRPML1, is a cation channel ubiquitously expressed on the membranes of late endosomes and lysosomes (LELs) in mammalian cells. TRPML1 activity is vital for proper functioning of these organelles. The TRPML1 protein is encoded by the gene MCOLN1. TRPMLs are also conserved in Drosophila melanogaster and Caenorhabditis elegans, each having one TRPML gene, trpml and cup-5, respectively.

Mutations in MCOLN1 lead to a lysosomal storage disorder, mucolipidosis type IV (MLIV), a neurodegenerative disease characterized by psychomotor retardation and ophthalmological abnormalities. MLIV was classified as a mucolipidosis after electron microscopy data revealed the lysosomal storage of lipids and complex carbohydrates as well as water-soluble, granulated substances in these patients. At the cellular level, lack or dysfunction of TRPML1 impairs endocytic trafficking and lysosomal function, resulting in the accumulation of enlarged LEL compartments (vacuoles) in which heterogeneous material accumulate.

TRPML1 has a 6 transmembrane domain structure, which closely resembles TRPML2 and TRPML3. TRPML1 is a 580 amino acid long channel, with both the N- and C-termini exposed.
to the cytoplasm. The structure of TRPMLs differs from the rest of the TRP family in that they contain a large cytosolic loop between membrane domains one and two and have short C-terminal tails\(^9\). The di-leucine motifs found in both the N- and C-termini promote TRPML1 transport from the trans-Golgi network to LEL compartments, which are the primary location of TRPML1 in mammalian cells\(^10\).

1.5.2.1 TRPML1 cation selectivity
Numerous studies have cited TRPML1 as permeant to multiple cations, including Ca\(^{2+}\). LaPlante et al. first characterized the cation selectivity of TRPML1 by expressing human MCOLN1 RNA in Xenopus oocytes. Patch-clamping across the plasma membrane recorded channel opening and conductance in response to various ions. They concluded that TRPML1 is permeable to Ca\(^{2+}\), K\(^+\) and Na\(^+\), and the open probability of the channel is enhanced with increasing cytosolic Ca\(^{2+}\) concentrations\(^10\). Shen et al. later confirmed the Ca\(^{2+}\) permeability of TRPML1 with endolysosomal electrophysiology. Vacuolin-1, a cell-permeable triazine, was used to increase the size of endosomes and lysosomes, and then whole-endolysosome recordings were performed using patch-clamping. Here, they showed that Ca\(^{2+}\) currents through TRPML1 increase in response to the channel’s agonist, mucolipin synthetic agonist 1 (MLSA1)\(^10\). This paper also confirmed that TRPML1 is an inwardly rectifying current channel, which transports cations from the lumen of the lysosome to the cytosol. TRPML1 has also been shown to be permeable to other divalent cations, such as Fe\(^{2+}\) and Zn\(^{2+}\)\(^10\).

1.5.3 TRPML1 and lysosomal function
1.5.3.1 TRPML1 and lysosomal biogenesis, positioning and nutrient adaptation
TRPML1-mediated lysosomal calcium release regulates many aspects of lysosomal function, such as lysosomal degradation, catabolite export, and lysosomal trafficking, including autophagosome-lysosome fusion\(^10\). Lack of TRPML1 in cells results in defects in the LEL compartment, which may be due to impairments in vesicular fusion/fission due to a reduction in lysosomal Ca\(^{2+}\) release\(^10\).
TRPML1 also regulates lysosome size, reformation, biogenesis and positioning in the cell\textsuperscript{106,107}. Following autophagy induction, TRPML1 activity is required for the centripetal movement of lysosomes to the perinuclear region\textsuperscript{108}. TRPML1-mediated Ca\textsuperscript{2+} release activates the Ca\textsuperscript{2+}-binding protein, ALG-2, which binds to TRPML1 and recruits the dynein-dynactin complex for retrograde transport of lysosomes. With this enhanced mobility of lysosomes to the perinuclear region (where autophagosomes are), vesicular fusion is promoted, and the formation of autolysosomes ensues.

TRPML1 has also been implicated in the quality-control process of ensuring enhanced lysosome function and biogenesis matches the increase in autophagosome number upon autophagy induction. Wang \textit{et al.} showed that hours after nutrient starvation, TRPML1 channel activity was dramatically upregulated to increase lysosomal Ca\textsuperscript{2+} release, thereby promoting both lysosome reformation from autolysosomes and autophagosome-lysosome fusion\textsuperscript{109}. Furthermore, the presence of a nutrient starvation effect (either by inhibiting mTORC1 or activating TFEB) resulted in an increase in lysosomal proteostasis and enhanced clearance of lysosomal storage. Thus, lysosomal adaptation to the environment requires the upregulation of TRPML1 channels and subsequent Ca\textsuperscript{2+} signaling. Overall, these findings suggest that the upregulation of the TFEB-TRPML1 pathway could serve as a potential therapeutic target for lysosomal storage disorders and metabolic diseases.

1.5.4 TRPML1 and the induction of autophagy

Previous research has also connected TRPML1 to the induction of autophagy. Lack or dysfunction of TRPML1 has been related to defective autophagy, indicated by impairments in lysosomal pH as well as the accumulation and aggregation of p62 and autophagomes\textsuperscript{110,111}. In 2008, Vergarajauregui \textit{et al.} demonstrated that TRPML1 is required for fusion of autophagosomes and late endosomes with lysosomes\textsuperscript{45}. In another study, this same group showed that neurons isolated from the cerebellum of TRPML1 -/- mouse embryos had defective LC3 clearance, suggesting inefficient lysosomal function\textsuperscript{112}. In contrast, overexpression of TRPML1 resulted in a significant increase in autophagic flux in HeLa cells\textsuperscript{88}. 
Recent studies have also shown that lysosomal calcium regulates autophagy through calcineurin and TFEB\textsuperscript{87,109}. In conditions of nutrient starvation, lysosomal Ca\textsuperscript{2+} release via TRPML1 is triggered. Calcium activates the lysosomal Ca\textsuperscript{2+}-calmodulin-dependent calcineurin phosphatase, which binds and dephosphorylates TFEB\textsuperscript{88}. Dephosphorylated TFEB enters the nucleus and drives a transcriptional program to activate and sustain autophagy as described in section 1.4.4\textsuperscript{88}. Importantly, TRPML1 is a direct transcriptional target of TFEB, resulting in a positive feedback loop that increases TRPML1-TFEB response\textsuperscript{83}. TFEB activation is impaired upon depletion of TRPML1 or calcineurin, demonstrating that these three proteins coordinate starvation-mediated autophagy via a lysosome-to-nucleus signaling pathway\textsuperscript{88}.

Interestingly, TRPML1 has also been noted as a target of mTORC1 and rapamycin, further elucidating the complex role of this calcium channel in autophagy regulation. mTORC1 directly phosphorylates and inactivates TRPML1 thereby inhibiting autophagy\textsuperscript{113}. Rapamycin is commonly known to activate autophagy by inhibiting mTORC1; however, a recent study identified TRPML1 as another direct target of rapamycin\textsuperscript{114}. Through path-clamping of isolated lysosome membranes, Zhang et al. revealed that micromolar concentrations of rapamycin activated lysosomal TRPML1 directly and specifically. Interestingly, they found that rapamycin subsequently induced autophagic flux and lysosomal biogenesis via nuclear translocation of TFEB, thus demonstrating that rapamycin-dependent autophagy promotion occurs through a TRPML1-dependent mechanism.

1.5.5 TRPML1 agonism via synthetic agonist MLSA1

MLSA1 is a true agonist of mammalian TRPML1\textsuperscript{115} (Figure 1.10). MLSA1 induces conformational changes in the channel structure that ultimately leads to the opening of the selectivity filter, which allows cations such as Ca\textsuperscript{2+} to flow through the channel\textsuperscript{99}. MLSA1-mediated activation of TRPML1 channels has been directly linked to autophagy induction and reduction of lipid accumulation within lysosomes in cellular models of lysosomal storage diseases, such as MLIV and Niemann-Pick’s disease type C\textsuperscript{88,102}. In pathological conditions characterized by defective autophagy and lipid accumulation, as is the case in steatotic livers, pharmacological activation of TRPML1 may be a useful strategy to activate autophagy in an attempt to ameliorate the extent of steatosis.
Figure 1.10. TRPML1 is activated by MLSA1.

The lysosomal calcium channel, TRPML1, changes its conformation state from closed to open upon activation with MLSA1. Calcium can now freely flow through the channel, leading to successive autophagy activation via TFEB.
Chapter 2: Research Aims and Hypothesis

2.1 Hypothesis

We hypothesize that activation of TRPML1 via MLSA1 will attenuate hepatic steatosis by increasing autophagic flux and lysosomal function. More specifically, we predict that the introduction of MLSA1 in an in vitro model of steatosis will significantly reverse LD accumulation by increasing autophagic flux.

2.2 Research Aims Summary

The aim of this thesis is to explore the effect of TRPML1 activation on autophagy induction and potential concurrent reduction of hepatic steatosis in an in vitro fatty liver model.

2.3 Research Aim 1

To define a relevant model of in vitro hepatic steatosis in a hepatocyte cell line (Hep3B cells) and primary mouse hepatocytes.

2.3.1 Research Aim 1: Rationale and Significance

Lipid accumulation in hepatocytes is a defining pathologic hallmark of hepatic steatosis and is believed to be caused by increased uptake of FFAs from the circulation and/or increased de novo synthesis. Therefore, it is important for an in vitro model of steatosis to induce lipid accumulation so hepatocellular consequences of this can be further studied. Oleic acid (OA) and palmitic acid (PA) are the most frequently used FFAs in in vitro models of steatosis as they are the most abundant FFAs in Western diets and in liver triglycerides in both patients with NAFLD and normal subjects. Moreover, previous literature suggests that an in vitro model of steatosis using an FFA mixture that consists of a low portion of palmitic acid (i.e. oleate/palmitate 2:1 ratio) induces maximal fat over-accumulation with minimal toxicity. These findings were taken into consideration when designing the present in vitro experiments. With this aim, we will first try to create a physiologically relevant model of hepatic steatosis in vitro. We expect lipid accumulation to be significantly greater in cells treated with FFA.

Since hepatic steatosis is defined by an accumulation of LDs in the cytoplasm of hepatocytes, if our in vitro model system induces significant lipid accumulation in our cell line and in primary
hepatocytes then we will have a reductionist model of hepatic steatosis in vitro that we can employ for our studies.

2.4 Research Aim 2

To assess whether FFA-induced lipid accumulation impairs autophagy.

2.4.1 Research Aim 2: Rationale and Significance

Previous studies demonstrate that autophagy regulates LD accumulation in hepatocytes. Therefore, alterations in autphagic function might influence the development of hepatic steatosis. Through the autophagic process, portions of or entire LDs are enclosed in autophagosomal vesicles and degraded in the lysosome by lysosomal hydrolases. While lipophagy is activated and able to clear acute lipogenic stimuli, sustained lipogenic or acute elevated abnormal lipid levels may compromise lipophagic function. Moreover, impairments in autophagosomal maturation and/or autophagosome-lysosome fusion steps of the autophagy pathway may contribute to the excessive LD accumulation seen in steatotic liver diseases. As such, it is imperative to assess whether our in vitro models of hepatic steatosis also negatively impact autophagic function.

These results would indicate whether FFA-induced hepatic steatosis dysregulates or activates autophagy in cultured Hep3B cells and in mouse primary hepatocytes providing a basis and rationale for further studies to activate autophagy to reverse hepatic steatosis.

2.5 Research Aim 3

To determine whether addition of the TRPML1 agonist, MLSA1, will reduce hepatic steatosis and whether this occurs by increasing autophagic flux.

2.5.1 Research Aim 3: Rationale and Significance

If autophagy is impaired in conditions where hepatocytes are faced with a lipid challenge, as in our in vitro fatty liver model. activating autophagy may serve to reduce steatosis. MLSA1 activates TRPML1 Ca\(^{2+}\)-permeable channels, increasing calcium efflux from the lysosome to the cytoplasm to activate calcineurin-dependent TFEB pathways, which ultimately leads to increased
transcription of autophagy- and lysosomal-related genes. Therefore, MLSA1-mediated activation of TRPML1 may increase autophagic flux and reduce steatosis in our in vitro steatosis model.

Taken together, these results would fill a pre-existing gap in the literature by recognizing the activation of autophagy via TRPML1 channels as a mediator of hepatic steatosis. In future, MLSA1 addition may be used ex vivo to defat steatotic liver grafts, making these allografts more suitable for transplantation. This is especially imperative now, as a great discrepancy exists between the number of livers available for transplant and the number of liver transplant candidates.
Chapter 3: Methods

3.1 Cell Cultures

3.1.1 Hep3B cells

Human hepatocellular carcinoma cells (Hep3B) were kindly provided by the lab of Dr. Jorge Filmus (Sunnybrook Research Institute, Toronto, Canada). Hep3B cells were cultured in Eagle’s Minimum Essential Medium (EMEM; Wisent, St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent) and incubated at 37°C in a humidified atmosphere with 5% CO₂. For sub-culturing and experimental plating purposes, cells were detached with treatment of 0.25% trypsin/0.02% EDTA (Thermo Fisher Scientific, Waltham, MA, USA). Cells were used at ~70% confluency.

3.1.2 Primary hepatocytes

Primary hepatocytes from 10-week old male C57BL/6 mice were isolated by the MacParland lab. Once isolated, hepatocytes were plated on either collagen I coated multi-well plates (Thermo Fisher Scientific) or on collagen I coated coverslips (collagen I rat protein - tail, Thermo Fisher Scientific) to promote maximal cell adherence. To coat coverslips, collagen was diluted to 10µg/mL with PBS. Diluted collagen was added to wells (24 well plate) to cover the surface (up to 500µL). The plate was then incubated for 1 h at 37°C. Wells were washed 3X with PBS before cell cultures were added.

Primary hepatocytes were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific), media supplemented with 100units/mL penicillin and 100ug/mL streptomycin (Wisent). Right before hepatocyte culturing, this base media was supplemented with 2mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), 10% human serum (Sigma-Aldrich), 1% Insulin-Transferrin-Selenium-Sodium Pyruvate (Thermo Fisher Scientific), and 0.004% epidermal growth factor (Sigma-Aldrich). Hepatocytes were plated at a density of 1x10⁶ cells/well (24 well plate). Hepatocytes were grown in the incubator at 37°C with 5% CO₂. Four hours after seeding, the media was changed, and the cells left for one hour. Primary cells were then used for experiments 5 h after seeding and kept in culture for a maximum of 24 h.
3.2 Bacterial Cultures and Growth Conditions

*Helicobacter pylori* (*H. pylori*) strain 60190 (*cagA+, vacA+ s1m1i1*) and the corresponding isogenic *vacA*- mutants were provided by Dr. Richard Peek Junior (Vanderbilt University of Medicine, Nashville, TN, USA). *H. pylori* was grown on Colombia agar plates supplemented with 5% sheep blood (Thermo Fisher Scientific) for 2-3 days at 37°C in microaerophilic conditions (5% oxygen, 10% carbon dioxide, 85% N₂). Then, *H. pylori* was transferred to Brucella broth (Sigma-Aldrich) supplemented with 10% FBS (Wisent) and grown in the same microaerophilic conditions with shaking for 16-24 h. Broth cultures of *H. pylori* were grown to an optical density (OD) of 1.0 at 600nm (1 OD = 2x10⁸ bacteria/mL). Viability of bacteria was assessed by viewing its helical morphology and active motility under a light microscope.

3.2.1 Preparation of conditioned culture media supernatants

Wild-type and *vacA* mutant *H. pylori* culture supernatants (optical density [OD] = 1.0 at 600 nm) were filtered through a 0.22-µm-cutoff membrane filter and concentrated 50 times using a 30-kDa-cutoff Amicon Ultra centrifugal filter (Millipore, Billerica, MA, USA). In experiments, conditioned culture media supernatant (CCMS) was used at 1X final concentration. Cells were treated with CCMS for 14 h.

3.3 Fat-overloading induction in cells

3.3.1 Preparation of FFA stock solutions

Oleic acid (C18:1) and palmitic acid (C16:0) (Sigma-Aldrich) stock solutions were prepared to a final concentration of 200mM in 50% ethanol (volume/volume) by heating at 55°C and vortexing until dissolved and solutions were clear (~2 h).

3.3.2 Preparation of FFA-BSA conjugates

In order to increase the solubility of FFAs *in vitro*, they must be bound to albumin since they have poor solubility in aqueous solutions. To accomplish this, first a 10.5% (1.5mM) bovine serum albumin (BSA) solution was made by dissolving 1.6g of low-endotoxin, FFA-free BSA (Proliant Biologicals, Boone, IA, USA) in 15mL of serum-free advanced MEM (AMEM; Wisent). The BSA solution was prepared in a rocking water bath at 42°C for 30 min. Then, the lipids were conjugated to BSA by adding 200µL of the 200mM FFA stock solution to 4.8mL of
BSA solution (1:25 dilution, molar ratio lipid: BSA of 5:1). Control BSA was prepared by adding the same amount of 50% ethanol into the 10.5% BSA solution. This was used as a negative control in experiments (denoted as 0mM FFA). FFA-BSA conjugation was done in a shaking water bath at 40ºC for 2 h until turbid solutions became clear. The final stock solution had a concentration of 8mM and was aliquoted and stored at -20ºC; only to be used once after thawing. Stock solutions were further diluted in culture media to achieve desired concentrations and filter-sterilized using a 0.22-µm-cutoff membrane filter.

### 3.3.3 Inducing lipid overloading in cells

Fatty acid-BSA complexes were further diluted in culture medium to a final concentration of 1 mM, with OA:PA in a 2:1 ratio. We chose to use these two long-chain FFAs in this ratio as this has been shown in previous studies to induce maximal lipid loading with minimal cytotoxicity in *in vitro* models of hepatic steatosis\(^{120,127}\). Moreover, oleic and palmitic acid are the most abundant FFAs in the Western diet, and are elevated in serum of NAFLD patients\(^{118}\).

These solutions were then filter-sterilized and added to Hep3B cells 16 h after seeding, or to primary hepatocytes 5 h after seeding. Hep3B cells were either incubated for 8 h or 24 h with FFAs, whereas primary hepatocytes were incubated for 8 h or 16 h to adhere to cell culture requirements.

### 3.3.4 Pharmacological Treatments

#### 3.3.4.1 Treatment with MLSA1

At the end of treatment with VacA+ or VacA- CCMS, MLSA1 (Tocris) or DMSO (vehicle control) was added directly to cell culture wells at a final concentration of 20µM for 4 h.

In Hep3B cells, following both 8 and 24 h FFA treatments, cells were washed 1X with PBS and fresh media was added containing 50µM MLSA1 (or DMSO). MLSA1 was added either once for 8 h (single dose), or twice every 8 h for 8 h (double dose). In the double dose condition, the first dose of MLSA1 was added with fresh media once the FFA-supplemented media was removed, but the second dose was added directly to the well containing media which had already been supplemented with MLSA1. In primary hepatocytes, following both 8 and 16 h FFA
treatments, MLSA1 was added only at single doses for either 4 h or 8 h, in fresh media following a wash with PBS.

3.3.4.2 Treatment with bafilomycin

Since an increase in LC3-II can be indicative of either the activation of autophagy or blockage of downstream steps of autophagy (i.e. insufficient autophagosomal-lysosomal fusion), it is important to use autophagic flux assays to understand the entire dynamic process of autophagy. Therefore, in order to assess whether FFA supplementation alters autophagy flux, LC3 detection experiments were completed in the presence and absence of lysosomal inhibitor, bafilomycin (BAF).

Bafilomycin effectively inhibits the completion of autophagy by inhibiting fusion between autophagosomes and lysosomes by inhibiting vacuolar H+-ATPase. Under homeostatic conditions and bafilomycin treatment, LC3-II levels should increase due to inhibition of autophagosome maturation. Under FFA treated conditions and bafilomycin treatment, a further increase in LC3-II levels, in comparison with bafilomycin alone, indicates that autophagic flux is increased following FFA treatment. In experiments where BAF (Cayman Chemical Company, Ann Arbor, MI, USA) was required, it was added at the end of the respective treatment conditions for 2 h at a 100nM final concentration.

3.4 Antibodies

All antibodies used in this thesis are summarized in Tables 1 and 2. Table 1 lists all antibodies used for immunoblotting. Table 2 lists all antibodies used for immunofluorescence.

3.5 Immunoblotting

Cells were collected following the end of the treatment period and washed twice with PBS. Cells were then harvested and lysed in 50-60uL of radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl pH 8.0, 150mM sodium chloride, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with the following protease and phosphatase inhibitors: 1mM sodium orthovanadate, 50mM sodium fluoride, 1% protease inhibitor cocktail, and 0.5mM phenylmethylsulfonyl fluoride for 30 min on ice (all Sigma-Aldrich). Cell suspensions were then centrifuged at 13,000 rpm for 15 min at 4°C to pellet
cellular debris. Supernatants were collected and protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Supernatants were heated at 95°C with 1X Laemmli Sample Buffer for 5-10 min. Equal protein concentrations were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis at 135 volts for 1 h 30 min. Proteins were transferred to nitrocellulose membrane at 70 volts for 1 h 30 min at 4°C. Membranes were blocked in 5% w/v non-fat milk powder prepared in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h. Primary antibody incubations were performed overnight at 4°C in blocking solution (Table 1). Horseradish peroxidase (HRP)-conjugated secondary antibody incubations were performed for 1-2 h at room temperature in blocking solution (Table 1). Western blots were visualized by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnologies, Dallas, TX, USA) using the Bio-Rad ChemiDoc Touch imaging system.

3.6 Densitometry Analysis

Densitometry analysis was performed using ImageLab 6.0 Software (Bio-Rad). Densities of proteins of interest were measured and compared to loading control (i.e. LC3-II/actin). These ratios were graphed.
<table>
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<th>Antibodies</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Concentration</th>
</tr>
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<td>Novus Biologicals</td>
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</table>

Table 1. Antibodies used for immunoblotting.
3.7 Immunofluorescence

For imaging, cells were seeded on cover glass (cover glass was collagen-coated for primary hepatocytes). Following the end of treatment, cells were washed three times with PBS. Cells were then fixed for 15-20 min at room temperature with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA). Cells were washed again with PBS, and then permeabilized for 15 min in ice-cold methanol. After permeabilization, cells were blocked for 1 h in 1% BSA in PBS, followed by 1 h primary antibody incubations in 5% BSA in PBS (all staining was done at room temperature) (Table 2). Cells were washed three times with PBS after primary antibody incubations. Secondary antibody incubations were performed in the dark for 1 h in 5% BSA in PBS (Table 2). Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, 1µg/mL, Thermo Fisher Scientific) for 10 min in PBS. Cells were washed twice with PBS before coverslips were mounted on coverslip slides using DAKO fluorescent mounting medium (Agilent Technologies, Santa Clara, CA, USA). Images were acquired using a Quorum Spinning Disk Confocal Microscope using either 63x or 40x oil immersion objectives with identical imaging settings between conditions. Image acquisition and analysis was performed using Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA, USA).

Lipid droplets were stained using 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Catalogue No. D3922, Thermo Fisher Scientific). For BODIPY staining, cells were washed and fixed using the same methods described in the paragraph above after their respective treatments. After fixation, cells were washed with PBS and incubated with a DAPI/BODIPY solution (1µg/mL for both) in PBS for 30 min in the dark at room temperature. Following fixation, cells were washed, and coverslips were mounted onto slides using DAKO fluorescent mounting medium. Image acquisition and analysis was performed as stated above.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Concentration</th>
</tr>
</thead>
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<tr>
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<td>Developmental Studies Hybridoma Bank (DHSB)</td>
<td>H4A3</td>
<td>1:300</td>
</tr>
<tr>
<td><em>Rabbit α-LC3B</em></td>
<td>Novus Biologicals</td>
<td>NB600-1384</td>
<td>1:300</td>
</tr>
<tr>
<td><em>Rat α-LAMP-1</em></td>
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Table 2. Antibodies used for immunofluorescence.
3.8 Oil Red O Staining

A stock solution of Oil Red O (ORO) was prepared by dissolving 0.3g ORO powder (Catalogue No. O0625; Sigma-Aldrich) in 100mL of 100% isopropanol overnight on a stir plate, and then filtered with a 0.22-µm-cutoff membrane filter. Right before cells were ready to be stained, a working solution was made by dissolving the stock solution 3:2 in ddH2O. This was mixed well and allowed to sit for 10 min before filtering it again with a 0.22-µm-cutoff membrane filter.

Cells were seeded on cover glass. At the end of treatment, cells were washed three times with PBS. Cells were then fixed with 10% formalin in PBS for 10 min at room temperature. Cells were incubated with the working solution for 15 min. Then, excess dye was eluted quickly with one wash of 60% isopropanol in ddH2O, followed by one wash with PBS. Cells were then counterstained with hematoxylin solution (Gill No.1, Catalogue No. GHS132, Sigma-Aldrich) for 30-45 seconds. Then, cells were washed once with warm tap water. Cells were then washed once with PBS for 30 seconds, and then washed three more times with distilled ddH2O (3x 3min washes). Coverslips were then mounted using DAKO mounting medium and imaged with a Leica DMC5400 microscope camera (Leica Biosa systems, Wetzlar, Germany).

3.9 Statistical Analysis

All data represent three independent replicates, unless otherwise stated. All data are presented as mean ± standard error of the mean (SEM). Two-tailed unpaired Student’s t-tests were used to assess whether treated groups differed significantly from control groups. In experiments where there were multiple treatment groups, a one-way analysis of the variance (ANOVA) followed by Tukey’s post-hoc test was used to determine statistical significance. All statistical analysis was conducted using GraphPad Prism (GraphPad Software Prism 6, La Jolla, California, USA).
4.1 TRPML1 is functional in Hep3B cells and primary hepatocytes and MLSA1 rescue is effective

4.1.1 TRPML1 immunoblot analysis

In order to determine if Hep3B cells expressed TRPML1, immunoblot analysis was performed using whole cell lysates prepared from Hep3B cells and human gastric epithelial AGS cells, which we have previously shown have functional TRPML1\textsuperscript{129}. In addition to TRPML1 expression validation, the effect of various concentrations of MLSA1 (0 µM, 20µM and 50µM) on protein levels was determined. Immunoblotting results revealed that Hep3B cells exhibited a band at the molecular weight of interest for TRPML1 (Figure 4.1A).

We next determined if the band of interest was indeed TRPML1 by performing immunoblotting in lysates obtained from murine stomach tissues from Trpml1-/- (genotype was verified using PCR; data not shown) and WT mice. A band at the molecular weight of interest was identified in both Trpml1-/- and WT mice demonstrating that the TRPML1 antibody bound non-specifically (Figure 4.1B). Therefore, we next performed a functional assay to assess TRPML1 activity in these cell lines (described in section 4.1.2).
Figure 4.1. TRPML1 immunoblot analysis did not validate TRPML1 expression.
(A) Hep3B or AGS cells were incubated for 4 h with indicated concentrations of MLSA1. Whole cell lysates were subjected to immunoblot analysis. (B) Murine stomach lysates from Trpm1/- or WT mice were subjected to immunoblot analysis.
4.1.2 TRPML1 functional assay: disrupting autophagy by *H. pylori*

We next performed functional assays for TRPML1 utilizing both a bacterial toxin that inhibits TRPML1 activity, as well as a small molecule agonist of TRPML1, MLSA1. The vacuolating cytotoxin A (VacA) toxin is a key virulence factor that is secreted by the gastric pathogen *H. pylori*. Our laboratory has previously shown that VacA specifically targets TRPML1 to disrupt endolysosomal trafficking to generate an intracellular reservoir that promotes bacterial survival *in vitro*. Prolonged exposure to the toxin inhibits TRPML1 thereby disrupting autophagy by preventing maturation of the autolysosome.

Accordingly, VacA-mediated vacuoles display characteristics of late endocytic compartments, such as lysosomal-associated membrane protein 1 (LAMP-1). As such, VacA-mediated vacuolation can be assessed by immunolabeling for LAMP-1. Since toxigenic VacA results in the production of dysfunctional lysosomes, this subsequently impairs autophagosome maturation, resulting in the accumulation of LC3-II protein. Therefore, VacA-mediated disruptions in autophagy can also be visualized by an increase in LC3-II protein via immunoblotting.

In addition, we have demonstrated that TRPML1 agonism with MLSA1 restores VacA-disrupted endolysosomal and autophagy pathways. Therefore, we determined if VacA treatment of Hep3B cells and primary hepatocytes phenocopied the altered endolysosomal trafficking defects observed in AGS cells. Furthermore, we determined if vacuole formation and autophagic defects were reversed with MLSA1 treatment indicating that the TRPML1 channel is functional in these cells.

4.1.3 Activation of TRPML1 via MLSA1 reverses accumulation of VacA-induced LAMP-1-positive vacuoles

First, TRPML1 function was assessed via the reversal of VacA-induced LAMP-1-positive vacuoles upon addition of MLSA1. Bacteria-free conditioned culture media supernatants (CCMS) were collected from either wildtype *H. pylori* (VacA+) or VacA mutant *H. pylori* (VacA−). Cells were treated with CCMS for 14 h, and subsequently treated with MLSA1 (20 μM) for 4 h. Cells were labelled with anti-LAMP-1 antibodies to visualize lysosomal markers, as well as DAPI to visualize cell nuclei. Large vacuoles containing LAMP-1 accumulated in
cells treated with VacA+ CCMS, which was reversed with MLSA1 addition in both Hep3B cells (Figure 4.2) and primary hepatocytes (Figure 4.3) as assessed by immunofluorescence microscopy. TRPML1 activation reversed VacA-induced LAMP-1-positive vacuoles in both cell types, thereby providing evidence that TRPML1 is present, functional, and responsive to small molecule agonism in hepatocytes.
A.

VacA- DMSO

VacA+ DMSO MLSA1

Normal

Folder 13μm

Zoom

DAPI LAMP-1

B.

Average LAMP-1-Positive Vacuole Diameter (μm)

Relative to VacA-/DMSO Control

DMSO MLSA1 DMSO MLSA1

VacA- VacA+

***

***

ns
Figure 4.2. TRPML1 activation reverses the accumulation of VacA-mediated LAMP-1-positive vacuoles in Hep3B cells.

(A) LAMP-1 staining of Hep3B cells after overnight VacA- or VacA+ culture supernatant treatments, followed by 4 h MLSA1 (20µM) or vehicle (DMSO) treatment. A zoomed image of the outlined area is shown at the bottom. (B) The graph shows quantification of vacuole diameter for a minimum of 10 cells per treatment condition (n=3 independent experiments). Data are presented as the average vacuole diameter per condition, relative to control (VacA-/DMSO).

All data are mean ± SEM. ***p<0.001, by one-way ANOVA with Tukey’s post-hoc test.
### A.

#### Normal

- **DMSO**
- **MLSA1**

#### Zoom

- **DAPI**
- **LAMP-1**

### B.

**Average LAMP-1-Positive Vacuole Diameter (μm)**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>MLSA1</th>
<th>DMSO</th>
<th>MLSA1</th>
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<tr>
<td><strong>VacA+</strong></td>
<td></td>
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</tbody>
</table>

- **DMSO**
- **MLSA1**

**Relative to VacA-/DMSO Control**

- **ns**
- ****
- ****

- **VacA-**
- **VacA+**
Figure 4.3. TRPML1 activation also reverses the accumulation of VacA-mediated LAMP-1-positive vacuoles in primary hepatocytes.

(A) LAMP-1 staining of primary cells after overnight VacA- or VacA+ culture supernatant treatments, followed by 4 h MLSA1 (20µM) or vehicle (DMSO) treatment. A zoomed image of the outlined area is shown at the bottom. (B) The graph shows quantification of vacuole diameter for a minimum of 10 cells per treatment condition (n=3 independent experiments). Data are presented as the average vacuole diameter per condition, relative to control (VacA-/DMSO).

All data are mean ± SEM. **p<0.001, by one-way ANOVA with Tukey’s post-hoc test.
4.1.4 MLSA1-mediated TRPML1 activation does not reverse autophagy defect following VacA treatment

To verify that VacA disrupted autophagosome maturation and that MLSA1 reversed the VacA-disrupted autophagy in hepatocytes, immunoblotting for LC3-II was performed in cell lysates. Hep3B cells were treated with CCMS from wild-type or VacA mutant *H. pylori* for 14h, followed by treatment with MLSA1 (20 µM) for 4 h. Cell lysates were then subjected to Western blotting.

In Hep3B cells, LC3-II was significantly increased in the VacA+/DMSO condition (Figure 4.4). When cells were treated with VacA+/MLSA1, LC3-II was still significantly increased compared to the control. Densitometric ratios also demonstrated minimal differences between LC3-II in VacA+/DMSO and VacA+/MLSA1 conditions. This finding suggests that MLSA1 is not capable of rescuing autophagic defects following VacA+ treatment in Hep3B cells.

Primary hepatocytes were treated in the same manner described for Hep3B cells. In primary hepatocytes, there were no statistically significant differences between any of the treatment conditions (Figure 4.5). An increase in LC3-II following VacA+ CCMS treatment was observed in two experiments (Figure 4.5A & B), however basal autophagy was significantly elevated in the two other experiments (Figure 4.5 C&D), introducing significant variability.

This result, in combination with the immunofluorescence data presented in section 4.1.3, proves that MLSA1 rescues endolysosomal trafficking – but not autophagic – defects caused by prolonged VacA exposure in both primary hepatocytes and in Hep3B cells. In sum, these findings in hepatocytes differ from what our lab has previously published in AGS cells, where MLSA1 restored the autophagy pathway; assessed by a reduction in LC3-II protein levels and in the size of LAMP-1-positive vacuoles.129.
A.

B.

C.

D.

LC3-II/actin

VacA- VacA+

0.0 0.5 1.0 1.5 2.0

DMSO MLSA1 DMSO MLSA1

VacA- VacA+

**

*
**Figure 4.4. MLSA1 does not rescue VacA-induced autophagy disruption in Hep3B cells.**

Following overnight treatment with either VacA- or VacA+ culture supernatant, Hep3B cells were treated for 4 h with MLSA1 (20µM) or vehicle (DMSO). Cells were then subjected to immunoblot analysis. (A-C) Western blots for each independent experiment are shown, with densitometry (LC3-II/actin) ratios below. (D) Quantification plot of LC3-II/actin ratios.

All data are mean ± SEM. **p<0.01, *p<0.05, by one-way ANOVA with Tukey’s post-hoc test.
Figure 4.5. MLSA1 does not rescue VacA-induced autophagy disruption in primary hepatocytes.

Following overnight treatment with either VacA- or VacA+ culture supernatant, primary hepatocytes were treated for 4 h with MLSA1 (20µM) or vehicle (DMSO). Cells were then subjected to immunoblot analysis. (A-D) Western blots for each independent experiment are shown, with densitometry (LC3-II/actin) ratios below. (D) Quantification plot of LC3-II/actin ratios.

All data are mean ± SEM. Statistical significance evaluated by one-way ANOVA with Tukey’s post-hoc test.
4.2 Developing an *in vitro* hepatic steatosis model

4.2.1 FFA treatment induces lipid droplet accumulation

Following the verification of the functionality of TRPML1 in Hep3B cells and primary hepatocytes, we next sought to establish an appropriate *in vitro* hepatic steatosis model for the basis of the remaining experiments. Since hepatic steatosis is defined by an accumulation of LDs in the cytoplasm of hepatocytes, we sought to define the experimental conditions of lipid exposure that lead to significant intracellular fat accumulation, in the absence of overt cytotoxicity.

The concentrations and ratios of FFAs employed were based on a previous study aimed at establishing appropriate *in vitro* models to investigate steatosis in human hepatocyte cell lines and human hepatocytes. This study concluded that an FFA mixture containing a low proportion of palmitic acid (oleate/palmitate, 2:1 molar ratio) is associated with lipid droplet accumulation and only minor toxic effects, thus representing a cellular model that mimics benign chronic steatosis. For our experiments, we used oleate/palmitate in a 2:1 ratio for a total concentration of 1 mM (BSA alone was used as a 0mM FFA control). Cells were subjected to either short-term (ST, 8 h) or long-term (LT, 16 h in primary hepatocytes/24 h in Hep3B cells) incubations with this FFA mixture.

To assess whether this FFA mixture led to an increase in LDs, Hep3B cells were first split and left overnight to establish a monolayer. Once a monolayer was established, the medium was replaced with fresh medium supplemented with OA and PA. After 8 h or 24 h FFA incubations, LD accumulation was assessed via BODIPY and ORO staining. BODIPY analysis was performed by quantifying a minimum of 50 cells per treatment condition for the following parameters: number of LDs and volume of LDs. Thresholds for quantitation were set based on fluorescence intensity and LD size. These parameters were then measured and averaged by dividing by the number of cells in each respective image.

After 8 h of FFA treatment, ORO staining demonstrated an increase in LD accumulation (*Figure 4.6A*). BODIPY staining (*Figure 4.6B*) and subsequent analysis demonstrated significant increases in the average number of LD per cell (*Figure 4.6C*) as well as the average LD volume per cell (*Figure 4.6D*).
LD accumulation also occurred following LT FFA supplementation (Figures 4.7A&B). Following 24 h of FFA treatment, there was a statistically significant increase in the average number of LD per cell as well as in the average LD volume per cell (Figure 4.7 C&D). Preliminary experiments revealed that FFA supplementation to 48 h or 72 h had no further effect on LD accumulation, thus 24 h was chosen as our LT time point.
Figure 4.6. ST FFA incubation induces LD accumulation in Hep3B cells.

(A) ORO staining demonstrates an accumulation in LDs following 8 h FFA treatment. A zoomed image of the outlined area is shown at the bottom. (B) BODIPY staining also shows an increase in LDs following exposure to FFA treatment for 8 h. (C) Quantification of BODIPY staining reveals an increase in the average number of LDs per cell in FFA-treated cells. (D) The average LD volume per cell was also increased. All graphs show quantification of the indicated parameters per cell, averaged.

Figure 4.6 shows data from n=3 independent experiments. All data are mean ± SEM. Statistical significance evaluated by two-tailed unpaired Student’s t-test.
Figure 4.7. LT FFA incubation induces LD accumulation in Hep3B cells.

(A) ORO staining demonstrates an accumulation in LDs following 24 h FFA treatment. A zoomed image of the outlined area is shown at the bottom. (B) BODIPY staining also shows an increase in LDs following LT FFA treatment. (C, D) Following 24 h FFA treatment, the average number of LDs as well as average LD volume per cell was significantly increased in treated cells. All graphs show quantification of the indicated parameters per cell, averaged.

Figure 4.7 shows data from n=3 independent experiments. All data are mean ± SEM. Statistical significance evaluated by two-tailed unpaired Student’s t-test.
This same FFA mixture (oleate/palmitate, 2:1 ratio, 1mM final concentration) was added to primary hepatocytes and LD accumulation was assessed. Primary cells were isolated and allowed to attach to collagen-coated coverslips for 4 h, followed by a media wash for 1 h. The media was replaced with fresh FFA-supplemented media, and treatments were started 5 h post-isolation. After 8 h or 16 h FFA incubations, LD accumulation was assessed via BODIPY staining. BODIPY analysis was performed as described for Hep3B cells. The same parameters were assessed for a minimum of 50 cells per treatment condition.

In primary hepatocytes there was a significant accumulation of LDs in control untreated cells. There was a further accumulation in LDs after 8 h FFA treatment as assessed by BODIPY staining (Figure 4.8A). Quantification showed significant increases in the average number of LD per cell as well as average LD volume per cell (Figure 4.8B & C). Following 16 h FFA supplementation, the average LD number and volume per cell was also significantly increased in primary hepatocytes in comparison with control cells (Figure 4.9).

Taken together, these results suggest that the employed model of treatment with FFA was representative of in vitro hepatic steatosis in Hep3B cells and in primary hepatocytes. More specifically, both ST and LT FFA supplementation induced LD accumulation in both cell types.
A.  

0mM FFA  

1mM FFA  

B.  

Average No. LDs/Cell  

$p=0.0497$  

0mM FFA  

1mM FFA
Figure 4.8. ST FFA incubation induces LD accumulation in primary hepatocytes.

(A) BODIPY staining shows an increase in LDs following ST FFA treatment. (B, C) Following 8 h FFA treatment, the average number of LDs and LD volume per cell were significantly increased. All graphs show quantification of the indicated parameters per cell, averaged.

Figure 4.8 shows data from n=4 independent experiments. All data are mean ± SEM. Statistical significance evaluated by two-tailed unpaired Student’s t-test.
A.

![Images of cells with DAPI and BODIPY staining for 0mM and 1mM FFA concentrations.](image_url)

B.

![Graph showing average number of LDs per cell for 0mM and 1mM FFA concentrations.](image_url)

\[ p = 0.0132 \]
Figure 4.9. LT FFA incubation induces LD accumulation in primary hepatocytes.

(A) BODIPY staining shows an increase in LDs following LT FFA treatment. (B, C) Following 16 h FFA treatment, the average number of LDs and average LD volume per cell was significantly increased. All graphs show quantification of the indicated parameters per cell, averaged.

Figure 4.9 shows data from n=5 independent experiments. All data are mean ± SEM. Statistical significance was evaluated by two-tailed unpaired Student’s t-test.
4.2.2 FFA treatment does not impair autophagic flux

Previous studies suggest that intracellular lipid accumulation impairs autophagic clearance. Singh et al. found that in hepatocytes cultured with lipids, there was a decrease in LD/LAMP-1 co-localization and a lack of autophagic upregulation. In another study, HFD-fed mice had impaired lysosomal acidification, impaired autophagic degradation, and less association of autophagic vacuoles with LDs upon autophagy induction. In order to determine the effect of FFA incubation on autophagy, immunoblotting for LC3 was performed in cells with FFA supplementation in the presence or absence of bafilomycin, which inhibits autophagosome-lysosome fusion. Under homeostatic conditions and bafilomycin treatment, LC3-II levels should increase due to inhibition of autophagosome maturation. Under conditions of FFA treatment and bafilomycin treatment, a further increase in LC3-II levels, in comparison with bafilomycin alone, indicates that autophagic flux is increased following FFA supplementation.

In the last 2 h of 8 or 24 h FFA supplementation, bafilomycin (100 nM) was added to Hep3B cells. Cells were then either harvested and subjected to immunoblot analysis or were immunolabelled by probing for LC3. The results from the immunoblot analyses showed that LC3-II in 0mM and 1mM FFA/BAF conditions were significantly increased compared to 0mM FFA/DMSO control following both ST (Figure 4.10E) and LT (Figure 4.11F) FFA treatments. Immunofluorescence for LC3 puncta revealed similar results, whereby LC3-II was significantly increased in the 1mM FFA/BAF condition compared to the 0mM FFA/DMSO control (Figure 4.10G & 4.11H).

Differences in LC3-II between 0mM FFA/BAF and 1mM FFA/BAF were statistically significant following both ST and LT FFA treatments (Figure 4.10A-E & Figure 4.11A-F). Similarly, raw densitometric ratios demonstrated an increase in LC3-II/actin with FFA treatment and bafilomycin treatment, compared to bafilomycin alone. These results suggest that short- or long-term FFA supplementation does not disrupt autophagic flux in Hep3B cells, as LC3-II was elevated in the treatment plus BAF conditions compared to BAF alone; indicating successful autophagosome maturation. Unfortunately, due to limitations in cell number and availability, this same assay could not be performed in primary hepatocytes.
Figure 4.10. Autophagic flux assays with bafilomycin reveal that ST FFA supplementation does not disrupt autophagy.

Hep3B cells were treated with 0 or 1mM FFA for 8 h; during the last 2 h of treatment BAF (100nM) was added. (A-D) Individual Western blots from n=4 independent experiments with densitometry (LC3-II/actin) ratios below (some images are sliced, but from the same blot). (E) Quantification plot of LC3-II/actin ratios. (F) LC3 immunostaining of Hep3B cells from the same set of experiments. (G) A minimum of 20 cells per condition were quantified for n=3 independent experiments. The graph shows quantification of these experiments, with the number of LC3 puncta divided by cell number in each respective image, then averaged. This data is presented as a fold change compared to the 0mM FFA/DMSO control.

All data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, by one-way ANOVA with Tukey’s post-hoc test.
Figure 4.11. Autophagic flux assays with bafilomycin reveal that LT FFA supplementation does not disrupt autophagy.

Hep3B cells were treated with 0 or 1mM FFA for 24 h; during the last 2 h of treatment BAF (100nM) was added. (A-E) Individual Western blots from n=5 independent experiments with densitometry (LC3-II/actin) ratios below (some images are sliced, but from the same blot). (F) Quantification plot of LC3-II/actin ratios. (G) LC3 immunostaining of Hep3B cells from the same set of experiments. (H) A minimum of 20 cells per condition were quantified for n=3 independent experiments. The graph shows quantification of these experiments, with the number of LC3 puncta divided by cell number in each respective image, then averaged. This data is presented as a fold change compared to the 0mM FFA/DMSO control.

All data are mean ± SEM. *p<0.05, **p<0.01, by one-way ANOVA with Tukey’s post-hoc test.
4.3 MLSA1 induces LC3 accumulation in Hep3B cells

Autophagy enhancement has been linked to LD clearance\textsuperscript{136}. Here, we showed that basal autophagy is enhanced following FFA supplementation though is not sufficient to clear LDs (data presented in section 4.2.1). Since autophagy induction occurs via TRPML1 activation, and enhanced autophagy has been shown to clear LDs in previous studies, we next assessed the effects of TRPML1 activation in our \textit{in vitro} model of FFA treatment\textsuperscript{72,73,134}. Specifically, we activated TRPML1 via MLSA1, which has been implicated in the regulation of autophagy through downstream TFEB activation and successive upregulation of autphagic and lysosomal gene expression\textsuperscript{88}. Following MLSA1 treatment, we assessed whether autophagy was induced and whether this resulted in a subsequent clearance of LDs (data presented in section 4.4).

First, in order to assess whether MLSA1 was inducing autophagy following FFA supplementation in Hep3B cells, we performed immunoblotting for LC3-II. If autophagy was in fact being induced, we would expect to see an increase in LC3-II protein. For these experiments, Hep3B cells were incubated with either 0 or 1 mM FFA for 8 or 24 h. Following this, cells were treated with a single dose of MLSA1 (50 µM for 8 h), a double dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). Following 8 h FFA treatment, LC3-II was significantly increased following both single and double dosing of MLSA1 (\textbf{Figure 4.12}). Following 24 h FFA treatment and subsequent MLSA1 treatment, LC3-II was significantly increased only in the 1mM FFA/M+M condition; however, individual immunoblots demonstrated a visible increase as well as increased densitometric ratios for LC3-II/actin in all MLSA1-treated groups (\textbf{Figure 4.13}). Thus, we have illustrated that LC3-II was increased upon single and repeat dose MLSA1 administration, following both 8 and 24 h FFA treatments. This finding suggests that autophagy was upregulated following MLSA1 addition, however future experiments with bafilomycin are needed to properly assess autophagic flux and verify this result.
Figure 4.12. MLSA1 addition following ST FFA supplementation results in an increase in LC3-II.

Hep3B cells were treated with 0 or 1mM FFA for 8 h, followed by treatment with a single dose of MLSA1 (50 µM for 8 h), a double dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). (A-E) Individual immunoblots for n=5 independent experiments with densitometry (LC3-II/actin) ratios below (some images are sliced, but from the same blot). (E) Quantification plot of LC3-II/actin ratios.

All data are mean ± SEM from n=5 independent experiments. **p<0.01, ***p<0.001, by one-way ANOVA with Tukey’s post-hoc test.
Figure 4.13. MLSA1 addition following LT FFA supplementation results in an increase in LC3-II.

Hep3B cells were treated with 0 or 1 mM FFA for 24 h, followed by treatment with a single dose of MLSA1 (50 µM for 8 h), a double dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). (A-E) Individual immunoblots for n=5 independent experiments with densitometry (LC3-II/actin) ratios below (some images are sliced, but from the same blot). (F) Quantification plot of LC3-II/actin ratios.

All data are mean ± SEM from n=5 independent experiments. *p<0.05, by one-way ANOVA with Tukey’s post-hoc test.
4.4 MLSA1 addition does not rescue FFA-induced lipid droplet accumulation

Since MLSA1 induced autophagy in our in vitro model system and autophagy enhancement has been associated with an increased clearance of LDs\(^{136}\), we wanted to assess whether autophagic induction via TRPML1 activation had an effect on LD reduction following FFA supplementation.

Before proceeding with these experiments, we aimed to determine a concentration and time point for MLSA1 dosing that would be ideal for clearing LDs that resulted in minimal toxicity. First, we employed the same concentration and timepoint that we used in our TRPML1 functional validation experiments (20µM for 4 h). However, this MLSA1 dosing was not sufficient to clear LDs. We next measured toxicity of various concentrations and timepoints of MLSA1 to determine the maximal dose to use. From these experiments, we concluded that 100µM of MLSA1 was toxic to cells at the 8 h timepoint, whereas 50µM was not toxic, even at the 16 h timepoint. Moreover, in the 100µM condition some crystals were seen visible through the light microscope, suggesting that MLSA1 had precipitated out of solution as it was not completely soluble at this concentration. Therefore, we first assessed whether MLSA1 at 50µM was sufficient to reduce LDs following both ST and LT FFA supplementation. Preliminary experiments revealed that 4 h of MLSA1 at 50µM concentration was not sufficient to clear LDs. Therefore, we assessed longer timepoints of 8 h, as well as repeat dosing of MLSA1 in 8 h intervals.

Hep3B cells were incubated with either 0 or 1mM FFA for 8 or 24 h. Following this, cells were treated with either a single dose of MLSA1 (50 µM for 8 h), a double dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). At the end of these treatments, cells were fixed and stained for BODIPY and LD number and volume were assessed. BODIPY staining and subsequent analysis showed that single and repeated dosing of MLSA1 did not have an effect on LD parameters (Figure 4.14 & 4.15).
A.  

DMSO  
MLSA1  
M+M  

0mM FFA  
1mM FFA  

0mM FFA  
1mM FFA  

0mM FFA  
1mM FFA  

11µm  

B.  

Average No. LDs/Cell  
Fold Increase Over Control  

0mM FFA  
1mM FFA  
0mM FFA  
1mM FFA  
0mM FFA  
1mM FFA  

DMSO  
MLSA1  
M+M  

78
Figure 4.14. MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in Hep3B cells.

Hep3B cells were incubated with either 0 or 1mM FFA for 8 h. Cells were then treated with a single dose of MLSA1 (50 µM for 8 h), a repeat dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). (A) BODIPY staining following treatment. (B) Graphs show quantification of the indicated parameters per cell, averaged, and normalized to control (0mM FFA/DMSO). Data represents the mean ± SEM for n=3 independent experiments. A one-way ANOVA with Tukey’s post-hoc test to compare means between treatment groups to determine statistical significance.
A.

B.

Average No. LDs/Cell

Fold Increase Over Control

0mM FFA
1mM FFA
0mM FFA
1mM FFA
0mM FFA
1mM FFA

DMSO
MLSA1
M+M

0mM FFA
1mM FFA
0mM FFA
1mM FFA
0mM FFA
1mM FFA

DMSO
MLSA1
M+M

0
10
20
30

*
Figure 4.15. MLSA1 treatment does not decrease LD accumulation following 24 h FFA incubation in Hep3B cells.

Hep3B cells were incubated with either 0 or 1mM FFA for 24 h. Cells were then treated with a single dose of MLSA1 (50 µM for 8 h), a repeat dose of MLSA1 (M+M; 50 µM for 2x 8 h intervals), or DMSO (vehicle control). (A) BODIPY staining following treatment. (B) Graphs show quantification of the indicated parameters per cell, averaged, and normalized to control (0mM FFA/DMSO).

All data are mean ± SEM for n=3 independent experiments. *p<0.05, by one-way ANOVA with Tukey’s post-hoc test.
The effect of MLSA1 addition on LD accumulation was also assessed in primary hepatocytes. Here, cells were incubated with FFA-supplemented media 5 h post-plating. Cells were incubated with FFAs for 8 or 16 h, followed by 4 or 8 h MLSA1 (50µM). The 16 h FFA supplementation could only be followed by 4 h not 8 h MLSA1 since primary hepatocytes lose their morphology, liver-specific functions, accumulate stress fibers, and die following 24 h of cell culture\textsuperscript{137}. This same limitation meant we were unable to perform MLSA1 double dosing experiments in these cells. At the end of these treatments, cells were fixed and stained for BODIPY and LD number and volume were assessed.

BODIPY staining and subsequent analysis showed that 4 h MLSA1 treatment did not have an effect on LD following either 8 h (\textbf{Figure 4.16}) or 16 h FFA supplementation (\textbf{Figure 4.18}). There were no differences in average LD number per cell or average LD volume per cell. Similarly, longer treatment with treatment with MLSA1 for 8 h following 8 h FFA supplementation did not have an effect on LD parameters (\textbf{Figure 4.17}).
Figure 4.16. MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in primary hepatocytes.

Primary hepatocytes were treated 5 h post-isolation with either 0 or 1mM FFA for 8 h. Cells were then treated with a single dose of MLSA1 (50 µM for 4 h) or DMSO (vehicle control). (A) BODIPY staining following treatment. (B) Graphs show quantification of the indicated parameters per cell, averaged, and normalized to control (0mM FFA/DMSO).

All data are mean ± SEM for n=5 independent experiments. *p<0.05, by one-way ANOVA with Tukey’s post-hoc test.
Figure 4.17. Longer MLSA1 treatment does not decrease LD accumulation following 8 h FFA incubation in primary hepatocytes.

Primary hepatocytes were treated 5 h post-isolation with either 0 or 1mM FFA for 8 h. Cells were then treated with a single dose of MLSA1 (50 µM for 8 h) or DMSO (vehicle control). (A) BODIPY staining following treatment. (B) Graphs show quantification of the indicated parameters per cell, averaged, and normalized to control (0mM FFA/DMSO).

All data are mean ± SEM for n=4 independent experiments. ***p<0.001 by one-way ANOVA with Tukey’s post-hoc test.
A.

Control

Treated

DMSO

MLSA1

20μm

DAPI

BODIPY

B.

Average No. LDs/Cell

Fold Increase Over Control

Average LD Volume (μm³)/Cell

Fold Increase Over Control

0 mM FFA

1 mM FFA
Figure 4.18. MLSA1 treatment does not decrease LD accumulation following 16 h FFA incubation in primary hepatocytes.

Primary hepatocytes were treated 5 h post-isolation with either 0 or 1mM FFA for 16 h. Cells were then treated with a single dose of MLSA1 (50 µM for 4 h) or DMSO (vehicle control). (A) BODIPY staining following treatment. (B) Graphs show quantification of the indicated parameters per cell, averaged, and normalized to control (0mM FFA/DMSO).

All data are mean ± SEM for n=5 independent experiments. *p<0.05, by one-way ANOVA with Tukey’s post-hoc test.
Chapter 5: Discussion

There currently exists a discrepancy between the number of livers available for transplant and the number of liver transplant candidates\(^2\). Thus, transplant centers resort to utilizing suboptimal grafts, such as steatotic livers, which result in worse transplant outcomes\(^{24}\). Mechanisms to defat steatotic grafts during the *ex vivo* preservation period prior to transplantation could augment the use of steatotic grafts and increase the number of livers available for transplant. Autophagy regulates lipid metabolism and dysfunctional autophagy is linked to the development of hepatic steatosis\(^{66,72,138}\). The autophagy enhancer, rapamycin, is not safe to use in the peri-transplant setting, demanding the identification of other agents that upregulate autophagy and defat the liver without adverse effects\(^{78}\). In this thesis, I sought to explore the effect of increased autophagic flux and lysosomal function via activation of the lysosomal calcium channel, TRPML1, on hepatic steatosis using an *in vitro* model system.

5.1 TRPML1 functionality in Hep3B cells and in primary hepatocytes

TRPML1 is an essential endolysosomal and lysosomal calcium channel that is responsible for maintaining adequate ion homeostasis and membrane trafficking\(^{139,140}\). Studies have confirmed the role of lysosomal calcium release via TRPML1 in autophagy regulation through calcineurin and TFEB\(^{87,109}\). Since we sought to activate autophagy via TRPML1 activation, we first performed studies assessing whether the channel was functional in Hep3B cells and in primary hepatocytes. These functional studies were based on our previous studies with the VacA toxin, secreted by *H. pylori*, which we have shown targets TRPML1 to impair host lysosomal and autophagy pathways to promote intracellular bacteria survival\(^{129,130,141}\). In AGS cells, VacA treatment results in the accumulation of large vacuoles that represent very enlarged dysfunctional lysosomes\(^{129}\). VacA-disrupted lysosomal function subsequently impairs autophagosome maturation, which is demonstrated by an accumulation of the LC3-II protein\(^{129}\). In VacA-treated AGS cells, administration of the TRPML1 agonist, MLSA1, reverses cell vacuolation and restores the autophagy pathway, as shown by a reduction in the LC3-II protein. We aimed to test TRPML1 functionality by evaluating the resolution of these same VacA-mediated endolysosomal and autophagic defects in hepatocytes.
In Hep3B cells and in primary hepatocytes, VacA treatment induced the formation of vacuoles similar to AGS cells indicating that VacA disrupted endolysosomal trafficking\textsuperscript{129}. MLSA1 administration markedly reduced the size of VacA-mediated vacuoles in both cells, proving that TRPML1 agonism in hepatocytes restored vesicular trafficking (Figure 4.2 & 4.3).

Next, we assessed whether the production of VacA-mediated dysfunctional lysosomes impairs autophagosome maturation by assessing for an increase in the LC3-II protein. In Hep3B cells, LC3-II was significantly increased in the VacA+/DMSO condition, however MLSA1 addition did not restore the accumulation of LC3-II (Figure 4.4). This finding suggests that MLSA1 treatment does not rescue VacA-mediated autophagy defects in Hep3B cells.

In primary hepatocytes, VacA did not have a significant effect on LC3-II levels (Figure 4.5). No further difference in LC3-II was observed upon MLSA1 administration. This results suggest that TRPML1 reactivation is not capable of rescuing autophagic defects following exposure to VacA in primary hepatocytes.

These results are different to what was previously published in AGS cells\textsuperscript{129}; however, there may be some potential explanations for these discrepancies. First, Capurro et al. were able to induce autophagic defects in AGS cells using 10x concentrated CCMS, and MLSA1 (20µM for 4h) was enough to reverse these effects. Here, preliminary experiments showed that this concentration was not enough to impair autophagy in Hep3B cells, as it did not result in an accumulation of LAMP-1 positive vacuoles, nor did it increase LC3-II protein levels. For our experiments in hepatocytes, we needed to use 40-50x concentrated VacA in order to see an autophagic response. We then used the same concentration and timepoint of MLSA1 in previous studies with AGS cells which may not have been sufficient to reverse all VacA effects. Though MLSA1 addition reversed vacuolation, autophagic defects were only slightly resolved in Hep3B cells and did not occur in primary cells. Since cells were incubated with a higher VacA concentration, future experiments may need to optimize MLSA1 dosing to see complete resolution in VacA-induced autophagic defects. TRPML1 overexpression experiments in hepatocytes could also be performed to further assess the effects of TRPML1 agonism on VacA-mediated defects in the autophagy pathway.
The differences between the current experiments and our previous studies could also be explained by the differences in cell type. Hep3B cells or primary hepatocytes could have altered TRPML1 expression than AGS cells which may explain the differences in their response to VacA and MLSA1. We attempted to measure TRPML1 expression using WB in hepatocytes, however, the TRPML1 antibody lacked specificity. Interestingly, Northern blots performed on murine tissues showed that the highest levels of TRPML1 were present in the brain, kidney and liver\textsuperscript{142}. Despite this finding, no quantitative measures of TRPML1 exist in isolated primary mouse hepatocytes, nor in AGS or Hep3B cells. Therefore, TRPML1 expression in these cell types should be assessed and compared using real-time polymerase chain reaction (RT-PCR)\textsuperscript{143} before further conclusions can be made.

We observed an increase in LC3-II following MLSA1 treatment in Hep3B cells treated with VacA+ CCMS. An increase in LC3-II may be explained by an induction of autophagy or by blockage of downstream steps of autophagy\textsuperscript{69,124}. MLSA1 may have induced autophagy in the VacA+ condition, meaning the increase in LC3-II seen here may be a result of autophagy induction via TRPML1 activation rather than ineffective clearance of VacA-induced autophagic defects. Preliminary experiments performed with MLSA1 in the presence of bafilomycin have demonstrated that MLSA1 increases autophagic flux in this cell line, however future experiments will need to compare the effects of MLSA1 in the presence of both VacA and bafilomycin to assess whether MLSA1 activates autophagy in this condition.

Finally, in primary hepatocytes there were no significant differences in LC3-II between any of the treatment conditions. A potential limitation of primary cell lines is the potential variability in the cells over time following isolation as described previously\textsuperscript{137} (Figure 4.5). We observed a variable response in LC3-II levels following VacA+ CCMS treatment in primary hepatocytes. In addition, the level of basal autophagy was quite inconsistent in these cells compared to AGS cells and Hep3B cells, whereby LC3-II levels in the control condition (VacA-/DMSO) were minimal and only increased with VacA treatment\textsuperscript{129}. Very high levels of basal autophagy in some of our experiments may be explained by a few factors. In a previous study, LC3-II protein levels in control conditions were elevated in primary mouse hepatocytes compared to a hepatic carcinoma cell line\textsuperscript{144}. This suggests that the autophagic machinery in primary cells may differ from that which is seen in cancer cell lines, and perhaps will require different amounts of VacA.
to see an autophagic defect. Autophagy also promotes cellular longevity in times of metabolic stress\textsuperscript{145}, which the hepatocytes could have been experiencing post-isolation\textsuperscript{146}, thereby offering another potential explanation for increased LC3-II in control conditions. A complementary method, such as immunofluorescence for LC3-positive puncta, should be performed to verify this result\textsuperscript{129}. It is important to note that flux assays must be performed to determine which conditions disrupt or induce autophagy. As previously mentioned, primary hepatocytes may have different TRPML1 expression compared to the cell lines tested which may account for these effects. Future experiments should attempt to classify the differences in TRPML1 expression between these cell types using RT-PCR in order to better understand the basis of the differences seen in these functional assays.

5.2 Effect of FFA supplementation on LD accumulation

Short- and long-term FFA treatments induced LD accumulation in both cell types (Figures 4.5-4.8). Specifically, we assessed LD accumulation on the following parameters: LD number and LD volume. These measures were significantly increased following both ST and LT FFA treatment in primary hepatocytes and in Hep3B cells. In Hep3B cells, the average number of LDs and average volume of LDs was greater following LT FFA supplementation. These results are consistent with what has been previously published, as LD accumulation increased in hepatocytes with oleic and palmitic acid supplementation\textsuperscript{120,147,148}.

Here, it is also important to acknowledge the difference seen in LD accumulation between Hep3B cells and primary hepatocytes. For example, following ST FFA incubations the average number of LDs per cell nearly doubled in both Hep3B cells and in primary hepatocytes. However, there were more lipid droplets in the control condition in primary hepatocytes, indicating that these cells contained more fat to begin with. In fact, the control condition for primary hepatocytes had more than three times the number of LDs compared to the control condition for Hep3B cells, despite the mice being fed a normal chow diet. Interestingly, the accumulation of LDs seen in the control conditions of primary hepatocytes may be explained by a diminished oxygen supply as static \textit{in vitro} cultures of hepatocytes receive much less oxygen via diffusion from the air-liquid interface to the cell surface compared to blood circulation \textit{in vivo}\textsuperscript{146}. This oxidative stress may contribute to hypoxic conditions and subsequently alter metabolism in cultured hepatocytes\textsuperscript{146}. Gilgioni \textit{et al.} compared isolated murine hepatocytes
cultured under static or shaken conditions in a collagen sandwich and found that hepatocytes in shaken cultures showed lower triglyceride accumulation than static cultures as a result of improved oxygen delivery and improved mitochondrial energy metabolism. Thus, future experiments should utilize shaken culture techniques to allow for optimal oxygen delivery to cells in an attempt to minimize hypoxic effects on cell metabolism. LD amounts at the time of hepatocyte isolation should also be assessed to determine the effect of culture methods on LD accumulation.

5.3 Effect of FFA supplementation on autophagic flux

Previous studies have shown that abnormal increases in intracellular lipid impair autophagic clearance. For example, Singh et al. observed a decrease in LD/LAMP-1 co-localization and a lack of autophagic upregulation in hepatocytes cultured with lipids. In accordance with this data, preliminary experiments demonstrated that FFA supplementation resulted in an increase in LC3-II. However, by employing bafilomycin, an inhibitor of autophagosome-lysosome fusion, we showed that in Hep3B cells, FFA addition did not result in defective autophagic flux, as there was a significant increase in LC3-II in 1mM FFA/BAF conditions compared to 0mM FFA/DMSO (control), following both ST and LT FFA treatments (Figure 4.10 & 4.11). Moreover, the difference in LC3-II between 0mM FFA/BAF and 1mM FFA/BAF was statistically significant, and individual immunoblots for these experiments also showed visible increases in LC3-II with 1mM FFA/BAF compared to 0mM FFA/BAF. In addition, densitometric ratios demonstrated an increase in LC3-II/actin with FFA treatment and bafilomycin treatment, compared to bafilomycin alone. This finding suggests that FFA supplementation increased autophagic flux. Due to limitation in the number of primary hepatocytes we obtained, we did not assess the effect of FFA on autophagic flux in primary hepatocytes. If autophagic flux was indeed impaired by the addition of FFAs in primary hepatocytes, we could postulate that MLSA1 dosing used in Hep3B cells may not be sufficient to reverse LD accumulation here as TRPML1 activation would have to compensate for both impaired autophagic machinery as well as an increase in LD abundance. Future experiments are needed in primary hepatocytes to assess whether FFAs affect autophagic flux.
5.4 Effect of MLSA1 on autophagy following FFA supplementation

Autophagy regulates intracellular lipids by delivering LDs to lysosomes for degradation by lysosomal acid hydrolases\(^{44,134}\). Our data revealed that FFA supplementation induces autophagy (data presented in section 4.2.2); however, this basal level of autophagy was not sufficient to clear lipid droplets (data presented in section 4.2.1). TRPML1 activation increases autophagic flux in HeLa cells\(^{88}\) and in AGS cells\(^{129,130}\). The release of lysosomal calcium through TRPML1 increases autophagy through the activation of TFEB, which promotes the expression of genes related to autophagy and lysosomal biogenesis\(^{87,109}\). Since TRPML1 activates autophagy and autophagy activation has been associated with lipid droplet clearance, we aimed to clear LDs in our \textit{in vitro} model of hepatic steatosis via TRPML1 agonism.

First, we assessed whether MLSA1, a synthetic TRPML1 agonist, activates autophagy in our \textit{in vitro} system. We assessed LC3-II accumulation via immunoblotting following either a single or double dose of MLSA1. Here, we showed that LC3-II increased following both single and repeat doses of MLSA1, with no difference between the two (Figure 4.12 \& 4.13). This increase was seen following both 8 and 24 h FFA incubation. It is important to note that following LT FFA supplementation, the LC3-II increase observed with MLSA1 treatment was only statistically significant in the 1mM FFA/M+M condition; however, we observed visible increases as well as increases in densitometric ratios of LC3-II/actin across all conditions with MLSA1. The increase observed in LC3-II suggests that there is an increase in autophagosome formation following MLSA1 treatment, and a potential upregulation of autophagy. Future experiments in the presence and absence of BAF are required to verify that this increase in LC3-II is indicative of an activation of autophagy. Moreover, these experiments need to be repeated in primary hepatocytes to determine their autophagic state following both FFA and MLSA1 treatments. If MLSA1 does activate autophagy in our \textit{in vitro} hepatic steatosis model and a subsequent reduction in LD accumulation is observed, this would offer TRPML1-mediated autophagy activation as a potential therapeutic target in reducing LDs seen in hepatic steatosis.
5.5 Effect of MLSA1 addition following FFA supplementation on LD accumulation

As previously mentioned, TRPML1 has been linked to autophagy induction\textsuperscript{88}, and autophagy induction has been shown to decrease LDs\textsuperscript{134}. Thus, we tested whether MLSA1 reduces LD accumulation in our \textit{in vitro} steatosis model. In Hep3B cells, there were no significant differences in LD parameters with MLSA1 addition following both ST and LT FFA treatments (\textbf{Figure 4.14 & 4.15}). Similarly, MLSA1 had no effect on LDs in primary hepatocytes (\textbf{Figure 4.16-4.18}). If the average number and volume of LDs per cell was significantly reduced with MLSA1 addition, this would suggest that TRPML1 agonism may play a role in LD clearance.

Though trends were observed in the reduction of LD volume and number with the addition of MLSA1 in Hep3B cells, none of these differences were statistically significant. This could be explained by the apparent paradoxical increase in LD accumulation that can be observed with increased autophagy. Interestingly, previous studies have shown that LD biogenesis can occur in the setting of increased autophagic flux\textsuperscript{150,151}. In mouse embryonic fibroblasts (MEFs) exposed to conditions of nutrient starvation, although lipolytic degradation was occurring, LDs increased in abundance\textsuperscript{151}. This increase in LDs was not seen in MEFs lacking ATG5, suggesting that autophagic breakdown of organelles releases lipids that are re-esterified and packaged into new LDs. Consistent with these reports, Nguyen \textit{et al.} showed that starvation in MEFs as well as in a few cell lines (i.e. Huh7, HeLa) led to an increase in LDs. Here, they also determined that LD biogenesis is necessary to prevent the accumulation of acyl-carnitines; important FA-conjugates that are formed at the mitochondria and are required for FA transport into the mitochondrial matrix for β-oxidation. These acyl-carnitines directly cause selective lipotoxic mitochondrial dysfunction. Thus, LD biogenesis in response to autophagy induction serves a protective role for mitochondria. Since MLSA1 has been shown to induce autophagy\textsuperscript{129,152}, this may explain why there were no changes in LDs seen following MLSA1 addition; perhaps LD degradation and subsequent biosynthesis was occurring simultaneously as a result of autophagy induction. Future studies could perform live cell imaging to assess whether MLSA1 is degrading FFAs produced by FFA-supplementation, or whether MLSA1 addition further enhances \textit{de novo} LD synthesis.

In primary hepatocytes, MLSA1 did not have an effect on LD number or volume, which may be explained by a number of factors. Firstly, primary hepatocytes had elevated cytoplasmic LDs
even in the control condition - potentially as a cause of altered lipid metabolism due to hypoxic stress\textsuperscript{146,153}, as further described above in section 5.2. This difference in the number and volume of LDs may describe why the same dosing of MLSA1 used in Hep3B cells was not sufficient to clear LDs in this setting. We were unable to double dose MLSA1 due to limitations in cell availability and number as well as timing limitations in standard culture\textsuperscript{137}, however it would be interesting to see the effect of MLSA1 double dosing on LD accumulation.

5.6 Translational relevance

Hepatic steatosis is common among liver donors, and rates of steatotic grafts are only expected to increase in parallel with the rise in the prevalence of obesity\textsuperscript{22,23}. As steatotic grafts have been linked to risk of graft failure, they are frequently discarded, which further contributes to the organ shortage\textsuperscript{24}. Therefore, there is a need for \textit{in vivo} or \textit{ex vivo} manipulations to defat steatotic liver grafts prior to transplantation to increase the quality and use of these grafts and improve post-transplant outcomes. Experimental studies have already reported macrosteatotic liver defatting using \textit{ex vivo} perfusion\textsuperscript{36,37}. As lipophagy mediates the breakdown of LDs in hepatocytes\textsuperscript{134}, this suggests a novel pathway to target during the peri-transplant period to ameliorate steatosis. TRPML1-mediated autophagy activation may mediate a reduction in LD accumulation\textsuperscript{88,134,154}. In Hep3B cells, we observed an induction of autophagy with TRPML1 agonism, though this did not have a significant effect on LDs (Figure 4.14 & 4.15). This preliminary data suggests that we may see more significant LD reductions with increased optimization of MLSA1 dosing, use of more potent TRPML1 agonists, or TRPML1 overexpression. Activation of autophagy through lysosomal calcium release via the TRPML1 channel may be safer to use in the transplant setting compared to another autophagy enhancer which works by inhibiting mTORC1, rapamycin, which has been prohibited during the peri-transplant period\textsuperscript{78}. At present, no studies have assessed to safety of MLSA1 \textit{in vivo}. Therefore, future studies will need to characterize the safety of MLSA1 \textit{in vivo}. It is important to consider that an increase in the permeability of calcium from the lysosome may alter concentration gradients of other ions since lysosomes contain a large number of other ion transporters in a small-sized vesicle; thus, the increased permeability of one ion may affect ion homeostasis\textsuperscript{155}. Overall, the future of improving marginal graft viability may lie in the coupling of \textit{ex vivo} preservation techniques with pharmacologic agents.
5.7 Conclusions

In summary, this thesis provided evidence for the functionality of TRPML1 in hepatocytes via resolution of VacA-mediated endolysosomal trafficking with MLSA1 addition and showed that TRPML1 agonism activates autophagy in an in vitro hepatic steatosis model. Trends were observed in the reduction of LD volume and number with the addition of MLSA1 in Hep3B cells; however, the differences were not statistically significant. This may have been attributed to the fact that MLSA1 significantly induced autophagic flux, which has been to shown in previous studies to increase LD biogenesis as a mito-protective effect\textsuperscript{150,151}. Future experiments should be performed to assess whether MLSA1 addition contributes to de novo LD synthesis or whether MLSA1 decreases the LD accumulation seen following FFA supplementation. If MLSA1 in fact reduces LD accumulation in hepatocytes, this may allow for advances to be made in reducing fat in steatotic liver grafts thus rendering them viable for transplantation. Presently, in vivo models of MLSA1-mediated hepatic steatosis rescue are limited by the bioavailability and potency of this TRPML1 agonist. Despite this limitation, future applications of MLSA1 may include administering MLSA1 during ex vivo liver perfusion before a steatotic liver is transplanted. This may increase the number of suitable ECD organs and shorten LT waitlists. Moreover, the present work can underlie future experiments, in working to elucidate the role of TRPML1-mediated autophagy activation in the rescue of hepatic steatosis.
Chapter 6: Future Directions

6.1 Can we further characterize TRPML1 channels?

In order to gain insight into the biological functions of TRPML1, it is important to know the relative expression levels of this isoform in specific tissues. Unfortunately, the antibodies for TRPML1 that are currently available are inadequate for this purpose, therefore published data has had to instead measure TRPML1 mRNA expression levels with nucleotide-based techniques such as RT-PCR. While this thesis began to explore the functionality of TRPML1 in both primary hepatocytes as well as in Hep3B cells using VacA assays, a more in-depth understanding of TRPML1 expression levels are needed to further characterize the physiological relevance of this channel in the liver. Though TRPML1 has a ubiquitous expression pattern and is expressed in every tissue, it would be useful to compare the expression levels of TRPML1 in the liver and in the stomach (the organ that was the basis of previous experiments on TRPML1 in our lab). Different cell lines or types may also possess varying levels of TRPML1, making it necessary to characterize expression patterns here as well. To further verify TRPML1 expression levels in hepatocytes, and to compare these levels across cell lines, we could measure differences in TRPML1 gene expression using RT-PCR.

6.2 Would more potent TRPML1 agonists be able to reduce LD accumulation?

MLSA1 is a weak activator of TRPML1. More potent TRPML1 activators have been recently developed, including MLSA3 (Figure 6.1). Wang et al. performed experiments that directly compared MLSA1 and MLSA3 on the basis of lysosomal calcium release, measured with the Fura-2 ratio. Here, authors serum-starved Cos-1 cells for 4 h, and then treated with 20µM of either MLSA3 or MLSA1. They concluded that MLSA3 induced greater calcium release than MLSA1. Zhang et al. showed that MLSA3 induced robust LC3 puncta formation and TFEB nuclear localization. The authors also showed that MLSA3 led to an increase in LC3-II levels, suggesting an upregulation of autophagy via TRPML1 activation.

While these data suggest that a more potent TRPML1 agonist could induce greater intracellular calcium release via TRPML1, there still exists a paucity in the literature in terms of a direct
comparison of these agonists’ autophagic induction potential. This provides an exciting avenue to pursue in future. For example, it would be interesting to observe whether MLSA3 more strongly induces autophagic flux, and whether this leads to greater LD clearance when compared to MLSA1. If this more potent TRPML1 agonist reverses LD accumulation while concomitantly inducing autophagy, this may delineate a potential mechanism of TRPML1-mediated autophagic clearance of LDs.
Figure 6.1. MLSA1 and MLSA3 possess very different chemical structures. The differences in these agonists’ chemical makeup and structure could very well underlie the differences observed in their potency.
6.3 Is TRPML1-mediated autophagy induction the mechanism behind LD breakdown?

As mentioned in the discussion, we only briefly touched upon the effects of MLSA1 on autophagy following FFA supplementation in this thesis. Future experiments are required to further explore the potential of TRPML1-mediated autophagy induction on ameliorating hepatic steatosis. These experiments should compare the different TRPML1 agonists for their autophagic induction potential as well as their ability to clear LDs. First, these experiments should compare autophagy induction for all TRPML1 agonists by assessing LC3-II protein levels, p62 turnover, and an increase in LC3 puncta. Lysosomal calcium release following TRPML1 activation subsequently activates TFEB. Therefore, to evaluate whether autophagy induction is TRPML1-dependent, TFEB activation should be studied via a decrease in the ratio of phosphorylated to total TFEB as well as an increase in TFEB nuclear translocation. In addition, an increase in lysosome number should be detected as TFEB transcriptionally regulates lysosomal biogenesis. These experiments should also be performed in systems that lack or inhibit TRPML1, such as in hepatocytes isolated from Trpml1−/− mice or by blocking TRPML1 using a pharmacological inhibitor, to further understand this biological process.

From these experiments, the most effective TRPML1-mediated autophagy inducer could be used in future experiments to assess for LD clearance. Since autophagy activation has been shown to reduce LD accumulation, by selecting the most potent TRPML1 agonist - and by consequence, autophagy enhancer - we may be able to see further LD reduction than what was observed with MLSA1. LD reduction could be assessed using similar methods used in this thesis. It would also be important to perform these experiments in the presence of synthetic TRPML1 inhibitors, such as mucolipin synthetic inhibitor 3 (MLSI3). This would provide further evidence that the beneficial effect of LD reduction is attributed to TRPML1. If these agonists reduce LD accumulation, this would offer TRPML1-activation as a possible therapeutic target for ameliorating hepatic steatosis in steatotic liver grafts, thus potentiating their use.

To prove that the mechanism involved in TRPML1-mediated LD reduction is autophagy-dependent, we could perform these same experiments in a system that inhibits autophagy, such as in cell lines that lack Atg5. If an inhibition of autophagy reverses the beneficial effects of
LD reduction via TRPML1 activation, this would identify a critical function for autophagy
TRPML1-mediated LD clearance.

6.3.1 Does TRPML1 activation mediate changes in fat catabolism?
Future experiments should also incorporate additional readouts of fat catabolism. The breakdown
of lipids through autophagy leads to the release of degradation products into the cytosol, such as
FFAs. FFAs sustain rates of mitochondrial β-oxidation in the generation of ATP to maintain
cellular energy homeostasis\textsuperscript{154,158}. In addition to upregulating lysosomal and autophagic gene
expression, TFEB has shown to govern lipid catabolism by regulating gene expression of
peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α); an important factor for
mitochondrial biogenesis and subsequent mitochondrial fatty acid β-oxidation\textsuperscript{74}. In a recent
study, Chao et al. demonstrated the role of TFEB in lysosome biogenesis and autophagy in
mouse models of alcoholic liver disease\textsuperscript{75}. The authors showed that TFEB markedly increased
the expression of PGC1α, as well as improved mitochondrial bioenergetics, and increased
expression of fatty acid β-oxidation genes (i.e. ACOX1, LCAD, PPARα)\textsuperscript{75}. A similar approach
could be taken to future experiments in order to evaluate whether the activation of TFEB induces
changes in fatty acid metabolism, in addition to inducing autophagy and enhancing lysosomal
biogenesis.

6.3.2 Does a lack of TRPML1 expression increase LD accumulation in the
liver?
Another potential future direction involves assessing the extent to which a lack of TRPML1
contributes to hepatic steatosis. This could be accomplished by evaluating the livers of mice
from Trpml1\textsuperscript{-/-} mice, as well as livers of MLIV patients. Though lysosomal cytoplasmic
inclusions in the livers of MLIV patients have been observed, it has not been noted whether these
livers are in fact steatotic\textsuperscript{159}. It has been shown that loss of effectors of TRPML1 (i.e. mice with
liver-specific TFEB KO) have impaired lipid metabolism and imbalances in metabolic pathways
in obese mouse models\textsuperscript{160}. Liver-specific Atg7 KO mice have been shown to have an increase of
lipid droplets, cholesterol and triglycerides in the liver\textsuperscript{136}. Since TRPML1 has been implicated in
activating TFEB and subsequently inducing autophagy, and loss of TFEB/ATG genes have been
implicated in the development of fatty liver, it would be interesting to examine the extent to
which TRPML1 deficiencies contribute to fatty liver. More specifically, liver-specific loss and
gain of functional studies could be used to elucidate the role of TRPML1 in lipid metabolism. These data would add to preexisting literature as it would delineate the extent to which TRPML1 and TRPML1-mediated TFEB activation are involved in lipid metabolism.

6.4 Would it be advantageous to target multiple routes simultaneously to defat livers?

There may be a significant advantage to targeting multiple metabolic pathways in the defatting of steatotic livers during the ex vivo preservation period. This thesis explored the effects of activating autophagy through TRPML1 to reduce steatosis. While further reductions in LD accumulation may be seen with the use of more potent TRPML1 agonists, an additional benefit may be observed if another autophagy enhancer is used in combination. For example, torin-1 is a catalytic inhibitor of the kinase activity of mTOR, and a subsequent autophagy enhancer. When the kinase activity of mTOR is inhibited, TFEB remains in its dephosphorylated form where it can translocate into the nucleus to bind to the CLEAR sequence and upregulate lysosomal biogenesis and autophagy. If torin-1 is used in combination with TRPML1, together this may allow for a greater amount of TFEB in its activated state, which may further promote an increase in autophagy and a greater reduction in LDs by consequence. Administration of torin-1 has been shown to increase liver levels of TFEB and decrease steatosis and liver injury induced by ethanol. Torin-1 has also been shown to strongly induce autophagosome formation. In Cos-1 and CHO cells, torin-1 in combination with MLSA1 significantly increased TRPML1 currents, compared to rapamycin and MLSA1-treated cells alone. Together, this evidence suggests that TRPML1 agonism in combination with another autophagy enhancer such as torin-1 may prove beneficial in LD reduction.

Increasing autophagy would increase the number of LDs that are broken down, thus increasing the amount of FFAs in the cytoplasm. Another approach to promote defatting would be to increase transportation of FFAs to mitochondria where they undergo β-oxidation to generate ATP. The transport of FFAs into the mitochondria requires the conjugation of acyl-coA with L-carnitine by carnitine palmitoyltransferase-1. Previous studies have shown that L-carnitine supplementation promotes a reduction in hepatic triglycerides and an increase in β-oxidation.
Increasing β-oxidation through PPARα agonism has also proven to be effective in reducing steatosis\cite{34}.

In addition to the autophagy pathway, other cellular pathways that decrease TG storage should also be considered. Cytosolic lipolysis involves the coordination of perilipins on the surface of LDs and lipases in the breakdown TGs\cite{36}. Protein kinase A activation via forskolin has been shown to result in an increased phosphorylation of perilipin 5 on LD surfaces, which promotes lipolysis\cite{164}. Inducing LD breakdown via both acid lysosomal hydrolases through lipophagy as well as through cytosolic lipases via lipolysis may assist in further LD breakdown.

Therefore, concomitant targeting of multiple metabolic pathways may prove most beneficial in reducing LD accumulation in steatotic grafts. A further understanding of the interactions between these metabolic pathways and any off-target effects in steatotic hepatocytes is needed prior to implementation of this approach in the transplant setting. For example, rapamycin, an mTORC1 allosteric inhibitor and autophagy inducer, has been linked to an increased risk of hepatic artery thrombosis in the peri-transplant period\cite{78}. The mechanism of this association is unknown; thus, it is unclear whether mTORC1 inhibition is unsafe in this setting and whether torin-1 will also induce this negative off-target effect.

6.5 What is the effect of TRPML1 activation in more physiologically relevant models?

6.5.1 Development of macrosteatotic culture systems

Macrosteatosis is characterized by more than 30% of hepatocytes possessing a single fat vacuole which displaces the nucleus to cell periphery, and is a common cause of liver donor ineligibility\cite{165}. In particular, these livers have been linked to greater rates of primary non-function, increased morbidity and mortality, as well as greater sensitivity to I/R injury\cite{166}. Suitable cell culture methods that recapitulate a macrosteatotic environment are required in the evaluation of agents aimed towards reducing macrosteatosis in transplanted steatotic livers. While microsteatotic hepatocyte culture systems have been characterized in the literature\cite{120,127}, and used in this thesis, the clinical relevance of these model systems is unclear since macrosteatotic – and not microsteatotic – livers are associated with worse transplant outcomes, and frequently discarded\cite{165}.
In 2013, Nativ et al. developed a novel macrosteatotic hepatocyte culture system in rat hepatocytes\textsuperscript{167}. Long-term culture of these hepatocytes was made possible by culturing them in a collagen sandwich configuration, which allows for cellular morphology to stay intact for more than a month, with albumin and urea secretion (measurements of liver function) rising and stabilizing after 10 days in culture\textsuperscript{137}. Here, steatosis was induced using medium supplemented with 2mM oleic acid and 2mM of linoleic acid for a total of 6 days. Macrosteatosis was assessed using Nile red staining and an algorithm that allowed for measurements of LD size as well as distribution per cell. Utilizing this type of culture system would allow us to assess whether TRPML1 agonists are capable of reversing LDs seen in macrosteatosis as well, further supporting the use of TRPML1-mediated autophagy induction in a model system that is more clinically-relevant.

Despite being able to recapitulate in vitro macrosteatosis, this system has some drawbacks. For example, previous studies have shown that albumin and urea secretion only stabilize after 10 days post-isolation when cultured in a collagen sandwich conformation\textsuperscript{137}, however Nativ et al. began their experiments 4-5 days post-isolation\textsuperscript{167}. This calls into question the liver-specific functions of these rat hepatocytes in culture and highlights potential limitations of this culture technique.

Furthermore, we may be able to work with primary hepatocytes that are isolated from animals with macrosteatotic livers, as is seen in NAFLD animal models. For example, we could use hepatocytes isolated from mice that are leptin deficient (ob/ob) or from mice than have been fed a methionine and choline deficient diet\textsuperscript{168,169}. Finally, culturing of primary hepatocytes from a steatotic human liver that was deemed unacceptable for transplantation would allow for the optimal in vitro environment. Addition of TRPML1 agonists to these hepatocytes in culture would allow us to determine whether this method of autophagy activation would be suitable in the reduction of macrosteatosis.

6.5.2 Liver organoids

Organoid cultures have rapidly emerged as an alternative in vitro system that more authentically capitulates human tissues in culture. Organoids have been able to replicate key aspects of human liver tissue, including its metabolic functions and complicated architecture; something that mouse models and cell lines cannot do\textsuperscript{170}. Takebe et al. have even gone as far as recapitulating
some aspects of NAFLD and NASH in vitro\textsuperscript{171}. FFA supplementation led to lipid accumulation and fibrosis in hepatocyte organoids that were differentiated from induced pluripotent stem cells (iPSCs). This new technique offers the opportunity to study steatosis and potential new therapeutics in a more physiologically relevant model system.

### 6.6 The use of MLSA1 in the transplant setting

Currently, MLSA1 is not suitable for \textit{in vivo} studies due to its lack of solubility and potency. In future, as drug development studies continue to work on optimizing TRPML1 agonists, this may allow for its use in the \textit{in vivo} setting. If further data suggests that TRPML1 activation reduces steatosis, we could attempt to perfuse animal livers \textit{ex vivo}, adding a TRPML1 agonist to the perfusate to see if this has any effect on LDs. In addition, the combination of TRPML1 activation and activation of other metabolic pathways in parallel to reduce LD abundance would need to be further evaluated.

This data would allow us to prove that TRPML1 agonism is a potential therapeutic target to reduce steatosis in cadaveric livers prior to transplantation. Future studies should also determine whether TRPML1 agonism is protective against injury in macrosteatotic livers. Before this can be translated, much investigation will be needed to assess the effects of accelerated macrosteatosis reduction in hepatocytes. The impact of this on the viability and function of hepatocytes will be important, as these parameters are necessary for successful transplant outcomes\textsuperscript{36}. In sum, the combination of more physiologically-relevant methodologies as well as future development of more soluble, potent TRPML1 agonists may highlight TRPML1-mediated autophagy induction as a potential mechanism for reducing steatosis in steatotic liver grafts.
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