ANALYSIS OF

PHOSPHATIDYLINOSITOL 3-

PHOSPHATE BINDING TO THE

ERLIN COMPLEX

Fanghui Hua

A Dissertation in the Department of Pharmacology

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies of State University of New York, Upstate Medical University.

Approved

Richard J.H. Wojcikiewicz, Ph.D.

Date 8-22-23
# Table of Contents

**Table of Contents** ........................................................................................................... i

**List of Figures and Tables** ............................................................................................. vii

**List of Abbreviations** ................................................................................................. ix

**Acknowledgments** ...................................................................................................... xii

**Abstract** ...................................................................................................................... xiv

**Chapter 1. Introduction** ............................................................................................. 1

1.1. *Overview of the SPFH superfamily proteins* ......................................................... 2

1.1.1. Stomatins and stomatin-like proteins ................................................................. 4

1.1.2. Flotillins ............................................................................................................. 5

1.1.3. Prohibitins ......................................................................................................... 7

1.1.4. HflK/C complex ............................................................................................... 8

1.2. *Overview of erlins* ............................................................................................ 12

1.2.1. Lipid microdomains and localization of erlins ............................................... 14

1.2.2. Current structure model of the erlin complex .................................................. 14

1.2.3. Current cellular function of erlins ................................................................. 17

1.2.3.1. Erlins in IP$_3$R ERAD ................................................................................. 17

1.2.3.2. Erlins in lipid binding and metabolism ....................................................... 21

1.2.3.3. Erlins in ER morphology ............................................................................ 21

1.2.3.4. Erlins in autophagy .................................................................................... 21

1.2.3.5. Erlins in viral infections ............................................................................. 22

1.2.3.6. Erlins in cancer .......................................................................................... 22

1.2.3.7. Erlins in neurodegenerative disease ......................................................... 24
1.2.3.8. Potential mechanism for erlin mutation related neurodegenerative disorder ................................................................. 25
1.2.4. Conclusions ............................................................................ 29

1.3. **Overview of phospholipids and cellular functions** .................... 29
   1.3.1. Membrane phospholipids and the subcellular localization .......... 29
   1.3.2. Phosphoinositide-binding domains ........................................... 33
      1.3.2.1. Pleckstrin homology (PH) domain ..................................... 35
      1.3.2.2. FYVE domains ................................................................. 36
      1.3.2.3. Phox homology (PX) domains ........................................... 37
   1.3.3. Metabolism and functions of main phosphoinositides ............... 40
      1.3.3.1 Cellular regulation of PI(3)P and PI(3,5)P2 .......................... 40
      1.3.3.2 Cellular Regulation of PI(3,4,5)P3 and PI(3,4)P2 ................... 44

1.4. **Overview of phosphoinositides in endosome system** ................. 45
   1.4.1. Clathrin-mediated endocytosis and phosphoinositides conversion ... 46
   1.4.2. Clathrin-independent endocytosis and phosphoinositides conversion .... 46
   1.4.3. Phosphoinositides in dynamics of endosomes ............................. 47
   1.4.4. Phosphoinositides in endocytic cargo recycling and degradative sorting .... 48

1.5. **Overview of phosphoinositides in autophagy-lysosomal system** .... 49
   1.5.1 Phosphoinositides in lysosome ................................................. 50
   1.5.2. Phosphoinositide regulation of autophagy ............................... 51
   1.5.3. Phosphoinositide regulation of autophagosome–lysosome fusion ...... 56
   1.5.4. Phosphoinositide in lysosomal homeostasis and reformation .......... 56
   1.5.5. Transcriptional and epigenetic regulation of autophagy-lysosomal system ... 60
1.5.5.1 Transcriptional regulation of autophagy-lysosomal system .......... 60
1.5.5.2 Epigenetic regulation of autophagy-lysosomal system ............... 64

1.6. Summary and aims of this dissertation ........................................... 64

1.7. References ...................................................................................... 65

Chapter 2. Materials and Methods .......................................................... 94

2.1. Materials .......................................................................................... 95
2.1.1. Cells .............................................................................................. 95
2.1.2 Cell culture and transfection materials ............................................ 95
2.1.3. Plasmids and molecular biology materials ...................................... 95
2.1.4. Antibodies .....................................................................................100
2.1.5. Materials for protein purification and in vitro experiments ............ 102
2.1.6. Materials for cell lysis and immunoprecipitation ............................. 102
2.1.7. Materials for electrophoresis and immunoblotting ......................... 103
2.1.8. Materials for calcium measurements ............................................. 104
2.1.9. Materials for Fluorescence Microscopy .......................................... 104
2.1.10. Materials for Cathepsin activity assay .......................................... 104
2.1.11. Chemicals and Miscellaneous Materials ...................................... 105

2.2. Methods .......................................................................................... 105
2.2.1. Universal procedures in PCR ....................................................... 105
2.2.2. Generation of His- SUMO (HS) constructs ..................................... 108
   2.2.2.1. Gibson assembly procedure .................................................. 108
   2.2.2.2. Generation of HS constructs and mutagenesis ......................... 108
2.2.3. E.coli in Luria Broth (LB) agar/growth ............................................ 109
2.2.4. Cell culture ................................................................. 110
   2.2.4.1. Cell passage and routine maintenance ....................... 110
   2.2.4.2. Freezing and Thawing Cells ..................................... 111
2.2.5. Transfection .............................................................. 111
   2.2.5.1 HeLa cells .......................................................... 111
   2.2.5.2 SH-SY5Y cells ..................................................... 112
2.2.6. Generation of E1, E2 and E1/E2 knock-out cell lines .......... 113
2.2.7. Cell lysis and immunoprecipitation ................................. 116
2.2.8. SDS-PAGE and immunoblotting ..................................... 117
2.2.9. Blue native PAGE ...................................................... 118
2.2.10. Coomassie blue staining ............................................. 119
2.2.11. Bacterial expression and purification of HS-proteins .......... 119
   2.2.11.1. Transformation and the Subsequent E. coli growth ........ 120
   2.2.11.2. Protein purification .......................................... 121
2.2.12. Immunopurification erlin complex from αT3 cells .......... 122
2.2.13. Reconstitution of immunopurified erlin complex with the membrane scaffold protein ......................................................... 124
2.2.14. Lipid overlay assay ................................................... 125
2.2.15. Fluorescence Polarization assay .................................... 126
2.2.16. Confocal microscopy .................................................. 126
   2.2.16.1. Image acquisition ............................................. 126
   2.2.16.2. Image analysis ................................................. 127
2.2.17. ELISA assay ........................................................... 128
2.2.18. VPS34 complex immunoprecipitation and activity assay .......................... 129

2.2.19. Cathepsin activity assay ........................................................................ 131

2.2.20. Statistical analysis .................................................................................. 131

2.3. References ................................................................................................. 132

Chapter 3. Phosphatidylinositol 3-phosphate binding to the erlin1/erlin2 complex
sustains autophagy and lysosome function ....................................................... 133

3.1. Preface ........................................................................................................ 134

3.2. Summary ..................................................................................................... 135

3.3. Introduction ............................................................................................... 135

3.4. Results ....................................................................................................... 137

3.4.1. Recombinant erlins bind selectively to PI(3)P ......................................... 137

3.4.2. PI(3)P binds to a juxta-membrane domain on E2 ................................... 137

3.4.3. Erlns regulate cellular PI(3)P levels ....................................................... 138

3.4.4. Erlns regulate PI(3)P metabolism .......................................................... 140

3.4.5. Erlin KO does not affect the endocytic pathway ..................................... 141

3.4.6. Erlin KO inhibits autophagy ................................................................. 141

3.4.7. Erlin KO inhibits lysosome acidification and function ........................... 142

3.4.8. VPS34 inhibition impairs autophagy and lysosome function ................. 143

3.5. Discussion ................................................................................................. 143

3.6. Experimental Procedures .......................................................................... 147

3.6.1. Cells and reagents .................................................................................. 147

3.6.2. Cell lysis, Electrophoresis and Immunoblotting ..................................... 148

3.6.3. Recombinant protein expression and purification ................................. 148
3.6.4. Lipid overlay assay ................................................................. 149
3.6.5. Fluorescence polarization (FP) assay ................................. 150
3.6.6. Generation and analysis of E1, E2 and E1/E2 KO cell lines .......... 150
3.6.7. Determination of PI(3)P levels by ELISA .......................... 151
3.6.8. VPS34 complex IP and analysis ........................................ 152
3.6.9. Fluorescence microscopy ................................................... 153
3.6.10. FITC-NP uptake ............................................................... 153
3.6.11. Cathepsin activity assay .................................................. 153
3.6.12. Data Presentation, imaging, and statistical analysis .............. 154

3.7. Reference .................................................................................. 155

Chapter 4. Ancillary Data ................................................................ 180

Introduction .................................................................................. 181

4.1. Structural studies of the erlin complex binds to PI(3)P ................. 181
  4.1.1. Determine the PI(3)P binding region on G domain E2 .......... 181
  4.1.2. Correlation between the erlin complex's assembly and PI(3)P interaction .... 190
  4.1.3. Generating the erlin complex structural model ....................... 194
  4.1.4. Discussion .......................................................................... 198

4.2. Erlin KO does not affect cell proliferation and ER morphology in HeLa cells ...... 203
  4.2.1. Erlin KO does not affect cell proliferation in HeLa cells .......... 203
  4.2.2. Erlin KO does not affect ER morphology in HeLa cells .......... 204

4.3. Reference ................................................................................. 207

Chapter 5. General Discussion and future directions .......................... 210

5.1. Introduction .............................................................................. 211
5.2. Erlins and PI(3)P interaction .......................................................... 211
5.3. Erlins and PI(3)P metabolism ...................................................... 213
5.4. Erlins in autophagy ................................................................. 218
5.5. Erlins in lysosome function ...................................................... 220
5.6. Concluding remarks ............................................................... 221
5.7. References ............................................................................. 222

List of Figures and Tables

Chapter 1

Figure 1.1. Domain structure of SPFH domain-containing proteins ............... 3
Figure 1.2. The overall structure of the HflK/C complex .................................. 11
Table 1.1. The current cellular function of E1 and E2 .................................. 13
Figure 1.3. TEM and image analysis of the erlin complex .............................. 16
Figure 1.4. The key elements of IP_{3}R ERAD ...................................... 19
Figure 1.5. Model of how an activated IP3R1 tetramer and the erlin complex interact... 20
Table 1.2. The current literature review of diseases associated with mutations in E1 and E2 ................................................................. 28
Figure 1.6. Distribution of phosphoinositides in various organelles in the cell ...... 32
Table 1.3. Phosphoinositide-binding domains ........................................... 34
Figure 1.7. The structures of main phosphoinositide-binding domains ............ 39
Figure 1.8. Phosphoinositide metabolism ................................................ 42
Figure 1.9. Autophagy and the essential protein complexes .......................... 54
Figure 1.10. Summary of the regulatory pathway of ALR tubule scission .......... 59
Figure 1.11. Autophagy regulation at different levels .................................... 62
Chapter 2

Table 2.1. Oligonucleotides for PCR and sequencing ................................................. 90
Table 2.2. Antibodies and other reagents used in Western blotting and immunoprecipitations .......................................................... 101
Table 2.3. The PCR cycling parameters ................................................................. 107
Table 2.4. List of oligonucleotides designed to generate gRNA for CRISPR/Cas9 mediated gene editing ................................................................. 114

Chapter 3

Figure 3.1. Recombinant erlins bind selectively to PI(3)P ............................... 164
Figure 3.2. PI(3)P binds to a juxta-membrane domain on E2 ............................. 166
Figure 3.3: Erlins regulate cellular PI(3)P levels ............................................. 168
Figure 3.4. Erlins regulate PI(3)P metabolism ................................................ 170
Figure 3.5. Erlin KO does not affect the endocytic pathway ......................... 172
Figure 3.6. Erlin KO inhibits autophagy ......................................................... 173
Figure 3.7. Erlin KO inhibits lysosome acidification and function ................. 174
Figure 3.8. VPS34 inhibition impairs autophagy and lysosome function .......... 175
Figure 3.9. Model for how erlins regulate autophagy and lysosome function .... 176
Figure 3.S1. PI(3)P levels in E2KO SH-SY5Y cells and E1/E2KO αT3 cells .......... 177
Figure 3.S2. Characterization of the VPS34 complex isolated from WT and E1/E2KO HeLa cells .............................................................. 178
Figure 3.S3. Effect of PI(3)P depletion on IP3R1 downregulation ................. 179

Chapter 4

Figure 4.1. Structure perdition of G domain E2 ............................................. 184
Figure 4.2. PI(3)P binding affinity on different regions of G domain E2 ............... 185
Figure 4.3. G domain E2 point mutations do not affect PI(3)P binding .............. 188
Figure 4.4. Correlation between the erlin complex’s assembly and PI(3)P interaction ................................................................................................................. 192
Figure 4.5. TEM and image analysis of the immunopurified erlin complex ............ 196
Figure 4.6. Structure prediction of the erlin complex ........................................... 202
Figure 4.7. Erlin KO does not affect cell proliferation and ER morphology ............ 206

Chapter 5

Figure 5.1. Model of erlins regulating autophagy and lysosome function .............. 216

List of Abbreviations

ALR ................................................................. Autophagic lysosome reformation
ALS ................................................................. Amyotrophic lateral sclerosis
AMBRA1 ......................................................... Autophagy and beclin1 regulator 1
ATG ................................................................. Autophagy-related protein
Aβ ................................................................. Amyloid β-peptide
BRD4 ............................................................. Bromodomain-containing protein 4
CCPs .............................................................. Clathrin-coated pits
CL ................................................................. Cardiolipin
DAPP1 .............................................................. 3-phosphoinositides-1
DFCP1 .......................................................... Double FYVE domain-containing protein 1
E1 ................................................................. Erlin1
E2 ................................................................. Erlin2
ER ................................................................. Endoplasmic reticulum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box transcription factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)</td>
</tr>
<tr>
<td>HSP</td>
<td>Hereditary spastic paraplegia</td>
</tr>
<tr>
<td>INSIG1</td>
<td>Insulin-induced gene 1 protein</td>
</tr>
<tr>
<td>IP3Rs</td>
<td>Inositol 1,4,5-trisphosphate receptors</td>
</tr>
<tr>
<td>MAMs</td>
<td>Mitochondria-associated ER membranes</td>
</tr>
<tr>
<td>MTMs</td>
<td>Myotubularins</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAS</td>
<td>Phagophore assembly site</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHB1</td>
<td>Prohibitin 1</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PI(3)P5K</td>
<td>Class III PI(3)P 5-kinases</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PI(3,5)P2</td>
<td>Phosphatidylinositol 3,5-bisphosphate</td>
</tr>
<tr>
<td>PI(4)P</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
</tbody>
</table>
PI(4,5)P$_2$ ........................................ Phosphatidylinositol 4,5-bisphosphate
PIKfyve .................................. FYVE finger-containing phosphoinositide kinase
PLS .............................................. Primary lateral sclerosis
PM ............................................. Plasma membrane
PPI ............................................. Phosphoinositides
PS ............................................. Phosphatidylserine
PX ............................................. Phox homology
siRNAs ........................................ Small interfering RNAs
SPFH ........................................ Stomatin/ Prohibitin/ Flotillin/ HflK/C
TEM ........................................ Transmission Electron Microscopy
TFEB ........................................ Transcription factor EB
TGN ........................................... Trans-Golgi network
TM ............................................. Transmembrane domain
ULK1 ........................................ UNC-51-like kinase 1
VPS ........................................... Vacuolar protein sorting
WIPI ........................................... WD repeat domain phosphoinositide-interacting protein
Acknowledgment

I would like to begin by expressing my deepest appreciation for Dr. Richard Wojcikiewicz, my mentor, whose exceptional dedication to excellence has profoundly influenced my research journey. His unwavering commitment has shaped my personal and professional growth, providing invaluable guidance during the challenging stages of my Ph.D.

I would also like to extend my gratitude to the current and former members of the Wojcikiewicz lab, Caden, Katherine, Jenson, and Laura. Their assistance in preparing my manuscript and engaging in insightful discussions about experimental design, optimization, and result analysis has enriched my knowledge and experience. Participating in lab activities like boating on Otisco Lake has made my Ph.D. life enjoyable.

I am thankful to the Department of Pharmacology, especially Kay, Katy, and Kelly, for their daily support. Furthermore, I express my gratitude to the College of Graduate Studies for inviting me to Upstate and organizing various events and gatherings with delightful food, enabling me to forge meaningful friendships.

I extend my deepest appreciation to my dissertation defense committee members, Dr. Patty Kane, Dr. Stephan Wilkens, Dr. Alaji Bah, Dr. Juntao Luo, and Dr. Forrest Wright for their invaluable contributions to my research. Thank you to Dr. Stephan Wilkens and Dr. Alaji Bah for their tremendous support and guidance throughout the year greatly appreciate their crucial guidance in generating the structural model of the erlin complex and assisted me in numerous erlin preparations for negative staining. I am also grateful to Dr. Rebecca Oot from Dr. Stephan Wilkens' lab for her assistance in purifying MSP. Dr. Alaji Bah provided valuable suggestions on protein purification and optimization. I would like to acknowledge Dr. Xinjie Chen and his wife for their gracious hospitality and for inviting me and my
husband to their home during the summers. Their invaluable suggestions and support during my adjustment to life in the US are greatly appreciated.

I offer my heartfelt gratitude to my parents for their support, encouragement, and sacrifices throughout my life. Their love and guidance have been instrumental in shaping the person I am today. I would also like to express my special appreciation to my amazing dog, Elmo, whose wagging tail, big smile, and endless cuddles have brought immense joy and comfort into my life. His unconditional love is truly a blessing.

Lastly, I want to express my profound gratefulness to my husband for being my rock throughout my research journey. He has been my sounding board, my partner in brainstorming, and a constant source of practical solutions to overcome challenges. He has provided emotional support and taken care of my physical well-being by preparing nutritious meals and organizing outdoor activities to help me rejuvenate and clear my mind. I consider myself incredibly fortunate to have such a loving and supportive husband by my side. I know that I couldn't have come this far without him, and I am forever grateful for his presence in my life.
Abstract

Analysis of phosphatidylinositol 3-phosphate binding to the erlin complex

Author: Fanghui Hua

Sponsor: Richard J.H. Wojcikiewicz

The Endoplasmic reticulum (ER) membrane lipid raft-associated proteins erlin 1 (E1) and erlin2 (E2) are ~40 kDa proteins, and they are members of a superfamily of stomatin/prohibitin/ flotillin/HflK/C (SPFH) domain-containing proteins. E1 and E2 form a massive (~2 MDa) hetero-oligomeric complex that is an essential mediator of inositol 1,4,5-trisphosphate receptor (IP₃R) ubiquitination and degradation. Mutations of E1 and E2 are involved in many pathological processes in neurological disorders, such as Hereditary Spastic Paraplegia (HSP), with unknown molecular mechanisms.

The Wojcikiewicz laboratory has previously provided evidence that the erlin complex, immunopurified from mammalian cells binds to phosphatidylinositol 3-phosphate (PI(3)P), a key player in membrane dynamics and trafficking regulation in endocytosis and autophagy. In addition, the erlin complex may be involved in different cellular processes beyond IP₃Rs degradation, but the exact nature of these roles has remained elusive.

My research described in this thesis has uncovered intriguing new insights into the erlin complex and its role in previously unknown aspects of cellular biology. Through the application of diverse biochemical and molecular biology assays, I successfully identified specific regions on E2 that are essential for its binding to PI(3)P. Additionally, my research revealed that the erlin complex plays an important role in regulating cellular PI(3)P levels.
through its interaction and stabilization with this lipid. This binding and stabilization of PI(3)P are crucial for the regulation of autophagy and lysosome function. These findings contribute to our understanding of the erlin complex’s importance in cellular biology and provide valuable knowledge about related processes that have implications for human health and disease.
Chapter 1. Introduction
1.1. Overview of the SPFH superfamily proteins

The SPFH protein family encompasses various proteins, including Stomatins, Prohibitins, Flotillins, HflK/C, Erlin1 (E1), and Erlin2 (E2)\textsuperscript{1,2}. These proteins share the highly conserved SPFH domain (Fig. 1.1) \textsuperscript{2,3}. Functional studies across species have demonstrated SPFH superfamily proteins have crucial roles in a range of physiological processes\textsuperscript{3}. The family is also associated with various disease pathology, and drug-like small molecules have been developed to target these proteins to treat conditions like neuropathic pain and cancer\textsuperscript{4}.

The SPFH proteins share two prominent and conserved characteristics: the ability to form oligomers and to localize in cholesterol-rich microdomains within different membranes. These proteins are also associated with different intracellular compartments and bind to specific classes of lipids. For instance, stomatin binds cholesterol\textsuperscript{5}, while stomatin-like protein 2 binds cardiolipin (CL)\textsuperscript{6}, and prohibitin links to phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P\textsubscript{3})\textsuperscript{7}. Erlins bind to cholesterol\textsuperscript{8} and phosphatidylinositol 3-phosphate (PI(3)P)\textsuperscript{9}. The presence of SPFH proteins in lipid raft-like microdomains results in the formation of a unique environment that recruits specific lipids and proteins. This microenvironment is distinct from the rest of the membrane and can potentially provide platforms for certain receptor signaling pathways. This, in turn, can lead to the establishment of specific cellular functions\textsuperscript{10,11}.
Figure 1.1. Domain structure of SPFH domain-containing proteins.

1.1.1. Stomatins and stomatin-like proteins

The human genome encodes five stomatin genes: Stomatin (STOM), and three types of stomatin-like proteins (STOML1, STOML2, STOML3), as well as podocin (NPHS2) 12. The major member, Stomatin, a 31 kDa integral membrane protein, forms high-order homo-oligomers of approximately 300 kDa, comprising 9- to 12-mers and is linked to the membrane through a TM domain (Fig. 1.1). It is primarily located in cholesterol-rich membrane domains on the plasma membrane, late endosomes, and lipid droplets. Stomatin is expressed widely across all human tissues, with hematopoietic cells exhibiting high levels of expression and the brain showing relatively low levels 13,14.

Structural studies have revealed that the human stomatin protein has an N-terminal basic domain followed by a hydrophobic domain associated with the cytoplasmic face of the membrane at Cys-3015. However, the mechanism driving the oligomerization state of the SPFH domain in stomatin and the high-resolution structure of the complex have not been solved. The crystal structure of prokaryotic stomatin (p-stomatin) showed that the SPFH domain forms a stable trimer16, while the eukaryotic SPFH domain assembles into a banana-shaped dimer in the first crystal structure of the mouse stomatin 4. A structure of the SPFH domain of another p-stomatin PH0470 showed that the SPFH domain formed various oligomers and a multimer even without the coiled-coil region at the C-terminal end16. However, mutational studies of human stomatin suggest that the coiled-coil domain is essential for oligomerization, and the cholesterol recognition/interaction amino acid consensus domain is also involved in oligomerization 17. Recently, a study showed whether stomatin SPFH domains form dimers or trimers depending on specific residues 18.
Stomatin and its related proteins interact with different ion channels in cholesterol-rich membrane domains and they regulate the activities of these channels in a cholesterol-dependent manner\textsuperscript{19,20}. Human stomatin interacts with the glucose transporter GLUT1, thereby regulating glucose and dehydroascorbate influx into erythrocytes\textsuperscript{21}. These findings suggest a function for stomatin as an integral scaffolding protein. According to a recent report, stomatin participates in the cytokinesis stage of cell division\textsuperscript{22}. Although it is not a lipid biosynthetic enzyme, depletion of stomatin results in alterations to certain lipid species such as ether lipids, phosphatidylcholines (PC), and phosphatidylinositol (PI)\textsuperscript{22}. Increased stomatin promotes lipid droplet growth or enlargements by facilitating lipid droplet fusion\textsuperscript{23}. Another report shows stomatin as an integral membrane protein that contributes to the uptake of microbes, e.g., spores of the human-pathogenic fungus\textsuperscript{24}.

1.1.2. Flotillins

Flotillin-1 and -2, also referred to as Reggie-2 and Reggie-1 respectively, are proteins that are expressed ubiquitously and have a molecular weight of \(~48\) kDa\textsuperscript{25}. These proteins are highly conserved, with a homology of approximately \(64\%\) between humans and flies\textsuperscript{26,27}. Flotillin-1 and -2 exhibit a similar domain architecture and share sequence identity of up to \(50\%\)\textsuperscript{28} and consist of an SPFH domain that starts a few amino acids downstream of the N-terminus and encompasses around 180 amino acids. They also have an adjacent flotillin domain of similar size and several distinct lipids and protein-binding motifs\textsuperscript{29} (Fig. 1.1). It is noteworthy that flotillins lack a transmembrane domain, and both C- and N-termini face the cytosol\textsuperscript{30,31}.

Flotillins tend to form hetero- as well as homo-oligomers like other SPFH domain-containing proteins and this oligomerization requires the flotillin domain and Tyr-163 of
flotillin-2. Flotillins stabilize each other, as deleting either flotillin-1 or -2 results in a concomitant decrease in the expression of the other one. Flotillin-1 is more dependent on flotillin-2 than vice versa since flotillin-1 depletion usually leads to a moderate or even no depletion of flotillin-2 29.

The three-dimensional structure of the Band 7 domain (also known as SPFH or PHB domain) of the mouse Flotillin 2 Protein (PDB:1WIN) has been resolved (Miyamoto K, publication unfinished), showing SPFH domain of Flotillin is a compact, ellipsoid-globular structure containing four to five α-helices and six β-strands. The flotillin domain, which has not yet been solved, is predicted to harbor several α-helices, some of which might be involved in coiled-coil formation. The membrane binding and oligomerization of flotillins are mediated by the SPFH and flotillin domains, respectively.

Flotillins are primarily found at the plasma membrane and are often considered markers of this membrane due to their association with the inner leaflet via acylations 32. Although flotillin proteins are primarily located in the plasma membrane, they can also be found in various vesicular compartments within the cell. In many types of cells, both flotillin proteins are predominantly found in the pericentrosomal area, where they sometimes overlap with recycling endosomes. Additionally, flotillins are present in multi-vesicular bodies and co-localize with markers for lysosomes. Like other proteins associated with rafts, they also localize to lipid-rich droplets or lipid bodies 33.

Flotillins are expressed ubiquitously, and their expression is particularly high in the brain, heart, lung, and placenta but relatively low in the pancreas and liver. Despite their ubiquitous tissue distribution, the expression of flotillins undergoes transcriptional regulation and can be regulated by different transcription factors 34.
Flotillins have been implicated in the development of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's. Evidence shows that senile plaques and primitive plaques, which are characteristic features of these diseases even in non-demented individuals, exhibit strong labeling for flotillin-2. Furthermore, a detailed examination at the cellular level revealed an accumulation of flotillin-2 in lysosomes of neurons containing neurofibrillary tangles, another hallmark of AD. In AD patients, both flotillins are significantly elevated in the cortex. Recent findings also indicate an increase in flotillin-2 expression in Parkinson's patients. Additionally, flotillin microdomains have been associated with the progression of prion diseases, which are caused by the aggregation of misfolded cellular prion protein Prpc.

1.1.3. Prohibitins

Prohibitin 1 (PHB1) and Prohibitin 2 (PHB2) are proteins with molecular weights of 32 and 34 kDa, respectively. They form a ring-shaped structure, consisting of approximately 12-20 PHB heterodimers, at the mitochondrial inner membrane, with a total size of approximately 1 MDa. PHB1 and PHB2 contain an N-terminal transmembrane domain called the PHB domain, which is evolutionarily conserved and present in other SPFH superfamily proteins (Fig. 1.1). Additionally, they have a C-terminal coiled-coil domain that facilitates protein-protein interactions.

While detailed structural information remains incomplete, yeast studies have provided initial insights into the subunit arrangement of prohibitin complexes within the inner membrane of mitochondria. Electron microscopy images of purified yeast prohibitin complexes indicate a ring-like shape with a diameter of approximately 20-25 nm.
PHB1 and PHB2 are present in the nucleus, mitochondria, and cytosol, and interact with certain cell membrane receptors\textsuperscript{43}. In the nucleus, PHBs act as transcriptional co-regulators by interacting with transcription factors, DNA-modifying associated enzymes, cell cycle-associated proteins, and RNA-binding proteins\textsuperscript{44}. In the cytosol, PHB proteins are highly expressed in cells with high energy demand and are involved in various mitochondrial functions such as respiratory chain subunit degradation, assembly, and activity of the oxidative phosphorylation system, mitochondrial biogenesis, mitochondrial networks, mitochondrial apoptosis, and mitophagy\textsuperscript{45}. In mice, loss of the PHB complex leads to embryonic lethality, degeneration of adult neurons postnatally\textsuperscript{46}, and loss of β cells\textsuperscript{47}. At the cellular level, depletion of the PHB complex results in proliferation defects and increased sensitivity to apoptosis\textsuperscript{48–50}. In addition, PHBs are involved in age-related diseases such as AD, Parkinson's disease, diabetes, and cancer\textsuperscript{44,47,51–53}.

1.1.4. HflK/C complex

HflK and HflC are bacterial homologous proteins of mitochondrial prohibitins. They form a super complex with the AAA+ protease FtsH, which is called the KCF complex\textsuperscript{16}. These proteins are in the inner membrane and have a single N-terminal transmembrane domain and a large C-terminal domain that is exposed to the periplasm. The KCF complex is anchored in the bacterial inner membrane and plays a role in regulating the quality control and homeostasis regulation of membrane proteins. Additionally, the HflK/C complex interacts with FtsH and is known to regulate its degradation of different substrate proteins, such as SecY and the lambda cII protein\textsuperscript{54–56}. Therefore, HflK/C may serve as an FtsH specificity factor.
A recent high-resolution structure (3.3 Å) of cryo-EM data has revealed that HflK/C is a heterodimer with a cage-like structure consisting of twelve HflK molecules, twelve HflC molecules, and four FtsH hexamers, creating a transmembrane hetero-twenty-four polymer. The periplasmic domain of the FtsH hexamer is fully enclosed within the cage structure created by HflK/C in the periplasmic space, and it interacts directly with the SPFH domain of HflK molecules in the complex (Fig. 1.2). The SPFH domain can be divided into two domains: SPFH1 and SPFH2. The SPFH1 domain serves as a membrane insertion domain, with about half of the structure inserted into the outer leaflet of the cell membrane. The adjacent SPFH1 domains closely interact with each other, creating a 20 nm diameter microdomain on the outer leaflet that is entirely separated from the surrounding lipids. Apart from the interacting SPFH1 and SPFH2 domains, other domains, such as the single α helix, CC1, and CC2, also closely interact with each other, forming a cage-like wall through the right-handed helix. The molecular mechanism by which HflK and HflC regulate bacterial membrane protein homeostasis through FtsH has been proposed. In normal physiological conditions, the cage-like structure formed by HflK/C isolates FtsH proteases in space, preventing the degradation of functional membrane proteins on the cell membrane. This is crucial for maintaining the stability of membrane proteins on the crowded cell membrane.

Importantly, the structure of typical SPFH family proteins was analyzed based on sequence and the domain organization of all SPFH family proteins was highly like that of HflK/C. Other members of the SPFH family are likely to form cage-like structures on lipid microdomains of subcellular membranes in a similar way. Therefore, the form of HflK/C cage structure recruiting and isolating FtsH hexamers provides a novel model for other
SPFH family proteins: SPFH proteins interact with membrane proteins (AAA+ proteases, ion channels, membrane receptors, etc.) and form a cage-like heteromeric complex, anchoring on the different membrane microdomains\textsuperscript{57}. 
Figure 1.2. The overall structure of the HflK/C complex.

A. Top and bottom views of the HflK/C 24 heteromer, with the N-domain hexamers of four encased FtsH shown in surface representation (magenta). The membrane footprint, indicated by a dashed box, has an approximate area of 16 nm × 16 nm.

B. Close-up view of the HflK/C heterodimer, showing the domain architecture.

1.2 Overview of Erlins

E1 and E2 are transmembrane glycoproteins with a molecular weight of 41 kDa and 43 kDa, respectively\textsuperscript{59,60}. They belong to the SFPH domain-containing protein family and are primarily found in the ER.

The main functions of erlins include promoting the degradation of ER-associated proteins, mediating inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptors\textsuperscript{60–63}, and participating in lipid metabolism processing and regulation\textsuperscript{8,64}. Recent advancements have shed light on their involvement in various cellular processes, including regulating calcium channel IP\textsubscript{3}R, cell cycle progression, and lipid homeostasis. They also play a crucial role in the regulation of viral infections, cell fate determination by protecting cells from ER-induced cell death, and the control of the autophagy process (\textbf{Table 1.1}). Mutations in erlins have significant implications in the development of various human diseases (\textbf{Table 1.2}).
**Table 1.1. The current cellular function of E1 and E2.**

The checkmark (√) indicates which member of the erlin complex plays a role in the cellular function.

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>Citation</th>
<th>E1</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediate inositol 1,4,5-trisphosphate receptors degradation by recruiting E3 ligase RNF170</td>
<td>Pearce, et, al, J Biol Chem. 2008</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Interacts with AMBRA1 within MAM raft-like microdomains and promotes the formation of autophagosomes</td>
<td>Manganelli V, et,al. Autophagy. 2021.</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>Interact with Rhomboid protease RHBDL4 and p97, initiate degradation by an ERAD pathway parallel to Hrd1-dependent retrotranslocation, and cleave aggregation-prone ER-luminal proteins</td>
<td>J Bock et,al. Cell Reports. 2022</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Bind to ER transmembrane J-protein B12 and facilitates B12 mobilization to specific foci in the ER, aiding polyomavirus SV40 penetrate the ER membrane to cytosol and cause infection</td>
<td>Inoue T, et,al. PLoS Pathog. 2017</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Host factor required for HCV life cycle, regulates both early steps of RNA and protein accumulation and later steps of virus production</td>
<td>Whitten-Bauer C, et,al. Cells. 2019</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>Supports breast cancer cell growth by promoting the activation of the key lipogenic regulator SREBP1c and the production of cytosolic lipid droplets</td>
<td>Wang G, et,al. Biochem J. 2012</td>
<td></td>
<td>✔</td>
</tr>
</tbody>
</table>
1.2.1. Lipid microdomains and localization of erlins

Lipid raft-like microdomains are one of the specific compartmentalized regions on the membranes that include specific lipids and proteins, such as cholesterol, and sphingolipids, and preferentially contain lipid-modified proteins, such as glycosylphosphatidylinositol-anchored proteins\(^2,10\). These components create a tightly packed lipid environment, which is resistant to detergents\(^2,65\) and is distinct from the rest of the membrane regions\(^10,11,59\).

Proteins containing the SPFH domain are associated with lipid raft-like microdomains on various intracellular membranes\(^45,59\). These membranes include mitochondria (prohibitin), the trans-Golgi network (flotillins), endosomes, and the plasma membrane (flotillin and stomatin). The SPFH domain-containing proteins play a role in maintaining the integrity and function of these microdomains.

The erlin complex is a large ring-shaped complex with a molecular weight of approximately 2 MDa\(^60–62\). E1 and E2 have been identified explicitly as protein markers of the ER and are localized within ER lipid raft-like microdomains. They were initially discovered in detergent-resistant membrane fractions isolated from myelomonocytic cells and are considered components of ER membrane lipid rafts\(^59\).

1.2.2. Current structure model of the erlin complex

E1 and E2 are highly similar proteins, sharing about 89% amino acid similarity\(^59\). They have juxta-membrane globular domains (G domains) that make up roughly 50% of each protein and exhibit approximately 90% identity (residues 27-179 in E1 and 25-176 in E2), with a molecular weight of around 17 kDa. Apart from the G domain, both E1 and E2 possess several distinctive features, including a hydrophobic transmembrane domain (TM)
at their N-termini (residues 6-26 in E1 and 4-24 in E2), an N-linked glycosylation site (Asn108 in E1 and Asn106 in E2), and an α-helical stretch predicted to form coiled-coil motifs (residues 179-276 in E1 and 177-274 in E2). Additionally, they have an α/β domain (residues 277-300 in E1 and 275-298 in E2), an assembly domain (residues 301-311 in E1 and 299-309 in E2), and a C-terminal domain (residues 312-348 in E1 and 310-340 in E2).

Utilizing negative stain Transmission Electron Microscopy (TEM) and single-particle image analysis techniques, our lab previously reconstructed a three-dimensional model of the erlin complex (Fig. 1.3) ⁶⁰. The model reveals that the erlin complex is composed of two ring-shaped structures with an outer diameter of approximately 250 Å and an inner diameter of about 125 Å. The rings are connected by weak densities to a smaller disc with a diameter of approximately 125 Å. While this domain appears as a disc, it may also be ring-shaped, as stain-excluding detergent micelles and/or associated lipids could fill a putative central cavity. The larger ring likely represents the luminal domains of E1 and E2 and exhibits 2-fold symmetry, with two thicker densities connected by a thinner density on one side. Nevertheless, the accuracy of this model could be compromised by the constraints of TEM and the negative staining technique. Therefore, we have developed an alternative homology model for the erlin complex. This new model draws inspiration from recently published high-resolution cryo-EM structure of the bacterial SPFH domain-containing proteins HflK/C, which share homology with E1 and 2, which is described in section 1.1.4. A detailed discussion of this model will be presented in section 4.1.3.
Figure 1.3. TEM and image analysis of the erlin complex.

Three-dimensional model of the erlin complex, determined at a resolution of ~33 Å and contoured at a volume corresponding to a calculated molecular mass of ~2 MDa (scale bar: 100 Å). Putative side (1), top (2), and bottom views (3) are shown, with membrane and luminal domains indicated by arrows and arrowheads, respectively. Reproduced from reference 60.
1.2.3. Current cellular function of erlins

E1 and E2 are evolutionarily conserved with homologs in C. elegans, plants, and vertebrates. The strong evolutionary conservation supports the vital roles of the erlin complex in the cell (Table 1.2).

1.2.3.1. Erlins in IP₃R ER-associated degradation (ERAD)

By far, the most widely accepted function of erlins is to mediate inositol 1,4,5-trisphosphate receptor (IP₃R) degradation. There are three subtypes of IP₃ receptors: IP₃R1, IP₃R2, and IP₃R3. These subtypes are expressed in different proportions in various cell types and can form homo- or heterotetramers. IP₃Rs are huge Ca²⁺ channel that localizes at the ER membranes and are important regulators of many cell-signaling pathways that release Ca²⁺ from the ER to the cytosol. Stimulation of certain cell surface receptors, such as G-protein-coupled receptors, triggers IP₃ formation at the plasma membrane.

The binding of IP₃ and Ca²⁺ molecules in a coordinated manner triggers the opening of the channel, enabling the release of Ca²⁺ from the ER lumen into the cytosol, which promotes Ca²⁺-dependent downstream cellular events, such as secretion, neurotransmitter release, fertilization, cell division, and apoptosis. Hence, it is important for cells to maintain the homeostasis of cellular IP₃Rs levels. Our lab discovered the erlin complex binds to activated IP₃Rs and initiates their ERAD by recruiting an E3 ubiquitin ligase RNF 170, and deletion of erlin proteins increases basal levels of IP₃R, indicating the erlin complex is crucial for IP₃R turnover. The recently acquired structure of the bacterial HflK/C complex provides valuable insights into the potential interaction mechanism between the erlin complex and IP₃R1. In this context, HflK/C assemble into a cup-shaped...
complex, firmly anchored to the bacterial inner membrane by their N-terminal TM regions. The connection point between their SPFH1 and SPFH2 domains lies proximal to the membrane, serving as the binding site for client FtsH hexamers that are encircled by the HflK/C complex. Given the erlin complex’s similar ~2 MDa architecture associated with the ER membrane, it becomes feasible to construct models illustrating its interactions with activated IP₃Rs (Fig. 1.5) with two possible orientations. A more comprehensive understanding of the binding interface will emerge when high-resolution structures become available for the erlin complex in the future.

On the other hand, the erlin complex is an essential element in the ERAD pathway, which is responsible for the degradation of misfolded proteins in the ER and some metabolically regulated ER membrane proteins, including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase⁷⁸,⁷⁹. Furthermore, recent reports indicate that the erlin complex exhibits interaction with Rhomboid protease RHBDL4 and AAA + ATPase p97, resulting in the formation of an ERAD complex. This complex functions in parallel with the Hrd1-dependent retrotranslocation pathway, initiating degradation processes and cleaving ER-luminal proteins that are prone to aggregation⁸⁰.
**Figure 1.4. The key elements of IP₃R ERAD.**

The erlin complex (dark gray) and bound RNF170 (red, with the critical RING domain gold. Only 2 of the 4 subunits of the IP₃R are depicted (colored green and red). RNF170 catalyzes IP₃R ubiquitination, resulting in the attachment of 2 chains of ubiquitin (red ellipses). These chains are recognized by the p97/Ufd1/Npl4 complex that facilitates the transfer of proteins to the proteasome for degradation. *Reproduced from reference*⁶³.
Figure 1.5. Model of how an activated IP₃R1 tetramer and the erlin complex interact.

Diagrams of IP₃R1 tetramers and erlin complexes, based on the dimensions and membrane topology of HflC/K with one AlphaFold-derived E2 subunit fitted into each complex. Activated IP₃R1 tetramers could bind with the erlin complex through interactions with the outer surface of the complex (left) or even be encircled by the complex (right). Reproduced from reference 81.
1.2.3.2. Erlins in lipid binding and lipid metabolism

In addition to their ERAD-associated functions, erlins have been found to have additional cellular roles. Like other proteins in the SPFH superfamily, erlins possess lipid-binding properties and have been studied for their involvement in lipid metabolism. Our lab’s previous research shows E2 is the primary partner in the erlin complex and interacts with PI(3)P through the T65 region on the G domain. This region is also critical for binding to activated IP3R and ERAD processing. Additional studies have revealed that erlins bind to cholesterol and play a crucial role in regulating cholesterol homeostasis protein: SREBP–Scap–Insig. Furthermore, other studies have indicated that E2 is linked to gp78 and facilitates sterol-accelerated ERAD of the HMG-CoA reductase, an enzyme essential for cholesterol biosynthesis.

1.2.3.3. Erlins in ER morphology

Erlins might affect ER morphology by regulating the stability of ER-shaping protein CLIMP63 and showing a regulatory role in CLIMP63 cleavage. Loss of the erlin complex leads to decreased ER tubulation and proliferation of ER sheets to the cell periphery (Schumacher, 2018, unpublished data).

1.2.3.4. Erlins in autophagy

It is interesting to note that erlin proteins have been found to associate with ER raft-like microdomains, indicating their potential role as ER-anchoring factors. Manganelli et al. identified the presence of raft-like microdomains in the mitochondria-associated ER membranes (MAMs) and E1 is on the MAMs and plays a role in the initial stages of autophagosome formation. The study also revealed a physical interaction between E1 and
autophagy and beclin1 regulator 1 (AMBRA1) once autophagy was triggered. The disruption of AMBRA1-E1 interaction hinders autophagy and silencing E1 results in a huge decrease in the number of both autophagosomes and autolysosomes during starvation. Furthermore, E1 has been shown to influence autophagy-dependent survival during chemotherapy\(^8\). However, the study overlooked exploring the role of E2 in autophagy since E1 and E2 not only form an endogenous complex but also share a 83% sequence identity\(^5\).

1.2.3.5. Erlins in viral infections

Some studies have explored the involvement of erlins in viral infections, suggesting their relevance in this context. A study conducted by Inoue and Tsai\(^8\) indicates that erlin facilitates the non-enveloped polyomavirus SV40 to penetrate the ER membrane to cytosol and triggers the infection. During this process, SV40 recruits an ER transmembrane J-protein B12, as well as other membrane components, into distinct puncta in the ER membrane. Erlins bind to B12 and facilitate its reorganization into the SV40-induced foci and act as anchors that restrict B12 to the ER.

Furthermore, a recent article has revealed a role for E1 in hepatitis C virus (HCV) infection\(^4\). E1 is considered a host factor required for the HCV life cycle, and depletion of E1 leads to decreased infection efficiency. Given the dependence of HCV on lipid metabolism and the ER for its life cycle, E1 may regulate not only the early steps leading to RNA and protein accumulation but also the later steps affecting virus production.

1.2.3.6. Erlins in cancer
Ongoing research is currently investigating the involvement of erlins in the regulation of cancer. Notably, the E2 gene is located on chromosome 8p11.2, a region that is commonly subject to mutations in human breast cancer. Moreover, E2 exhibits high expression levels in aggressive forms of human breast cancer, including luminal and HER2+ subtypes.

Several studies have suggested that erlins may have a crucial role in promoting breast cancer. E2 plays a role in regulating the progression of human breast cancer specifically during the G2/M phase of the cell cycle. It achieves this by interacting with and stabilizing α-tubulin, a component of microtubules, as well as the mitosis-promoting complex Cyclin B1/Cdk132.

Furthermore, E2 facilitates the K63-linked ubiquitination of Cyclin B1, thereby promoting the progression of breast cancer cells. In addition, downregulation of E2 results in cell cycle arrest represses breast cancer proliferation and malignancy and increases the sensitivity of breast cancer cells to anti-cancer drugs.

The same group also showed that E2 interacts with the ER-resident insulin-induced gene 1 protein (INSIG1) to regulate the activation of SREBP 1c, the key regulator of de novo lipogenesis. Through this regulation, E2 may help breast cancer cells maintain high levels of cytosolic lipid content and gain growth advantage under oncogenic stress conditions.

Another study has indicated that E2 is an oncogenic factor associated with the ER stress response pathway. The IRE1α/XBP1 axis in the ER stress pathway modulated the expression of E2 levels in breast cancer cells and overexpression of E2 facilitated the
adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cells from ER stress-induced cell death. 

1.2.3.7. Erlins in neurodegenerative disease

Mutations in E1 or E2 genes have been linked to rare neurodegenerative diseases, such as sclerosis, paraplegia, and sensory ataxia (Table 1.3). The most prevalent loss-of-function mutations in E2 are associated with the development of both juvenile primary lateral sclerosis (PLS) and complicated, autosomal recessive hereditary spastic paraplegia (HSP).

PLS specifically affects upper motor neurons and causes their degeneration, leading to various phenotypes such as weakness in the legs, spasticity, urinary dysfunction, and cognitive impairment. A case report showed that PLS is caused by a c.499-1G>T mutation in the E2 gene that affects splicing, resulting in the introduction of an in-frame stop codon that truncates two-thirds of the encoded protein and decreases the patient's mRNA level by approximately 15%, which drastically reduces the cellular complement of functional E2.

HSP is a large genetic group of inherited neurologic disorders characterized by the degeneration of cortical moto-neuron axons, resulting in progressive spasticity and weakness of the lower limbs. In 2011, a Turkish group reported the first case of a novel autosomal recessive disease caused by a homozygous two-nucleotide insertion in E2, which was later identified as HSP. That same year, Alazami et al. reported a Saudi family with five HSP-affected members who had a 20 kb transposition upstream of exon 2 in the SPG18 locus. Furthermore, a dominant form of pure HSP has been recently linked to a
heterozygous mutation in E2, highlighting the complexity of E2 mutations that can result in cognitive and speech impairment phenotype\textsuperscript{94}.

In addition, mutations in E2, such as V168M, D300V, N125S, A309V, and D69V\textsuperscript{95,96}, have been identified as potential causes of rapidly progressive amyotrophic lateral sclerosis (ALS), a more severe form of motor neuron disease characterized by the extensive degeneration of both upper and lower motor neurons. ALS is characterized by severe symptoms such as tetraplegia, bulbar palsy, respiratory insufficiency, and ultimately, patient mortality. This underscores the significance of E2 point mutations, which have been implicated in the development of various debilitating neurodegenerative disorders.

Apart from rare neurodegenerative diseases like HSP, PLS, and ALS, erlins have been found to be directly associated with Alzheimer's disease. The Teranishi group utilized tandem mass spectrometry to identify E2 as a new binding partner of brain \( \lambda \)-secretase-associated protein. Silencing of E2 resulted in a decrease in the production of neurotoxic amyloid \( \beta \)-peptide (A\( \beta \)), providing additional evidence for its involvement in Alzheimer's disease. These findings highlight the potential role of E2, and possibly the erlin complex, in regulating A\( \beta \) and suggest that targeting the interaction between E2 and \( \gamma \)-secretase could be a viable pharmacological strategy for treating Alzheimer's disease\textsuperscript{103}.

1.2.3.8. Potential mechanisms for erlin mutation related neurodegenerative disorder

Erlin mutations in neurodegenerative diseases can involve multiple pathological mechanisms, including dysregulation of \( \text{Ca}^{2+} \) levels and ERAD. Neurons are particularly sensitive to intracellular \( \text{Ca}^{2+} \) levels, and disruptions in \( \text{Ca}^{2+} \) homeostasis can impair neuronal function, impacting their normal activities, structural integrity, and vitality.
Research suggests that Ca\textsuperscript{2+} dysregulation is a common underlying mechanism in neurodegenerative disorders like Alzheimer's disease, Huntington's disease, and spinocerebellar ataxias\textsuperscript{104,105}.

The IP\textsubscript{3}Rs in the ER membrane play vital roles in maintaining proper intracellular Ca\textsuperscript{2+} levels. The IP\textsubscript{3}R-erlin complex-RNF170 axis, as a crucial component of IP\textsubscript{3}R ERAD, is involved in regulating the life cycle and cellular function of IP\textsubscript{3}Rs and therefore affecting cellular Ca\textsuperscript{2+} homeostasis. Mutations in IP\textsubscript{3}R, erlins, and RNF170 genes have been implicated in several neurodegenerative disorders, particularly autosomal dominant sensory ataxia in cases of RNF170 mutations\textsuperscript{106,107}. RNF170 mutations are known to disrupt Ca\textsuperscript{2+} regulation\textsuperscript{75} and contribute to autosomal dominant sensory ataxia. Similarly, loss-of-function variants in the erlins can lead to increased IP\textsubscript{3}-dependent signaling through IP\textsubscript{3}Rs, prolonging Ca\textsuperscript{2+} release from the ER and contributing to disease pathogenesis. Additionally, recent studies have revealed that gain-of-function HSP-related E2 mutation results in the degradation of IP\textsubscript{3}Rs, impeding Ca\textsuperscript{2+} release from ER and triggering ER stress-mediated apoptosis\textsuperscript{108}. Consequently, both gain-of-function and loss-of-function variants in these genes disrupt intracellular Ca\textsuperscript{2+} homeostasis, highlighting the IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} pathway as a potential therapeutic target for future interventions in motor neuron disorders.

On the other hand, E2 is important in the ERAD pathway, responsible for the degradation of misfolded proteins within the ER, such as major histocompatibility complex class I heavy chain\textsuperscript{80}. E2 is also associated with the degradation of cholesterol biosynthetic enzyme HMG-CoA reductase\textsuperscript{78}. Mutations in E2 may disrupt the function of ERAD, leading to the accumulation of abnormal proteins, a characteristic feature observed in
various neurological disorders, including Alzheimer's disease, Huntington's disease, Parkinson's disease, and ALS\textsuperscript{109}.

In addition to the pathways described above, previous research has highlighted erlins’ importance in maintaining proper ER shape and regulating autophagy\textsuperscript{82}. Studies on HSP pathogenesis have shown a strong correlation between proteins responsible for coordinated ER dynamics and auto-lysosome function, accounting for over 50\% of HSP cases\textsuperscript{100}. Therefore, erlin mutations may affect ER shape, autophagy, and lysosomal functions\textsuperscript{110,111}, leading to the manifestation of HSP\textsuperscript{100,110,111}.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutations</th>
<th>Species</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlin 2</td>
<td>T65I</td>
<td>Human</td>
<td>Hereditary Spastic Paraplegia</td>
<td>Darios et al.2018</td>
</tr>
<tr>
<td>Erlin 2</td>
<td>Mutation in Intro7, altered splicing</td>
<td>Human</td>
<td>Primary Lateral Sclerosis</td>
<td>Al-Saif, et al. Neurology 2012</td>
</tr>
<tr>
<td>Erlin 2</td>
<td>S129T</td>
<td>Human</td>
<td>Hereditary Spastic Paraplegia</td>
<td>Rydning SL, et al. 2018</td>
</tr>
<tr>
<td>Erlin 1</td>
<td>I291V</td>
<td>Human</td>
<td>Non-alcoholic Fatty Liver Disease</td>
<td>Feitosa, et al. 2013</td>
</tr>
<tr>
<td>Erlin 1</td>
<td>V94A</td>
<td>Human</td>
<td>Amyotrophic lateral sclerosis</td>
<td>Tunca , et al. 2018</td>
</tr>
</tbody>
</table>

Table 1.2. The current literature review of diseases associated with mutations on E1 and E2.
1.2.4. Conclusions

Recent findings suggest that the erlin complex is involved in multiple ER-associated cellular pathways and has diverse roles within the cell. This could explain why disruptions to the erlin complex have been linked to several neurodegenerative disorders. However, the full extent of the complex's contribution to cellular functions is not yet clear. Future research is necessary to explore the role of erlins in the related cellular pathways responsible for neurodegenerative diseases. A deeper understanding of the molecular mechanisms by which erlins coordinate these pathways and their potential involvement in the pathogenesis of diseases may lead to the identification of new therapeutic approaches for treating various neurodegenerative diseases.

1.3. Overview of phospholipids and cellular functions

1.3.1. Membrane phospholipids and their subcellular localization

Phospholipids, also known as glycerophospholipids, make up the lipid bilayer of cellular membranes, serving as a barrier between the cell and its surroundings and between different cellular compartments.112,113 The major glycerophospholipids in eukaryotic membranes include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA). These lipids consist of a hydrophilic head group attached to a glycerol backbone and a hydrophobic tail made up of two fatty acyl chains of varying lengths and degrees.114

The composition of lipids in organelles differs due to variations in cellular lipid synthesis.115 Lipidomics has shown that there is an uneven distribution of lipid classes among organelles. The ER is the primary location for lipid synthesis, producing significant
amounts of structural phospholipids and cholesterol, as well as ceramide and galactosylerceramide. The ER membrane has a high concentration of PC, PE, PS, and PA, but low concentrations of cholesterol and sphingolipids. Golgi is another major location for lipid synthesis, producing sphingolipids, SM, and lactosylerceramide, with a similar lipid composition to the ER, but with unique lipids involved in signaling pathways, like phosphatidylinositol 4-phosphate (PI(4)P). The plasma membrane (PM) maintains high levels of PC, SM, cholesterol, and PI, receiving sphingolipids and sterols from the ER and Golgi. Although PM is not a location for structural lipid synthesis, it has many lipid-synthesizing or degrading reactions involved in signaling transduction. Mitochondrial membranes are mostly composed of PE, phosphatidylglycerol (PG), CL, and PA, which are synthesized within the mitochondria. CL exhibits exclusivity to the mitochondria, and its biogenesis undergoes a process wherein PA is transferred from the outer mitochondrial membrane to the inner mitochondrial membrane through the intermembrane space. The sterol content of mitochondria is generally low, except in cells involved in steroid hormone synthesis. Early endosomes have a lipid composition like the PM, but during maturation to late endosomes, the sterols, and PS decrease, and bis-monoacylglycerol phosphate (BMP) dramatically increases. BMP functions in multivesicular body generation, fusion processes, and sphingolipid hydrolysis.

In cells, the presence of various PIs plays a crucial role, and this section will specifically focus on them. These include PI(4)P and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) found on the PMs, PI(3)P on early endosomes and autophagosomes, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) on late endosomes and lysosomes, and PI(4)P on the trans-Golgi network (TGN). The diverse distribution of these PIs within the cell relies on a
specialized system of kinases and phosphatases responsible for their synthesis and degradation. These enzymes ensure the precise regulation and maintenance of specific phosphoinositide pools in different cellular compartments. (Fig. 1.6).
Figure 1.6. Distribution of phosphoinositides in various organelles in the cell.

The majority of PI(4,5)P\(_2\) is located in the PM, and that is where it is converted to PI(3,4,5)P\(_3\). PI(4)P is also found in the PM, but it is also highly enriched in the Golgi and in some endosomes that are part of the TGN. PI(4)P can be converted to PI(4,5)P\(_2\) in exocytic vesicle when recycling back to PM. PI(3)P is initially found on early endosomes and undergoes conversion to PI(3,5)P\(_2\) on late endosomes. Late endosomes have two possible fates: they either recycle back to the plasma membrane or transform into lysosomes. PI(3)P is also located on the phagophore, which originates from ER. The phagophore converts to mature autophagosome, which ultimately fuses with the lysosome to form autolysosome, which is enriched PI(3,5)P\(_2\). PI is generated in ER-derived compartments that can reach every other membrane. The figure was prepared with BioRender.
1.3.2. Phosphoinositide-binding domains

Phosphoinositides (PPI) are a class of lipids that are phosphorylated versions of PI. The only difference between PPI and PI is the phosphorylation status of their inositol head group, as shown in Table. 1.3\textsuperscript{116}. PPI act as lipid second messengers, as they are synthesized and rapidly turned over from the abundant precursor PI within specific membrane microdomains. This allows many distinct proteins to be targeted to a particular membrane without saturating binding sites, due to the relatively large ratio of PI to the binding partner. Moreover, different structurally distinct PIs can activate different downstream effectors. The rapid and sequential interconversion between phosphorylated forms of PI enables processivity in membrane signaling events\textsuperscript{117}. Various PPI-binding domains/motifs, including FYVE, phox homology (PX), pleckstrin homology (PH), ENTH, and FERM domains, have been identified and characterized\textsuperscript{117,118} (Table 1.3).
<table>
<thead>
<tr>
<th>Name of Domain</th>
<th>Lipid Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleckstrin homology (PH) domain</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td></td>
<td>PI(4)P</td>
</tr>
<tr>
<td></td>
<td>PI(3,4,5)P₃</td>
</tr>
<tr>
<td></td>
<td>PI(3,4)P₂</td>
</tr>
<tr>
<td>FYVE domain</td>
<td>PI(3)P</td>
</tr>
<tr>
<td>Phox homology (PX) domains</td>
<td>PI(3)P</td>
</tr>
<tr>
<td>ENTH (epsin NH2-terminal homology)</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>ANTH (AP180 NH2-terminal homology)</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>FERM domains</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>PTB domain</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>GLUE domain</td>
<td>PI(3,4,5)P₃</td>
</tr>
<tr>
<td>Eps15 homology (EHD) domains</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td></td>
<td>PI(4)P</td>
</tr>
<tr>
<td>BAR (Bin–Amphipysin–Rvs)</td>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>PDZ domain</td>
<td>PI(4,5)P₂</td>
</tr>
</tbody>
</table>

Table 1.3. Phosphoinositide-binding domains.
1.3.2.1. Pleckstrin homology (PH) domain

PH domains found in certain molecules like protein kinase B (PKB), Bruton's tyrosine kinase, the general receptor for phosphoinositides-1 (Grp1), and the dual adaptor for PS and 3-phosphoinositides-1 (DAPP1). Previously, it was believed that these domains were exclusively bound to PI(3,4,5)P$_3$. However, a recent single-molecule pulldown assay shows PH domains binding to other PPIs (Table 1.3) and only a small portion of PH domains (perhaps 5-10% in the human proteome) are capable of specifically recognizing PI(3,4,5)P$_3$.

PH domains, which are structural modules composed of approximately 120 amino acids, have been extensively characterized. All PH domains share a common core fold that is comprised of a 7-stranded β-sandwich formed by two nearly orthogonal β-sheets (Fig. 1.7. A). The β-sandwich has four corners, and because of the twist in the antiparallel β-sheets, two of them are open while the other two are closed. The characteristic C-terminal α-helix of PH domains covers one of the open corners, while the remaining open corner is covered by three interstrand loops that form the PPI-binding site.

There are around 20 PH domain proteins that can recognize PI(3,4,5)P$_3$, which all contain a sequence motif with basic residues in the loop between the first two β-strands. Within the binding pocket, the PH domains demonstrate a strong binding affinity towards the PI(3,4,5)P$_3$ headgroup. This interaction is facilitated by the formation of numerous hydrogen bonds between the PH domains and each accessible phosphate group of PI(3,4,5)P$_3$. The PH domains containing this motif exhibit significant conformational changes in response to the binding of the PI(3,4,5)P$_3$ headgroup, as revealed by their crystal structures. The insertion of PI(3,4,5)P$_3$ induces conformational changes in both the
highly positively charged loop at the entrance of the PIP3 binding pocket and the entire β barrel of the PH domain. This suggests that the motif plays a role in arranging basic side chains in a way that maximizes hydrogen bonding interactions with the phosphate groups of the headgroup.

1.3.2.2. FYVE domains

The FYVE domain received its name from the initial proteins in which it was discovered, namely Fab1p, YOTB, Vac1p, and EEA1. It is composed of approximately 60-70 amino acids and features a zinc finger-like structure consisting of two Zn$^{2+}$ β-binding clusters that hold together two β-hairpins and a small C-terminal α-helix (Fig. 1.7. B). The primary feature of the FYVE domain's surface is a shallow, positive charged PI(3)P binding pocket.

In the FYVE domain structure model of EEA1, which was crystallized in a complex with Inositol 1,3-bisphosphate, the PI(3)P headgroup is primarily bound to the FYVE domain via hydrogen bonds with side chains derived from this fundamental motif. In comparison to the PH domain in Grp1, which binds the PIP3 headgroup via 19 hydrogen bonds with a $K_d$ of ~27 nM, the EEA1 FYVE domain binds the PI(3)P headgroup weaker, with a $K_d$ of ~71 nM at pH 6.0. A later study has shown that $K_d$ is pH-dependent: ~150 nM at pH 6.8; ~1.5 mM at pH 7.4; and ~10 mM at pH 8.0. Although the specific recognition of PI(3)P is a significant differentiating factor of the FYVE finger, the overall mechanism of membrane anchoring is multivalent and includes non-specific electrostatic contacts with acidic lipids other than PI(3)P, activation of the histidine switch, hydrophobic insertion into the bilayers, and, in certain cases, dimerization. Each element within the multistep binding mechanism contributes uniquely to the FYVE domain's specificity and affinity for PI(3)P present in membranes. Upon binding PI(3)P,
the FYVE domain's membrane insertion loop, located within the domain, penetrates into the phospholipid bilayer. Through dimerization and lowering cytosolic pH, the FYVE domain exhibits increased PI(3)P affinity\textsuperscript{128,129}.

FYVE domains play a crucial role in directing a broad range of cytosolic proteins to membranes during various signaling and trafficking events by binding PI(3)P. For example, FYVE-containing proteins are associated with regulating the vacuolar/lysosomal membrane trafficking pathway and the regulation of TGF β-receptor signaling\textsuperscript{130,131}.

Given its prominence as the predominant binding domain for PI(3)P, it is common to integrate the FYVE domain in tandem configurations such as 2xFYVE or even in double tandem arrangements like 4xFYVE. Numerous research efforts have effectively utilized GFP (or RFP)-tagged PI(3)P binding domains to visualize the spatial distribution of intracellular PI(3)P within cells via fluorescence microscopy under fixed conditions. This staining technique has proven valuable in tracking PI(3)P within the autophagic and endocytic pathways, facilitating the exploration of PI(3)P pool dynamics during the initial stages of autophagosome formation\textsuperscript{132}.

1.3.2.3. Phox homology (PX) domains

The PX domain is a region of homology that has 130 amino acids and can be found in two components of the phagocyte NADPH oxidase complex (p40phox and p47phox), as well as many other proteins that have diverse functions\textsuperscript{118}. Most of these PX domain-containing proteins are involved in processes such as vesicular trafficking, protein sorting, or lipid modification. A significant portion of the PX domain-containing proteins is sorting nexins, also known as SNX proteins, which have been implicated in the endocytic pathway\textsuperscript{133}.
The PX domain has been identified as a binding module for PI(3)P\textsuperscript{133}, however, some PX domains, such as those found in p47phox and C2 domain PI3-kinase, have been reported to bind other phosphoinositides as well\textsuperscript{134}.

The crystal structure of the p40phox PX domain bound to dibutyl-PI(3)P shows the 120 amino-acid domain consisting of an N-terminal 3-stranded β-sheet that is adjacent to a helical subdomain\textsuperscript{135}. The pocket between the β-sheet and the helical subdomain is where the PI(3)P headgroup binds (Fig. 1.7.C)\textsuperscript{136}. A total of 9 hydrogen bonds are formed between the PI(3)P and PX domain, with 3 involving the 3-phosphate and 2 involving the 1-phosphate\textsuperscript{136}. In the complex between p40phox-PX and dibutyl-PI(3)P, the glycerol backbone of the PPI appears to have additional Van Der Waal's contact with a tyrosine side-chain (Y94)\textsuperscript{137}. This tyrosine is found in the 'membrane interaction loop', where substantial NMR chemical shift differences are observed upon PX domain binding to PI(3)P-containing micelles\textsuperscript{137}. Similar to FYVE domains, penetration of the bilayer may be a crucial factor in increasing the affinity of the PX domain binding to PI(3)P-containing membranes\textsuperscript{138}. 
Figure 1.7. The structures of the main phosphoinositide-binding domains.

A. Ribbon representations of the DAPP1-PH structures. The C-terminal amphipathic α helix is colored blue. β strands that form the core β sandwich of the PH domains are colored green and are labeled 1–7. The phosphate positions in the bound inositol phosphates are numbered in red. N and C termini are labeled.

B. Structure of protein FYVE domain of the FYVE domain-containing protein 1; Zn atoms are shown in red and coordinating cysteines in yellow (PDB code 2yw8).

C. Bem1p PX domain is depicted in stereo as a ribbon representation colored rainbow from the N to the C terminus. (PDB code 2v6v). A, B, and C are reproduced from references 120, 122 and 136 respectively.
1.3.3. Metabolism and functions of main phosphoinositides

PPIs are produced through a series of enzymatic reactions involving phosphorylation or dephosphorylation, by specifically modifying one position on the inositol ring (Fig. 1.8). Due to their integration within the lipid bilayer, PPIs are confined to domains of the cellular membrane and their mobility within the cytoplasm is constrained to alterations in the surrounding lipid composition\textsuperscript{139}. PPIs frequently recruit adaptor proteins, which trigger molecular interactions and activate phosphorylation cascades, and adaptor proteins play a crucial role in this regulation, as they facilitate enzyme activity and direct them to the appropriate subcellular location. The levels of PPI species are regulated by these enzymes' activity and their localization within the cell\textsuperscript{139}.

1.3.3.1 Cellular regulation of PI(3)P and PI(3,5)P\textsubscript{2}

In mammals, PI(3)P is generated mainly by the Class III phosphatidylinositol 3-kinase VPS34/PIK3C3, which phosphorylates the 3-OH position of PI (Fig. 1.8). In certain cellular conditions, such as shear stress, class II PI3Ks like PI3KC2\textalpha can also synthesize autophagic pools of PI(3)P. Two types of PI3KC complexes have been identified in yeast and mammals\textsuperscript{140}. Each of the two complexes consists of a set of core components, including vacuolar protein sorting (VPS)34, VPS15, and Beclin 1, which are bound to a specific component. Complex-I is bound to autophagy-related protein (ATG) 14L (ATG14 in yeast), while complex-II is bound to UVRAG (VPS38 in yeast). The presence of ATG14, in conjunction with ATG6, facilitates the localization of complex I and other ATG proteins to the phagophore assembly site (PAS). On the other hand, UVRAG is the only difference between these two complexes and is responsible for their localization to different membrane compartments. This is because UVRAG acts as a bridge between VPS34-
VPS15 and VPS30, and it specifically localizes complex II to the endosome. Studies have shown that VPS34 complex I and II both have a Y-shaped structure, with one arm bearing VPS34 and VPS15 as the catalytic arm and the other arm bearing VPS30 and VPS38 as the adaptor arm\textsuperscript{141,142}.

Regulation of intracellular PI(3)P level is important for diverse processes for the nervous system\textsuperscript{143,144}, including dendritic arborization and post-natal development of the mouse brain, control of axonal transport and growth, and GABAergic neurotransmission at inhibitory post-synapses. Importantly, the deletion of VPS34, which results in the depletion of PI(3)P levels specifically in sensory neurons, leads to accelerated neurodegeneration\textsuperscript{143}. 
Figure 1.8. Phosphoinositide metabolism.

Addition and removing phosphate groups at the 3’-hydroxy, 4’-hydroxy, and 5’-hydroxy positions of PIs through the action of kinases (upper box) and phosphatases (lower box). These enzymatic activities lead to the generation of seven distinct PPI species. The interconversion of these PPIs forms a network in which the formation of one species is reliant on the presence of another. Consequently, the cellular levels of specific PPIs are interdependent. This dynamic interplay between phosphoinositides contributes to the intricate regulation of cellular processes. Reproduced from reference¹⁴⁵.
The VPS34 complex I plays an important role in the formation of autophagosomes during times of starvation\textsuperscript{146,147} on ER sites known as omegasomes, which are recognized by PI(3)P-specific binding proteins such as double FYVE domain-containing protein (DFCP)\textsuperscript{148–150}. Complex I is activated by being recruited to specific membrane sites, with the help of upstream ULK1 complex \textsuperscript{151}, the detail will be discussed in section 1.5.2. Complex II of VPS34, on the other hand, plays a role in regulating endocytic pathways by co-localizing with RAB5- and RAB7-positive endo-lysosomal compartments, as well as Rab9-positive compartments that are important for the endosome to TGN pathway\textsuperscript{140}. Recruitment of complex II to early endosomes and its activation is aided by RAB5\textsuperscript{152}, although the exact mechanism is not well characterized. PI(3)P produced by complex II leads to the recruitment of various PI(3)P binding proteins such as EEA1, and sorting nexins, which regulate endocytic pathways\textsuperscript{153,154}.

Specialized phosphatases, including myotubularins (MTMs), lipid kinases, and lysosomal lipases, control the turnover of PI(3)P, leading to PI, PI(3,5)P\textsubscript{2}, and lipid degradation, respectively (Fig. 1.8). The myotubularin family of PI(3)P 3-phosphatases are lipid phosphatases that specifically target PI(3)P and PI(3,5)P\textsubscript{2} as substrates by removing the 3-phosphate, and play an essential role in controlling the levels of PI(3)P and PI(3,5)P\textsubscript{2}. In humans, 14 members of the MTMs family have been identified, and mutations in these genes have been linked to various neurodegenerative diseases\textsuperscript{155,156}.

Class III PI(3)P 5-kinases phosphorylate PI(3)P to PI(3,5)P\textsubscript{2} (Fig. 1.8) and play a crucial role in endosomal trafficking. FYVE domain-containing proteins, such as FYVE finger-containing phosphoinositide kinase (PIKfyve), possess PI(3)P\textsubscript{5K} activity and regulate retrograde traffic from endosomes to the TGN\textsuperscript{157}. SAC domain-containing protein 3, serves
as the primary phosphatase that hydrolyzes the 5-phosphate of PI(3,5)P₂ to form PI(3)P (Fig. 1.8), which interacts with FAB1 and VAC14 in a protein complex that controls the overall level of PI(3,5)P₂. Although the detailed molecular mechanism of this regulation remains to be clarified, the loss of function of FIG4 causes a decrease in the levels of PI(3,5)P₂, which ultimately results in several neuronal degeneration.

1.3.3.2 Cellular Regulation of PI(3,4,5)P₃ and PI(3,4)P₂

PI(3,4,5)P₃ and PI(3,4)P₂ are the two least abundant PPIs in most cells. PI(3,4,5)P₃ is primarily produced through the phosphorylation of PI(4,5)P₂ by one of four Class I PI3-Kinases (α, β, δ, and γ), encoded by PIK3CA, PIK3CB, PIK3CD, and PIK3CG, respectively (Fig. 1.8). These kinases interact with subunits that regulate their activity. For example, the Class IB PI3K, p110γ, is activated by G protein-coupled receptor (GPCR) signaling and is crucial in inflammation. The regulatory subunits p55 and p85 form complexes with the three Class I PI3K isoforms and contain SH2 domains that bind to PS. Upon tyrosine phosphorylation, this binding helps recruit the catalytic p110 enzymes to receptors or receptor-associated adapter proteins. In addition to PS binding, other protein-protein interactions may activate the regulatory subunits, such as with small GTPases Ras and RAB5, or the 3-phosphatase PTEN. After being recruited to tyrosine phosphorylated receptors, the Class I PI3K enzymes phosphorylate PI(4,5)P₂ to PI(3,4,5)P₃, initiating the subsequence signaling events.

PI(3,4,5)P₃ levels are typically transient because the phospholipid is rapidly degraded after its synthesis. There are two main mechanisms that can reduce PI(3,4,5)P₃ levels, each of which requires the activity of a lipid phosphatase at the 3 or 5 positions of the inositol ring (Fig. 1.8). The most well-studied negative regulator of PI(3,4,5)P₃ is 3-phosphatase PTEN,
which was initially identified as a tumor suppressor\textsuperscript{166}. PTEN catalyzes the conversion of \( \text{PI}(3,4,5)P_3 \) to \( \text{PI}(4,5)P_2 \). Additionally, \( \text{PI}(3,4,5)P_3 \) levels can be decreased by 5-phosphatases, such as SHIP-2 (encoded by INPPL1) or SKIP (encoded by INPP5K), to generate \( \text{PI}(3,4)P_2 \), as depleting either SHIP or INPP5K has been shown to increase \( \text{PI}(3,4,5)P_3 \) levels and enhance cancer-related AKT signaling\textsuperscript{167}.

\( \text{PI}(3,4)P_2 \) is dephosphorylated from \( \text{PI}(3,4,5)P_3 \) by 5-Phosphatases SHIP or phosphorylated from \( \text{PI}(4)P \) by Class I PI3-Kinase (Fig. 1.8). Furthermore, Class I PI3-Kinase can also activate AKT through interactions with the kinase's PH domain\textsuperscript{168}. The 4-phosphatases INPP4A/B, and 3-phosphatase PTEN can degrade \( \text{PI}(3,4)P_2 \) to generate \( \text{PI}(3)P \) and \( \text{PI}(4)P \), respectively (Fig. 1.8). It is interesting to note that depletion of the 3-phosphatase PTEN and the 4-phosphatase INPP4B can enhance AKT signaling\textsuperscript{169,170}, which highlights maintaining the level of \( \text{PI}(3,4)P_2 \) is crucial for AKT signaling due to its role as a signaling molecule\textsuperscript{145}. However, it is currently unclear whether the mechanism of AKT activity regulated by certain levels of \( \text{PI}(3,4)P_2 \) differs from the \( \text{PI}(3,4,5)P_3 \)-related pathway. The functional differences between the negative regulation of AKT by different lipid phosphatases need to be determined.

1.4. Overview of phosphoinositides in the endosome system

Endocytosis is a fundamental cellular process that encompasses the organization of molecules, proteins, lipids, and fluids within the cell through the generation of vesicles at intermediate stages\textsuperscript{171}. \( \text{PI}(3)P \) plays a crucial role as a component of the endosomal system\textsuperscript{144}. Additionally, \( \text{PI}(4,5)P_2 \), a characteristic lipid found in the PM, is involved in the formation of endocytic vesicles. The conversion of \( \text{PI}(4,5)P_2 \) to endosomal \( \text{PI}(3)P \) is a
critical step in the process of endocytosis \(^{172}\) (Fig. 1.6) and will be discussed in the following sections.

1.4.1. Clathrin-mediated endocytosis and phosphoinositides conversion

Clathrin-mediated endocytosis is a complex process that involves the formation of endocytic clathrin-coated pits (CCPs) promoted by PI(4,5)P\(_2\). Cargo proteins are recruited into the CCP through the simultaneous recognition of both cargo and PI(4,5)P\(_2\) by clathrin adaptor complexes (such as AP2) that also bind to PI(4,5)P\(_2\). The nascent CCP acquires more curvature, leading to the formation of an endocytic vesicle. As CCPs mature, there is a gradual depletion of PI(4,5)P\(_2\) from the coat and synthesis of PI(3,4)P\(_2\)\(^{173}\). This phosphoinositide conversion mechanism involves the recruitment of various enzymes such as the PI(4,5)P\(_2\) 5-phosphatase synaptojanin 1 (p170) and SHIP2\(^{174}\). In this process, clathrin recruits the PI(4,5)P\(_2\)-activated PI3KC2α58, which can also synthesize PI(3,4)P\(_2\) from PI(4)P. PI(3,4)P\(_2\) formation is known to promote the constriction of the neck of invaginated CCPs through its effectors, including sorting nexin 9 (SNX9), SNX18, and FCHSD2\(^{175}\). This ultimately recruits the co-chaperone protein auxilin or GAK to remove the clathrin. As newly formed vesicles undergo uncoating, the 5-phosphatase OCRL and synaptojanin 1(p145) hydrolyze PI(4,5)P\(_2\) and generate PI(4)P, leading to the further depletion of PI(4,5)P\(_2\)\(^{176}\). The final conversion of PI(3,4)P\(_2\) towards the endosomal signature PI(3)P is completed by the action of 4-phosphatases INPP4A, INPP4B, and SAC2\(^{177}\).

1.4.2. Clathrin-independent endocytosis and phosphoinositides conversion

Clathrin-independent endocytic pathways are context-dependent or tissue-specific\(^{171}\). The internalization of large amounts of extracellular fluid (macropinocytosis) requires initial
membrane curvature, which involves the formation of PI(4,5)P₂ by actin polymerization. PI(4,5)P₂ produced by phosphatidylinositol-4-phosphate 5-kinase (PIPKI). The extension and closure of the macropinocytic cup is then driven by class I PI3K-mediated synthesis of PI(3,4,5)P₃ from PI(4,5)P₂, which mediates the coordinated activation of Rho family GTPases through modulation of their guanine-nucleotide exchange factors and GTPase-activating proteins. This is accompanied by depletion of PI(4,5)P₂ and resolution of the actin meshwork starting from the base of the cup, to which PI3Ks, the activation of PLCγ, recruitment of 5-phosphatases, and focal exocytosis contribute. After the macropinosome is closed, the sequential activities of phosphatases SHIP1/2 and INPP4A/B convert PI(3,4,5)P₃ to PI(3)P, which may finally be cleared by the phosphoinositide 3-phosphatase MTMR6. These phosphoinositide conversions enable the recruitment of the dual PI(3)P and PI(4)P binding protein Phafin 2, which is also required for actin remodeling.

1.4.3. Phosphoinositides in dynamics of endosomes

Following the uptake of cargo from endocytic vesicles, early endosomes undergo a sorting process wherein the specific cargo is selected and packaged into recycling vesicles for transport back to the cell surface or to the Golgi (Fig. 1.6). Membrane cargo designated for disposal is internalized into intraluminal vesicles, ultimately leading to its degradation within endolysosomes. As these sorting endosomes progress into late endosomes, they cease to receive cargo from the cell surface but instead acquire characteristics essential for their interaction with lysosomes. These characteristics include an acidic and hydrolytic environment that facilitates the degradation of the remaining cargo and enables lysosome regeneration.
As endosomes mature, they undergo significant changes to aid their divergent functions. The selective recruitment of GTPases: RAB5 and RAB7 to early and late endosomes, respectively, ensures specificity of interaction with other organelles such as endocytic vesicles and other early endosomes for RAB5-positive endosomes, and lysosomes for RAB7-positive endosomes. Furthermore, early endosomes are characterized by the presence of the signaling lipid PI(3)P, which is phosphorylated to PI(3,5)P₂ in late endosomes, thereby serving as identity molecules for the organelles (Fig. 1.6). These lipids are crucial in facilitating the recruitment of factors like sorting and tethering proteins necessary for the proper functioning of endosomes. Additionally, the endosomal acidification process is also crucial for endosomes to mature, with early, late, and lysosomal endosomes exhibiting pH levels of approximately 6.5, 5.5, and 4.5, respectively. These changes in GTPase recruitment, PPIs composition, and acidification status are precisely coordinated to ensure the adjustments to endosome identity and purpose for the endocytic system to function in a unidirectional and aligned manner¹⁷².

1.4.4. Phosphoinositides in endocytic cargo recycling and degradative sorting

The proper sorting of cargo within endosomes is dependent on the presence of PI(3)P. GTPase RAB5 is critical in the maintenance and facilitation of early endosomal function. The activity of RAB5 on early endosomes is modulated by the RAB5-GTP-associated VPS34 complex II, along with its lipid product, PI(3)P, as well as the RAB5 effector Rabaptin 5. The latter forms a tightly bound complex with the VPS34 complex II¹⁷²,¹⁷⁸.

The fusion of early endosomes is reliant on the recognition of both RAB5-GTP and PI(3)P, which are essential for maintaining endocytic pathway cargo transfer and preserving early endosomal membrane stability. RAB5 has various regulators, including 5-phosphatases.
OCRL and INPP5B, as well as PI(3,4)P₂ 4-phosphatases INPP4A and INPP4B. These effectors convert PPIs on incoming endocytic vesicles to PI(3)P, which plays a crucial role in determining whether cargo should be sorted for recycling or degradation.

During the recycling process, the membrane is returned from PI(3)P-containing endosomes to the PI(4)P/PI(4,5)P₂-enriched PM through the aid of protein complexes such as retromer, retriever, and ESCPE. On the other hand, during the degradation process, cargo is ubiquitylated and sorted into vesicles that bud into the lumen of maturing endosomes. This process is facilitated by four subcomplexes collectively referred to as the 'endosomal sorting complexes required for transport (ESCRT) 0–III'. ESCRT-0 is targeted to the early endosomal compartment through the PI(3)P-binding FYVE domain of HRS. The sequential activity of ESCRT-I to ESCRT-III causes the inward budding of the endosomal membrane, eventually leading to the inclusion of degradative subdomains in intraluminal vesicles. Increasing intraluminal vesicle formation results in endosomal maturation to late endosomes/multivesicular bodies and is accompanied by the endosomal conversion of RAB5 to RAB7. The effectors of RAB7–GTP characterize the late endosomal compartment, including VPS34 complex II and the retromer complex, which ensure continued PI(3)P formation and cargo retrieval. Late endosomal PI(3)P serves as a substrate for the PIKfyve complex, a PI(3)P 5-kinase that is essential for late endosomal and lysosomal function and homeostasis.

To sum up, whether endosomes recycle back to the cell surface or are sorted for degradation in late endosomes and lysosomes depend on whether there is a loss or consolidation of endosomal PI(3)P.

1.5. Overview of Phosphoinositides in the autophagy-lysosomal system
1.5.1 Phosphoinositides in the lysosome

Lysosomes play a crucial role as dynamic organelles responsible for degrading intracellular and exogenous substrates into their constituent building blocks through the action of acid hydrolases in the lumen. Apart from their degradative function, lysosomes also serve other roles such as secretion of cytotoxins and hydrolases, acting as a metabolic signaling hub that integrates nutrient sensing and metabolic adaptation, and facilitating lipid and metabolite exchange through membrane contact sites with other organelles\textsuperscript{186}.

Lysosomes possess varied kinds of PPIs (Fig. 1.6). Besides PI(3)P, lysosomes are known to harbor or be influenced by other phosphoinositide species such as PI(3,5)P\textsubscript{2} and, possibly, PI(5)P. These species are believed to be specific to lysosomes, late endosomes, and other lysosome-related organelles. Interestingly, lysosomes also contain minor amounts of PI(4)P and PI(4,5)P\textsubscript{2}, which are lipids that are primarily enriched at the Golgi complex and PM, respectively. Additionally, lysosomes contain PI(3,4)P\textsubscript{2}, which is a rare PIP species that may regulate lysosome function in response to cell signaling. Therefore, lysosomes exhibit a variety of PPIs that can adapt to the cell's functional status. Currently, it remains unclear why lysosomes possess such a diverse range of lipids, and whether and how different PPI pools may be associated with the functional state and intracellular distribution of lysosomes within cells\textsuperscript{186,187}.

The lysosome serves as the destination for two separate pathways: the endocytic pathway and the autophagy pathway (Fig. 1.6). The endocytic pathway facilitates the fusion of late endosomes with lysosomes to transfer extracellular materials to the lysosome. Autophagy is a catabolic process that is activated in response to stress (such as starvation) and involves the targeting of aggregated intracellular proteins, defective organelles, or pathogens to
lysosomes for degradation. Two nutrient-responsive kinases, mechanistic target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK)\cite{188}, rapidly respond to nutrient fluctuations and activate the whole autophagy pathway through the formation of double membrane-bounded autophagosomes, which ultimately merge with lysosomes. A unique feature of autophagy is its ability to invert the topology of the cytoplasm and create novel membrane identity by synthesizing PI(3)P from PI on specific sites, referred to as the omegasome, on the ER\cite{186,187}.

### 1.5.2. Phosphoinositide regulation of autophagy

Multiple complexes containing autophagy-related proteins (ATG proteins) coordinate the biogenesis of autophagosomes\cite{149,189,190}. These complexes initiate the formation of a pre-autophagosomal structure known as the "phagophore", which originates from the omegasome. The phagophore then elongates and closes to form a fully mature autophagosome, which ultimately fuses with the lysosome\cite{191} (Fig. 1.9).

In mammalian cells, in response to mTORC1 (negative) and AMPK (positive) signaling, autophagy induction is regulated by the ULK1 kinase complex \cite{189,190}, which consists of the catalytic subunit ULK1, regulatory scaffold proteins ATG13 and RB1CC1, and the stabilizing protein ATG101 at the PAS\cite{192} and triggers the downstream autophagy. Upon activation, ULK1 facilitates the recruitment of VPS34 complex I, which is responsible for generating local pools of PI(3)P\cite{191}, although the precise mechanism of recruitment remains incompletely understood\cite{193} (Fig. 1.9). This phosphorylation event plays a crucial role in the synthesis of PI(3)P and the initiation of autophagy, specifically during the early stages. Notably, the ULK1 complex plays a negative regulatory role in mTORC1 activity,
therefore, two upstream regulators of autophagy, the ULK1 complex and the mTORC1, mutually regulate each other.

PI(3)P serves as a signaling molecule that attracts various PI(3)P-binding proteins, including DFCP1, autophagy-linked FYVE protein, and the WD repeat domain phosphoinositide-interacting protein (WIPI) family of scaffold proteins with WD repeat domains at PAS, all of which contribute to autophagosome formation\textsuperscript{194}. Additionally, additional PI(3)P-binding partners have been discovered in the autophagy process, such as PI(3)P interacting with and activating TRPML3, resulting in Ca\textsuperscript{2+} release from the phagophore to promote autophagy\textsuperscript{195}.

Autophagosome formation involves two consecutive ubiquitin-like reactions (Fig. 1.9). The initial reaction utilizes the E1-like enzyme ATG7 and the E2-like enzyme ATG10, which conjugates the ubiquitin-like ATG12 to ATG5. This conjugate subsequently forms a complex with ATG16L1. The second set of reactions involves the ubiquitin-like LC3 protein family. LC3 family members are then conjugated to PE on the nascent autophagosomal membrane by ATG7, functioning as the E1-like enzyme, ATG3 as the E2-like enzyme, and the ATG5-12-16L1 complex acting as the E3-like enzyme. The lipidated LC3, known as LC3-II, is primarily associated with autophagosomes and autolysosomes\textsuperscript{189}. Simultaneously, WIPI2 binds to PI(3)P at the omegasome via its PROPPIN domain and recruits the ATG12-ATG5-ATG16L1 complex. This process involves a positive feedback loop between PI(3)P and LC3 lipidation, which is critical for phagophore expansion and closure. Autophagy receptors like p62, which are themselves autophagy substrates, often aid in this process\textsuperscript{196}. WIPI2 and VPS34 complex I mutually promote each other's recruitment, thereby strengthening the feedback loop. Furthermore,
WIPI4 binds to the lipid transfer protein ATG2 at PI(3)P-rich sites, allowing for the transfer of phospholipids, which is necessary for phagophore expansion\textsuperscript{191}.

PI(3)P plays a critical role in initiating autophagy, and its elimination is equally essential for facilitating lysosomal degradation and completing the autophagy-lysosome pathway. This occurs through its conversion to PI(3,5)P\textsubscript{2}\textsuperscript{197}, which will be discussed in section 1.5.4. Additionally, PI(3,5)P\textsubscript{2} plays a pivotal role in regulating autophagy through various mechanisms\textsuperscript{198}. Firstly, a decrease in PI(3,5)P\textsubscript{2} levels in yeast lead to impairments in autophagosome degradation within the vacuole, the organelle responsible for degradation. Secondly, inhibiting the activity of Fab1, an enzyme involved in the production of PI(3,5)P\textsubscript{2}, results in a decrease in mTORC1 activity and a simultaneous increase in autophagy\textsuperscript{198}. These findings strongly suggest that PI(3,5)P\textsubscript{2} acts as a downstream signaling molecule of autophagy. Furthermore, disruption of Fab1/PIKfyve function in mammalian cells also hinders autophagy, indicating an association between PI(3,5)P\textsubscript{2} and autophagy.
Autophagy is initiated by the activation of the ULK1 complex, which phosphorylates and activates the PI3K complex I, leading to the initiation of phagophore formation. The expansion of the phagophore is supported by two systems like ubiquitin, which facilitate the assembly of the ATG12-ATG5-ATG16L1 complex and the attachment of Atg8-family proteins to PE. As the expanding membrane closes, a fully formed autophagosome is created. The outer membrane of the autophagosome then merges with a lysosome, resulting in the formation of an autolysosome. The contents of the autophagosome are subsequently degraded and recycled.
1.5.3. Phosphoinositide regulation of autophagosome-lysosome fusion

Autophagosomes are enriched in PI(3)P derived from the VPS34 complex I, while lysosomes contain both PI(3)P synthesized by the VPS34 complex II and PI(3,5)P$_2$ synthesized by the PIKfyve complex. Both the synthesis and turnover of lysosomal PI(3,5)P$_2$ are critical for autophagosome-lysosome fusion$^{197}$. Effector proteins potentially regulated by lysosomal PI(3,5)P$_2$ include cortactin, a protein associated with actin, the calcium release channel mucolipin, twin pore channel 2 (TPC2)$^{199}$, and the PX domain-containing protein SNX14$^{200}$.

Autophagosome-lysosome fusion requires not only the presence of PI(3)P and PI(3,5)P$_2$, but also the synthesis of PI(4)P by lysosomal PI4KIIα. PI(4)P can be further converted into PI(4,5)P$_2$ on lysosomes, leading to the dissociation of the RAB7 effector PLEKHM1 through an unknown mechanism. Recent findings suggest that non-conventional PI(4,5)P$_2$ synthesis via PI(5)P 4-kinases may promote lipid catabolism and autophagosome clearance during fasting, but the function of this pool of PI(4,5)P$_2$ is not yet clear$^{201}$. Thus, it appears that parallel pathways of PI(3)P-PI(3,5)P$_2$ and PI(4)P-PI(4,5)P$_2$ synthesis and turnover control steps in autolysosome formation, with unknown mechanisms.

1.5.4. Phosphoinositide in lysosomal homeostasis and reformation

Lysosomes exhibit high plasticity and turnover due to various cellular processes$^{187}$. For instance, autophagosomes merge with lysosomes, leading to a significant reduction in the lysosomal pool. Lysosomes also frequently merge with the PM, resulting in a decline in functional lysosomes over time, while late endosomes constantly fuse with and mature into lysosomes, increasing the lysosomal membrane content. Therefore, endo-lysosomal
membrane and protein homeostasis mechanisms maintain the rates of trafficking, fusion, and secretion, which depend on extracellular factors such as nutrients and pH\textsuperscript{186,187}. PPIs play a vital role in regulating lysosomal membrane and protein homeostasis and can act as major signal transducers adapting to environmental changes\textsuperscript{197}.

The transportation of newly synthesized lysosomal enzymes from the TGN to maturing lysosomes is facilitated by PI(4)P through the sorting of mannose 6-phosphate receptors \textsuperscript{186}. Phosphatidylinositol 4-kinase III\(\beta\) (PI4KIII\(\beta\)) and II\(\alpha\) (PI4KII\(\alpha\)) are two PI4-kinase isozymes that are essential for the delivery of acidic hydrolases to lysosomes\textsuperscript{202}. PI4KII\(\alpha\) also interacts with multiple components of protein complexes that are required for the trafficking of lysosomes and lysosome-related organelles, such as melanosomes or lytic granules in T cells of the immune system\textsuperscript{203}.

Autophagic lysosome reformation (ALR) is a process where lysosomes and autolysosomes tubulate and bud off new nascent lysosomes, to balance lysosomal membrane homeostasis. Recent data suggest that multiple lysosomal PIP species, including PI(4)P, PI(3)P, PI(3,5)P\(_2\), and PI(4,5)P\(_2\), act at distinct stages of ALR\textsuperscript{204}. PI(4,5)P\(_2\) plays a significant role in ALR, as it is synthesized on autolysosomes and lysosomal tubules upon prolonged starvation by phosphatidylinositol 4-phosphate 5-kinase type I isoforms, which triggers the formation of lysosomal tubules by recruiting the kinesin 1 KIF5B. PI(4,5)P\(_2\) also triggers the assembly of a clathrin/AP-2 coat that mediates the budding of proto-lysosomes from these tubular intermediates, followed by OCRL-mediated PI(4,5)P\(_2\) hydrolysis\textsuperscript{205}. Despite its role as a precursor to PI(4,5)P\(_2\), PI(4)P has been found inhibits the formation of lysosome tubules in nutrient-rich conditions\textsuperscript{206}(Fig. 1.10, left panel).
During the final step of proto-lysosome formation, vesicles pinch off from lysosomal tubules by membrane scission, which is regulated by PI(3)P\textsuperscript{194,207,208}. mTORC1 enhances VPS34 activity by phosphorylating UVRAG, thereby increasing lysosomal PI(3)P levels\textsuperscript{207}. Blocking the phosphorylation of UVRAG or inhibiting the production of PI(3)P leads to the persistence of lysosome tubules even in nutrient-rich conditions, indicating that PI(3)P synthesis on proto-lysosomes is crucial for fission (Fig. 1.10. right panel). Spastizin with the PI(3)P binding FYVE domain, assists in the formation of lysosomal tubules by binding to its membrane anchor PI(3)P and the VPS34-complex II\textsuperscript{209}. Mutations in Spastizin, frequently observed in HSPs, lead to the disruption of its interaction with VPS34 complex II, consequently affecting ALR\textsuperscript{209,210}.

The ALR process also relay on the generation of PI(3,5)P\textsubscript{2}. When PIKfyve is inhibited, lysosome tubulation induced by starvation is also inhibited\textsuperscript{211}. The involvement of MCOLN1 as a potential effector of PI(3,5)P\textsubscript{2} in this process is still under debate\textsuperscript{197}. Other lysosomal effectors of PI(3,5)P\textsubscript{2} have been discovered, such as the V-ATPase and the sodium-selective twin pore channel 2 (TPC2)\textsuperscript{199,212}, but the mechanism of the interaction with PI(3,5)P\textsubscript{2} contributes to ALR or other lysosome-related functions is still unclear.
Figure 1.10. Summary of the regulatory pathway of ALR tubule scission.

Left: PI(4)P and PI(4,5)P$_2$ play essential roles in ALR and the localization of PI(4,5)P$_2$ on reformation tubules is required for tubule scission.

Right: when mTOR is reactivated at the termination of autophagy, the lysosome pool of PI(3)P increases due to activation of VPS34–Beclin1–UVRAG and results in the scission of ALR tubules. Reproduced from reference 208.
1.5.5. Transcriptional and epigenetic regulation of the autophagy-lysosomal system

1.5.5.1 Transcriptional regulation of the autophagy-lysosomal system

Increasing evidence suggests that nuclear transcriptional and epigenetic mechanisms play a crucial role in autophagy regulation (Fig. 1.11). Transcription factor EB (TFEB) is particularly important in coordinating the regulation of both autophagy and the formation of lysosomes\textsuperscript{213}. TFEB binds to a specific DNA sequence called the coordinated lysosomal expression and regulation (CLEAR) element, which is present in genes responsible for producing proteins involved in lysosomal biogenesis, autophagy, exocytosis, and endocytosis. Normally, TFEB is in the cytoplasm and is bound to the 14-3-3 protein scaffold due to the phosphorylation of two serine residues by TFEB kinases like ERK2 or mTORC1\textsuperscript{187}. However, when the cell undergoes stresses such as starvation or lysosomal dysfunction, the lysosomal nutrient sensing machinery, located on the cytoplasmic side of the lysosomal membrane, detects the nutrient levels within the lysosome and transmits this information to the nucleus. As a result, inhibition of mTORC1 and concomitant activation of the phosphatase calcineurin by TRPML1-mediated lysosomal calcium release induces TFEB dephosphorylation., causing it to move into the nucleus. Once in the nucleus, TFEB activates the transcription of genes containing the CLEAR element and triggers the downstream autophagy pathway\textsuperscript{214}.

Aside from being regulated by mTORC1, TFEB's movement to the nucleus is also controlled by other growth-regulating kinases, such as ERK2, which phosphorylates TFEB at Ser142, inhibiting its nuclear translocation, resulting in reduced transcriptional activation of its downstream genes\textsuperscript{215}. Alternatively, the calcium and calmodulin-dependent serine/threonine phosphatase calcineurin can also trigger TFEB's movement
into the nucleus\textsuperscript{213}. During starvation, the release of Ca\textsuperscript{2+} through the PI(3,5)P\textsubscript{2}-activated Ca\textsuperscript{2+} channel known as mucolipin 1 is initiated. This Ca\textsuperscript{2+} release subsequently activates calcineurin, which leads to the translocation of the TFEB into the nucleus.

The regulation of autophagy is a widely recognized function of the O class of the forkhead box transcription factors (FOXO) family. The activity of three out of four members (FOXO1, FOXO3, and FOXO4) is mainly controlled by AKT phosphorylation in response to growth factors and insulin stimulation\textsuperscript{216}. FOXO3 was the first member of this family identified as a transcriptional regulator of several autophagy genes (ATG4, ATG12, BECN1, BNIP3, LC3, ULK1, ULK2, and VPS34)\textsuperscript{217}. A combined analysis of ChIP-Seq, and RNA-Seq on mouse embryonic fibroblasts after DNA damage showed that P53 controls the expression of several genes essential for autophagy induction (LKB1, ULK1/2), and autophagosome maturation (ATG4, ATG7, and ATG10)\textsuperscript{218}. Additionally, transcription factors E2F1 and NF-kB regulate autophagy by controlling BNIP3 expression. BNIP3 is an autophagy activator induced by hypoxia that disrupts the inhibitory binding of B-cell lymphoma 2 to Beclin1, which promotes autophagosome biogenesis.
Figure 1.11. Autophagy regulation at different levels.

Upper panel: In the nucleus, autophagy can be regulated in the nucleus through epigenetic mechanisms, which involve modifications to DNA and histones, such as methylation and acetylation. Transcription factors play a crucial role in controlling the expression of autophagy-related genes.

Lower panel: In the cytosol, autophagic processes are further regulated at the post-transcriptional level by RNA binding proteins, non-coding RNAs, and modifications to mRNA. The translation of autophagy-related mRNA can be influenced by RNA-binding proteins and the core translation machinery, with certain proteins like EIF5A being required for the translation of ATG3. Once the ATG proteins are synthesized, they can
undergo post-translational modifications that impact their activity and stability.

1.5.5.2 Epigenetic regulation of the autophagy-lysosomal system

Besides transcription factors, histone post-translational modifications, such as methylation, acetylation, and deacetylation, influence the overall chromatin structure, thus affecting regulations of the autophagy pathway (Fig. 1.11. lower panel). Numerous examples of epigenetic regulations of the autophagy pathway have been reported to date. The epigenetic reader Bromodomain-containing protein 4 (BRD4) has been identified as a repressor of a transcriptional program that promotes autophagy and lysosome biogenesis\textsuperscript{219}. When nutrients are present, BRD4 inhibits the expression of multiple genes related to autophagy and lysosomes by enlisting G9a, a histone lysine methyltransferase, to introduce a repressive H3K9diMe into the promoters of lysosomal and autophagy genes. Conversely, when nutrients are depleted, the expression of these genes is promoted by an unknown transcriptional regulator through AMPK-mediated BRD4 inhibition.

A study by Füllgrabe and colleagues in 2013 reported a reduction in the levels of H4K16 acetylation in response to nutrient starvation and/or mTOR inhibition\textsuperscript{220}. This decrease results in the transcriptional repression of crucial autophagy genes, which helps prevent excessive autophagy induction that could be harmful. These responses are mediated by the histone acetyltransferase hMOF/KAT8/MYST1.

1.6. Summary and aims of this dissertation

This thesis examines the multifaceted role of the erlin complex, extending beyond its involvement in IP\textsubscript{3}R ERAD. Chapter 3 focuses on unraveling the crucial impact of the erlin complex's interaction with PI(3)P and its influence on autophagy and lysosomal function.
Furthermore, Chapter 4 presents additional investigations, including the identification of the PI(3)P binding region on E2. It also discusses the establishment of a structure model using negative staining, Cryo-EM preparation, and 3D model reconstitution with mammalian purified erlin complex integrated with membrane scaffold proteins. Moreover, Chapter 4 explores other cellular roles attributed to the erlin complex. Lastly, Chapter 5 delves into the implications of these studies and outlines future research directions.

### 1.7. References


64. Huber, M. D., Vesely, P. W. & Gerace, L. Erlins are part of an ER macromolecular assembly regulating cellular cholesterol levels. Mol Biol Cell (2012).


68. Wojcikiewicz, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. Journal of Biological Chemistry 270, (1995).


158. Sbrissa, D. et al. Core protein machinery for mammalian phosphatidylinositol 3,5-bisphosphate synthesis and turnover that regulates the progression of endosomal transport:


Chapter 2. Materials and Methods
2.1. Materials

2.1.1. Cells

The cell lines used were: αT3 mouse pituitary cells, a kind gift from Dr. P. L. Mellon, University of California, San Diego, CA, HeLa human cervical cancer cells, a kind gift from Dr. Y. Huang; SH-SY5Y human neuroblastoma cells, a kind gift from Dr. J. Beidler, Sloan-Kettering Cancer Center.

2.1.2. Cell culture and transfection materials

Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals or R&D Systems. For cell culture, Dulbecco's modified Eagle medium (DMEM), dimethylsulfoxide (DMSO), Opti-MEM I reduced serum medium, Lipofectamine 2000, CoolCell TM FTS30 Cell freezing containers, the Neon TM transfection system, and associated reagents were purchased from Thermo Fisher Scientific. Penicillin/streptomycin and trypsin/EDTA for cell maintenance were procured from Corning, BD Falcon, or Nest Biotechnology. Cell culture dishes were also obtained from these suppliers. The TC20 TM Automated Cell counter and its counting slides were acquired from Bio-Rad to facilitate cell counting and analysis.

2.1.3. Plasmids and molecular biology materials

The pcDNA3 vector containing erlin constructs was obtained from Invitrogen/Life Technologies. Specifically, the hE2HA^{651}, mE2HA^{305A}, mE1HA, hE2HA, and mE2HA constructs were previously described. For bacterial expression and protein purification, Homo sapiens E1 and E2 DNA sequences with an HA tag (GYPYDVPDYAG) at the C-terminus were designed and ordered through GenScript in the pUC57 vector. The
sequences were codon-optimized using GenScript's codon optimization tool and subcloned into a pET SUMO vector. The pET SUMO vector was a generous gift from Dr. A. Bah and was originally obtained from Invitrogen (#K300-01). This allowed the generation of His-SUMO (HS)-E1HA or E2HA constructs.

Several other vectors and constructs were acquired from different sources. GFP-Rab5b (#61802) and GFP-Rab7a (#28047) were purchased from Addgene. GFP-2xFYVE was a gift from Dr. N Ktistakis at the Babraham Institute, and GFP-LAMP1 was a gift from Dr. A Morgan at Oxford University. The DsRed2-ER vector was obtained from Clontech. The self-designed pCas-Guide-EF1a-GFP vectors, also known as erlin Crispr Cas9 gRNA vectors, were obtained from OriGene (#GE100018).

For immunopurification of the erlin complex, an E1 peptide, sequence [H]CEPSGESPIQNKENAG[OH] was obtained from Sigma (#50221-1, MW 1660). PreScission Protease was generously provided by Dr. S. Wilkens.

Various reagents and kits were used in the experimental procedures. PFU Ultra II high fidelity DNA polymerase, PFU Ultra II buffer, DH5α electrocompeotent cells, DH5α Mix 'N Go cells, T4 DNA ligase, T4 DNA ligase buffer, Hifi Gibson Assembly 2X Master Mix, dNTPs, DNA standard ladders, and restriction enzymes were obtained from New England Biolabs. Ampicillin (amp), puromycin (puro), and the Nanodrop One C UV-Vis spectrophotometer were from Thermo Fisher. Kanamycin (kan) was obtained from Millipore. The Zippy Plasmid mini-prep kit and ZymoPURE II plasmid maxi-prep kit were acquired from Zymo Research, Inc. The QiaQuick PCR purification kit, DNA agarose gel purification kit, and other DNA purification kits were obtained from Qiagen. The T100 Thermal Cycler and MJ MiniCycler were from Bio-Rad. DNA sequencing services were
provided by Genewiz, Inc. Oligonucleotides used for cDNA modifications (primers) are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name Forward/ Reverse</th>
<th>Sequence</th>
<th>Purpose</th>
<th>Template</th>
<th>Optimum anneal temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET vector</td>
<td>pET FWD (vector)</td>
<td>TAAACTGGAATCCATGGTGACCCTGAGTGTAAG</td>
<td>Linearize pET vector when performing Gibson ligation</td>
<td>pET vector</td>
<td>72</td>
</tr>
<tr>
<td>pET vector</td>
<td>pET RVS (vector)</td>
<td>ACCACACATCTTGTAATGGCCCTACAA</td>
<td>Linearize pET vector when performing Gibson ligation</td>
<td>pET vector</td>
<td>72</td>
</tr>
<tr>
<td>Gene of interest</td>
<td>pET FWD (gene)</td>
<td>GGCAAGAGAAGAGGATGGTTG</td>
<td>Linearize the gene of interest for insertion into pET vector performing Gibson ligation</td>
<td>gene (in pUC57 vector)</td>
<td>54-60</td>
</tr>
<tr>
<td>Gene of interest</td>
<td>pET RVS (gene)</td>
<td>CCTACTCCCTGGATCAGCTT</td>
<td>Linearize the gene of interest for insertion into pET vector performing Gibson ligation</td>
<td>gene (in pUC57 vector)</td>
<td>54-60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain E2+HA FWD *</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>make HS-21-177E2HA*</td>
<td>HS-E2HA</td>
<td>84.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain E2+HA RVS *</td>
<td>GCTGATGTCGCAAGAGAGAAGAAGAGG</td>
<td>make HS-21-177E2HA*</td>
<td>HS-E2HA</td>
<td>84.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain + CT FWD *</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-E2HA: 21-177E2HA*</td>
<td>HS-E2HA</td>
<td>86.2</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain + CT RVS*</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-E2HA: 21-177E2HA*</td>
<td>HS-E2HA</td>
<td>85.3</td>
</tr>
<tr>
<td>Human E2</td>
<td>5G domain FWD*</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-E2HA: 177E2HA</td>
<td>HS-E2HA</td>
<td>83.1</td>
</tr>
<tr>
<td>Human E2</td>
<td>hE2 RVS</td>
<td>GGGCTGGGCTGACGACGACGACGACGACGAG</td>
<td>make truncations of HS-E2HA, pair HS-E2HA with 5G domain FWD and 5CC2</td>
<td>HS-E2HA</td>
<td>84.4</td>
</tr>
<tr>
<td>Human E2</td>
<td>5CC2 FWD</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-217E2HA*</td>
<td>HS-E2HA</td>
<td>83.6</td>
</tr>
<tr>
<td>Human E2</td>
<td>5CT FWD</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-217E2HA*</td>
<td>HS-E2HA</td>
<td>82.4</td>
</tr>
<tr>
<td>Human E2</td>
<td>5CT RVS</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-217E2HA*</td>
<td>HS-E2HA</td>
<td>82.9</td>
</tr>
<tr>
<td>Human E2</td>
<td>5ACT RVS</td>
<td>GCAAGATGCTGGAGGACAGGAGATCTGGAAG</td>
<td>make truncations of HS-217E2HA*</td>
<td>HS-E2HA</td>
<td>82.4</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain (21-172) VETOR FWD</td>
<td>TACCGGTAAGATATTCCGACGACTCACG</td>
<td>For performing Gibson Assembly with G domain with or without HA FWD/RVS to make HS-21-172E2HA or HS-21-172E2</td>
<td>HS-E2HA</td>
<td>72.5</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain (21-172) VETOR RVS</td>
<td>ACCACATCTCCTGCTGCTGAGGCCTAC</td>
<td>For performing Gibson Assembly with G domain with or without HA FWD/RVS to make HS-21-172E2HA or HS-21-172E2</td>
<td>HS-E2HA</td>
<td>72</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain (21-172) no HA FWD</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>make HS-21-172E2HA*</td>
<td>HS-E2HA</td>
<td>71</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain (21-172) no HA RVS</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>make HS-21-172E2HA*</td>
<td>HS-E2HA</td>
<td>85</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain HA FWD</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>make HS-21-172E2HA*</td>
<td>HS-E2HA</td>
<td>84.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>G domain HA RVS</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>make any HS-E2HA truncations have HS-E2HA CT and HA tag</td>
<td>HS-E2HA</td>
<td>84.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>S58 to K172 FWR</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>Pair with G domain HA RVS to make HS-E2HA S58-172 E2HA</td>
<td>HS-E2HA</td>
<td>85.6</td>
</tr>
<tr>
<td>Human E2</td>
<td>S79 to K172 FWR</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>Pair with G domain HA RVS to make HS-E2HA S79-172 E2HA</td>
<td>HS-E2HA</td>
<td>84.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>V96 to K172 FWR</td>
<td>GAGGGTGCCAGAGACACAGAGATCTGGAAG</td>
<td>Pair with G domain HA RVS to make HS-E2HA V96-172 E2HA</td>
<td>HS-E2HA</td>
<td>84.8</td>
</tr>
<tr>
<td>Gene target</td>
<td>Primer name, Forward/Reverse</td>
<td>Sequence</td>
<td>Purpose</td>
<td>Template</td>
<td>Optimum anneal temp. (C°)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>---------</td>
<td>----------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Human E2</td>
<td>A109 to K172 FWR</td>
<td>GAAGCTTCAGAAGAACCAGAAGTTGAGCTCTGAGTACAGCAAAAGGCAGTTGGTTCAAG</td>
<td>Pair with G domain HA RVS to make H5-18S-E2HA</td>
<td>109-172 E2HA</td>
<td>85.8</td>
</tr>
<tr>
<td>Human E2</td>
<td>H131 to K172 FWR</td>
<td>GAAGCTTCAGAAGAACCAGAAGTTGAGCTCTGAGTACAGCAAAAGGCAGTTGGTTCAAG</td>
<td>Pair with G domain HA RVS to make H5-18S-E2HA</td>
<td>131-172 E2HA</td>
<td>84</td>
</tr>
<tr>
<td>pET vector</td>
<td>His sumo HA FWR1</td>
<td>CGTCACACAGGCCGACCTGGAGGAAACGAC</td>
<td>Pair with different His sumo HA RVS 2-18S-E2HA primer to linearize the pET vector when performing Gibson Assembly with products generated from His sumo HA FWR 1 and different His sumo HA RVS 2 primers</td>
<td>85.9</td>
<td></td>
</tr>
<tr>
<td>pET vector</td>
<td>His sumo HA RVS3</td>
<td>GCTCAAGGCTGGTCGGTGTCACG</td>
<td>Pair with His sumo HA FWR4 to linearize H5-E2HA vector when performing Gibson Assembly with products generated from His sumo HA FWR 1 and different His sumo HA RVS 2 primers</td>
<td>79.2</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>His sumo HA FWR4</td>
<td>TACCGGATGAGTGTCGGGACTACGCT</td>
<td>Pair with His sumo HA RVS 3 when H5-E2HA primers ends at 340 and the HA tag</td>
<td>79.5</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>A109-D155E2 RVS2</td>
<td>AGGCTATGGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when A109-K172E2HA</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>S21-T108E2 RVS2</td>
<td>AGGCTATGGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>A109-V130E2 RVS2</td>
<td>CGTTAGTCCGCAACATACACGCTAACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when A109-V130E2HA</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>21-J6E2 RVS2</td>
<td>AAGCTGATGGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>21-48E2 RVS2</td>
<td>AGGCTATGGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>K119A RVS2</td>
<td>GTGCTGATGGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>K119A FWR4</td>
<td>GCCGTATTGTCGACGGGACTACGCTAG</td>
<td>Pair with His sumo HA RVS 3 when K119A on S21-K172E2HA</td>
<td>79.5</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>H121A RVS2</td>
<td>ATCGAATTTGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>84.2</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>H121A FWR4</td>
<td>GCTGATTGTCGACGGGACTACGCTAG</td>
<td>Pair with His sumo HA RVS 3 when H121A on S21-K172E2HA</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>H122A RVS2</td>
<td>ATCGAATTTGCGGAAACATCTACACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA FWR 1 when S21-K172E2HA</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>H122A FWR4</td>
<td>GCTGATTGTCGACGGGACTACGCTAG</td>
<td>Pair with His sumo HA RVS 3 when H122A on S21-K172E2HA</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>S21-V130E2 RVS2</td>
<td>CGTAGTCCGCAACATACACGCTAACCCTATCTGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA RVS 3 when S21-V130E2HA</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>K119A, H121A, H122A E2</td>
<td>CGCTGACAGAATTTAACCCTACGGGGCGAGGGCTGTTGGAAATAGGCACGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with PET SUMO F1 when S21-K172E2HA</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>pET vector</td>
<td>PET SUMO F1 (FWR)</td>
<td>CCAGAACAGCTATACTGGCTCTGACACTGAGAAGGATTTTCGAACCGCAGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with PET SUMO F1 when K119A, H121A, H122A RVS2, H5-E2HA</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>K119A H121A H122A E2</td>
<td>F4 CGAAGCAGAATTTAACCCTACGGGGCGAGGGCTGTTGGAAATAGGCACGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with PET SUMO R3 when S21-K172E2HA</td>
<td>59.6</td>
<td></td>
</tr>
<tr>
<td>pET vector</td>
<td>PET SUMO R3 (RVS)</td>
<td>TCAGAAGCAGGCTAATACGGCGTCTCGACGCG</td>
<td>Pair with K119A, H121A, H122A E2 F4, H5-E2HA</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>T65E2 Reverse2</td>
<td>GTCCGGTTGCAAGGATGGCTCGCAGGCA</td>
<td>Pair with His sumo HA FWR 1 when S65E2HA</td>
<td>80.6</td>
<td></td>
</tr>
<tr>
<td>Human E2</td>
<td>T65I Forward 4</td>
<td>TCCGTGACAGAATTTAACCCTACGGGGCGAGGGCTGTTGGAAATAGGCACGCTGAAAGGATTTTCGAACCGCAGCTG</td>
<td>Pair with His sumo HA RVS 3 when S65E2HA</td>
<td>80.6</td>
<td></td>
</tr>
<tr>
<td>Gene target</td>
<td>Primer name, Forward/Reverse</td>
<td>Sequence</td>
<td>Purpose</td>
<td>Template</td>
<td>Optimum annual temp. (°C)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>---------</td>
<td>----------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-57 RVS2</td>
<td>TCCGATTGTTAAGGCTGAGCTGGAAAACATCATCAGGGGTGTGTTAAAGGCTAGCTATTAAGTG</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make 21-57E2HA</td>
<td>S21-K172E2HA</td>
<td>86.3</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-78 RVS2</td>
<td>GTAGTCCGGAACATCATACCAAGGGTAGTACCACACCGGACAGTTCTTT</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make HS-21-78E2HA</td>
<td>S21-K172E2HA</td>
<td>82.2</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-95 RVS2</td>
<td>TCCAGTTTGAAGCTGACATACATACACGGGCTGTACAAAAGCTTACTCCATTCAAT</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make HS-21-95E2HA</td>
<td>S21-K172E2HA</td>
<td>80.7</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-360RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make HS-21-360E2HA</td>
<td>S21-K172E2HA</td>
<td>85</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-480RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make HS-21-480E2HA</td>
<td>HS-E2HA</td>
<td>85.3</td>
</tr>
<tr>
<td>Human E2</td>
<td>A109-D155R2 RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make A109-D155E2HA</td>
<td>A109-K172E2HA</td>
<td>83.8</td>
</tr>
<tr>
<td>Human E2</td>
<td>S21-T108E2 RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make S21-T108E2HA</td>
<td>S21-K172E2HA</td>
<td>86</td>
</tr>
<tr>
<td>Human E2</td>
<td>A109-V130E2 RVS2</td>
<td>CTAGTACGGAGGATACGAGGAGGTTCTACGTCTACTACTACGTGACTAGTTACGCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make A109-V130E2HA</td>
<td>A109-K172E2HA</td>
<td>85</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-36E2 RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make 21-36E2HA</td>
<td>S21-K172E2HA</td>
<td>85</td>
</tr>
<tr>
<td>Human E2</td>
<td>21-48E2 RVS2</td>
<td>AGCGATGGACGGAACATCATACACCGGTAAGCGTAAATGAAACGCCCAATATTG GCC</td>
<td>Pair with His same HA Fw/R 1 when performing Gibson Assembly to make 21-48E2HA</td>
<td>S21-K172E2HA</td>
<td>85</td>
</tr>
</tbody>
</table>

| pET vector | His sumo F1 (new) (FW) | CGTGCACAGCACGCCGAGTTGGA | Pair with R36A Reverse2 when performing Gibson Assembly to make R36A on K199H22EI2AS21-K172E2HA | R36A-K119H21I122A S21-K172E2HA | 76.8 |

Table 2.1. Oligonucleotides for PCR and sequencing

All sequences are listed in the 5’ to 3’ orientation

* Indicates G domain is 21-177 region.
2.1.4. Antibodies

The antibodies utilized in the research presented in this dissertation and the sources are listed in Table 2.2. All antibodies which were not generated by the Wojcikiewicz Lab, in collaboration with the SUNY Upstate Medical University Department of Animal Laboratory Research facility, were either purchased from commercial sources or kind gifts from the indicated labs, which are greatly appreciated.
<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Source</th>
<th>Lab/Commercial Source with catalog #</th>
<th>Optimum dilution for immunoblot</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-IP3R1 (CT1)</td>
<td>mouse/human IP3R1</td>
<td>Rabbit</td>
<td>Dr. R. Wojcikiewicz</td>
<td>1:50</td>
</tr>
<tr>
<td>Sammy (erlin2)</td>
<td>mouse/human erlin2</td>
<td>Rabbit</td>
<td>Dr. R. Wojcikiewicz</td>
<td>1:100</td>
</tr>
<tr>
<td>Seymour (erlin1)</td>
<td>mouse erlin1</td>
<td>Rabbit</td>
<td>Dr. R. Wojcikiewicz</td>
<td>1:100</td>
</tr>
<tr>
<td>4c11 (erlin1)</td>
<td>human erlin1</td>
<td>mouse</td>
<td>A gift from Dr Stephen Robbins, University of Calgary, Canada</td>
<td>1:200</td>
</tr>
<tr>
<td>7D3 (erlin1)</td>
<td>human erlin1</td>
<td>Mouse</td>
<td>A gift from Dr Stephen Robbins, University of Calgary, Canada</td>
<td>1:200</td>
</tr>
<tr>
<td>anti-p97</td>
<td>p97</td>
<td>Mouse</td>
<td>#10R-P104A, Fitzgerald</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-HA-11</td>
<td>HA tag (YPYDVPDYA)</td>
<td>Mouse</td>
<td>MMS-101R, Covance</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-PIK3R4 (VPS15)</td>
<td>VPS15</td>
<td>Rabbit</td>
<td>#14580, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-ATG14</td>
<td>ATG14</td>
<td>Rabbit</td>
<td>#5504, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-UVRAG</td>
<td>UVRAG</td>
<td>Rabbit</td>
<td>#5320, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-PI3Kinase Class III</td>
<td>VPS34</td>
<td>Rabbit</td>
<td>#3811, Cell Signaling Technology</td>
<td>1:1000, used for immunoblot</td>
</tr>
<tr>
<td>anti-Beclin1</td>
<td>Beclin1</td>
<td>Rabbit</td>
<td>#3495, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-LC3 A/B</td>
<td>LC3</td>
<td>Rabbit</td>
<td>#4108S, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-hVPS34</td>
<td>VPS34</td>
<td>Rabbit</td>
<td>#Z-R016, Echelon Biosciences</td>
<td>primarily used for IP</td>
</tr>
<tr>
<td>anti-GAPDH</td>
<td>GAPDH</td>
<td>Mouse</td>
<td>#G2320, Santa Cruz</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-SQSTM1/p62</td>
<td>SQSTM1/p62</td>
<td>Rabbit</td>
<td>#5114, Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.2. Antibodies and other reagents used in Western blotting and immunoprecipitations.
2.1.5. Materials for protein purification and in vitro experiments

For bacterial protein expression, the necessary materials were obtained from various suppliers. The BL21-CodonPlus (DE3)-RIPL *E. coli* strain was obtained from Agilent Technologies. Kanamycin and chloramphenicol were purchased from Millipore and VWR, respectively. Isopropyl β-D-1 thiogalactopyranoside (IPTG), used for protein induction, was obtained from either Promega or GoldBio. Thermo Fisher provided protease inhibitor tablets, while Roche supplied DNase I. Lysozyme was purchased from Thermo Scientific™, and columns from Pierce were used in the experiments. Nickel-nitriloacetic acid (Ni-NTA) beads were obtained from GoldBio, and benzamidine and imidazole were sourced from BioBasic and Alpha Aesar, respectively. All other buffer components were purchased from Sigma.

Phosphatidylinositol lipids including PI, PI(3)P, PI(4)P, PI(5)P, PI(3.5)P<sub>2</sub>, and PI(4,5)P<sub>2</sub> (all diC<sub>16</sub>) were acquired from Echelon Biosciences (#P-0016, P-3016, P-4016, P-5016, P-3516, and P-4516, respectively). These lipids were utilized in the lipid overlay assay. Fluorescence polarization (FP) assays were conducted using a Flex Station 3 instrument from Molecular Devices. BODIPY-phosphatidylinositol lipids for FP assay including PI, PI(3)P, PI(4)P, PI(5)P, PI(3.5)P<sub>2</sub>, and PI(4,5)P<sub>2</sub> were acquired from Echelon Biosciences (#C-00F6, C-03F6, C-04F6, C-05F6, C-35F6 and C-45F6, respectively).

2.1.6. Materials for cell lysis and immunoprecipitation

Tris, NaCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub>, 1% NP-40, and protease inhibitors (pepstatin, soybean trypsin inhibitor (SBTI), phenylmethyl-sulphonyl fluoride (PMSF)), Ethylenediaminetetraacetic acid (EDTA), 3-[(3-Cholamidopropyl) dimethylammonio]-1-
propanesulfonate hydrate (CHAPS), Triton X-100, and DTT were procured from Sigma for the experiments. Protein A-Sepharose C1-4B beads for immunoprecipitation (IP) were obtained from GE Healthcare. The specific antibodies used in the IP assays are listed in Table 2.2.

2.1.7. Materials for electrophoresis and immunoblotting

For SDS-PAGE electrophoresis experiments, Bio-Rad provided a comprehensive range of reagents including 30% bis-acrylamide, ammonium persulfate, N, N, N', N'-tetramethylethylenediamine (TEMED), Mini-PROTEAN 3 and Tetra electrophoresis chambers and electrodes, pre-stained Precision Protein Plus standards, Mini-Protean TGX gels, and the Transblot Turbo Transfer system.

For Blue native PAGE experiments, Invitrogen/Life Technologies supplied the necessary components, such as the NativePAGETM Bis-Tris Gel (3-12%), 20X NativePAGETM Running Buffer, Cathode Buffer additive, NativeMarkTM unstained protein standards, 4X NatitePAGETM Sample Buffer, NatitePAGETM G-250 sample additive (5%), and the XCell SureLock Mini-Cells electrophoresis system.

To facilitate protein transfer and detection, GE Healthcare provided nitrocellulose, polyvinylidene fluoride (PVDF) blotting membranes, and gel blot paper. Antibodies used for immunoblotting experiments were obtained from various sources listed in Table 5. Thermo Fisher supplied the SuperSignal West Pico Plus and SuperSignal West Dura enhanced chemiluminescence (ECL) kits. The ChemiDocTM imager from Bio-Rad was used for visualizing immunoblots, and ImageJ software (https://imagej.nih.gov/ij/) was utilized for the quantification of the obtained data.
2.1.8. Materials for calcium measurements

The 96-well black polystyrene microplate with a clear bottom, was obtained from Corning. The FLIPR® Calcium6 assay kit, Flex Station 3 Multi-Mode Microplate Reader, compound plates, and instrument tips were purchased from Molecular Devices. Phenol red-free DMEM and Hank's Balanced Salt Solution were obtained from Gibco, while ethylene glycol-bis (β-aminoethyl ether)-N,N,N, N-tetraacetic acid (EGTA) was purchased from Sigma. The EVOS Cell Imaging System was purchased from Thermo Fisher Scientific.

2.1.9. Materials for Fluorescence Microscopy

The micro cover glasses (18 mm circle, #48680046) and 29 mm glass bottom dishes used in the experiment were obtained from VWR and Cellvis, respectively, while microscope slides (25 x 75 x 1 mm, #12-544-2) were purchased from Fisher Scientific. A Poly-D-Lysine solution (#633307, 1.0 mg/mL) from Sigma was diluted with PBS to a concentration of 0.1 mg/mL prior to use. Phenol Red-free DMEM was obtained from Gibco. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (#D1306) was purchased from Thermo Fisher. Prolong™ Gold antifade reagent with DAPI, originally from Invitrogen (#2336639), was kindly provided by Dr. L Kotula. LysoTracker™ Red DND-99 was purchased from Invitrogen. FITC-nanoparticle was a generous gift from Dr. J Luo and Dr. D Guo. Cells were imaged using a Leica SP8 confocal microscope equipped with a ×63 oil-immersion objective lens and analyzed using Image J software.

2.1.10. Materials for Cathepsin activity assay

96-well black polystyrene microplate (clear bottom) was purchased from Corning and Greiner. Cathepsin B- Magic Red™ reagent was purchased from BIO-RAD. Phenol red-
free Dulbecco's modified Eagle's medium (DMEM) was from Sigma. Fluorescent signals were detected using a FlexStation 3 Microplate Reader.

2.1.11. Chemicals and Miscellaneous Materials

VPS34-IN1 (#17392) was from Cayman Chemical. Apilimod (#2974) and Bafilomycin A1 (#B1793) was from Sigma. Gonadotropin-releasing hormone (GnRH), trypsin, carbachol, and other unlisted chemicals were purchased from Sigma. Bio-Beads (#1528920) were from BIO-RAD.

2.2. Methods

2.2.1. Universal procedures in PCR

PCR and mutagenesis reactions were conducted using the primers listed in Table 2.1. The PCR reaction mixtures were prepared as follows: 50 ng of template plasmid DNA was combined with 5 µL of forward and reverse primers (5 µM each), 5 µL of PFU Ultra II Buffer (10X stock), 1 µL of dNTPs (40 mM), 1 µL of PFU Ultra II enzyme, and ddH2O to reach a final volume of 50 µL. As shown in Table 2.3, the cycling parameters were set and adjusted based on the desired product size. The reactions were performed using either the T100 Thermal Cycler or the MJ MiniCycler. During the annealing step, a gradient was applied to four PCR tubes containing the same components. After completion of the cycling, the samples were retrieved, and 5 µL from each tube was combined with gel loading buffer and analyzed on a 1% agarose gel in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 120 V for approximately 45 minutes. The agarose gels were stained with EtBr in ddH2O (10 µL/100 mL) for approximately 20 minutes and imaged using the EtBr program on the ChemiDoc system. Once the successful PCR
products were confirmed, tubes containing the desired product were pooled, and any remaining template DNA was removed through DpnI digestion: 1-2 µL of the enzyme was directly added to the PCR mixture, which was then incubated at 37°C for 30 minutes. The mixture was subsequently purified using the QiaQuick PCR purification kit, and elution was performed with approximately 50 µL of ddH$_2$O.
Table 2.3. The PCR cycling parameters

Note: two different optimized cycling conditions were used based on the size of target DNAs
2.2.2. Generation of His-SUMO (HS) Constructs

2.2.2.1. Gibson assembly

Gibson assembly reactions were prepared according to the manufacturer's instructions. Multiple reactions were typically set up using different vector/insert ratios, such as 100/20 ng, 100/40 ng, and 100/100 ng. After determining the DNA concentration using a Nanodrop, the Gibson reactions were assembled as follows: the insertion plasmid DNA was mixed with the vector plasmid DNA in a 1:1 volume ratio. Then, 10 µL of Hifi Gibson Assembly 2X Master Mix and ddH₂O were added to achieve a final volume of 20 µL. The reaction mixture was incubated at 50°C for 15 minutes and immediately cooled on ice for approximately 2 minutes. Subsequently, the ligation product was transformed either by electroporation or by incubating the ligated product on ice with DH5α Mix 'N Go cells. For electroporation, 2 µL of the ligation product was added to 18 µL of DH5α electrocompetent cells, followed by plating on LB + antibiotic agar plates. Alternatively, for the incubation method, 2 µL of the ligated product was mixed with 50 µL of DH5α Mix 'N Go cells, and the mixture was directly plated on LB + antibiotic agar plates. Individual colonies were selected and cultured at 37°C, 200-250 rpm, in 3-6 mL LB + appropriate antibiotic cultures until the OD600 reached a range of 1.6-1.8. Plasmid mini-preps were then performed using the Zippy Plasmid Miniprep kit to isolate the plasmids.

2.2.2.2. Generation of HS constructs and mutagenesis

The generation of HS-E2HA or HS-E1HA constructs involved PCR amplification of DNA segments using the primers listed in Table 2.1. The PCR recipes and cycling parameters specified in Table 2.3 were followed for amplifying both the pET SUMO vector and the
gene of interest (insert). The incorporation of the insert into the pET SUMO vector was achieved using Gibson assembly without the introduction of additional restriction enzyme sites. Subsequently, HS-E2HA served as a template for generating various HS-E2HA mutants through inverse PCR and Gibson assembly, using the primers listed in Table 2.1. After digestion of PCR products with DpnI and purification, the Gibson quick change reaction was performed following the methodology outlined in section 2.2.1.2. The resulting DNA constructs were visualized on a 1% agarose gel, and potentially correct constructs were selected for sequencing using T7 primers. Sequencing services were provided by Genewiz, Inc. Once validated, the confirmed preparations were re-plated, and a colony was cultured in 300 mL of LB + kanamycin medium until reaching an optical density at 600 nm (OD600) ranging from 1.6 to 1.8. Maxi-prep DNA isolation was then performed using the ZymoPURE II plasmid maxi-prep kit.

To ensure the quality of the maxi-preps, rigorous quality control measures were implemented, including confirmatory sequencing and repeated diagnostic digestion. It is important to note that the transformation of pET vector constructs requires the use of BL21-CodonPlus (DE3)-RIPL E. coli cells for subsequent protein expression and purification, as detailed in section 2.2.9.

2.2.3. E coli in Luria Broth (LB) agar/growth

For plating constructs, Luria Broth (LB) agar plates were commonly used and supplemented with specific antibiotics based on the vector preparation or cell type: Ampicillin (50 µg/mL), kanamycin (50 µg/mL), or kanamycin/ Chloramphenicol (50 µg/mL and 34 µg/mL, respectively, for BL21 DE3 E. coli, used in His-SUMO protein
expression and purification). The antibiotic concentrations in LB for liquid cultures matched those in the LB plates. Unless otherwise specified during IPTG induction, all cultures were grown at 37°C. The shaking speed during growth was typically set at 250 rpm, but it could be adjusted within the range of 180-300 rpm depending on the incubation time. Slower shaking speeds were suitable for overnight growth, while higher shaking speeds were appropriate for day growth. To ensure adequate aeration and optimal growth conditions, culture tubes and flasks were usually filled to less than 50% of their capacity. Constructs were typically grown until they reached an OD600 of 1.6-1.8.

To amplify DNA from an existing preparation without the need for re-plating, glycerol stocks were used as a storage and propagation method. Glycerol stocks were prepared by combining equal volumes of LB growth and a 30% glycerol solution in a cryovial. The sample was then rapidly frozen by immersing it in 100% ethanol with dry ice and stored at a temperature of -80°C.

2.2.4 Cell culture

2.2.4.1. Cell passage and routine maintenance

αT3 cells were cultured in DMEM supplemented with 5% FBS and antibiotics (100 units of penicillin and 100 μg of streptomycin per mL). HeLa cells and SH-SY5Y cells were cultured in DMEM supplemented with 10% FBS and the same antibiotics. All cells were maintained at a temperature of 37°C with 5% carbon dioxide.

For adherent cells, they were typically grown until reaching confluence or near confluence. Sub-culturing was performed by rinsing the cell monolayers with trypsin/EDTA, followed by a brief treatment with trypsin to detach the cells from the culture dishes. The trypsin
activity was stopped by adding fresh culture medium, and the cells were then pipetted approximately 20 times before being divided for stock culture or experiments as required.

Regular screening for mycoplasma infection was conducted using a Plasmotest™ detection kit to ensure the absence of infection in the cells. In the case of a positive mycoplasma result, the infected cell line was transferred to a 6-well dish and treated with 25 µg/mL of Plasmocin™ for a duration of 2 weeks. This treatment helped eliminate the mycoplasma infection.

2.2.4.2. Freezing and Thawing Cells

For long-term storage, cells were frozen at -80°C. The cells were pelleted, resuspended in ice-cold DMEM supplemented with 20% FBS, and incubated for 20 minutes. DMSO was added to the cell solution to achieve a final concentration of 10%, and the cells were aliquoted into cryovials. The cells were initially frozen at -20°C for 1 hour before being transferred to a -80°C freezer or directly to a -80°C freezer using CoolCell freezing containers. The viability of the frozen cells was typically assessed after 48 hours. For extended storage (more than 12 months), cells were placed in a liquid nitrogen tank. Cells sorted by the SUNY Upstate Medical University Research Flow Core were screened for mycoplasma infection using Plasmotest™ before being frozen. To thaw the cells, cryovials were placed in a 37°C water bath for approximately 5 minutes with or without pelleting and then transferred into a 10 cm culture dish with fresh medium.

2.2.5. Transfection

2.2.5.1. HeLa cells
Apart from CRISPR-Cas9-mediated gene editing, HeLa cells were transfected using polyethyleneimine (PEI). Approximately 6 x 10^5 cells per well were seeded into individual wells of 6-well dishes. After an incubation period of approximately 8 hours, 1-2 μg of the desired cDNAs and 6 μL of PEI was added to each Eppendorf tube containing 50 μL of serum-free culture medium. The mixture was gently vortexed for 4 seconds and incubated for 10 minutes at room temperature. Following the incubation, 1 mL of fresh medium was added to the same tube, mixed well by pipetting, and the resulting ~1 mL mixture was used to replace the medium in the corresponding well of the 6-well plates. The cells were typically incubated in the transfection solution for 16 hours before being sub-cultured and transferred to other cell culture plates for subsequent experiments.

For CRISPR-Cas9-mediated gene editing, HeLa cells were transfected using Lipofectamine 2000. The transfection process involved seeding HeLa cells into 10 cm dishes at a density of 2 x 10^6 cells per dish. The transfection solution was prepared by combining 90 μL of Lipofectamine with 1 mL of OptiMEM. Firstly, 90 μL of Lipofectamine was mixed with 500 μL of OptiMEM in one eppendorf tube and incubated for 5 minutes. Simultaneously, 15 μg of DNA was mixed with 500 μL of OptiMEM in another eppendorf tube. The contents of the two tubes were then combined and briefly vortexed, followed by centrifugation and incubation at ambient temperature for 10 minutes. After incubation, approximately 1 mL of the solution was added dropwise to the dish, gently swirling the dish. The cells were typically incubated in the transfection solution for 16-20 hours before further processing.

2.2.5.2 SH-SY5Y cells
For CRISPR/Cas9-mediated gene editing, SH-SY5Y cells were transfected using Lipofectamine 2000. The cells were initially seeded at a density of $8 \times 10^6$ cells in a 10 cm dish, specifically targeting exon 3 of E2. After 24 hours, a transfection solution was prepared by combining 90 µL of Lipofectamine with 500 µL of OptiMEM, which was then incubated at room temperature for 5 minutes. Simultaneously, tubes containing 10 µg of guide DNA were prepared with 500 µL of OptiMEM. After 5 minutes, the 500 µL Lipofectamine solution was mixed with the 500 µL DNA solution in each tube, followed by a brief vortexing, centrifugation, and incubation at room temperature for 10 minutes. Once the incubation period was completed, 1 mL of the transfection solution was added dropwise to the 10 cm dish, a gentle swirling to thoroughly mix the solution within the medium. After approximately 16 hours, the cells were subjected to GFP sorting, as described in the corresponding section.

**2.2.6. Generation of E1, E2 and E1/E2 knock-out cell lines**

Initially, gRNA design was conducted using an online software tool (benchling.com). The selection of target sequences was based on the algorithm scores provided by the software, aiming to maximize the activity and minimize the off-target effects of CRISPR/Cas9. Subsequently, the chosen target sequences (listed in Table 2.4) were cloned into pCas-Guide-EF1a-GFP vectors.
<table>
<thead>
<tr>
<th>Gene target</th>
<th>Exon</th>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlin1</td>
<td>exon2</td>
<td>Homo sapiens</td>
<td>GATATGATAGCCTGGTCCACTGG</td>
</tr>
<tr>
<td>Erlin1</td>
<td>exon6</td>
<td>Homo sapiens</td>
<td>GACTTAAACCTCATGGCCCCAGG</td>
</tr>
<tr>
<td>Erlin2</td>
<td>exon3</td>
<td>Homo sapiens</td>
<td>GCCCTGCTGACTTCGACCAGCGG</td>
</tr>
<tr>
<td>Erlin2</td>
<td>exon6</td>
<td>Homo sapiens</td>
<td>TGCAGAACTGGTTTCAGTCGTTG</td>
</tr>
</tbody>
</table>

Table 2.4. List of oligonucleotides designed to generate gRNA for CRISPR/Cas9 mediated gene editing.

All sequences are listed in the 5’to 3’ orientation.
HeLa cells were transfected with gRNA construct using Lipofectamine 2000 as described in 2.2.5.1, and the medium was changed on the second day. On the third day, approximately 48 hours after transfection, the cells were detached from the culture dishes through trypsinization and resuspended in a culture medium. Cell counting was performed using an automated cell counter while the suspension was centrifuged to obtain a cell pellet. The pellet was then re-suspended in PBS containing 1% FBS at a concentration ranging from 1 to 5 x 10^6 cells/mL. The cells were transferred to 15 mL conical centrifuge tubes and transported to the SUNY Upstate Medical University Research Flow Core.

At the Flow Core facility, the cells underwent sorting based on forward scatter side scatter (SSC) parameters, which enabled the identification of desired cells based on size and granularity. Specifically, GFP-positive cells were sorted while non-transfected cells served as a GFP-negative control. The sorted cells were subsequently seeded at a density of 1 cell per well in 96-well dishes. These cells were cultured for a period of 2-4 weeks until single colonies formed in the wells. Upon colony formation, the cells were sub-cultured with a 25%/75% split into two wells of a 24-well dish.

Once the sub-cultured wells reached near-confluency, the cells were harvested and lysed using an appropriate volume of 1% Triton lysis buffer, as described in section 2.2.7. The resulting cell lysates were subjected to SDS-PAGE and immunoblotting following the procedures outlined in section 2.2.8. However, protein concentrations were not determined using the Bradford assay, and 15 µL of all samples were loaded onto the SDS-PAGE gel.

Subsequently, all successful KO clones and control clones that still expressed the gene of interest underwent sub-culturing and were subjected to another round of screening through lysis and SDS-PAGE. Protein concentrations were measured during this second round of
screening. Typically, 5-6 KO clones and 2 control clones were maintained for each targeted exon.

**2.2.7. Cell lysis and immunoprecipitation**

To prepare cells for lysis and immunoprecipitation, the following steps were carried out. The cells were routinely grown to confluence. The medium was first removed from the tissue culture dishes, and then the cells were briefly washed once with ice-cold PBS. Subsequently, the cells were detached using HBSE solution (150 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4) and transferred to tubes. The tubes were centrifuged for 1 minute at 8,000 xg to pellet the cells. The supernatant was discarded, and the cells were resuspended in an ice-cold lysis buffer.

For lysates and select immunoprecipitations (IPs), the lysis buffer consisted of 150 mM NaCl, 50 mM Tris HCl, 1 mM EDTA at pH 8.0, and 1% Triton at pH 8.0. For IPs, the lysis buffer contained 150 mM NaCl, 50 mM Tris HCl, 1 mM EDTA at pH 8.0, and 1% CHAPS. Both lysis buffers were supplemented with 1 mM DTT and protease inhibitors (0.2 μM SBTI, 10 μM pepstatin, 0.2 mM PMSF). The cell lysates were briefly vortexed and incubated on ice for approximately 30 minutes with intermittent vortexing. Subsequently, the lysates were subjected to centrifugation at 16,000 xg for 10 minutes at 4°C.

The supernatant, containing soluble proteins (lysates), was transferred to new tubes, and the pellets were discarded. For Bradford assays, a volume of 5 μL was taken from each sample and mixed with 2 mL of ddH2O and 1 mL of Bradford reagent. The resulting mixture was measured using a Nanodrop and compared to a standard BSA curve. Following clarification, the lysates were supplemented with 4X gel-loading buffer (GLB),
which consisted of 200 mM Tris-HCl, 400 mM DTT, 8% SDS, 0.4% Bromophenol Blue, and 40% Glycerol at pH 8.0. In the case of IP experiments, 45 µL of the supernatant was collected after centrifugation and mixed with 15 µL of 4X GLB, serving as the "pre-IP" sample. The remaining solution was incubated with 5 mg/50 µL of lysate Protein A-Sepharose CL-4B beads and the appropriate antibody at 4°C for 16-20 hours. After incubation, the beads were collected by gentle centrifugation (1 minute, ~1300 xg, 4°C). A volume of 45 µL of the supernatant was taken as the "post-IP" sample, and the beads were subsequently washed 3-4 times with ice-cold lysis buffer. For both lysates, 40-60 µL of 2X or 1X GLB was added to the washed beads and the bead solution. The lysates were then incubated at 50°C for approximately 10 minutes, or the IPs were boiled for 5 minutes. Finally, the samples were subjected to SDS-PAGE following the procedures described in the corresponding section 2.2.8.

2.2.8. SDS-PAGE and immunoblotting

The samples were loaded onto either homemade or Mini-protean TGX gels and electrophoresed for 30-45 minutes at 500 mA and 250 V (constant volts) in Gel Running Buffer (25 mM Tris-base, 250 mM glycine, 1% SDS, pH 7.4), followed by transfer to nitrocellulose membranes at 25 V and 0.7 A (constant A) for 1 hour in blotting buffer (20% methanol, 48 mM Tris-base, 39 mM glycine, 1.3 mM SDS). After transfer, the nitrocellulose membranes were incubated in TBS-T (20 mM Tris-base, 137 mM NaCl, 0.2% Tween-20, pH 7.5) containing 5% nonfat milk for approximately 40 minutes and were washed three times in TBS-T for 5 minutes each before being incubated with the desired primary antibodies. The primary antibodies were diluted in TBS-T containing 4% BSA and 0.1% NaN3, and the incubation took place for 16-24 hours at 4°C.
Following the incubation with primary antibodies, the antibodies were removed, and the membranes were washed three times in TBS-T. Then, the membranes were incubated with secondary antibodies, typically diluted at 1:1000 in TBS-T containing 5% nonfat milk, for 45 minutes at room temperature. After incubation, the secondary antibodies were discarded, and the membranes were washed three times in TBS-T. Immunoblots were developed by incubating the membranes with chemiluminescent substrates for 5 minutes. Immunoreactivity in immunoblots was quantitated using ImageJ (https://imagej.nih.gov)

2.2.9. Blue native PAGE

To prepare purified Bactrail-expressed proteins for Native PAGE, 75 µL of 4X NativePAGE Sample Buffer and 5 µL of 5% Coomassie Blue G250 (final concentration: 0.25% w/v) were added. NativePAGE Anode Buffer was prepared by diluting 20X Running Buffer stock in ddH$_2$O. Two types of Cathode Buffers, Light and Dark, were prepared by adding 1 mL (Light) or 10 mL (Dark) of 20X Cathode Buffer Additive to 1X Running Buffer, with a final volume of 200 mL. Prior to use, all samples, buffers, gels, equipment, and reagents were chilled to 4°C.

The gel was placed in the XCell SureLock Mini-Cell's electrophoresis apparatus. The wells were washed twice with cold ddH$_2$O and then filled with Dark Cathode Buffer. Native Unstained Protein Standards and samples were loaded into 3-12% Bis-Tris gels. The inner chamber was filled with Dark Cathode Buffer, and the outer chamber was filled with 1X Running (Anode) Buffer. Electrophoresis was conducted at 4°C and 150V (constant voltage) for 1 hour. Subsequently, the Dark Cathode Buffer within the inner chamber was replaced with Light Cathode Buffer, and electrophoresis was continued at 4°C and 185V (constant voltage) until the dye front reached the end of the gel.
After completing the electrophoresis, a PVDF membrane was soaked in MeOH for 30-60 seconds, rinsed twice with ddH₂O, and equilibrated in a transfer buffer for 10 minutes. Simultaneously, the gel was also equilibrated in a transfer buffer for 10 minutes. The samples were then transferred to the PVDF membrane at 85mA (constant current) for 1 hour. The PVDF membrane was rinsed in de-stain buffer (40% MeOH, 10% Acetic Acid in water) for 10 minutes. During the de-staining process, protein standards became visible and were marked with a pencil. Subsequently, the PVDF membrane was washed twice with ddH₂O and incubated in TBS-T buffer for 10 minutes. It was then incubated in 5% nonfat milk in TBS-T, followed by subsequent immunoblotting procedures as described in section 2.2.8.

2.2.10. Coomassie blue staining

The standard protocol involved loading samples onto homemade gels and conducting SDS-PAGE following the instructions outlined in section 2.2.7. To visualize the proteins, a microwave-assisted staining method was utilized. Initially, the gels were placed in a tray and rinsed with ddH₂O to remove any excess SDS that could interfere with the staining process. The ddH₂O was then discarded, and the gels were subjected to two cycles of microwave treatment for 45 seconds each, using a Coomassie staining solution (composed of 10% ethanol, 3 mL of HCl, and 0.1 g of Coomassie Blue G-250 per liter of solution). Subsequently, the gels were de-stained by either microwaving them in ddH₂O for two cycles of 45 seconds or by leaving them overnight in ddH₂O with gentle shaking. Finally, the gels were imaged using the ChemiDoc system and analyzed with ImageJ software using the Coomassie Blue setting.

2.2.11. Bacterial expression and purification of HS-proteins
2.2.11.1. Transformation and the subsequent *E. coli* growth

pET SUMO plasmids were transformed into BL21-CodonPlus (DE3)-RIPL *E. coli* for protein expression. To perform the transformation in BL-21-Codon Plus (DE3)-RIPL *E. coli*, the following steps were followed: pET SUMO plasmids were diluted to approximately 30 ng/μl (avoid exceeding 50 ng). BL-21-Codon Plus (DE3)-RIPL *E. coli* were thawed on ice. Then, 1 μl of DNA was added to approximately 30 μl of cells (incubate on ice), gently flicked 4-5 times, and incubated on ice for 30 minutes. Meanwhile, SOC medium (Invitrogen™) was warmed up. The cells were heat-shocked at 42°C for 20 seconds and immediately placed back on the ice for 2 minutes without mixing. Next, 900 μl of pre-warmed SOC medium was added to the cells, gently resuspended, and allowed to recover at 37°C and 250 rpm for 1 hour. Simultaneously, the kanamycin/chloramphenicol (kan/chlor) agar plate was pre-warmed at 37°C. Finally, the cells were spun down at approximately 1000 rpm for 1 minute (low speed, time can be adjusted) to obtain a pellet, which was then resuspended with 150 μl of SOC medium and plated on kan/chlor agar plates.

The following day, a starter culture was prepared by inoculating 5 colonies from the plate into the 5 mL LB culture. The culture was shaken at 37°C until the OD reaches 0.4-0.8, which typically takes 4-5 hours. 500 μL of culture was taken from the 5 mL culture and transferred to 50 mL of LB with 1% glucose and shaken overnight at 37°C. Approximately 10 mL (or as desired) cultures were taken from the 50 mL overnight culture and transferred to a larger volume of LB media (1-2 L) shaken at 250 rpm at 37°C. The OD was routinely checked until it reached 0.6-0.8 (ideally 0.7). A 30 μL sample was taken for pre-induction analysis, and a 1 mL sample was taken for a glycerol stock (the samples were mixed in a
1:1 volume-to-volume ratio with a 30% glycerol solution, and the resulting mixture was rapidly frozen and stored in a freezer at -80°C. The culture was induced by adding 1 mM IPTG and was continued to be shaken at 16°C overnight (16-19 hours). The OD was checked the next day, and it was expected to be above 1.5 (optional). 30 μL sample was taken for post-induction analysis. Both samples were mixed with 4X GLB, incubated briefly at 37°C, and protein expression was confirmed by Coomassie Blue staining as described in section 2.2.10.

The *E. coli* were harvested by centrifugation at 6300 rpm for 15 minutes at 4°C. Pelleted bacteria were re-suspended in 20-30 mL of an ice-cold phosphate buffer (300 mM NaCl, 50 mM Na2HPO4/NaH2PO4, 10 mM imidazole, 1 mM benzamidine, 5 mM beta-mercaptoethanol, 100 mM arginine-HCl, 100 mM glutamine, 5% glycerol, 0.2% CHAPS, pH 7.4) and were then transferred to 50 mL canonical tubes. If not proceeding on the same day, the tubes were stored at -20°C. If starting from a frozen pellet, it was thawed at room temperature in ddH₂O.

### 2.2.11.2. HS-protein purification

Ensure that everything was kept on ice or maintained at ice-cold temperatures throughout the process. The resuspended pellet was transferred to a 250 mL glass beaker, and the volume was increased to ~100 mL with phosphate buffer described above in 2.2.10.1. The beaker was placed in an ice bucket filled with ice. A protease inhibitor tablet, ~0.1mg/ml lysozyme, and DNase were added to the lysate, and the mixture was stirred thoroughly. The sample was sonicated for 5 minutes using the desired program (2s on, 4s off), and a 15 μL sample is saved after sonication as the "total protein" fraction. Then, the lysate is centrifuged at 16,000 xg for 30 minutes at 4°C. The supernatant is collected, and 15 μL is...
saved as the "soluble fraction." Approximately 100 mL of the supernatant is loaded onto a clean column containing Ni-NTA beads. The column has been pre-washed twice with the phosphate buffer. The column is inverted and mixed well and then incubated on its side at 4°C for at least 20 minutes (alternatively, it can be shaken slowly). The flow-through is collected, and 15 μL is saved as the "flow-through" fraction. If desired, steps of incubation and flow-through can be repeated. The column is washed three times with 30 mL phosphate buffer, thoroughly mixing and inverting the column after each wash. 15 μL of each wash fraction is saved. Then elute the bound proteins with the same phosphate buffer enriched with 400 mM imidazole (10 mL each time) by mixing and inverting the column thoroughly. 5 elution fractions were collected and 15 μL of each elution fraction was saved. The quality of the purification was analyzed by taking 15μL of each fraction of all the steps described above and mixing with 5μL 4X Gel Loading Buffer and performing Coomassie Blue staining as described in section 2.2.10.

2.2.12. Immunopurification erlin complex from αT3 cells

Ensure that everything was kept on ice or maintained at ice-cold temperatures throughout the process. Typically, αT3 cells at confluence were used, with at least 8 large dishes (150 mm x 20 mm) employed. Firstly, the culture medium was removed, and the dish was rinsed once with ~5 mL of ice-cold PBS. The dishes were then placed on ice, and ~3 mL of ice-cold lysis buffer (composed of 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and 1% Triton, pH 8.0) was added for cell lysis, which was carried out for 30 minutes.

Afterward, the cell lysate was centrifuged at 16,000 xg for 10 minutes at 4°C. The clarified lysate was then transferred to a 50 mL canonical tube, and 1 mL of anti-E1 was added. The mixture was gently rocked for 1 minute, and a 30 μL sample was taken and labeled as "pre-
IP." Next, approximately ~3 mg of Protein A-Sepharose C1-4B beads (PA-beads) per 1 large dish were added (pre-washed twice with the same lysis buffer), and incubation was performed overnight with gentle rocking at 4°C.

The next day, the 50 mL canonical tube was slow spun at 1200 xg for ~3 minutes, and a 30 μL sample was taken from the supernatant and labeled as "post-IP." All the supernatant was then removed, and the PA beads were washed three times using the same lysis buffer (first wash with 25 mL, followed by the remainder with 1 mL). Afterward, the PA beads were washed once with detergent-free lysis buffer (150 mM NaCl, 50 mM Tris base, 1 mM EDTA at pH 8.0), and the liquid was completely removed from the beads. One aliquot of 1 mg/mL E1 peptide (~100 μL) was added, mixed thoroughly, and incubated for at least 36 hours with gentle rotation at 4°C. The mixture was then centrifuged at maximum speed (16,000 xg for 1 minute at 4°C), and approximately ~80 μL of the supernatant was collected as the eluted purified erlin complex. Around 15 μL of eluted erlin complex was saved as "elution" for purification analysis. The beads were washed once with detergent-free lysis buffer, and ~80 μL of 1x Gel loading buffer was added, referred to as "beads." All the samples, except for the beads with 1x Gel loading buffer, were added to 4x Gel Loading Buffer. The samples were boiled for 5 minutes. The quality of the purification was analyzed by taking 15 μL of the elution fraction and beads fraction, along with standard BSA samples, performing Coomassie Blue staining as described in section 2.2.10. Simultaneously, approximately 15 μL of the "pre-IP" and "post-IP" samples, along with 1 μL of the elution fraction and beads fraction, were loaded onto an SDS-PAGE gel. The gel was then transferred onto nitrocellulose membranes, followed by subsequent anti-E2 immunoblotting as described in section 2.2.8.
2.2.13. Reconstitution of immunopurified erlin complex with membrane scaffold protein

Approximately 8 large dishes of confluent aT3 cells were collected. The cells were lysed using 20 mL of 1% Triton lysis buffer according to the procedure outlined in section 2.2.12. After centrifugation, the supernatant containing the whole cell lysate was carefully collected. The protein concentration of the lysate was determined using the Bradford assay. It was found that the membrane protein constituted approximately 30% of the total protein content. The ratio of the purified erlin complex to MSP was approximately 1:1. To proceed with the purification process, the corresponding amount of biotin-tagged MSP was added to the lysate, and the mixture was gently rocked at 4°C for 1 hour. Subsequently, bio-beads were added to the mixture at a concentration of 0.2 g/mL, and the incubation was continued for 2 hours at 4°C. After the incubation, the bio-beads were removed from the mixture. Next, approximately 500 μL of anti-E1 was introduced to the mixture, and a 30 μL sample was collected as the pre-IP fraction for further analysis. Following that, approximately 25 mg of protein-A beads were added to the lysate. The mixture was then incubated overnight at 4°C. From this point onward, the steps outlined in section 2.2.12 were followed for further purification and analysis.

The subsequent streptavidin pull-down of biotin-MSP was performed as an optional step, intended for concentrating the volume of the eluted erlin complex and achieving a second round of purification for the erlin complex. The experiment was carried out as follows: 20 μL of streptavidin beads were washed three times with 100 μL of detergent-free lysis buffer at low speed. Subsequently, the washed streptavidin beads were transferred to the purified ~80 μL erlin elution and rotated at 4°C for 1 hour. The mixture was then centrifuged at
1500 xg for 1 minute at 4°C, and the resulting supernatant (100 μL) was collected. To the streptavidin beads, 19 μL of 1 mM DTT with detergent-free lysis buffer was added, followed by the addition of 1 μL of 3 mg/mL PreScission Protease. The beads were then subjected to overnight rotation at 4°C. Afterward, the mixture was centrifuged at maximum speed for 3 minutes, and approximately 16 μL of the supernatant was collected for Cryo-EM preparation, while 3 μL was taken for gel electrophoresis, as described in section 2.2.8.

2.2.14. Lipid overlay assay

The experimental protocol used in this study was adapted from¹, with slight modifications. Lipid overlay arrays were prepared by spotting 200 pmol of PI(3)P, PI(4)P, PI(5)P, PI, PI(3.5)P2, and PI(4,5)P2, dissolved in chloroform/methanol/water (1:2:0.8, by volume) onto Hybond C or Protran Supported 0.2-μm nitrocellulose membranes and dried overnight. Bacteria-expressed proteins or immunopurified mammalian proteins were incubated and washed with PBS containing 1% nonfat dried milk at 4 °C using the following steps: 1-hour preincubation for blocking, 2 times two-minute washes, overnight incubation with purified protein (0.5-2 μl for bacteria-expressed proteins and approximately 10 μl for immunopurified mammalian proteins in ~600ul PBS containing 1% milk), followed by 2 times two-minute washes, 2-hour incubation with anti-E2 or anti-HA antibody, 3 times two-minutes washes, 1-hour incubation with a secondary antibody, 3 times two-minute washes, 1 times two-minute wash with PBS, and exposure to enhanced chemiluminescence reagents.

To ensure the presence of equivalent proteins in the lipid overlay assay, the following steps were taken for the immunoblotting analysis. After incubating the purified proteins with PBS containing 1% milk, approximately 50 μL of the mixture was collected and added to
2x gel loading buffer. The final concentrations of the components in the loading buffer were as follows: 1% SDS, 0.05% bromphenol blue, 5% glycerol, 100 mM DTT, and 50 mM Tris-HCl pH 6.8. Approximately 10 µL of the sample was loaded onto an SDS-PAGE gel, as described in 2.2.8.

2.2.15. Fluorescence Polarization assay

Experiments were conducted under conditions of dark or near dark due to the light-sensitive nature of the BODIPY-phosphatidylinositol lipids. Whenever necessary, samples were protected from light by covering them with aluminum foil. The FP³ experiments were performed in a phosphate buffer solution (300 mM NaCl, 50 mM Na₂HPO₄/NaH₂PO₄, 10 mM imidazole, 1 mM benzamidine, 5 mM beta-mercaptoethanol, 100 mM arginine-HCl, 100 mM glutamine, 5% glycerol, pH 7.4). An increasing amount of purified protein (~0-30 µM final concentration) was mixed with 5 nM of BODIPY-phosphatidylinositol lipids, resulting in a total volume of 150 µL. Measurements were promptly taken using a FlexStation 3 Microplate Reader (Molecular Devices) with excitation at 475 nm, a cut-off filter at 495 nm, and emission detection at 513 nm. The K_d values were determined using GraphPad Prism software after subtracting the non-specific signal (through nonlinear regression, Binding-Saturation, and one site-specific binding).

2.2.16. Confocal microscopy

2.2.16.1. Image acquisition

HeLa cells were seeded at approximately 2 x 10⁵ cells on micro cover glass coated with a 0.1 mg/mL poly-D-lysine solution (the coated cover glass was prepared as follows: the poly-D-lysine stock was diluted 10 times with PBS, and the micro cover glass was
incubated with the ~500 µL diluted solution for 20 minutes, followed by air drying for at least 2 hours). After approximately 24 hours, the cells were transiently transfected using PEI with plasmids containing GFP-2xFYVE, GFP-Rab5b, GFP-Rab7a, GFP-LAMP, and DS-Red ER, following the procedure described in section 2.2.3. From this step onwards, precautions were taken to minimize exposure to light. Following an 18-hour post-transfected incubation period, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 minutes. Subsequently, the cells were mounted onto glass slides using Prolong TM Gold antifade reagent with DAPI.

For Lysotracker Red staining, the cells were seeded using the same procedure as described above. The next day, cells were then incubated with 3 µM Lysotracker™ Red DND-99 in the culture medium for 30 minutes at 37 °C. The fixing and mounting processes were carried out in the same manner. Imaging was performed using a Leica SP8 confocal microscope with a 63x oil-immersion objective lens, using 488 nm excitation/510 nm emission for GFP-containing constructs and 577 nm excitation/590 nm emission for Lysotracker Red staining.

In the FITC-NPs cellular uptake assay, HeLa cells were seeded at approximately 1 x 10^5 cells onto a 29 mm glass bottom dish and cultured overnight. The culture medium was then replaced with a medium containing FITC-NPs at a final concentration of 500 µg/mL. After a 2-hour incubation, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 minutes. Subsequently, the cells were stained with DAPI for 6 minutes and briefly washed twice. They were kept in PBS in the dish and imaged using the same Leica SP8 confocal microscope as above, with 495 nm excitation/519 nm emission.

2.2.16.2. Image analysis
For imaging analysis, multiple images (individual planes) from a Z-stack (3-D) of a single cell were imported into ImageJ and then projected into one 2-D image. To quantify GFP-Rab5b, GFP-Rab7a, and Lysotracker red fluorescence, intensity thresholding was applied to identify fluorescent structures, and the mean intensity was obtained for each cell. To measure the GFP-LAMP1, GFP-2xFYVE, Lysotracker red, and FITC-NPs puncta number (the cutoff for particles was set to 0.1-3 µm), intensity thresholding was applied to identify GFP-LAMP1, GFP-2xFYVE, Lysotracker red and FITC-NPs fluorescence puncta, and puncta were counted within the cell. At least two separate cell lines were used for all cell types to ensure the accuracy and reproducibility of results.

### 2.2.17. ELISA assay

PI(3)P and PI(4,5)P$_2$ levels were determined using PI(3)P and PI(4,5)P$_2$ ELISA kits. HeLa cells were seeded into six-well plates, while αT3 and SH-SY5Y cells were seeded into 10 cm dishes one day prior. Typically, approximately 4 x 10$^6$ HeLa cells (three wells of a six-well plate) or approximately 12 x 10$^6$ αT3 and SH-SY5Y cells were harvested and lysed in a single Eppendorf tube, which could then be split into triplicates for ELISA analysis.

To prepare the lipid extract, cells were detached by scraping using approximately 1 mL (for one well of a six-well plate) or 2 mL (for a 10 cm dish) of ice-cold 0.5 M trichloroacetic acid (TCA), followed by centrifugation at 1000 x g for 7 minutes at 4°C. The resulting pellets were vortexed for 30 seconds and subjected to two washes with approximately 500 µL of 5% TCA containing 1 mM EDTA at room temperature. Subsequently, the pellets were mixed with approximately 400 µL of MeOH:CHCl$_3$ (2:1) and vortexed for 10 minutes, repeating this step twice. The supernatant was discarded, and the pellets were obtained. For the extraction of acidic lipids, the pellets were incubated with approximately 350 µL of a
solution containing MeOH:CHCl₃:12 M HCl (80:40:1) and vortexed for 25 minutes, and centrifuged at 1000 xg for 5 minutes. The resulting supernatant, containing the acidic lipids, was mixed with 180 µL of CHCl₃ and 80 µL of 0.1 M HCl, vortexed for 30 seconds, and centrifuged again at the same speed. The organic phase was collected and divided into two portions, with a ratio of 5:1 (v/v), for the measurement of PI(3)P and PI(4,5)P₂, respectively. Finally, the samples were vacuum dried and stored in a -20°C freezer if further processing was not performed on the same day.

ELISA was conducted following the manufacturer's instructions. To prepare the PI(3)P/PI(4,5)P₂ lipid standard, PBS-T 3% Protein Stabilizer was added to the vial containing the standard lipids, while the dried organic extract samples were reconstituted using the same PBS-T + 3% Protein Stabilizer buffer through water bath sonication for 10 minutes. The reconstituted samples were then divided into triplicates and incubated with PI(3)P/PI(4,5)P₂ detector proteins in the plate for 1 hour. Subsequently, the samples were added to a PI(3)P/PI(4,5)P₂ coated microplate for an additional 1 hour for competitive binding. After three washes, a peroxidase-linked secondary detector was added and incubated for 1 hour to enable the detection of the bound protein. The colorimetric substrate was measured at 450 nm using a microplate reader. The concentrations of PI(3)P/PI(4,5)P₂ were determined from standard curves, generated by standard lipids, and the relative PI(3)P level was calculated by dividing the amount of PI(3)P by the amount of PI(4,5)P₂ (used as an internal control).

2.2.18. VPS34 complex immunoprecipitation and activity assay

Three 10 cm dishes of (~ 6 x 10⁷) HeLa cells were harvested with 3 mL ice-cold NP-40 lysis buffer (20 mM Tris, 137 mM NaCl, 1 mM MgCl₂, 1mM CaCl₂, 1% NP-40 (Sigma),
10% Glycerol, 10 μM pepstatin A, 0.2 μM soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, pH 7.5), incubated on ice for 30 minutes, and then centrifuged at 16,000 x g for 10 minutes at 4°C. The supernatants referred as "pre-IP" were then incubated with 10 μl (2.5 μg) anti-hVPS34 and 8 mg protein A-Sepharose CL-4B beads for approximately 16 hours at 4°C with gentle rotation, the mixture was centrifuged (1500 xg) to separate the supernatant (referred as "post-IP") and the IPs were collected as described in 2.2.5 and subjected to SDS-PAGE and immunoblotting with anti-VPS34 as described in 2.2.6. section or for kinase activity as follows.

The IPs underwent a series of washes using specific buffers. Firstly, IPs were washed three times with 1% NP-40 supplemented ice-cold PBS. This was followed by two washes with a buffer containing 100 mM Tris-HCl and 500 mM LiCl at pH 7.5. Subsequently, two washes were performed using a buffer composed of 10 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA at pH 7.5. Lastly, two washes were carried out with VPS34 kinase reaction buffer (20 mM Tris, 200 mM NaCl, 2 mM EDTA, 20 mM MnCl₂, and 100 μM ATP), with each wash consisting of 1 mL.

The IPs were then divided into duplicates, and activity reactions were initiated. This involved combining the IPs with 25 μL of VPS34 kinase reaction buffer, 8 μL of 500 μM PI, and 17 μL of H₂O to achieve a total volume of 50 μL, ensuring coverage of all the IP beads. The reaction mixture was incubated at 30°C for either 20 minutes or 1 hour with gentle shaking. To halt the reaction, 10 μL of 100 mM EDTA was added, resulting in a total volume of 60 μL. The samples were then centrifuged, the supernatant was collected, and it was frozen at -20°C.
For ELISA analysis, the samples were thawed, and 340 μL of PBST + 3% Protein stabilizer buffer was added to reach a total volume of 400 μL (8x dilution). The samples were divided into triplicates for ELISA detection. The quantification of the generated PI(3)P was conducted using the PI(3)P Mass ELISA kit, following the procedure outlined in section 2.2.14. Graphs were generated using GraphPad Prism software, utilizing nonlinear regression with curve fitting (one site-total).

2.2.19. Cathepsin activity assay

HeLa cells were seeded in duplicates or triplicates at a density of approximately 5-6 x 10^4 cells per well, while SH-SY5Y cells were seeded at 8 x 10^4 cells per well in a 96-well black-well, clear-bottom plate. After 24 hours of incubation at 37 °C, the cells reached confluence and were then incubated with 100 μL of phenol red-free DMEM containing 4 μL of Magic Red™ CTSB reagent (prepared by diluting a 10x working stock with ddH2O). In one well, the medium with the reagent was included as a non-specific signal control without cells. Fluorescent signals were immediately detected at time = 0 using a FlexStation 3 Microplate Reader with excitation at 592 nm, a cut-off filter at 610 nm, and emission at 628 nm. The plate was then returned to 37 °C, and measurements were taken at different time points. The traces presented represent the average values of duplicates or triplicates from an individual representative experiment. Graphs were generated using GraphPad Prism by subtracting non-specific signals and normalizing them to the protein amount (nonlinear regression, curve fit, one site-specific).

2.2.20. Statistical analysis
The numerical data obtained in this study were primarily analyzed using GraphPad Prism software or Microsoft Excel. The results are typically presented as mean ± S.E.M. with n representing the number of independent experiments (n ≥ 3). For microscopy experiments, at least 15 cells from a minimum of 2 independent experiments were analyzed. Statistical significance was assessed using t-tests for comparing two samples or one-way ANOVA for comparing multiple samples. A P-value of less than 0.05 was considered statistically significant.

2.3. References


Chapter 3.

Phosphatidylinositol 3-phosphate binding to the erlin1/erlin2 complex sustains autophagy and lysosome function
3.1. Preface

This chapter is submitted for publication in the Journal of Biological Chemistry

Fanghui Hua\textsuperscript{1}, Caden G. Bonzerato\textsuperscript{1*}, Katherine R. Keller\textsuperscript{1*}, Dandan Guo\textsuperscript{1*}, Juntao Luo\textsuperscript{1*} and Richard J.H. Wojcikiewicz\textsuperscript{1*}

\textsuperscript{1} Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY 13210, USA

\textsuperscript{*}The following individuals contributed to the data presented in this chapter.

D.G and J.L (generated the raw data for Figure. 3.5.A), C.G.B (analyzed data for Figure. 3.1. D, E; Figure 3.2.E; Figure 3.3.D; Figure 3.6 and repeated experiments for Figure. 3.5; Figure.3.7), K.R.K (repeated experiments for Figure. 6, Figure 8A, Figure S1, left panel), and R.J.H.W (principal investigator and editorial input)
3.2 Summary

The erlin1/erlin2 (E1/E2) complex is an endoplasmic reticulum membrane-located assemblage of the proteins erlin1 and erlin2, with a well-defined role in mediating the degradation of inositol 1,4,5-trisphosphate receptors by the ubiquitin-proteasome pathway. Here, we demonstrate the direct and selective binding of phosphatidylinositol 3-phosphate (PI(3)P) to recombinant erlins, and that binding is mediated by a juxta-membrane globular domain. Remarkably, CRISPR/Cas9-mediated disruption of the E1/E2 complex reduces cellular PI(3)P levels by ~50%. This was not due to an effect on VPS34 kinase activity, which is critical for maintaining steady-state levels of PI(3)P, but rather due to the loss of PI(3)P binding sites and, most likely, enhanced PI(3)P dephosphorylation. The reduction in PI(3)P levels correlated with a decrease in autophagic flux and lysosome function but had no effect on the early stages of the endocytic pathway. Pharmacological inhibition of VPS34 activity and suppression of PI(3)P levels caused a similar reduction in autophagic flux and lysosome function. Overall, these data indicate that by binding to PI(3)P, the E1/E2 complex plays an important role in maintaining the steady-state levels of PI(3)P and thus, sustains some key PI(3)P-dependent processes.

3.3. Introduction

Erlin1 (E1) and erlin2 (E2) are ~40 kDa endoplasmic reticulum (ER) membrane-located proteins that oligomerize to form an ~2 MDa complex (1–3). A variety of studies indicate that E1 and E2 have a single transmembrane domain located at their N-termini and that the remainder of the polypeptides lie within the ER lumen (1, 2, 4). Erlins are members of the SPFH domain-containing protein family, named for the founding members Stomatin, Prohibitin, Flotillin and HflC/K (1). These proteins display diverse subcellular
localizations and putative cellular functions and have a propensity to oligomerize into high molecular mass complexes and interact with membrane lipids (1, 5, 6).

The E1/E2 complex has a well-defined role in mediating the degradation of activated inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) by the ubiquitin-proteasome pathway (2, 7–10). It does this by acting as a ‘recognition factor’, binding to activated IP$_3$Rs and recruiting the ubiquitin ligase RNF170, which catalyzes the ubiquitination of IP$_3$Rs and their subsequent transfer to the proteasome for degradation (9). Additional roles for erlins have been proposed, e.g., evidence has been presented that erlins bind cholesterol and regulate proteins that control cholesterol metabolism (11, 12), that E1 regulates autophagy by associating with autophagy and beclin1 regulator1 (AMBRA1) (13), that erlins interact with rhomboid protease RHBDL4, facilitating the degradation of aggregation-prone proteins (14) and erlins can mediate virus exit from the ER membrane and replication (15, 16). Remarkably, mutations in the erlins are linked to neurodegenerative disorders, particularly to hereditary spastic paraplegia (HSP) (5, 8, 17). However, the direct functional consequences of the mutations and why they should lead to neurodegeneration are not yet resolved.

With regard to the lipid binding properties of erlins, our previous studies using E1/E2 complex immunopurified from mammalian cells have indicated that the erlins bind to monophosphoinositides, and most strongly to phosphatidylinositol 3-phosphate (PI(3)P) (8). Here, we have better defined this interaction using recombinant, bacterially-expressed erlins, and remarkably, find that in mammalian cells, the E1/E2 complex is critical to maintaining PI(3)P levels with downstream consequences on autophagy and lysosome function.
3.4. Results

3.4.1. Recombinant erlins bind selectively to PI(3)P

Previous research on immunopurified, mammalian E1/E2 complex showed selective binding to PI(3)P and that PI(3)P binds more strongly to E2 than E1 (8). To eliminate the possibility that co-immunopurifying contaminants might mediate or affect PI(3)P binding, the interaction between the erlins and PI(3)P was studied using bacterially-expressed, recombinant proteins tagged at the N-terminal with His-SUMO (HS) and at the C-terminal with the HA epitope tag (Fig. 3.1A and B). The phosphoinositide binding capacity of the recombinant proteins was initially tested with a “lipid overlay assay” (8) (Fig. 3.1C), showing that HS-E2HA selectively binds PI(3)P, similarly to immunopurified mammalian E1/E2 complex, and that HS-E2HA binds much more strongly than does HS-E1HA, suggesting that in the E1/E2 complex, E2 is the primary mediator of PI(3)P binding. Binding was also analyzed using BODIPY-tagged phosphoinositides in a Fluorescence Polarization (FP) assay (18). HS-E2HA bound with high affinity to BODIPY-PI(3)P (K_d: 1.6 ± 0.3 µM), with HS-E1HA exhibiting lower affinity binding, and with negative control HS-HA not binding at all (Fig. 3.1D). Furthermore, among the different phosphoinositides, HS-E2HA bound most strongly to PI(3)P, as judged by the lowest K_d and highest binding capacity (Fig. 3.1E). Overall, these data show that the erlins bind directly and specifically to PI(3)P and suggest that in the E1/E2 complex, E2 is the primary mediator of PI(3)P binding.

3.4.2. PI(3)P binds to a juxta-membrane domain on E2

To identify the PI(3)P binding site in E2, deletion mutants were created, guided by the known domain organization of E2 and the predicted three-dimensional E2 structure (Fig.
3.2 A-C. Interestingly, E2 contains a juxta-membrane globular (G) domain, consisting of the SPFH domain with characteristic β-strands connecting with two α-helices (Fig. 3.2B) (6, 19). Using the lipid overlay assay, the constructs with the 21-172 region (G domain), i.e. HS-E2HA<sup>1-177</sup> and HS- E2HA<sup>21-172</sup>, bound selectively to PI(3)P, similarly to HS-E2HA, while HS-E2HA<sup>178-340</sup>, which lacks the G domain, failed to associate (Fig. 3.2D). Parallel FP assays showed that HS-E2HA, HS-E2HA<sup>1-177</sup> and HS-E2HA<sup>21-172</sup> bound to PI(3)P better than HS-E2HA<sup>178-340</sup>, as indicated by relatively high affinity values and binding capacity (Fig. 3.2E). Overall, these data indicate that the G domain is the primary determinant of PI(3)P binding.

3.4.3. Erlins regulate cellular PI(3)P levels

The data in Fig. 1 and 2 reveal a robust interaction between E2 and PI(3)P, suggesting that erlins may regulate PI(3)P-dependent cell functions, or perhaps modulate PI(3)P levels. To investigate the effect of the erlins on PI(3)P levels, HeLa cells were engineered using CRISPR/Cas9 to generate E1 knockout (E1KO), E2 knockout (E2KO) and E1/E2 knockout (E1/E2KO) cell lines (Fig. 3.3A, left panel) and PI(3)P levels were measured using ELISA (Fig. 3.3A, right panel). Remarkably, PI(3)P levels were significantly lower in E1KO, E2KO and E1/E2KO cells, as compared to WT cells (Fig. 3.3A, right panel). Similar decreases in PI(3)P levels were also seen in E1/E2KO αT3 cells and E2KO SH-SY5Y cells, showing that this effect is not cell type-specific (Fig. 3.31). To visualize PI(3)P, cells were transfected to express a GFP-2xFYVE construct, which binds PI(3)P (20) and is commonly used to localize where PI(3)P is concentrated (21). Compared to WT cells, E1/E2KO cells had significantly fewer GFP-2xFYVE-positive puncta per cell (23±1 versus
16±2), a reduction of ~30% (Fig. 3.3.B). This is consistent with the ~50% decrease in PI(3)P levels seen in E1/E2KO cells (Fig. 3.3.A).

To show that the reduction in PI(3)P levels upon E1 and E2 deletion is caused by erlin loss and not by off-target effects of gene editing, exogenous hE2HA_{WT} (human E2 with an HA tag on the C terminal) was expressed in E1/E2KO HeLa cells at levels similar to endogenous E2, or overexpressed ~ five-fold (Fig. 3.3.C, left panel, lanes 1 and 2). This restored PI(3)P levels in a manner that correlates with the hE2HA_{WT} expression level (Fig. 3.3.C, right panels), showing that the effects of erlin KO are specific. To further explore the determinants of PI(3)P restoration, different constructs were expressed in E1/E2KO HeLa cells (Fig. 3.3.D, upper panels). To investigate the role of PI(3)P binding in PI(3)P restoration, cells were transiently transfected to express hE2HA_{T65I}, which binds PI(3)P poorly (8). This failed to restore PI(3)P levels (Fig. 3.3.D, lower panel, columns 3 versus 4), showing that ability to bind PI(3)P is needed for PI(3)P restoration. The ability of E1 to restore cellular PI(3)P levels was examined by expressing mouse (m) E1HA_{WT} in E1/E2KO cells. mE1HA_{WT} increased PI(3)P levels, but mE2HA_{WT} was more effective (Fig. 3.3.D, lower panel, columns 5 versus 6), confirming the correlation between PI(3)P binding ability and PI(3)P restoration. Finally, mE2HA_{F305A}, which does not assemble into high molecular weight complexes (3, 22), was able to restore PI(3)P levels similarly to mE2HA_{WT} (Fig. 3.3.D, lower panel, columns 5 versus 7), indicating that lower-order E2 assemblies (22) are sufficient to restore PI(3)P levels, and that high molecular weight complexes are not needed. In summary, these findings indicate that disruption of the E1/E2 complex by deleting E1, E2, or both, reduces PI(3)P levels and that the ability of exogenous E2 to restore PI(3)P levels correlates with PI(3)P binding capacity.
3.4.4. Erlins regulate PI(3)P metabolism

To investigate how the E1/E2 complex regulates cellular PI(3)P levels, PI(3)P metabolism in HeLa cells was examined. Two key kinases, PI3KC3 (VPS34) and PIKfyve, are responsible for converting phosphatidylinositol (PI) to PI(3)P and PI(3)P to phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), respectively, and are balanced by the activity of myotubularin-related proteins (MTMRs) and SAC3 that dephosphorylate the lipids (Fig. 3.4.A) (23–26). To determine the importance of these kinases in governing PI(3)P levels, cells were exposed to the inhibitors of VPS34 and PIKfyve, VPS34-IN1 and Apilimod, respectively (27, 28). Treatment of WT HeLa cells with VPS34-IN1 for 30 min dramatically and significantly reduced PI(3)P levels, while in contrast, Apilimod had no significant effect on PI(3)P levels (Fig. 3.4.B). Thus, VPS34 activity, but not PIKfyve activity, is key to controlling steady-state PI(3)P levels. These data also suggest that PI(3)P is subject to rapid dephosphorylation since PI(3)P levels fall so abruptly when VPS34 is inhibited. Imaging of GFP-2xFYVE confirmed the profound decrease in PI(3)P levels in cells treated with VPS34-IN1 (Fig. 3.4.C).

To measure whether VPS34 kinase activity might be erlin-dependent, VPS34 was immunoprecipitated (IP) from WT and E1/E2KO HeLa cells (Fig. 3.4.D, left panel). However, VPS34 activity was the same for both WT and E1/E2KO HeLa cells (Fig. 3.4.D, right panel), indicating that VPS34 activity is unaffected by erlin KO. Additional experiments showed that key components of the VPS34 complex, such as Beclin1 and ATG14L, which are modulators of VPS34 kinase activity (29), were unaffected by erlin KO (Fig. 3.52). To further investigate whether the ability to bind to PI(3)P is crucial for maintaining PI(3)P levels, the effects of GFP-2xFYVE on PI(3)P levels were examined.
Remarkably, this revealed that GFP-2xFYVE was as effective as hE2HA\textsuperscript{WT} in restoring PI(3)P levels (Fig. 3.4E, columns 3 and 4). These data, together with those from Fig. 3D, underscore the need for PI(3)P binding sites (either E2 or GFP-2xFYVE) to maintain steady-state PI(3)P levels. The PI(3)P binding sites provided by erlins may sequester PI(3)P strongly enough to slow its metabolism, presumably by limiting the dephosphorylation of PI(3)P to PI.

3.4.5. Erlin KO does not affect the endocytic pathway

Since PI(3)P is important for the endocytic pathway (23–25, 30), the possible effects of erlin KO were explored. The initial endocytic step was examined using a FITC-loaded nanoparticle (FITC-NP), since previous studies (31) have shown that FITC-NP is taken up into endocytic vesicles after internalization, as indicated by fluorescent puncta within cells. However, WT and E1/E2KO HeLa cells displayed a similar uptake profile (Fig. 3.5A), indicating that the early endocytic pathway is not affected by erlin KO, or the resulting decrease in PI(3)P levels. Subsequent steps in the endocytic pathway were also assessed by examining the early and late endosome markers Rab5b and Rab7a (32, 33), respectively. Although fluorescent puncta were not readily detectable, the distribution and mean fluorescence intensity of Rab5b and Rab7a were the same in WT and E1/E2KO HeLa cells (Fig. 3.5B and C), again indicating that the endocytic pathway is not impaired by erlin KO. Given the known role of PI(3)P in the endocytic pathway (23–25, 30), these findings are somewhat surprising but suggest that PI(3)P levels within the endocytic pathway are unaffected by erlin KO, and that PI(3)P levels may be reduced elsewhere in the cell, e.g., at sites of autophagosome biogenesis.

3.4.6. Erlin KO inhibits autophagy
Autophagy initiation depends on PI(3)P, which plays a fundamental role in the formation of pre-autophagosomal structures (e.g., phagophores) and mature autophagosomes (34–36). To investigate how erlin KO might affect autophagy, the levels of the autophagy marker LC3-II were examined (37). In WT cells, starvation (incubation with EBSS) increased the levels of LC3-II, indicating that autophagy is triggered (Fig. 3.6, lanes 1-3). This increase was enhanced by the lysosome inhibitor bafilomycin A1 (BafA) (Fig. 3.6, lanes 4-5), with most of the accumulation being attributed to the effect of BafA, rather than starvation (Fig. 3.6, lane 6). Interestingly, in the E1/E2KO cells, there was significantly less LC3-II under all conditions (Fig. 3.6, lanes 7-12 and histogram), indicating that erlins play an important role in the initiation of autophagy, either directly, or indirectly by sustaining PI(3)P levels at sites of autophagosome biogenesis.

3.4.7. Erlin KO inhibits lysosome acidification and function

Lysosomes play a pivotal role in metabolism by engaging with either autophagosomes or late endosomes to facilitate the degradation of internal and external substrates (38). The effect of erlin KO on lysosome abundance and properties was assessed using Lysotracker red, which labels acidic organelles, mostly lysosomes and late endosomes (39), and the lysosome membrane marker LAMP1 (40). Lysotracker red showed less fluorescence intensity and puncta number in E1/E2KO cells, indicating a reduction in lysosome acidification compared to WT cells (Fig. 3.7.A), while LAMP1 showed a very similar distribution in WT and E1/E2KO HeLa cells, indicating that lysosome number is not altered (Fig. 3.7.B). To determine whether lysosome function was altered in E1/E2KO cells, the activity of Cathepsin B, the key lysosomal protease (41), was examined using a fluorescently tagged substrate. This revealed that Cathepsin B activity was reduced by ~50%
in the E1/E2KO cells (Fig. 3.7.C). These findings suggest that while erlins do not regulate
lysosome number, they do have an impact on lysosomal acidification and catalytic function.

3.4.8. VPS34 inhibition impairs autophagy and lysosome function

To examine whether the inhibitory effect of E1/E2KO on autophagy and lysosome function
is directly due to erlin loss, or indirectly due to the resulting reduction in PI(3)P levels, WT
HeLa cells were incubated with VPS34-IN1 for 30 min, which reduced PI(3)P levels by
~80% (Fig. 3.4.B). Interestingly, this inhibited the LC3-II levels, in the absence or presence
of BafA, indicating that inhibition of PI(3)P levels inhibits autophagy (Fig. 3.8.A). Additionally, Cathepsin B activity was strongly inhibited by 30 min pre-incubation with
VPS34-IN1 (Fig. 3.8.B), indicating PI(3)P depletion dramatically reduces lysosome
function. Overall, the data suggest that the effect of erlin KO on autophagy and lysosome
function are due to the associated reduction in PI(3)P levels.

3.5. Discussion

To summarize, our findings indicate that (i) E2 binds directly to PI(3)P through a juxta-
membrane globular (G) domain, (ii) the E1/E2 complex is crucial for maintaining cellular
PI(3)P levels, most likely by stabilizing PI(3)P, and (iii) by maintaining PI(3)P levels, the
E1/E2 complex plays a key role in sustaining the autophagy pathway and lysosomal
function. These discoveries identify novel roles for erlins in mammalian cells.

Previously, we reported that immunopurified mammalian E1/E2 complex binds to PI(3)P
and that this binding is inhibited by a T65I mutation in E2 (8). However, a potential caveat
with this approach is that the immunopurified E1/E2 complex may contain co-purifying
contaminants that could impact PI(3)P binding. Here, using bacterially expressed erlins,
we show that the binding is direct, is strongest for E2, and is mediated by the G domain.

Interestingly, the G domain region contains many point mutations responsible for HSP (42–46), including the T65I mutation (8). While it has been observed that SPFH domain-containing proteins bind to lipids (e.g., stomatin binds cholesterol and prohibitin associates with phosphatidylinositol 3,4,5-trisphosphate) (5, 47, 48), no definitive lipid binding motif has been identified in the SPFH domain and no canonical PI(3)P binding domains, such as FYVE or PX domains, have been identified in erlins (6, 20, 49, 50). Furthermore, mutagenesis of positively-charged amino acids in the E2 G domain that could possibly form a PI(3)P-binding site (e.g., K119, H121, H122) did not impact binding to PI(3)P (data not shown). Thus, the amino acid cluster or motif responsible for the binding of the G domain to PI(3)P remains to be determined.

Interestingly, the G domain of E2 is predicted to reside within the ER lumen (2, 51), while PI(3)P is found in the cytosol-facing leaflet of the ER membrane (52, 53). Thus, how PI(3)P and erlins interact remains a puzzle. However, erlins are reported to interact with various components within the ER membrane and on the cytosolic side of the ER, such as AMBRA1, ER transmembrane J-protein B12, RHBDL4, and cholesterol (13, 14, 16, 54), suggesting that some erlins may be "flipped" to be oriented towards the cytosol, which would expose G domains that could interact with PI(3)P. Alternatively, “flippases” could translocate PI(3)P to the luminal side of the ER membrane (55). Clearly, a high-resolution structural model of the E1/E2 complex together with PI(3)P is needed to identify the binding determinants for PI(3)P on the G domain and to understand how erlins and PI(3)P interact in living cells.
A remarkable discovery is that disruption of the E1/E2 complex has a profound inhibitory effect on cellular PI(3)P levels in multiple cell lines, and that PI(3)P levels are restored when E1/E2KO cells are reconstituted with PI(3)P binding proteins like E2, or even GFP-2xFYVE. Interestingly, the activity of VPS34, the key kinase that produces PI(3)P, is not affected by the knockout of erlins. Instead, erlin binding to PI(3)P appears to be crucial for maintaining PI(3)P levels, most likely by sequestering PI(3)P and preventing dephosphorylation to PI by MTMRs. The deletion of other SPFH family proteins, such as stomatin, also appears to impact the levels of various lipid species (phosphatidylcholines, phosphatidylethanolamines and phosphoinositides), although the underlying mechanism remains unknown (56).

Interestingly, the erlin KO-induced reduction in PI(3)P levels has distinct effects on different PI(3)P-dependent cellular processes. While PI(3)P is known to act as a crucial regulator in the endosomal system (26, 30), surprisingly, the depletion of PI(3)P resulting from erlin knockout does not affect endocytic pathway. In contrast, the reduction in PI(3)P levels has a significant effect on the autophagy pathway, as expected, given the well-established role of PI(3)P in autophagy initiation and phagophore formation (36, 57, 58). This observation is consistent with findings that E1, and presumably the E1/E2 complex, plays a pivotal role in promoting the formation of autophagosomes through interaction with AMBRA1 at MAM raft-like microdomains (13). The differential effects of erlin KO on the autophagy and endosomal systems implies the existence of distinct PI(3)P pools (“autophagic” and “endocytic”), with only the ER-proximal autophagic pool being affected by erlin KO (Fig. 3.9).
Surprisingly, our research also shows that the absence of erlins significantly impacts lysosomal acidification and function, an effect mimicked by pharmacological inhibition of VPS34 activity, suggesting that PI(3)P levels have a profound effect on lysosomal function.

It has been reported that the production of lysosomal PI(3,5)P$_2$ by PIKfyve is critical for autophagosome-lysosome fusion (59), and that PI(3,5)P$_2$ stabilizes and activates the lysosomal V-ATPase in yeast (60). As PI(3)P serves as the primary precursor for PI(3,5)P$_2$ (61), the reduction of PI(3)P levels, seen in E1/E2KO cells could impact the levels of PI(3,5)P$_2$. Also, recent studies have indicated that lipid transport proteins, such as VPS13, facilitate the transport of lipids at contact sites between the ER and lysosomes (62). Thus, in E1/E2KO cells, PI(3)P transfer from the ER to lysosomes may be diminished with consequently, less lysosomal PI(3,5)P$_2$ generation, acidification, and function (Fig. 3.9).

PI(3)P has also been suggested to be a cofactor for the recognition and downregulation of activated IP$_3$Rs by the E1/E2 complex since the T65I mutation impairs both PI(3)P binding and the recognition of activated IP$_3$Rs (8). However, our experiments revealed that inhibiting cellular PI(3)P levels with VPS34-IN1 had no impact on IP$_3$R downregulation in αT3 cells (Fig. 3.S3), which suggests that PI(3)P is not involved in the erlin-dependent IP$_3$R degradation process.

Based on our findings, we propose a model (Fig. 3.9) to explain the role of the E1/E2 complex in controlling PI(3)P levels and associated cellular functions. In this model, the absence of the E1/E2 complex eliminates key ER-proximal PI(3)P binding sites, resulting in rapid dephosphorylation of PI(3)P, specifically in the autophagic pool. This inhibits the autophagy pathway and by reducing PI(3,5)P$_2$ levels, impairs lysosomal function.
Mutations in the E1/E2 complex have been linked to several neurodegenerative disorders, such as HSPs, which are caused by dysfunction of various cellular processes, including the lysosomal and autophagic pathways (63, 64). Our research reveals that the E1/E2 complex plays a critical role in maintaining PI(3)P levels and is essential for both autophagy and lysosomal acidification and function. These findings may provide insights into the pathological mechanisms underlying erlin mutations that cause HSP.

3.6. Experimental procedures

3.6.1. Cells and reagents

αT3 cells (8), HeLa cells (4) and SH-SY5Y cells (65) were cultured as described. Rabbit antibodies used were: anti-E2 (4), anti-mE1 (2), anti-IP3R1 (65), anti-hVPS34 (#Z-R016, used for IP, Echelon Biosciences) and from Cell Signaling Technology, anti-PIK3R4 (VPS15, #14580), anti-ATG14 (#5504), anti-UVRAG (#5320), anti-PI3Kinase Class III (VPS34, #3811, used for immunoblot), anti-Beclin1 (#3495), anti-LC3 (#4108S). Mouse monoclonal antibodies used were: anti-hE1 (3) (a gift from Dr. Stephen M Robbins, University of Calgary), anti-HA clone HA11 (MMS-101R, Covance), anti-p97 (#10R-P104A, Fitzgerald), anti-GAPDH (#G2320, Santa Cruz). Protease inhibitors, Triton X-100, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), NP-40, Apilimod (#2974), Bafilomycin A1 (#B1793), ATP (#A7699) and secondary antibodies were from Sigma. Reagents for SDS-PAGE were from Bio-Rad. VPS34-IN1 (#17392) was from Cayman Chemical. DNase I (#10104159001) was from Roche. Lysozyme (#89833), EBSS (#24010043), Lipofectamine 2000 (#1634547), and Opti-MEM (#217767) were from ThermoFisher. Protein A-Sepharose CL-4B was from GE Healthcare. Linear, MW ~25,000 polyethyleneimine (PEI) was from Polysciences Inc. GFP-Rab5b (#61802) and
GFP-Rab7a (#28047) were from Addgene. GFP-2xFYVE was a gift from Dr. Nicholas Ktistakis (Babraham Institute) and GFP-LAMP1 was a gift from Dr. Anthony Morgan (Oxford University).

3.6.2. Cell lysis, Electrophoresis, and Immunoblotting

Essentially as described (10), for cell lysis to assess protein expression level, cells were collected with HBSE (155 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4), resuspended in ice-cold Triton lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 10 μM pepstatin A, 0.2 μM soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, pH 8.0), incubated on ice for 30 min, centrifuged at 16,000 x g for 10 min at 4 °C, and supernatants were resuspended in gel loading buffer (final concentration: 1% SDS, 0.05% bromophenol blue, 5% glycerol, 100 mM DTT, 50 mM Tris-HCl, pH 6.8). Lysates were incubated at 55°C for 15 min, and subjected to SDS-PAGE, transfer to nitrocellulose membranes and immunoblotting. Immunoreactivity was detected with Pico Chemiluminescent Substrates (ThermoFisher #34579) and a ChemiDoc imager (Bio-Rad).

3.6.3. Recombinant protein expression and purification

Human E1 and E2 cDNA sequences with a C-terminal HA epitope tag were designed and ordered through GenScript using the codon optimization tool (available at genscript.com). Sequences were subcloned into a pET SUMO vector (Invitrogen, #K300-01), which contains an N-terminal His-SUMO (HS) tag, using Gibson assembly (primers sequence available upon request). HS-E2HAWT was utilized as a template for generating HS-E2HA mutants by inverse PCR and Gibson assembly (primer sequences available upon request).
All constructs were confirmed by DNA sequencing (Genewiz). Essentially as described (66), proteins were expressed in BL21-CodonPlus (DE3)-RIPL E. coli (Agilent Technologies) in Luria broth containing kanamycin and chloramphenicol and cultured at 37°C until OD$_{600}$ 0.6~0.7. After incubation with 1 mM IPTG for ~18 h at 16°C, cells were pelleted (3,000 x g for 15 min at 4°C), were re-suspended in phosphate buffer (300 mM NaCl, 50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM imidazole, 1 mM benzamidine, 5 mM beta-mercaptoethanol, 100 mM arginine-HCl, 100 mM glutamine, 5% glycerol, 0.2% CHAPS, pH 7.4) supplemented with ~0.1 mg/mL DNase I and lysozyme, were sonicated for 5 min (2 sec on, 4 sec off, on ice), and were centrifuged (27,000 x g for 30 min at 4°C). HS-tagged proteins in the supernatant were purified using a nickel-nitrilotriacetic acid column (#H-350-100, GoldBio) and eluted using the same phosphate buffer supplemented with 400 mM imidazole.

3.6.4. Lipid overlay assay

Purified proteins were subjected to a lipid overlay assay as described (8), with slight modifications. Arrays were prepared by spotting 200 pmol of PI(3)P, PI(4)P, PI(5)P, PI, PI(3,5)P$_2$ and PI(4,5)P$_2$ (all diC16, Echelon Biosciences), dissolved in chloroform/methanol/water (1:2:0.8, by volume) onto Protran Supported 0.2-μm nitrocellulose membranes (GE Healthcare). Arrays were incubated and washed with PBS plus 1% nonfat dried milk at 4°C as follows: 1 h preincubation to block; 2 × 2 min washes; ~16 h incubation with bacterially-expressed proteins or immunopurified E1/E2 complex; 2 × 2 min washes; 1 h incubation with anti-E2 or anti-HA; 2 × 2 min washes; 1 h incubation with secondary antibody; 3 × 2 min washes; 1 × 2 min wash with just PBS, and finally exposure to Pico Chemiluminescent Substrates and immunoreactivity was detected using
a ChemiDoc imager. To ensure that equivalent protein concentrations were used in the lipid overlay assay, 50 μL of the incubation mixtures were collected and analyzed by immunoblotting.

3.6.5. Fluorescence polarization (FP) assay

As described (18), an increasing amount of purified protein (0-30 μM final concentration) was mixed with 5 nM BODIPY-phosphoinositide (Echelon Biosciences) in phosphate buffer without CHAPS. Measurements were taken using a FlexStation3 Microplate Reader (Molecular Devices; excitation 475 nm, cut-off filter 495 nm, emission 513 nm). K_d values were determined using GraphPad Prism after subtraction of non-specific signal (nonlinear regression, Binding-Saturation, one site–specific binding).

3.6.6. Generation and analysis of E1, E2 and E1/E2 KO cell lines

The CRISPR/Cas9 system was used to target exons within E1 and E2 genes, using guide RNA (gRNA) oligonucleotides cloned into the pCas-Guide-EF1a-GFP vector (#GE100018, OriGene Technologies). The gRNAs for targeting exon 2 and exon 6 of E1 were GATATGATAGCCTGGTCCACTGG and GACTTAAACCTCATGGCCCCAGG, respectively. The gRNAs for targeting exon 3 and exon 6 of E2 were GCCCTGCTGACTTCGACCAGCGG and TGCAGAACTGGTTCAGTTCGTGG, respectively. HeLa cells (2 x 10^6) were seeded into 10 cm dishes and the next day, cells were transfected with 15 μg of each gRNA construct plus 90 μL of Lipofectamine 2000 and 1 mL of Opti-MEM. GFP-expressing cells were selected 48 h later by fluorescence-activated cell sorting and were distributed at 1 cell/well in 96-well plates. Colonies were expanded and screened in immunoblots for E1 or E2 immunoreactivity, which yielded
E1KO, E2KO and E1/E2KO HeLa cell lines (the latter, created by targeting E2 in E1KO cells). For reconstitution, various erlin cDNAs (hE2HAWT, hE2HAT65I, mE2HAWT, mE2HAF305A and mE1HAWT) (8, 22) and GFP-2xFYVE cDNA were transiently expressed in E1/E2KO HeLa cells (~5 x 10^5 in wells of 6 well plates) using 0.1-2.5 μg of plasmids plus 6 μL 1 mg/mL PEI, pre-mixed in 50 μL of serum-free culture media. After 18 h, cells were harvested and subjected to immunoblotting or ELISA. SH-SY5Y cells (8 x 10^6) were seeded into 10 cm dishes and 24 h later, were transfected with 10 μg of the exon 3 E2 gRNA construct plus 90 μL of Lipofectamine 2000 and 1 mL of Opti-MEM. GFP-expressing cells were cloned as for HeLa cells, yielding E2KO SH-SY5Y cell lines. Multiple independent cell lines from each exon target (HeLa), or from exon 3 (SH-SY5Y) were used for all experiments.

3.6.7. Determination of PI(3)P levels by ELISA

PI(3)P and PI(4,5)P_2 levels were assessed using ELISA kits, K-3300 and K-4500, respectively, and the protocols provided by the manufacturer (Echelon Biosciences). Briefly, HeLa cells (~4 x 10^6), αT3 and SH-SY5Y cells (~12 x 10^6) were lysed with ice-cold 0.5 M trichloroacetic acid and then centrifuged at 1,000 x g for 7 min at 4°C. The resulting pellets were vortexed for 30 sec and washed twice with 5% trichloroacetic acid, 1 mM EDTA at room temperature. The pellets were dissolved in MeOH:CHCl_3 (2:1), vortexed for 10 min, and centrifuged at 1,000 x g for 5 min at room temperature. Pellets were then dissolved in MeOH:CHCl_3:12 M HCl (80:40:1), vortexed for 25 min and centrifuged at 1,000 x g for 5 min at room temperature. CHCl_3 and 0.1 M HCl were then added to the resulting supernatant, which was then vortexed for 30 sec and centrifuged at 1,000 x g for 5 min at room temperature. The lower organic phase, containing the acidic
lipids, was collected and divided into two portions and vacuum dried for the measurement of PI(3)P and PI(4,5)P$_2$. For ELISA, the dried extracts were reconstituted, incubated with a PI(3)P/PI(4,5)P$_2$ detector, and added to a PI(3)P/PI(4,5)P$_2$ coated microplate for competitive binding. A peroxidase-linked secondary detector and colorimetric substrate were then used to detect the bound protein, and measured with a microplate reader (Synergy H1, BioTek) at 450 nm. The concentrations of PI(3)P/PI(4,5)P$_2$ were calculated from standard curves, and the relative PI(3)P level was calculated by dividing the amount of PI(3)P by the amount of PI(4,5)P$_2$ (internal control).

3.6.8. VPS34 complex IP and analysis

To IP endogenous VPS34, WT and E1/E2 KO HeLa cells were harvested with ice-cold NP-40 lysis buffer (20 mM Tris, 137 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1% NP-40, 10% Glycerol, 10 μM pepstatin A, 0.2 μM soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, pH 7.5), incubated on ice for 30 min, and then centrifuged at 16,000 x g for 10 min at 4°C. The supernatants (pre-IP) were then incubated with anti-hVPS34 and protein A-Sepharose CL-4B beads for approximately 16 h at 4°C, the mixture was centrifuged (1,500 x g for 1 min at 4°C) to separate the supernatant (post IP), and the IPs were collected and analyzed by immunoblot or for kinase activity. Briefly, IPs were washed three times with ice-cold PBS, 1% NP-40, followed by two washes with 100 mM Tris-HCl, 500 mM LiCl, pH 7.5, two washes with 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5, and two washes with kinase reaction buffer (20 mM Tris, 200 mM NaCl, 2 mM EDTA, 20 mM MnCl$_2$, 100 μM ATP). Reactions were performed at 30°C with the IPs, 25 μL kinase reaction buffer, 8 μL of 500 μM PI (diC8, P-0008, Echelon Bioscience) and 17 μL H$_2$O. Reactions were terminated by adding 10 μL of 100 mM EDTA.
The amount of PI(3)P generated was measured using the PI(3)P ELISA kit and graphs were plotted using GraphPad Prism (nonlinear regression, curve fit, one site–total).

3.6.9. Fluorescence microscopy

WT and E1/E2KO HeLa cells (~10^5) were seeded on micro cover glasses (VWR) coated with 0.1 mg/mL poly-D-lysine and ~24 h later, were transiently transfected using 0.1-2.5 μg of plasmids plus 6 μL 1 mg/mL PEI, pre-mixed in 50 μL of serum-free culture media. After 18 h, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min and then mounted using Prolong™ Gold antifade reagent with DAPI (Invitrogen) onto glass slides (ThermoFisher). For Lysotracker red staining, cells were incubated with 3 μM Lysotracker™ Red DND-99 (Invitrogen) for 30 min at 37°C and fixed and mounted in the same manner. Imaging was conducted using a Leica SP8 confocal microscope with a 63x oil-immersion objective lens with 488 nm excitation/510 nm emission (for GFP-containing constructs) or 577 nm excitation/590 nm emission (for Lysotracker red).

3.6.10. FITC-NP uptake

WT and E1/E2KO HeLa cells (~10^5) were seeded onto 29 mm glass bottom dishes (CellVis) and 24 h later, were incubated with 500 μg/mL FITC-NP. After 2 h, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min followed by staining with DAPI. Cells were imaged with a Leica SP8 confocal microscope with a 63x oil-immersion objective lens using 495 nm excitation/519 nm emission.

3.6.11. Cathepsin activity assay
Cathepsin B activity was detected using Magic Red™ according to the manufacturer’s protocol (Bio-Rad). Briefly, HeLa cells (6 x 10^4) were seeded into 96-well, black-wall, clear-bottom plates (Corning), and 24 h later, were incubated at 37°C with phenol red-free Dulbecco's modified Eagle's medium (Gibco) and Magic Red™ CTSB reagent (1/250, v/v). Fluorescent signals were detected at different time points using a FlexStation3 Microplate Reader (excitation 592 nm, cut-off filter 610 nm, emission 628 nm) and normalized to protein amount. Graphs were determined using GraphPad Prism after subtraction of non-specific signals (nonlinear regression, curve fit, one site–specific).

3.6.12. Data Presentation, imaging, and statistical analysis

All experiments were performed at least twice. For SDS-PAGE and immunoblotting, representative images are presented with molecular markers (in kDa) on the side. Immunoreactivity was quantitated using ImageLab (Bio-Rad). For microscopy, Z-stacks from individual cells were imported into ImageJ and then projected into one 2-D image for quantification. Fluorescence intensity of GFP-Rab5b, GFP-Rab7a and Lysotracker red was quantified by applying the same threshold in WT and E1/E2KO cells to identify fluorescent areas to obtain the mean intensity per cell. GFP-LAMP1, GFP-2xFYVE, Lysotracker red and FITC-NP puncta were quantified by applying the same threshold in WT and E1/E2KO cells and counting puncta number per cell. N=number of cells analyzed. All quantitated data are expressed as mean ± SEM (n = the number of independent experiments). Statistical analysis was performed using a Student’s t-test (with Welch’s correction in Fig. 3.6) and p-values of <0.05 and <0.01 are denoted with * and ** respectively. P-values of >0.05 were considered not significant and denoted with “ns”.

Data availability
All data described and discussed are located within the manuscript or available upon request.

This article contains supporting information.

Acknowledgments

The authors express their gratitude to Dr. Stephan Wilkens and Dr. Alaji Bah for their invaluable assistance in recombinant protein purification and deletion mutant design. Special thanks are also extended to Dr. Patricia Kane, Xiaokong Gao and Laura Szczesniak for their helpful advice.

Funding information

This work was primarily supported by National Institutes of Health Grants DK107944 and GM121621. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

3.7. References


(2022).


36. Axe, E. L. et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic


44. Amador, M.-D.-M. et al. Spastic paraplegia due to recessive or dominant mutations in ERLIN2 can convert to ALS. Neurol Genet 5, (2019).


46. Rydning, S. L. et al. A novel heterozygous variant in ERLIN2 causes autosomal


65. Wojcikiewicz, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. Journal of Biological Chemistry 270, (1995).
Figure 3.1. Recombinant erlins bind selectively to PI(3)P.

A. Diagram of recombinant erlin constructs expressed in bacteria. Erlin1 and Erlin2 are 348 and 340 amino acids in length, respectively. HS = His-SUMO tag, HA = HA epitope tag.

B. Coomassie blue-stained gel of purified bacterially-expressed HS-E2HA, HS-E1HA and HS-HA, which migrate at ~52, 54 and 19 kDa, respectively (indicated by white stars).

C. Binding to lipid arrays was assessed by probing with anti-E2 (for immunopurified
mammalian E1/E2 complex), or with anti-HA (for recombinant proteins). Right panel: the immunoblot, probed with anti-HA, shows that approximately equimolar amounts of recombinant proteins were used on the lipid arrays.

D. FP assay of BODIPY-PI(3)P binding to HS-E2HA, HS-E1HA and HS-HA, with $K_d$ values (mean ± SEM, n=2).

E. FP assay of BODIPY-phosphoinositide binding to HS-E2HA, with $K_d$ values (mean ± SEM, n=4, * designates p<0.05 and ** designates p<0.01 for differences from the PI(3)P $K_d$ values).
Figure 3.2. PI(3)P binds to a juxta-membrane domain on E2.

A. Diagram of recombinant E2 deletion mutants. Full-length E2 consists of the transmembrane (TM) domain, the juxta-membrane globular (G) domain, coiled-coil domains 1 and 2 (CC1 and 2), the α/β domain (α/β), the assembly domain (AD), and the C-terminal (CT) domain.

B. E2 structure created by Alphafold (https://alphafold.ebi.ac.uk/) and displayed by PyMOL. Region 21-172 (G domain) is shown in red.
C. Coomassie blue-stained gel of purified bacterially-expressed HS-E2HA, HS-E2HA$^{1-177}$, HS-E2HA$^{21-172}$ and HS-E2HA$^{178-340}$, which migrate at ~52, 35, 33 and 36 kDa, respectively (indicated by white stars).

D. Binding of HS-E2HA, HS-E2HA$^{1-177}$, HS-E2HA$^{21-172}$ and HS-E2HA$^{178-340}$ to lipid arrays was assessed by probing with anti-HA. Right panel: the immunoblot, probed with anti-HA, shows that approximately equimolar amounts of recombinant proteins were used on the lipid arrays.

E. FP assay of BODIPY-PI(3)P binding to HS-E2HA, HS-E2HA$^{1-177}$, HS-E2HA$^{21-172}$ and HS-E2HA$^{178-340}$ with $K_d$ values (mean ± SEM, n=4, * designates p<0.05 from the HS-E2HA $K_d$ values).
Figure 3.3. Erlins regulate cellular PI(3)P levels.

A. Effect of erlin KO on PI(3)P levels. Left panel: immunoblot of lysates for various HeLa cell lines, probed with anti-E1 and anti-E2, with p97 serving as a loading control. Right panel: PI(3)P levels in WT, E1KO, E2KO and E1/E2KO HeLa cells (mean ± SEM, n=2, ** designates p<0.01 for differences from WT values).

B. Representative confocal images of WT and E1/E2KO HeLa cells transiently expressing GFP-2xFYVE. Z-stack images were taken and projected (scale bars=20 µm). The graph shows the number of GFP-2xFYVE positive puncta per cell (mean ± SEM, ** designates p<0.01 for differences between WT and E1/E2KO cells; N=28 and 22 cells, respectively).

C. Restoration of PI(3)P levels in E1/E2 KO HeLa cells. Left panel: immunoblot of E1/E2KO HeLa cells transiently expressing exogenous hE2HAWT at levels similar to, or ~5x greater than endogenous E2 in WT cells, probed with anti-E2 to identify both hE2HAWT
and endogenous E2, and with p97 serving as a loading control. Right panel: PI(3)P levels in WT and E1/E2KO cells transiently expressing hE2HA\textsuperscript{WT} (mean ± SEM, n=4, ** designates p<0.01 for differences from PI(3)P levels in non-transfected E1/E2KO cells).

D. Restorative effects of various erlin constructs on PI(3)P levels. Upper panels: immunoblots of E1/E2KO HeLa cells transiently expressing erlin constructs, probed with anti-E2 to identify both hE2HA\textsuperscript{WT} and endogenous E2 (lanes 1-6), or anti-HA to identify mE2HA\textsuperscript{WT} and mE1HA\textsuperscript{WT} (lanes 7 and 8), with p97 serving as a loading control. Lower panel: PI(3)P levels in WT and E1/E2KO HeLa cells transiently expressing erlin constructs (mean ± SEM, n=3, * designates p<0.05 and ** designates p<0.01 from PI(3)P levels in non-transfected E1/E2KO cells).
Figure 3.4. Erlins regulate PI(3)P metabolism.

A. Diagram of PI(3)P-related kinase and phosphatase pathways. Kinases are indicated in red and phosphatases in blue (23-26).

B. The effect of kinase inhibitors on PI(3)P levels in WT HeLa cells. Cells were treated with VPS34-IN1 (left) and Apilimod (right) for 30 min. Cells were then assessed for PI(3)P levels (mean ± SEM, n=2, * designates p<0.05 for differences from control values).
C. Representative confocal images of WT HeLa cells transiently expressing GFP-2xFYVE in the presence or absence of VPS34-IN1 for 30 min. Z-stack images were taken and projected (scale bars=20µm). The graph shows the number of GFP-2xFYVE positive puncta per cell (mean ± SEM, ** designates p<0.01 for differences between DMSO and VPS34-IN1 conditions; N=29 and 19 cells, respectively).

D. VPS34 kinase activity in WT and E1/E2KO HeLa cells. Left panel: immunoblot of VPS34 IPs and pre IP and post IP lysates from both cell types. Right panel: IPs were incubated with PI and ATP and samples were assessed for PI(3)P levels using ELISA (mean ± SEM of triplicates from a representative experiment, n=2).

E. Restoration of PI(3)P levels in E1/E2KO HeLa cells with PI(3)P binding constructs. PI(3)P levels in E1/E2KO cells transiently expressing hE2HA WT or GFP-2xFYVE constructs (mean ± SEM, n≥2, * designates p<0.05 and ** designates p<0.01 for differences from PI(3)P levels in non-transfected E1/E2KO cells).
Figure 3.5. Erlin KO does not affect the endocytic pathway.

Representative confocal images of WT and E1/E2KO HeLa cells incubated with FITC-NP for 2 h (A), or transiently expressing GFP-Rab5b (B), or GFP-Rab7a (C). Z-stack images were taken and projected (scale bars=20 µm). Quantified data are expressed as mean ± SEM (N≥15 cells).
Figure 3.6. Erlin KO inhibits autophagy.

Upper Panel: WT and E1/E2KO HeLa cells were incubated with EBSS in the absence or presence of 100 nM BafA for the times indicated and immunoblots were probed for LC3-II, with p97 serving as a loading control.

Lower panel: Histogram of quantified LC3-II immunoreactivity in E1/E2 KO cells (mean ± SEM, n=4, * designates p<0.05 and ** designates p<0.01 for differences from WT cell values).
Figure 3.7. Erlin KO inhibits lysosome acidification and function.

A. Representative confocal images of WT and E1/E2KO HeLa cells incubated with Lysotracker red for 30 min. A single (2-D) plane and Z-stack images were taken and projected (scale bars=20µm). The graphs show quantified Lysotracker red fluorescence intensity per cell (mean ± SEM, ** designates p<0.01 for differences between WT and E1/E2 KO cells; N=42 and 44 cells, respectively) and Lysotracker red positive puncta per cell (mean ± SEM, ** designates p<0.01 for differences between WT and E1/E2KO cells; N=41 and 45 cells, respectively).

B. Representative confocal images of WT and E1/E2KO HeLa cells transiently expressing GFP-LAMP1. Z-stack images were taken and projected (scale bars=20µm). The graph shows quantified GFP-LAMP1 puncta number per cell (mean ± SEM; N=20 and 20 cells, respectively).

C. Cathepsin B activity in WT and E1/E2 KO HeLa cell (mean ± SEM, n=3, * designates p<0.05 and ** designates p<0.01 for differences between WT and E1/E2KO cells).
Figure 3.8. VPS34 inhibition impairs autophagy and lysosome function.

A. WT HeLa cells were incubated with 1 µM VPS34-IN1 in the absence or presence of 100 nM BafA for times indicated and the immunoblot was probed with anti-LC3-II, with p97 serving as a loading control.

B. Cathepsin B activity in WT HeLa cells with or without 30 min pre-incubation with 1 µM VPS34-IN1(mean ± SEM of triplicates, n=3).
Figure 3.9. Model for how erlins regulate autophagy and lysosome function.

In WT cells (left panel), the presence of the E1/E2 complex provides ER-proximal PI(3)P binding sites that maintain PI(3)P levels. PI(3)P is particularly abundant in the endocytic pathway (yellow), and the autophagic pathway (purple). By stabilizing PI(3)P, the E1/E2 complex maintains PI(3)P levels at the ER, supports the autophagy pathway, and may provide PI(3)P for transfer to lysosomes. In lysosomes, a fraction of PI(3)P is converted to PI(3,5)P₂, which supports lysosome acidification and function.

In E1/E2 KO cells (right panel), PI(3)P is dephosphorylated to PI due to the lack of the binding site provided by the E1/E2 complex. While the levels of PI(3)P remain unchanged in the endocytic pathway (yellow), they are reduced in the autophagic pathway (purple). This reduction in PI(3)P levels inhibits the formation of phagophores and autophagosomes, and may decrease transfer of PI(3)P to lysosomes, leading to reduced PI(3,5)P₂ levels, and compromised lysosomal acidification and function.
Figure 3.S1. PI(3)P levels in E2KO SH-SY5Y cells and E1/E2KO αT3 cells.

Upper Panels: Immunoblots of WT and E2KO SH-SY5Y cells (2 independent clones) and WT and E1/E2KO αT3 cells (8), probed with anti-E1 and anti-E2, with p97 serving as a loading control. Lower Panels: Cells were harvested, and lipid levels were measured by ELISA, as described in Figure 3A (mean ± SEM, n=2, * designates p<0.05 and ** designates p<0.01 for differences between WT and KO cells).
Figure 3.S2. Characterization of the VPS34 complex isolated from WT and E1/E2KO HeLa cells.

Anti-VPS34 IPs (lanes 5-7), pre-IP lysates (lanes 1-2) and post-IP lysates (lanes 3-4) were probed in immunoblots for the key elements of the VPS34 complex (VPS15, ATG14, UVRAG, Beclin1), with GAPDH serving as a loading control (the lane 7 sample contains anti-VPS34 only).
Figure 3.S3. Effect of PI(3)P depletion on IP₃R1 downregulation

Upper Panel: αT3 cells were incubated in the absence or presence of 1 µM VPS34-IN1 for 30 min then cells were treated with 100 nM GnRH for 0, 0.5, and 1h. Immunoblots were probed with anti-IP₃R1, with p97 serving as a loading control.

Lower panel: Quantitated IP₃R1 immunoreactivity graphed as a percentage of t=0 values (mean ± SEM, n=2).
Chapter 4.
Ancillary Data
**Introduction**

This chapter describes smaller projects that either played minor roles or serve to supplement the data presented in Chapter 3.

**4.1. Structural studies on erlin complex binding to PI(3)P**

**4.1.1. Determine the PI(3)P binding region on the G domain of E2**

In our previous study (Chapter 3), we conducted mutagenesis studies using bacterially expressed E2 to investigate the determinants responsible for PI(3)P binding. Our findings revealed that the G domain of E2 interacts with PI(3)P. To pinpoint the specific region or motif on the G domain responsible for PI(3)P binding, we obtained a homology structural model of the G domain from Phyre2 (Fig. 4.1.A). This model was based on the structure of SPFH domain-containing protein HflC, which shares approximately 19% sequence identity with the G domain and exhibits a confidence level of 99.97%. The homology model revealed that the G domain consists of signature β-sheets connected to α-helices. Leveraging this reliable homology prediction, we examined the secondary structure and disorder prediction of the G domain (Fig. 4.1.B).

Afterward, we proceeded to create a set of deletions at the N and C termini of the G domain (Fig. 4.2.A). Throughout this process, our priority was to maintain crucial structural components, such as α-helices and β-sheets, as indicated by the secondary structure prediction in Fig. 4.1.B. Our findings revealed that N-terminal deletions of the G domain, specifically HS-E2HA^{58-172}, HS-E2HA^{78-172}, HS-E2HA^{96-172}, and HS-E2HA^{109-172}, retained the ability to bind to PI(3)P. Interestingly, HS-E2HA^{131-172} showed no interaction with PI(3)P, indicating the essential role of the region spanning 110-130 in PI(3)P binding (Fig. 4.2.B).
4.2.B). On the other hand, C-terminal deletions on the G domain, which are independent of N-terminal deletions, including HS-E2HA\textsuperscript{21-48}, HS-E2HA\textsuperscript{21-57}, HS-E2HA\textsuperscript{21-78}, HS-E2HA\textsuperscript{21-96}, and HS-E2HA\textsuperscript{21-109}, exhibited PI(3)P binding, while HS-E2HA\textsuperscript{21-36} failed to bind (Fig. 2B). This indicates the significance of the amino acid region 37-48 in the binding of PI(3)P.

The parallel FP assays consistently supported this finding (Fig 4.2.C-D), demonstrating that the region HS-E2HA\textsuperscript{21-48} has a lower $K_d$ compared to HS-E2HA\textsuperscript{21-36}, suggest HS-E2HA\textsuperscript{21-48} bind to PI(3)P stronger than HS-E2HA\textsuperscript{21-36}. Similarly, HS-E2HA\textsuperscript{109-172} exhibited a lower $K_d$ value compared to HS-E2HA\textsuperscript{131-172}, suggesting HS-E2HA\textsuperscript{109-172} bind to PI(3)P stronger than HS-E2HA\textsuperscript{131-172}. These results validate the crucial involvement of the G domain in E2's interaction with PI(3)P and emphasize the contribution of two distinct and independent regions (37-48 and 110-130) to the binding affinity.

Upon thorough examination of the G domain structure model, we observed that amino acids 37 to 48 constitute a disordered region near the N terminal, while amino acids 110 to 130 form an $\alpha$-helical region towards the C terminal (Fig 4.1A, regions highlighted in red).

To gain a deeper understanding of the interaction between PI(3)P and E2, we focused on specific amino acids that could play a crucial role in this process. While members of the SPFH family do not possess lipid recognition domains, conventional PI(3)P binding domains like FYVE and PX domains typically contain a cluster of highly basic amino acids, forming a binding pocket within the recognized PI(3)P binding motifs\textsuperscript{1-4}. During our analysis, we identified a cluster of positively charged amino acids (K119, H121, H122) highlighted in blue in Fig 4.1A, located within the key PI(3)P binding $\alpha$-helical region from 109 to 130. Additionally, we found one additional amino acid, arginine 36, adjacent
to the key PI(3)P binding region from 37 to 48. Subsequent mutagenesis experiments were conducted to investigate the impact of the canonical PI(3)P binding clusters. Specifically, point mutations were introduced in amino acids K119, H121, and H122 (K119A, H121A, and H122A), as well as a triple mutation (K119K121K122A) in one construct. Surprisingly, none of these positive charge mutations on the key region has an impact on the binding to PI(3)P.
Figure 4.1. Structure prediction of G domain E2.

A. Phyre2 prediction (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) of G domain E2 homology model based on the model of the modulator of the FtsH protease HflC (PDB: c7vhG_) and displayed by PyMOL. Regions 37-48 and 110-130 are shown in red; K119, H121, H122, T65l, and R36 are highlighted in blue.

B. The secondary structure and disorder prediction of the G domain E2 were performed using Phyre2.
Figure 4.2. PI(3)P binding affinity on different regions of G domain E2.

A. Diagram of recombinant deletion mutants on G domain E2. A series of deletions from the G domain’s N and C termini were created. HS=His sumo, HA=HA epitope tag.

B. Upper panels: Left: HS-E2HA\textsubscript{21-172}, HS-E2HA\textsubscript{58-172}, HS-E2HA\textsubscript{79-172}, HS-E2HA\textsubscript{96-172}, and HS-E2HA\textsubscript{109-172} and HS-E2HA\textsubscript{131-172}; Right: HS-E2HA\textsubscript{21-36}, HS-E2HA\textsubscript{21-48}, HS-E2HA\textsubscript{21-57}, HS-E2HA\textsubscript{21-78}, HS-E2HA\textsubscript{21-96}, HS-E2HA\textsubscript{21-109} and HS-E2HA\textsubscript{21-130} binding to lipid arrays was assessed by probing with anti-HA. Lower panel: the immunoblot, probed with anti-HA, shows that approximately equimolar amounts of recombinant proteins were used on the lipid arrays.

C-D. FP assay of BODIPY-PI(3)P binding to HS-E2HA\textsubscript{21-36}, HS-E2HA\textsubscript{21-48} (C); HS-E2HA\textsubscript{109-172} and HS-E2HA\textsubscript{131-172} (D). Traces shown are the average of triplicate values for an individual representative experiment. K\textsubscript{d} values were determined from 2 independent
experiments using GraphPad Prism software after subtraction of non-specific signal (nonlinear regression, Binding-Saturation, one site – specific binding).
Both the lipid overlay assays and FP assays demonstrated that the PI(3)P binding affinity of HS-E2HA\textsuperscript{21-172 K119K121K122A} was comparable to that of HS-E2HA\textsuperscript{21-172}, as evident from the similar binding affinity observed in the lipid overlay assay and the comparable K\textsubscript{d} values for PI(3)P (Fig. 4.3.A and B). However, mutations on these positive charge clusters that could form canonical PI(3)P binding site (HS-E2HA\textsuperscript{21-172 K119K121K122A}) lost the binding selectivity to PI(3)P, as evidenced by similar K\textsubscript{d} values across different phosphoinositides (Fig. 4.3.B, right panel). This indicates that clusters of positively-charged amino acids in the G domain (K119, H121, H122), do not have a significant impact on PI(3)P binding, but it does affect the specificity of PI(3)P binding, possibly by altering the structural conformation of the binding pocket to accommodate the head group of PI(3)P.

Moreover, the incorporation of the R36A mutation within the G domain of E2 resulted in the protein's inability to retain its initial molecular size. Therefore, during the purification process, the protein was fragmented into multiple fragments, as evidenced by the elution fractions (Fig 4.3.C, lanes 10-13). Consequently, it was not feasible to evaluate the protein's ability to bind to PI(3)P. These findings suggest that the amino acid cluster or motif accountable for the G domain's binding is atypical and necessitates additional exploration through alternative methodologies.
Figure 4.3. G domain E2 point mutations do not affect PI(3)P binding.

A. Left panel: HS-E2HA, HS-E2HA<sup>21-172 K119A</sup>, HS-E2HA<sup>21-172 K121A</sup>, HS-E2HA<sup>21-172 K122A</sup>, HS-E2HA<sup>21-172</sup> and HS-E2HA<sup>21-172 K119K121K122A</sup> binding to lipid arrays was assessed by probing with anti-HA. Right panel: the immunoblot, probed with anti-HA, shows that approximately equimolar amounts of recombinant proteins were used on the lipid arrays.

B. Left panel: FP assay of BODIPY-phosphoinositides binding to HS-E2HA<sup>21-172</sup>. Right panel: FP assay of BODIPY-phosphoinositides binding to HS-E2HA<sup>21-172 K119K121K122A</sup>. Traces shown are the average of triplicate values for an individual representative experiment. K<sub>d</sub> values were determined from 2 independent experiments using GraphPad Prism software after subtraction of non-specific signal (nonlinear regression, Binding-Saturation, one site–specific binding).

C. Coomassie blue-stained gel of bacterially expressed HS-E2HA<sup>21-172 R36A</sup>. TP= Total protein, SF=soluble fraction, FT= flow through,
W = washes, E = elutions. Fragments are clearly observed in lanes 11-13 in elutions, shown by two bands above and below 20 kDa.
4.1.2. Correlation between the erlin complex's assembly and PI(3)P interaction

While investigating the binding of bacterially expressed E2 and its truncations to PI(3)P, we also evaluated their assembly capabilities. Since mammalian E1 and E2 have the ability to spontaneously form high molecular weight complexes\(^5\),\(^6\), we were curious to determine whether the bacterially expressed E1 and E2 exhibit similar characteristics and whether this assembly is related to PI(3)P binding.

A series of E2 truncations were made according to the known domain organization of E2\(^5\) and the predicted three-dimensional E2 structure in Fig. 3.2.B (as shown in Fig. 4.4A). With the blue-native (BN)-PAGE\(^7\), recombinant HS proteins that contain the G domain, such as HS-E2HA and HS-E2HA\(^{\Delta1-24}\) (lacking the transmembrane domain), as well as a series of C-terminal truncations including HS-E2HA\(^{\Delta177-310}\), HS-E2HA\(^{\Delta310-340}\), HS-E2HA\(^{\Delta299-340}\), and HS-E2HA\(^{\Delta177-340}\) were found to assemble into high molecular weight complexes similar to HS-E2HA (Fig. 4.4.C, lanes 1-2, 5-8). However, constructs lacking the G domain, such as HS-E2HA\(^{\Delta1-177}\) and HS-E2HA\(^{\Delta1-275}\), were unable to form high-order complexes (Fig. 4.4.C, lanes 3-4). These findings indicate the importance of the G domain in mediating both PI(3)P binding and assembly.

Further truncations of the G domain, including HS-E2HA\(^{\Delta58-172}\), HS-E2HA\(^{\Delta78-172}\), HS-E2HA\(^{\Delta96-172}\), and HS-E2HA\(^{\Delta109-172}\), as shown in Fig. 4.4.B, which retain the ability to bind to PI(3)P, demonstrated the capacity to form high molecular weight complexes (Fig. 4.4.D, lanes 1-5). In contrast, the truncation lacking the 110-130 region responsible for PI(3)P binding, HS-E2HA\(^{\Delta131-172}\), failed to assemble (Fig. 4.4.D, lane 6). To rule out the possibility of interference from the HS-HA in the assembly process, HS-HA was purified and subjected to BN-PAGE with HS-E2HA\(^{\Delta21-172}\) as a positive control, showing no impact on
the assembly ability (Fig. 4.4E, lane 2 versus 1). Furthermore, these findings suggest a potential relationship between the formation of high molecular weight complexes and the binding to PI(3)P. It is possible that the binding site for PI(3)P is located between different subunits of E2, and any disruption of the complex's integrity could impact the ability to bind to PI(3)P.
Figure 4.4. Correlation between the erlin complex's assembly and PI(3)P interaction

A-B. Diagram of recombinant HS-E2HA and deletion mutants on E2: HS-E2HA, HS-E2HA<sub>Δ1-24</sub>, HS-E2HA<sub>Δ1-177</sub>, HS-E2HA<sub>Δ1-275</sub>, HS-E2HA<sub>Δ177-310</sub>, HS-E2<sub>Δ310-340</sub>, HS-E2<sub>Δ177-340</sub>, and HS-E2<sub>Δ177-340</sub> (A); HS-E2HA<sub>21-172</sub>, HS-E2HA<sub>58-172</sub>, HS-E2HA<sub>79-172</sub>, HS-E2HA<sub>96-172</sub>, and HS-E2HA<sub>109-172</sub> and HS-E2HA<sub>131-172</sub> (B); along with their capacity to form high molecular weight complexes and exhibit PI(3)P interaction, indicated by positive (+) and negative (-) symbols.

C-E. Upper panel: Blue native (BN)-PAGE gel of recombinant deletion mutants on E2: HS-E2HA, HS-E2HA<sub>Δ1-24</sub>, HS-E2HA<sub>Δ1-177</sub>, HS-E2HA<sub>Δ1-275</sub>, HS-E2HA<sub>Δ177-310</sub>, HS-E2<sub>Δ310-340</sub>, HS-E2<sub>Δ299-340</sub>, and HS-E2<sub>Δ177-340</sub>(C); deletion mutants on G domain of E2: HS-E2HA<sub>21-172</sub>, HS-E2HA<sub>58-172</sub>, HS-E2HA<sub>79-172</sub>, HS-E2HA<sub>96-172</sub>, and HS-E2HA<sub>109-172</sub> and HS-
E2HA131-172(D); HS-E2HA$^{21-172}$ and HS-HA(E). Lower panel: the immunoblot, probed with anti-HA, shows that approximately equimolar amounts of recombinant proteins were used on BN-PAGE.
4.1.3. Generating erlin complex structural model

To gain a comprehensive understanding of the erlin complex and its interaction with PI(3)P, it is essential to obtain a high-resolution structure model, which would enable us to visualize the precise binding site of E2 with PI(3)P. We have successfully immunopurified the erlin complex from αT3 cells, which should allow us to obtain a high-resolution structure\(^5\). However, in the event that attaining a 1-3 Å resolution structure of the erlin complex proves to be challenging, a cryo-EM reconstruction at a resolution of 7-9 Å could still yield valuable insights. At this resolution, it would be feasible to identify the overall secondary structure elements such as α-helices and β-sheets, allowing for accurate positioning of the individual erlin subunits in the macromolecular assembly.

In our previous study, we were able to purify the endogenous erlin complex from a mammalian system using immunoprecipitation with immobilized anti-E1 polyclonal antibodies followed by elution with E1 peptide\(^5\). To generate a preliminary erlin structural model, we employed negative stain transmission electron microscopy (TEM)\(^5\). However, one limitation of this technique is that stain-excluding detergent micelles and/or associated lipids could potentially fill a putative central cavity, leading to uncertainties in the structural interpretation\(^5\). In our current study, we implemented a modification to our purification method. Instead of using detergent, we reconstituted the erlin complex with membrane scaffold protein (MSP) and endogenous lipids to form nanodiscs (ND)\(^8\). By utilizing lipid ND, we can maintain the integrity and stability of the complex while avoiding potential detergent-related artifacts or interference, providing a clearer view of the complex structure. Our result shows the Erlin complexes reconstituted with lipid ND revealed ring-shaped particles with a diameter of approximately 360 Å, which is similar to
the previous study using the same purification procedure but with 0.1% Triton X-100 (Fig. 4.5.A).

Using RELION 4.0 2D classification, we analyzed approximately 27,000 particles from 256 micrographs, generating two-dimensional projections and selecting five classes based on similar orientations. A total of 6,707 particles were classified into distinct classes based on their homologous sizes (Fig. 4.5.B). This classification provides a solid foundation for the future three-dimensional reconstruction of the erlin complex.

In order to obtain a three-dimensional model of the erlin complex, additional high-quality erlin complex particles images are required, and to further enrich the erlin complex on a carbon film, we employed a strategy to generate 2-D crystals by binding streptavidin (SA) to a biotinylated lipid monolayer and then attaching the erlin complex to the SA layer using biotinylated MSP (Fig. 4.5.C-D)\(^9,10\). However, the cryo images of the SA-2D crystals (Fig. 4.5.D, right panel) showed inefficient binding of the erlin complex in lipid ND to the SA film. To facilitate the 3-D reconstruction process, it is crucial to obtain a larger number of particle images, including top, side, and intermediate views.
Figure 4.5. TEM and image analysis of the immunopurified Erlin complex.

A. An immunopurified endogenous Erlin complex reconstituted in lipid nanodisc (ND) was visualized using negative staining electron microscopy, with 1% uranyl acetate (UA) for contrast. The representative micrograph shows the complex, and the boxed and circled regions indicate putative "top" and "side" views, respectively. (Scale bar = 100 nm).

B. Five class-averages, obtained from multiple iterations of 2-D alignment and classification starting form a dataset of 6707 particles.
C. Streptavidin (SA) was bound to a biotinylated lipid layer forming a 2-D crystal on the lipid monolayer, spanning a 1.2μm hole in a carbon film. Negative staining was performed using 1% UA.

D. The left panel shows cryo-electron microscopy images of the erlin complex reconstituted in a lipid ND on a SA carbon film. Putative "top" and "side" views are circled and boxed, respectively. The right panel displays a 2-D crystal of streptavidin that did not show any attached erlin complexes.
4.1.4. Discussion

Our experimental results have revealed an intriguing discovery: we have identified two small regions within the G domain that may play a role in PI(3)P binding. The ability to bind PI(3)P may be associated with the formation of high molecular complexes. However, due to the limitations of truncation mutagenesis, we were unable to pinpoint the exact binding sites for PI(3)P using this method. Truncating large regions of a protein or introducing mutations in these truncations can disrupt the protein's integrity, leading to instability, digestion, or even an inability to be expressed in bacterial systems. This is particularly significant for large complexes like the erlin complex, as truncations can result in the loss of biological activity. For example, in Figure 4.3.D, we observed that a point mutation (R36A) in the G domain region caused the protein to be digested or lose its overall functionality. Therefore, it is crucial to carefully evaluate the impact of truncations on both protein stability and function, as the observed effects may not be solely due to interference with the binding site but rather interference with the entire protein structure.

Moreover, our findings challenge the previous research suggesting that the interaction between the erlin complex and PI(3)P is not associated with the formation of high molecular weight complexes. The previous study demonstrated that reconstituting the F305AE2 mutation in the assembly domain in the mouse αT3 cells, which hinders the formation of high molecular weight complexes⁶, resulted in comparable binding ability to PI(3)P, similar to that of WTE2 (data not shown). Nevertheless, it is crucial to acknowledge that there might be variations in the assembly process between proteins expressed in bacterial systems and their mammalian counterparts. In our study, expressing human E2 in
a bacterial expression system may not fully replicate the behavior of mammalian E2 in αT3 cells.

Considering the information presented above, it highlights the necessity of generating a structural model of the erlin complex and its interaction with PI(3)P. During the generation of the model of the erlin complex, several challenges were encountered. Firstly, due to the immunopurification from mammalian cells, the yield of protein was low, and the quality varied from batch to batch. This inconsistency posed difficulties in obtaining consistent results. Additionally, the strategy of generating 2-D crystals of SA on biotinylated lipid monolayers to enrich the erlin complex required optimization. The binding efficiency of erlin ND with SA-2D was suboptimal, and the crystallization of SA-2D was inefficient (Fig. 4.5.D, right panel). Consequently, the amount of erlin material available was insufficient to acquire a substantial number of different viewing angles required for a reliable 3-D reconstruction, typically around 10,000 angles. These limitations highlight the need for further refinement of the experimental procedures to improve the yield and quality of the erlin complex samples for cryo-EM analysis.

The complexity of the erlin complex poses another challenge as we still lack precise information about the exact number of E1 and E2 subunits within the complex, as well as their orientations. A recent study on the high-resolution structure of the bacterial SPFH family protein: HflK/C complex provided valuable insights. This hetero oligomer, which shares a high sequence similarity with E1 and E2, assembles into a large molecular cage that encapsulates four AAA+ protease hexamers. This structural analysis shed light on how SPFH proteins confine their client proteins within specialized membrane microdomains.
Taking inspiration from the 3.3 Å resolution Cryo-EM density of an HflK/C 24-mer structure, we employed this model as a template and integrated the Alphafold-predicted E1 and E2 subunits to create a homology model of the erlin complex (Fig. 4.6.A). HflK and HflC exhibit a 16% sequence similarity with ERLIN1 and ERLIN2, while the predicted similarities for E1 and E2 individually are remarkably high, reaching 100%. This model allows us to simulate how twelve E1 and twelve E2 subunits assemble into a massive complex at a 1:1 ratio. The generated model provides insights into the positive charge clusters that could potentially serve as PI(3)P binding pockets between the two subunits.

In a zoomed-in view (Fig. 4.6.B), the composition of E1 and E2 is depicted in close proximity, with positively charged amino acids highlighted in the atomic structure, which provides a visual representation of the potential PI(3)P interactions between the subunits of E1 and E2. This approach may contribute to our understanding of the structural characteristics and functional implications of the erlin complex, particularly in relation to PI(3)P binding.

Artificial intelligence (AI) programs, such as Alphafold, have proven to be highly effective in predicting protein structures. Leveraging these programs, we were able to generate a 3D structure prediction of the E2 protein (Fig. 3.2. B). Alphafold-predicted E2 also allows us to conduct electrostatic analysis to identify the potential PI(3)P binding site (Fig. 6C). The electrostatic surface potentials of the predicted E2 structure revealed positively charged clusters, which may be indicative of the PI(3)P binding site.

It is worth noting that AI predictions in protein structure determination are primarily based on the amino acid sequence, making it challenging to accurately predict the oligomerization of multiple subunits and account for flexible or intrinsically disordered regions. As a result,
confidence in these areas of protein structure prediction is lower. Moreover, when it comes
to understanding the orientation of the erlin complex on the ER membrane and its precise
interaction with PI(3)P, the structural information obtained from Alphafold and similar
programs has limited utility. To enhance the accuracy of protein structure predictions,
future iterations of AI programs should incorporate additional information, such as the
presence of ligands and ions, post-translational modifications, and subcellular localization.
Acknowledging the significance of conventional analytical methods such as X-ray
crystallography, cryo-EM, and NMR is imperative as they remain indispensable in the field
of protein structure research. Hence, the integration of traditional techniques with AI
predictions will be vital for driving further progress in the study of protein structures.
Figure 4.6. Structure prediction of the erlin complex

A. Side view of the homology model of the erlin complex based on the Cryo-EM structure of the HflK/C 24 heteromer. The upper part shows the N-terminal TM domains, the middle part represents the G domain, and the bottom part depicts the coiled-coil domains.

B. Close-up view of the erlin complex heterodimer, highlighting the architecture of the G domain in red and the 37-48 and 110-130 regions in green. The atom structures of R36, T65 (in red), K119, H121, and H122 (in green) are shown. The adjacent E1 subunit is shown in blue, with positively charged residues K115, K121, H123, and H124 highlighted in red and depicted in atom structure.

C. Electrostatic surface potentials of the predicted 3D Structure of E2 by Alphafold. The N terminal is positioned on the left side, while the C terminal is on the right side. Negative charges are depicted in red, positive charges in blue, and neutral residues in white. *A, B and C are all displayed by PyMOL.*
4.2. Erlin KO does not affect cell proliferation and ER morphology in HeLa cells

4.2.1. Erlin KO does not affect cell proliferation in HeLa cells

In Chapter 1, section 1.5.3, we conducted a literature review to explore the various cellular functions attributed to erlin complex. One study highlighted the role of E2 in promoting the survival of breast cancer cells\(^\text{12}\), as E2 was shown to play a critical role in supporting cell growth and protecting breast epithelial cells from cell death induced by ER stress. Given the fact that high expression of E2 in aggressive breast cancer\(^\text{13}\), our study aimed to investigate its potential involvement in promoting cancer cell growth.

To assess the impact of the erlin complex on cell proliferation, we performed a cell proliferation assay with WT and erlin KO HeLa cells. Equal numbers of WT cells and cells with different erlin KO profiles (E1, E2, or both E1 and E2) were seeded into 96-well plates, and cell counts were recorded over several days. Surprisingly, no significant differences in cell proliferation were observed between the WT and the E1KO, E2KO, or E1/E2KO HeLa cells (Fig. 4.7.A). However, conducting a parallel MTT assay may provide more accurate insights into cell proliferation and further elucidate the potential role of erlin in cancer cell growth.

According to a recent study, it was found that a gain-of-function mutation in E2 resulted in inhibition of the MAPK signaling pathway, leading to a reduction in cell proliferation of neural progenitor cells\(^\text{14}\). It remains possible that erlin may regulate cell proliferation through distinct pathways in various cell lines or cell models due to loss-of-function, gain-of-function mutations, or deletions. Nevertheless, in the present study utilizing HeLa cell lines, no association between E2 and cell proliferation could be identified. To gain a better
understanding of the phenotype and mechanism associated with E2 deletion and mutants, additional cell models, cell lines, or in vivo models should be employed for evaluation purposes.

4.2.2. Erlin KO does not affect ER morphology in HeLa cells

Mutations in E2 have been strongly associated with several neurodegenerative disorders, particularly HSPs\textsuperscript{15–23}. A pathogenesis study of HSPs revealed that proteins involved in coordinated ER morphogenesis and microtubule dynamics are implicated in more than half of HSP cases\textsuperscript{24,25}. This suggests that pathways involved in ER morphogenesis may be linked to the progression of HSP. The role of the erlin complex, particularly E2 in maintaining ER morphology could be associated with cellular pathways that mediate HSPs. A previous study found that the erlin complex is essential for proper ER morphology, as its loss leads to the destabilization of CLIMP63, a protein involved in shaping ER sheets (Schumacher's thesis, unpublished)

To assess the impact of E2 on ER shaping, we employed microscopy techniques to visualize the ER structure using DsRed2-ER in both WT and E2 KO HeLa cells. Our initial observations (Fig. 4.7.B) suggest that the absence of E2 does not significantly alter ER morphology, as there were no notable changes in the overall ER structure. Additionally, we examined CLIMP63 levels in WT and E1/E2 KO HeLa cells, and no significant differences were detected between the two groups (Fig. 4.7.C).

Although the immunoblotting analysis did not reveal differences in the key ER shaping regulator CLIMP63 between WT and erlin KO cells, further investigations using immunofluorescence analysis may help identify potential expression and distribution
variations in the presence or absence of erlin. Future proteomic analyses with WT and erlin KO cells hold the potential to provide valuable insights into the identification of candidate ER regulatory proteins associated with the erlin complex, such as reticulons (tubule-forming proteins), atlastin, Sey1p/RHD3 (ER membrane fusion proteins), kinectin, p180 (ER sheet-regulating proteins), lunapark (ER junction formation protein), and protrudin (a protein involved in regulating the balance between sheets and tubules).
Figure 4.7. erlin KO does not affect cell proliferation and ER morphology

A. Cell growth curves were created for WT, E1KO, E2KO, and E1/E2KO HeLa cells, where cells were plated in 96-well plates with a density of 20,000 cells per well (Day 0). Cell counts were measured on days 1, 2, 3, and 4, and the quantified cell numbers were plotted as fold change compared to Day 0 values. The traces on the graph represent the mean ± SEM, n=2.

B. WT and E2KO HeLa cells were transfected with a plasmid encoding DsRed2-ER. The cells were imaged using a Nikon C2 microscope, scale bar =10 μM.

C. Immunoblot analysis was performed on lysates from WT and E1/E2KO HeLa cells. The blots were probed for CLIMP63, E1, and E2, with P97 serving as a loading control.
4.3. References


Chapter 5.
Summary and future direction
5.1. Introduction

This dissertation has further characterized the interaction between the erlin complex and PI(3)P, revealing novel cellular functions of this complex that were previously unknown. Chapter 3 describes the PI(3)P binding site on the G domain of E2 and erlin deletion disrupts cellular PI(3)P levels. By binding to PI(3)P and stabilizing PI(3)P levels, erlin plays a role in regulating autophagy and lysosome function. Chapter 4 presents additional data related to identifying PI(3)P binding regions on the G domain of E2, generating a 3-D structural model, and exploring other potential cellular roles of erlins. These findings have made significant progress towards creating a high-resolution structural model of the erlin complex and have opened new research avenues to investigate erlin's involvement in various cellular pathways, including autophagy and lysosome function. In the following discussion, I will explore the implications of these findings and propose future research directions.

5.2. Erlins and PI(3)P interaction

The Wojcikiewicz laboratory has been devoted to investigating the ERAD of IP₃R for decades¹⁻⁶. During the exploration of IP₃R stimulation, we identified two previously unknown proteins that bind to activated IP₃R. Through meticulous analysis using Mass spectrometry, we successfully determined these proteins as E1 and E2⁷. Following thorough characterization, we uncovered that these proteins are constituents of the erlin complex, which is a toroidal, 2 MDa hetero-oligomeric complex comprised of E1 and E2 subunits with a ratio ~1:2⁷. The primary cellular function of the erlin complex is to regulate the degradation of IP₃Rs through ERAD by recruiting an E3 ubiquitin ligase RNF170⁴⁻⁶.
Furthermore, through collaboration with a laboratory in France, the Wojcikiewicz laboratory made a remarkable discovery involving a novel point mutation of E2 associated with HSP. The T65I mutation was observed to have dual effects: it not only impairs the binding of PI(3)P but also inhibits the interaction with activated IP$_3$R and subsequent IP$_3$R ERAD$^8$. The study of erlins' lipid binding profile has paved the way for further investigations into the binding between erlins and PI(3)P, opening up new avenues of research in the lipid metabolism area.

In this study, I have successfully identified the G domain and two distinct regions within the G domain that play a role in the binding of PI(3)P based on \textit{in vitro} experiments (Fig. 3.2, and 4.2). To strengthen these conclusions and validate the interaction between E2 and PI(3)P within cellular contexts, conducting an \textit{in vivo} experiment targeting PI(3)P-enriched organelles like early endosomes would offer more compelling evidence. For instance, employing the GFP-G domain as a sensor to observe whether the fluorescence signal becomes concentrated in the PI(3)P-rich organelles could provide further support.

However, there are still several unanswered questions regarding the mechanism of erlin's interaction with PIP lipids. Specifically, it remains unclear how the interaction between E2 and PI(3)P occurs in the context of their topology within the lipid bilayer of the ER.

PI(3)P, a vital component in endocytosis and autophagy-related pathways$^9$, is primarily localized within the cytoplasmic leaflet of ER$^{10,11}$. However, proteolytic digestion experiments have revealed that almost the entire E2, including the G domain, is located within the ER lumen, with only the N-terminus exposed to the cytosol$^7$. This raises the possibility that the recognition of PI(3)P by E2 could occur through several mechanisms. These mechanisms may involve the flipping of PI(3)P to the luminal leaflet of the ER$^{12}$,
the contortion of the membrane during the initial stages of autophagosome formation (considering the high flexibility of the ER membrane)\textsuperscript{13}, or the potential different orientations of erlins, which is supported by the fact that erlins have been found to interact with various ER membrane proteins and components. To gain further insights into these questions, future research utilizing live cell imaging, combined with specific labeling of PI(3)P with quick-freezing and freeze-fracture replica labelling method\textsuperscript{11}, and high-resolution structural model of erlin-PI(3)P interaction (as described in section 4.1.3) could provide valuable information for the erlin complex-PI(3)P interaction and shed light on the underlying mechanisms.

5.3 Erlins and PI(3)P metabolism

I made an intriguing finding that erlin KO resulted in a significant decrease in the level of PI(3)P. It is important to note that PI(3)P is generated by VPS34 complex I and II, in ER and early endosome, respectively\textsuperscript{10}, and is involved in distinct cellular processes: autophagy and endocytosis\textsuperscript{14}. Interestingly, a recently discovered VPS34 complex I inhibitor selectively inhibits autophagy but not endocytosis\textsuperscript{15}, indicating the presence of distinct pools of PI(3)P within the cell. Moreover, additional research has identified an additional pool of PI(3)P on the outer leaflet of the plasma membrane in platelets\textsuperscript{16}, suggesting the existence of multiple pools of PI(3)P. Considering the information at hand and the observation that loss of erlin selectively affects autophagy, without influencing endocytosis (described in sections 3.4.5 and 3.4.6), it is reasonable to suggest that erlin KO specifically impacts the autophagic pool of PI(3)P, while leaving the PI(3)P pool associated with endocytosis unaffected.
The reduction in autophagic PI(3)P levels observed in erlin-deficient cells can be attributed to several potential factors. These include a decrease in VPS34 activity leading to reduced PI(3)P production, hyperactive PIKfyve kinase activity resulting in increased conversion of PI(3)P into PI(3,5)P₂, and enhanced dephosphorylation of PI(3)P back into PI mediated by myotubularins phosphatases. However, our findings using an ELISA-based \textit{in vitro} kinase reaction revealed that VPS34 immunoprecipitated from erlin-deficient cells did not exhibit significantly lower activity compared to control cells (\textbf{Fig. 3.4. D}). Additionally, erlin knockout did not seem to impact other components of the VPS34 complex (\textbf{Fig. 3. S2}). Furthermore, the activity of PIKfyve appeared normal in erlin-deficient cells, as indicated by the lack of significantly increased PI(3)P levels upon treatment with the PIKfyve inhibitor Apilimod (data not shown). Therefore, it can be concluded that erlin does not directly influence PI(3)P production via VPS34 complexes, nor does it affect PIKfyve activity in inhibiting PI(3)P phosphorylation to PI(3,5)P₂. Instead, erlin functions by binding to PI(3)P, thereby slowing down its dephosphorylation process, as discussed in \textbf{section 3.4.4}. However, it is unknown whether erlin regulates PI(3)P phosphatases MTMRs. Conducting a high-throughput proteomics screen, followed by co-immunoprecipitation experiments to identify potential interactions between erlins and MTMRs, would greatly contribute to our understanding of this regulatory process.

Furthermore, it would be beneficial to conduct a comprehensive lipidomic analysis to better understand the impact of erlins deletion on lipid composition. Measuring the levels of various PPI species, such as PI, PI(3)P, PI(4)P, PI, PI(3,5)P₂, PI(4,5)P₂, and PI(3,4,5)P₃ with high-performance liquid chromatography would provide valuable insights into the alterations in lipid metabolism. This expanded lipid analysis would enhance our
understanding of the broader effects of the erlin complex on cellular lipid homeostasis, and metabolism and potentially uncover additional lipid-related pathways affected by erlins deficiency.
Left panel: the erlin complex facilitates the binding of PI(3)P near the ER, maintaining PI(3)P levels. PI(3)P is abundant in the endocytic and autophagic pathways, supporting autophagy and potentially transferring PI(3)P to lysosomes. In lysosomes, PI(3)P is converted to PI(3,5)P$_2$, which aids in lysosome acidification and function. In addition, PI(3)P regulates lysosome regeneration from autolysosome.

Right panel: the absence of the erlin complex leads to a decrease in PI(3)P binding sites, resulting in the dephosphorylation of PI(3)P into PI. While the levels of PI(3)P in the endocytic pathway remain unaffected, they are reduced in the autophagic pathway. This reduction has several detrimental effects: it hinders the formation of phagophores and autophagosomes, reduces the transfer of PI(3)P to lysosomes, leading to a decrease in PI(3,5)P$_2$ levels. Therefore, lysosome acidification and function are compromised, and lysosome reformation is impaired. Furthermore, the absence of the erlin complex results in prolonged cellular Ca$^{2+}$ signaling and downregulation of transcription factors related to...
auto-lysosome processes. These combined effects contribute to impaired autophagy and compromised lysosome function.
5.4. Erlins in autophagy

An intriguing finding was made regarding impaired autophagy in erlin KO HeLa cells (section 3.4.6.) To investigate whether this impairment is directly related to the decreased level of PI(3)P, we used VPS34 inhibitor VPS34-IN1 to deplete PI(3)P level and replicated the effects observed in erlin KO cells, as indicated by a decrease level of LC3-II (Fig. 3.8.A). This suggested that the decreased PI(3)P level due to erlin KO could directly or indirectly impact autophagy via interfere with phagophore formation or autophagy signal transduction (Fig. 5.1, right panel). However, it is important to note that VPS34-IN1, being a potent inhibitor, may have additional effects beyond solely depleting PI(3)P levels. It could potentially interfere with the activation of the VPS34 complex by ULK1 during the initiation of autophagy, as well as the aggregation of the selective autophagy substrate p62, which we did not observe in the erlin KO cells (data not shown). To validate that the observed results were not caused by unintended off-target effects of the VPS34 inhibitor, employing VPS34 small interfering RNAs (siRNAs) could serve as a complementary approach to confirm that the reduction in PI(3)P levels is indeed a consequence of impaired autophagy in erlin KO cells. Furthermore, the investigation into whether the impact on autophagy is due to diminished PI(3)P levels or the erlin deletion can be elucidated by reintroducing erlin KO cells with a mutation (T65IE2) known to impede the restoration of cellular PI(3)P levels. This will allow testing whether the PI(3)P level-deficient mutant can effectively restore autophagy or not.

In addition, loss of erlin could also directly affects the autophagy process. Manganelli et al. reported that E1 interacts with AMBRA1 to promote autophagy. Our previous data (not shown) also indicate that E2 plays a significant role in autophagosome formation by
being concentrated at omegasomes on the ER and potentially interacting with early autophagosome formation proteins including LC3, ATG16, and PI(3)P effectors like DFCP (as observed through colocalization).

Moreover, extensive research has highlighted the significance of Ca\(^{2+}\)-dependent pathways and related effectors, including protein kinase C, Ca\(^{2+}\)/calmodulin-dependent kinase \(\beta\) (CaMKK\(\beta\) or CaMKK2), ERK, and VPS34\(^{20-22}\) in the initiation of autophagy. Based on the existing evidence pointing to the inhibitory effects of IP3R/Ca\(^{2+}\) on autophagy regulation, it can be inferred that the erlin complex, through its involvement in the degradation of calcium channels (IP\(_3\)Rs), may have a role in inhibiting autophagy when it is absent. In the absence of the erlin complex, the degradation of IP\(_3\)Rs is compromised, possibly leading to a prolonged intracellular Ca\(^{2+}\) signaling that ultimately hinders autophagy (Fig. 5.1, right panel).

To investigate the effects of erlin KO on autophagosome formation, a live cell imaging approach can be employed to observe the process at different stages. This would involve tracking the dynamics and localization of key autophagic regulators such as LC3, ATG16, VPS34, DFCP1, and WIPI proteins in both WT and erlin KO cells. Immunofluorescence staining can be used to visualize these proteins and examine any differences or alterations in their abundance, localization, or interactions between the two cell types.

In addition to live cell imaging, a range of techniques including high-throughput proteomics, and CRISPR-based screens can be utilized to identify potential interactions and mechanisms between erlins and autophagy-related regulators or pathways. Furthermore, RNA sequencing can help establish the relationship between erlins and the expression of autophagy-lysosome transcription factors and regulators, such as TFEB and...
TFEB kinases like ERK2 or mTORC1. By integrating these approaches, a comprehensive analysis can be conducted to gain a deeper understanding of the diverse molecular mechanisms involved in the impact of erlin on the autophagy pathway.

5.5. Erlins in lysosome function

Furthermore, erlin KO HeLa cells exhibit impaired lysosome function, characterized by alterations in lysosomal acidification and compromised overall catalytic activity (section 3.4.7.) We wanted to determine if the decreased levels of PI(3)P caused by erlin deletion directly contribute to this effect. To simulate the impact of erlin KO, we used a VPS34 inhibitor, which significantly reduced PI(3)P levels and led to a drastic decrease in lysosome catalytic function (Fig. 3.8.B). However, the specific mechanism by which reduced PI(3)P affects lysosome function is still unknown. In Chapter 3, we discussed a possible explanation involving the involvement of the lipid transporter VPS13C in facilitating the transfer of PI(3)P from the ER to the lysosome. Previous studies have shown that depletion of VPS13C in HeLa cells results in an altered lipid profile in lysosomes, including elevated levels of phosphoinositides\textsuperscript{23}. In recent studies that made use of AlphaFold predictions, the VPS13C architecture was employed to enhance the current understanding of VPS13’s structure. This advancement aided in constructing a comprehensive model of the entire human VPS13C, a paralog linked to Parkinson's disease. VPS13C is situated at the interface of the ER and endo/lysosomes. The resulting model presents an elongated, rod-like configuration measuring around 30 nm, distinguished by a hydrophobic groove that spans its complete length. Moreover, this model reinforces the conjecture that these proteins act as connectors, facilitating the exchange of lipids between opposing membranes\textsuperscript{24}.
Based on this study, a possible explanation can be suggested: In erlin KO cells, the reduced PI(3)P in the ER could lead to a decrease in PI(3)P transfer to the lysosome (Fig. 5.1, right panel). This reduction in PI(3)P levels on the lysosomal membrane could subsequently impact the generation of PI(3,5)P$_2$, which plays a crucial role in regulating the V-ATPase$^{25}$, Ca$^{2+}$ channel (via TRPML1) $^{26}$ and potassium transportation$^{27}$. Consequently, this alteration in PI(3,5)P$_2$ and disrupted pH level and ion channels within the lysosome could compromise lysosomal acidification, thereby contributing to the observed impaired lysosome function in erlin KO cells.

Studies have demonstrated that various pathways and genes can influence lysosome catalytic function. For instance, the transcription of lysosomal proteases mediated by STAT3 has been found to regulate lysosomal functions$^{28}$. As described in section 1.5.5, autophagy-lysosome transcription factor TFEB/TFE3, MYC, and ZKSCAN3$^{29-31}$ are significant regulators of lysosomal genes and erlin could be involved in downregulating these genes and pathways (Fig. 5.1, right panel).

In summary, there remain several unresolved inquiries regarding the regulatory role of erlin in shaping specific lipid profiles and the nature of its interactions within intracellular membrane compartments, particularly ER-lysosome contact sites. Further investigation including immunofluorescence staining, high-throughput proteomics screens, and transcriptome sequencing is required to determine the potential involvement of erlin in these regulatory mechanisms. These studies are crucial for unraveling the precise interplay between erlin, lipid transfer processes, autophagy-lysosome pathway, and lysosomal functions.

### 5.6. Concluding remarks
In the previous work, our lab made significant discoveries regarding the erlin complex by elucidating its role in regulating IP$_3$R ERAD and maintaining intracellular Ca$^{2+}$ homeostasis. Building upon these findings, I further investigated the erlin complex's interaction with PI(3)P and explored its functions beyond IP$_3$R ERAD in the broader context of cell biology.

My research findings have important implications for advancing our understanding of the erlin complex and the underlying mechanisms involved in neurodegenerative diseases. Specifically, I have uncovered the critical role of the erlin complex in maintaining cellular levels of PI(3)P and regulating important cellular processes, including autophagy and lysosome function. These findings have relevance to neurodegenerative disorders such as HSPs, as mutations in the erlin complex have been linked to these conditions and HSPs are characterized by the dysfunction of multiple cellular processes, including those involving lysosomes dysregulation and autophagy. Disruptions in intracellular PI(3)P levels and the impairment of autophagy and lysosomal pathways may involve in the pathological mechanisms underlying erlin mutations related diseases. By elucidating the role of the erlin complex in these processes, our research may contribute to a deeper understanding of these diseases and may pave the way for future therapeutic interventions.

5.7. References

1. Wojcikiewicz, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. Journal of Biological Chemistry 270, (1995).


8. Wright, F. A., Bonzerato, C. G., Sliter, D. A. & Wojcikiewicz, R. J. H. The erlin2 T65I mutation inhibits erlin1/2 complex-mediated inositol 1,4,5-trisphosphate receptor


