The Pennsylvania State University

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ENDOPHILIN B2:
AN ENDOCYTIC AND AUTOPHAGIC REGULATOR

A Dissertation in
Pharmacology

by

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ABSTRACT

This dissertation employed genetic approaches to study the impact of endophilin B2 on apoptosis, autophagy, and endocytic trafficking, which when disrupted can be associated with a variety of disorders including cancer and neurodegeneration. The endophilin family of proteins (including the endophilin A and endophilin B subfamilies) contains an amino-terminal Bin/Amphiphysin/Rvs (N-Bar) domain that induces membrane curvature, and a Src homology 3 (SH3) domain that facilitates protein-protein interactions. Endophilin B1 (SH3GLB1/Bif-1) is a positive regulator of endosome maturation, autophagy, apoptosis, and mitochondrial dynamics, but its major binding partner and fellow family member endophilin B2 (SH3GLB2) has not been as extensively studied.

To help understand the physiological role of endophilin B2 in vivo, we generated knock-out mice. Endophilin B2 was found not to be essential for embryonic development as mice were born at expected Mendelian ratios and did not exhibit any overt physiological defects during development. Regardless of the lack of disease phenotypes, further work revealed that tissue expression of endophilin B2 is similar to that of endophilin B1. We also found that isoforms of endophilin B2 are differentially expressed in a tissue specific manner.

We then tested the hypothesis that endophilin B2 is not redundant to the functions of endophilin B1. Using mouse embryonic fibroblasts (MEFs) isolated from knock-out mice, we explored the role of endophilin B2 in mitochondrial apoptosis, endocytosis, endosome maturation, and autophagy. We found that endophilin B2 was not essential for mitochondrial apoptosis resulting from over-expression of pro-apoptotic proteins or treatment of cytotoxic agents. Lack of endophilin B2 also failed to affect MEF mitochondrial dynamics studied via cell fusion assays. Using fluorescently-labeled dextran, we determined that ablation of endophilin B2 had no effect on endocytic internalization, but did decrease the acidification of endosomes assessed by dextran particles conjugated with a pH sensitive fluorescent dye. To further evaluate the role of endophilin B2 as a regulator of endocytic maturation, MEFs were infected with
influenza-A virus. Endophilin B2 could localize with incoming viral particles, and decreased endophilin B2 expression reduced the amount of viral genome replication that took place in the nuclei of cells. Finally, we used CRISPR-Cas9 gene editing technology in HeLa cells to delete endophilin B2. This attenuated the degradation of stimulated epithelial growth factor receptors (EGFR) by delaying the delivery of the receptor to lysosomes.

Since autophagy is highly dependent upon the endocytic pathway, we further sought to determine if endophilin B2 is an autophagic regulator. We induced autophagic flux through nutrient deprivation in endophilin B2 deficient HeLa cells where loss of endophilin B2 was observed to decrease autophagic flux potentially through the ineffective delivery of material to lysosomes. Re-expression of endophilin B2 in MEFs was observed to restore autophagic flux, confirming the impact of endophilin B2 on the autophagy pathway. In addition, we identified that the endophilin B2 can interact with the class-III phosphoinositide 3-kinase complex involved in phospholipid formation. This observation may explain, at least partially, the observed results with endosome and autophagosome maturation.

In summary, this dissertation has found new roles for endophilin B2 in endosome maturation and autophagic flux, while showing that it is not critical for endocytosis, mitochondrial apoptosis, and mitochondrial dynamics.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xiii</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Endocytic membrane trafficking</td>
<td>1</td>
</tr>
<tr>
<td> Endocytosis</td>
<td>1</td>
</tr>
<tr>
<td> Coating protein mediated endocytosis</td>
<td>2</td>
</tr>
<tr>
<td> Coating protein independent endocytosis</td>
<td>4</td>
</tr>
<tr>
<td> Endosome maturation</td>
<td>5</td>
</tr>
<tr>
<td> Early endosome sorting</td>
<td>5</td>
</tr>
<tr>
<td> Molecular mechanism of maturation</td>
<td>9</td>
</tr>
<tr>
<td> Endosomal acidification and lysosomal maintenance</td>
<td>12</td>
</tr>
<tr>
<td>Autophagy</td>
<td>14</td>
</tr>
<tr>
<td> Basic functions and regulation by mTORC1</td>
<td>14</td>
</tr>
<tr>
<td> Autophagosome maturation</td>
<td>15</td>
</tr>
<tr>
<td> Initiation</td>
<td>15</td>
</tr>
<tr>
<td> Elongation and closure of autophagosomes</td>
<td>16</td>
</tr>
<tr>
<td> Fusion and trafficking of autophagosomes</td>
<td>19</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>21</td>
</tr>
<tr>
<td> Extrinsic pathway</td>
<td>22</td>
</tr>
<tr>
<td> Intrinsic pathway</td>
<td>22</td>
</tr>
<tr>
<td>Mitochondrial dynamics</td>
<td>25</td>
</tr>
<tr>
<td> Cellular role</td>
<td>26</td>
</tr>
<tr>
<td> Molecular mechanisms: fission, fusion, and mitophagy</td>
<td>28</td>
</tr>
<tr>
<td>The endophilin family of proteins</td>
<td>30</td>
</tr>
<tr>
<td> Endophilin protein structure</td>
<td>30</td>
</tr>
<tr>
<td> Endophilin A family and functions</td>
<td>33</td>
</tr>
<tr>
<td> Endophilin B family and functions</td>
<td>33</td>
</tr>
<tr>
<td> Discovery, expression profile, and localization</td>
<td>33</td>
</tr>
<tr>
<td> Endophilin B1 in mitochondrial dynamics and apoptosis</td>
<td>34</td>
</tr>
<tr>
<td> Endophilin B1 in endosomal maturation</td>
<td>35</td>
</tr>
<tr>
<td> Endophilin B1 in autophagy</td>
<td>36</td>
</tr>
<tr>
<td> Endophilin B2</td>
<td>37</td>
</tr>
<tr>
<td>Summary</td>
<td>40</td>
</tr>
<tr>
<td>Chapter 2: Materials and Methods</td>
<td>41</td>
</tr>
</tbody>
</table>
Antibodies and plasmids.................................................................41
Generation of endophilin B2 knockout mice.................................42
Semi quantitative reverse transcriptase PCR (RT-PCR)......................43
Cell culture, viral transduction and generation of stable cell lines........44
Immunoblotting ........................................................................44
Fluorescence microscopy ................................................................45
Mitochondrial morphology and dynamics assay...............................45
Cell viability and apoptosis assays.................................................46
Endocytosis assays ......................................................................46
Autophagic flux assays ..................................................................47
Virus entry assay .........................................................................47
Immunoprecipitation ....................................................................48
Statistical analyses ......................................................................48

Chapter 3: Results ...........................................................................49

Preface ..........................................................................................49
Endophilin B2 is dispensable for embryonic development: ...............50
Endophilin B2 is dispensable for mitochondrial apoptosis and
mitochondrial dynamics: ..............................................................55
Endophilin B2 plays a critical role in endosome maturation: ............58
Endophilin B2 deficiency decreases autophagic flux upon nutrient
starvation: ..................................................................................66
Loss of endophilin B2 delays nuclear translocation and replication of
influenza A viruses: .....................................................................70
Endophilin B2 interacts with UVRAG ...........................................72

Chapter 4: Discussion ....................................................................73

References: ..................................................................................79
LIST OF FIGURES

Figure 1.1: Overview of coating protein mediated endocytosis ........................................... 3
Figure 1.2: Compartmentalization of phosphoinositides in endocytosis and endosome trafficking .................................................................................................................. 4
Figure 1.3: ESCRT mediated multi-vesicular body formation ................................................. 7
Figure 1.4: Overview of sorting mechanisms ........................................................................... 9
Figure 1.5: General overview of endosome maturation .......................................................... 13
Figure 1.6: LC3 lipidation ubiquitin-like reaction ................................................................... 18
Figure 1.7: Autophagy overview ............................................................................................. 21
Figure 1.8: Methods of apoptosis induction .......................................................................... 24
Figure 1.9: Interactions of the BCL-2 family of proteins in apoptosis induction ............... 25
Figure 1.10: Mitochondrial dynamics effects on cell physiology .......................................... 27
Figure 1.11: Mitochondrial fission and fusion proteins ......................................................... 30
Figure 1.12: N-BAR proteins structure induces membrane bending .................................... 32
Figure 1.13: Endophilin family protein alignment ................................................................. 39
Figure 3.1: Endophilin B1 and B2 share similar expression profiles in mice. ................. 51
Figure 3.2: Multiple isoforms of endophilin B2 exist in brain tissue .................................... 52
Figure 3.3: Endophilin B2 isoforms protein alignment ......................................................... 53
Figure 3.4: Semi-quantitative PCR for Endophilin B2 ......................................................... 54
Figure 3.5: Endophilin B2 is dispensable for apoptosis induction in MEFs and HeLa cells. ................................................................................................................................. 56
Figure 3.6: Endophilin B2 is dispensable for mitochondrial dynamics and morphology ......................................................................................................................... 58
Figure 3.7: Endophilin B2 colocalizes with endophilin B1 and is enriched in endosomal and autophagosomal compartments ......................................................... 60
Figure 3.8: Endophilin B2 deficiency impairs endosomal acidification ............................. 62
Figure 3.9. Loss of endophilin B2 suppresses EGFR degradation. .......................... 64

Figure 3.10. Endophilin B2 deficiency impairs the lysosomal delivery of internalized EGF. ........................................................................................................... 66

Figure 3.11. Loss of endophilin B2 suppresses autophagic flux. .......................... 69

Figure 3.12: Endophilin B2 deficiency attenuates nuclear trafficking of influenza A viral nucleoprotein. ................................................................. 71

Figure 3.13: Endophilin B2 interacts with UVRAG without stimulation .......... 72

Figure 4.1: Endophilin B2 expression in breast and gastric cancers affects patient survival ............................................................................................... 78
LIST OF TABLES

Table 2.1: Semi-quantitative PCR protocol .................................................. 44
LIST OF ABBREVIATIONS

MEFs: Mouse embryonic fibroblasts
PI(4,5)P2: phosphatidylinositol 4,5-bisphosphate
PIPKI: phosphatidylinositol 4-phosphate 5-kinase
PI(4,5)P3: phosphatidylinositol 3, 4, 5-trisphosphate
Class-I PI3K: class-I phosphatidylinositol 3-kinases
AP2: adaptor protein 2
Dnm1: Dynamin-1
Dnm2: Dynamin-2
GPI-linked proteins: glycosylphosphatidylinositol anchor linked proteins
EGFR: epithelial growth factor receptor
VEGF: vascular epidermal growth factor
PI3P: phosphoinositol 3 phosphate
INPP4A/B: inositol polyphosphate 4-phosphatase A or B
Class-III PI3K: class-III phosphoinositol 3 kinase
UVRAG: ultraviolet radiation resistance associated gene
ESCRT: endosomal sorting complex for transport
HRS: hepatocyte growth factor-regulated tyrosine kinase substrate
STAM: signal transducing adaptor molecule
AAA: ATPase associated with diverse cellular activities
MVB: multivesicular bodies
SNX: sorting nexin protein
BAR: Bin/Amphiphysin/Rvs domain
VPS: vacuolar protein sorting protein
DSCR3: Down’s syndrome critical region 3
M6P: mannose 6-phosphate receptors
PI(3,5)P2: phosphoinositol 3,5 bisphosphate
GEF: guanine exchange factor
CORVET: class C core vacuole/endosome tethering factor
HOPS: homotypic fusion and protein sorting
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
RILP: Rab interacting lysosomal protein
v-ATPase: vacuolar-ATPase
mTORC1: mechanistic (formally mammalian) target of rapamycin complex 1
mTORC2: mechanistic (formally mammalian) target of rapamycin complex 2
ATP: adenosine tri-phosphate
AMP: adenosine mono-phosphate
AMPK: AMP protein kinase
PKB, AKT: protein kinase B
ATG: autophagy related protein
ER: endoplasmic reticulum
ULK1: uncoordinated 51-like kinases 1
FIP200: focal adhesion kinase family kinase-interacting protein of 200 kDa
WIP1: WD-repeat protein interacting with phosphoinositides
ATG14L: ATG14-like protein
MAPLC3/ LC3: microtubule-associated protein 1 light chain 3
PE: phosphatidylethanolamine
LIR: LC3 interacting region
bNIP3: BCL-2 interacting protein 3
FUND1: FUN 14 domain containing 1
SNX17: syntaxin17
SNAP29: synaptosome associated protein 29
PLEKH1M1: pleckstrin homology and RUN domain containing M1
MOMP: mitochondrial outer membrane permeabilization
TNFR1: Tumor necrosis factor receptor 1
CD95: cluster of differentiation 95 receptor
DR: death receptor(s) DR3, DR4, DR5
TNF-α: tumor necrosis factor-α
FasL: first-apoptosis signal ligand
FADD: Fas-associated death domain
DISC: death inducing signaling complex
ROS: reactive oxygen species
UV: ultra-violet radiation
BCL-2: B-cell lymphoma-2 family
BH-3: BCL-2 homology-3
BAX: BCL-2 associated X
BAK: BCL-2 antagonist/killer
Smac/DIABLO: second mitochondria-derived activator of caspase/ direct inhibitor of apoptosis-binding protein with low pI
HtrA2/OMI: high temperature requirement protein A2
APAF1: apoptotic protease-activating factor 1
BID: BH3 interacting-domain death agonist
t-BID: truncated-BID
TCA cycle: tricarboxylic acid cycle
Mitophagy: selective autophagic clearance of damaged mitochondria
\( \Delta \Psi_m \): mitochondrial membrane potential
mtDNA: mitochondrial genome
Drp1: dynamin-related/-like protein 1
MiD49: mitochondrial dynamics protein of 49 kDa
MiD51: mitochondrial dynamics protein of 51 kDa
Mff: mitochondrial fission factor
Mfn1: mitofusin 1
Mfn2: mitofusin 2
Opa1: optic atrophy 1 protein
PINK1: PTEN-induced kinase-1
N-BAR: amino-terminal- Bin/Amphiphysin/Rvs domain
H0: Helix 0 amphipathic membrane insertion region (scaffolding)
H1: Helix 1 amphipathic membrane insertion region (membrane insertion)
SH3: Src-homology 3 domain
SH3GLB1/ BIF-1: Endophilin B/ BAX interacting Factor 1
SH3GLB2: Endophilin B2
TIP30: HIV-1 tat interactive protein 2, 30 kDa
ACSL4: acyl-CoA synthetase long chain family member 4
NGF: nerve growth factor
TrkA: Tropomyosin-related kinase A receptor
CPG2: candidate plasticity gene 2
MAPK: mitogen-activated protein kinase pathway
NF-\( \kappa \)B: nuclear transcription factor-kappa B
PLC-\( \gamma \): phospholipase C-\( \gamma \)
PTEN: phosphatase and tensin homologue deleted on chromosome 10
semi-quantitative RT-PCR: semi-quantitative reverse transcription PCR
BMF: BCL-2 Modifying Factor
MOI: multiplicity of infection
ActD: actinomycin D
STS: staurosporine
PEG: Poly-ethylene glycol
FITC: fluorescein
Baf A1: Bafilomycin A1
vRNP: viral ribonucleoprotein particle
vRNA: viral RNA genome
NP: viral nucleoprotein
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Dedication:

I dedicate this dissertation to my family.
Chapter 1: Introduction

Endocytic membrane trafficking

The plasma membrane is the barrier to the extracellular milieu, thus any non-diffusible forms of communication to and from the surrounding environment has to occur through receptors or ion channels embedded in the plasma membrane. In response to receptor stimulation, or solute changes in the extracellular milieu, the cell can change the abundance of receptors or ion channels on the plasma membrane through endocytosis or exocytosis. Endocytosis is the de novo production of internal vesicles from the plasma membrane to internalize stimulated cell receptors or extracellular material [1]. Exocytosis functions in opposition, whereby the internalized vesicle can fuse with the plasma membrane releasing the vesicle contents or replace receptors and membranes back to the cell surface. Whether or not the vesicle is budding or fusing with the plasma membrane, cells rely on a system of intracellular trafficking that dictates the fate of the contents of the vesicle [2-4]. As such, endocytosis and subsequent endosome sorting can greatly affect overall cell and organism homeostasis.

Endocytosis

The cell can internalize a wide array of substances through endocytosis including: ligand-bound receptors, other cells, bacteria, viruses, macromolecules, or simply sample the external milieu through random internalization of the surrounding fluid [1]. This can be accomplished via numerous mechanisms ranging from phagocytosis (bacteria, apoptotic cells), macropinocytosis (large portions of extracellular fluids and plasma membrane), pinocytosis (smaller scale), and endocytosis mediated through endosome coating proteins. Regardless of the method, cells require modification of the plasma membrane in order to internalize receptors or other material from the external milieu.
Coating protein mediated endocytosis

In general, the membrane coating proteins clathrin or caveolin assist in the internalization of budding endocytic vesicles by stabilizing (clathrin) or inducing membrane curvature (caveolin) and thereby subsequently recruit other downstream mediators of endocytosis (Fig. 1.1A). Before the membrane coating protein clathrin is recruited to the plasma membrane, upon receptor activation and ubiquitination, changes to the plasma membrane polarity are necessary. Production of negatively charged phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) from phosphatidylinositol-4-phosphate by members of the phosphatidylinositol-4-phosphate-5-kinase (PIPKI) and subsequently production of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) by class-I phosphatidylinositol 3-kinases (class-I PI3K) help recruit other effectors of signal transduction and membrane bending proteins (Fig. 1.2A) [5-7]. For example, PI(4,5)P₃ on the inner plasma membrane leaflet subsequently recruits other membrane effectors to induce membrane curvature such as Epsin, endophilin A proteins, and Amphypysin [5, 7-9]. Adaptor protein complexes, such as the adaptor protein 2 (AP2) complexes, are then recruited through interactions with the membrane curvature proteins and ubiquitin tags on receptors. Adaptor protein AP2 can then recruit clathrin to membranes to stabilize the budding endocytic vesicle [1, 6].

In a similar yet distinct pathway, the caveolin proteins (caveolin1, 2, 3) coat the plasma membrane to create caveolae, which affects receptor clustering on the plasma membrane and facilitate endocytosis [1, 10]. Expression of the caveolin proteins is tissue dependent: caveolin 3 is found primarily in muscle tissues, while caveolin 1 and 2 are more ubiquitous [1]. Unlike clathrin, caveolin proteins directly integrate into membranes through a transmembrane domain and subsequently recruits scaffolding proteins through a central scaffolding domain [10]. Caveolin is dependent upon cholesterol and lipid rafts instead of negatively charged head groups on lipids, therefore caveolae tend to be constitutively formed at the plasma membrane (Fig. 1.1A) [1, 10].

Both the clathrin and caveolin mediated endocytic pathways use the membrane scission GTPase protein dynamin (Dynamin-1 (Dnm1) for clathrin, Dynamin-2 (Dnm2)
for caveolin) to constrict the plasma membrane enough to separate the budding endocytic vesicle from the plasma membrane to create a new vesicle [10]. For both caveolin and clathrin, dynamin scission is essential for proper internalization and completion of endocytosis [10-13]. Dynamin, upon tubulation of the plasma membrane, is recruited to the vesicle neck portion of budding endosomes through interactions with membrane sensing/bending proteins such as endophilin A and amphiphysin (Fig 1.1B)[9, 14, 15].

**Figure 1.1: Overview of coating protein mediated endocytosis**

(A) General diagram of clathrin or caveolin coated pits observed at the plasma membrane. Clathrin assembles at the membrane upon receptor activation whereas caveolin proteins are constantly forming pits known as caveolae at the plasma membrane. Figure adapted from the review authored by Doherty, G.J. and McMahon, H.T [1].

(B) Temporal diagram of clathrin coating assembly beginning with receptor activation recruitment of clathrin at membranes after lipid polarity and adaptor protein recruitment to the plasma membrane. Clathrin assembly aids in stabilizing the invagination of the plasma membrane with the help of the actin cytoskeleton, although the actin cytoskeleton is not essential. Completion of vesicle formation occurs upon scission at the vesicle neck through N-Bar protein tubulation of the membranes and recruitment of the membrane scission protein dynamin 1. Figure adapted from the review authored by Merrifield, C.J. and Kaksonen, M [6].
Figure 1.2: Compartmentalization of phosphoinositides in endocytosis and endosome trafficking

Spatial diagram of the various phosphoinositides changes that occur during endocytosis (A) and endosome maturation processes (B, C). (A) Receptor activation at the plasma membrane (purple) results in the production $\text{PI}(3, 4, 5)\text{P}_3$ by the class-I PI3K (p110-blue). Production of $\text{PI}(3, 4)\text{P}_2$ can occur through dephosphorylation of $\text{PI}(3, 4, 5)\text{P}_3$ by the various phosphoinositide 5-phosphatases present, or by the class-II PI3Kα. $\text{PI}(3, 4)\text{P}_2$ at the plasma membrane is essential for the creation of clathrin coated pits (CCP) and the subsequent maturation into vesicles (CCV). (B) The endocytic vesicle, upon un-coating of clathrin, can then fuse with the early endosomes. $\text{PI3P}$ formation at the early endosome is primarily performed by the class-III PI3K complex II containing UVRAG, while dephosphorylation by inositol polyphosphate 4 phosphate A or B(INPP4A/B) from the incoming $\text{PI}(3, 4)\text{P}_2$ can contribute. $\text{PI3P}$ can be dephosphorylated by the Myotubularins (MTMs), to return back to phosphoinositol. $\text{PI3P}$ is important for the sorting process, which involves the formation of the multi-vesicular body (MVB) within late endosomes. (C) Maturation from early to late endosomes involves the production of $\text{PI}(3, 5)\text{P}_2$ at the early endosome by PIKfyve, while $\text{PI}(3, 4)\text{P}_2$ is observed at the early/late endosomes. The function is not quite clear. The formation of $\text{PI}(3, 5)\text{P}_2$ on late endosomes serves as a docking location for mTORC1, as well as an essential part for late endosome fusion to lysosomes. Figure adapted from Marat and Haucke [16].

Coating protein independent endocytosis

Although coating proteins have been well studied, there are other mechanisms of internalization that are devoid of coating proteins. As such, these endocytic pathways rely solely upon lipid composition particularly through cholesterol concentrations or the receptor protein composition such as having a glycosylphosphatidylinositol anchor (GPI-linked proteins) [1]. Although epithelial growth factor receptor (EGFR) or vascular epidermal growth factor (VEGF) are typically internalized by clathrin-mediated
endocytosis, a recent study identified endophilin A2 as being responsible for mediating internalization through a clathrin or caveolin independent mechanism [17]. Though this endophilin A2 mediated mechanism of “fast endocytosis” requires dynamin for internalization [17, 18], some of the coating protein independent forms of internalization can be dynamin independent [1].

The internalization of large macromolecules, pathogens, dead cells, or portions of extracellular fluids does not utilize the coating proteins. Instead, the plasma membrane is altered to enclose the material to be internalized through the actions of the actin cytoskeletal network [1]. Regardless of the method of internalization, the plasma membrane is altered to internalize the material either through modifications of polarity, lipid composition, or through physical means. The internalized material, receptors or otherwise, is consequently processed within the cell through endosome trafficking processes.

**Endosome maturation**

 Trafficking of the resultant endocytic vesicle within the cell depends on the cell type, type of receptor ubiquitination, or the particular amino-acid motifs of the receptor [3]. Receptors such as EGFR and/or the accompanying adaptor proteins (such as beta-arrestin for G-protein coupled receptors) are tagged with multiple ubiquitin proteins that serve as a destination and sorting tag for how the receptor is to be processed [19]. Ubiquitination can be at multiple locations on the receptor or in a poly-ubiquitin chain. Regardless, multiple ubiquitin tags are necessary for effective endocytic internalization [20]. Sorting of almost all incoming receptors or material occurs in the early endosome compartment [2, 3]. The internalized receptors, upon sorting, either undergo a maturation process that leads either to lysosomal degradation of receptors or trafficking back towards the plasma membrane.

**Early endosome sorting**

Simplistically, incoming endosomes are sorted at early endosomes to be either recycled back towards the plasma membrane or degraded by lysosomes. Similarly to
endocytosis, endosome trafficking also requires negatively charged phosphoinositides for recruiting accessory proteins necessary for sorting and trafficking of ubiquitin tagged receptors and material [5].

Early endosomes obtain phosphoinositol 3 phosphate (PI3P) from the incoming endocytic vesicles of the PI(3, 4)P2 variety through dephosphorylation by inositol polyphosphate 4-phosphatase (INPP4A/B) position on the inositol ring (Fig. 1.2B) [7, 16]. In addition, the majority of the negatively charged phosphatidylinositol head groups at the early endosome are phosphorylated by the class-III phosphoinositide 3 kinase (class III-PI3K) VPS34 to create PI3P (Fig. 1.2B) [5, 21]. VPS34 along with regulatory subunits p150 and Beclin-1 form the core of the class-III PI3K, while accessory protein ultraviolet radiation resistance associated gene (UVRAG) aids in early endosome localization and activity [22-24]. Regardless of how PI3P is produced, PI3P on early endosomes helps to recruit downstream effectors of sorting towards degradation or recycling [25, 26].

The endosomal sorting complex for transport (ESCRT) machinery helps to isolate and define the receptors and materials destined for lysosomal degradation at the early endosomes [26, 27]. The ESCRT machinery consists of four complexes (ESCRT 0, I, II, and III) that work together to capture the ubiquitin tagged receptors to prevent the recycling or retrograde trafficking by forming intralumenal vesicles within the early endosomes/late endosomes (Fig. 1.3) [3]. Recruitment of the ESCRT machinery begins with ESCRT-0 complex recognition of PI3P on the early endosome membrane. ESCRT-0 interacts with PI3P through the FYVE domain contained within the protein complex member hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) [26]. In addition to binding PI3P on the endosome, ESCRT-0, through the signal transducing adaptor molecule (STAM) and Epsin-15B proteins, interact with clathrin-coated vesicles facilitating the localization of incoming cargo to early endosomes [26]. Subsequently, ESCRT-0 can then recruit ESCRT-I that, in turn, recruits another PI3P interacting complex ESCRT-II. Ubiquitin recognition occurs through the ubiquitin binding domain found on the ESCRT(s)-0, I, and II [26, 27]. Once together, ESCRTs-0, I, and II then recruit ESCRT-III that functions to create the intralumenal vesicles through interaction
with the ATPase associated with diverse cellular activities (AAA) VPS4 which can mediate constriction and fission of intraluminal vesicles (Fig. 1.3) [26]. Formation of intraluminal vesicles, or multivesicular bodies (MVB), serves to effectively inactivate the receptor by removing it from the cytosol and to package the internalized receptors and glycosylated lipids for more effective degradation in lysosomes [3].

Figure 1.3: ESCRT mediated multi-vesicular body formation
Schematic of ESCRT recruitment and formation of multivesicular bodies (MVB) relies upon the presence of PI3P on early endosomes and late endosomes as well as ubiquitination of receptors. PI3P, along with ubiquitinated receptors, helps to recruit ESCRT-0 through the HRS protein found within the complex. ESCRT-0 then can recruit ESCRT-I which recognize more ubiquitin proteins and recruits ESCRT-II. ESCRT-II binds to PI3P as well as ubiquitinated receptors and recruits ESCRT-III to induce membrane invagination. ESCRT-III subsequently recruits VPS4 (not shown) to scission the newly formed intralumenal vesicle. Figure adapted from review authored by Raiborg, C. and Stenmark, H. [27].

Sorting towards recycling or retrograde trafficking towards the Golgi is performed by the retromer complex, which for mammals exists as two sub-complexes consisting of the sorting nexin (SNX) family [25]. The heterodimeric complex consists of either SNX1 or SNX2 and either SNX5, SNX6, or SNX32 in any possible combination. The complex
is known simply as the SNX-BAR dimer as the proteins contain an Bin/Amphiphysin/Rvs (BAR) domain which can sense or cause membrane curvature at membranes enriched in PI3P [2, 3, 25, 28]. The second sub-complex, or the retromer, exists as a heterotrimer of either vacuolar protein sorting (VPS) 26 (VPS26A or B) or Down’s syndrome critical region 3 (DSCR3) and VPS29 and VPS35 proteins [25].

Trafficking of components back to the Golgi from the early endosome is under control of the retromer, which is either in coordination with or independent of the SNX-BAR heterodimer. Thus, the retromer is responsible for recycling the mannose 6-phosphate receptors (M6P) back to the Golgi which are necessary for lysosomal function due to the receptors role in delivery of lysosomal hydrolases to lysosomes upon processing in the Golgi (Fig. 1.4) [25, 29]. Additionally, the retromer is observed to regulate the “fast recycling” of β2-adrenergic receptors towards the plasma membrane, or trafficking of other receptors towards the Golgi through interactions with SNX27 and SNX3, respectively (Fig. 1.4) [25]. Subsequently, the movement of these recycling or retrograde endosomes occur through the SNX-BAR heterodimer either in conjunction with or independent of the retromer through the microtubule or F-Actin cytoskeleton network through molecular motor proteins [25]. Additionally, VPS35 from the retromer can interact with the late endosomal small GTPase Rab7 and affect the early to late endosomal transition [25].
Figure 1.4: Overview of sorting mechanisms
Schematic of ESCRT sorting and retromer recycling/Golgi transport systems. Upon internalization, receptors like EGFR or β2AR are either sorted towards the plasma membrane to be recycled, or towards lysosomes for degradation. EGFR, depending upon the amount of stimulation, can undergo degradation upon sorting by the ESCRT proteins recognition of ubiquitin tags. In this schematic, β2AR is recycled back towards the plasma membranes by the SNX-BAR complex and the retromer along with help from fellow sorting nexin SNX27. Recycling of cation independent mannose 6 phosphate receptors (CI-MPR) is also under the control of the SNX-Bar-Retromer complexes. In addition, sorting of plasma membrane receptors towards the Golgi can occur as observed with the WNT receptor WNTless (WLS) through recognition of SNX3 and the retromer. Figure adapted from Gallon and Cullen [25].

Molecular mechanism of maturation

Endosome maturation is the trafficking of receptors from early to late endosomes and eventually the lysosomes for degradation. Trafficking of endosomes within the cell requires the functions of the small GTPase family of Rab proteins and their effectors...
The early endosome utilizes the active Rab5 protein to mediate the trafficking and recruitment of downstream effectors, while the active form of Rab7 does the same for late endosomes [2, 3]. The recruitment and activity of the Rab proteins and effectors, again, rely upon the formation of specific phosphoinositides at the endosome membrane [5, 29].

In endosome maturation the class-III PI3K complex is recruited to endosome membranes by active Rab5 through interactions with class-III PI3K core complex member p150, which also interacts with any form of Rab7 as well [22]. While PI3P formation is important, the endosomal system also relies upon the formation of phosphoinositol 3,5 bisphosphate (PI(3,5)P2) by the phosphoinositide 3 phosphate 5 kinase PIKfyve (Fig. 1.2C) [3, 22]. Production of PI(3,5)P2 is important for late endosome/lysosome fusion or function and can also interact with the ESCRT machinery as well [5]. The removal of PI3P on the endosomal membrane occurs either through the effects of conversion to PI(3,5)P2 by PIKfyve or by the 3-phosphatase myotubularin which removes the phosphate head of PI3P to return it back to phosphoinositol [3].

Endosome maturation from early to late endosomes requires the replacement of Rab5 to Rab7 on the endosome membrane. Rab proteins, in the active GTP bound form, are able to bind to endosome membranes and recruit downstream effectors that affect endosome sorting, trafficking, and fission/fusion events. Subsequent GTP to GDP hydrolysis by the Rab protein results in inactivation and dissociation of the Rab protein from the membrane. Rab5 is activated by the ubiquitin binding guanine exchange factor (GEF) Rabex-5 on the early endosome by exchanging the GDP for a GTP [3]. Ensuing GTP bound Rab5 can then recruit and stabilize other downstream effectors like the PI3P generating VPS34 complex, which in turn can recruit other PI3P binding proteins that affect sorting, the activity of Rab5, and recruitment of downstream Rab7 [30-32]. Dissociation of Rab5 occurs through the activity of SAND1/MON1-CCZ1 by displacing Rabex-5 and thus terminating the feedback loop of Rab5 activation, and subsequent recruitment of Rab7 to the membrane [2, 3]. Subsequently, the SAND1/MON1-CCZ1 complex acts as the GEF for Rab7 effectively activating and recruiting Rab7 to early endosomes [33]. Additionally, the recruitment of SAND1/MON1-CCZ1 to membranes
requires the formation of PI3P on membranes through the activity of active Rab5 [34, 35]. Thus, the replacement of Rab5 to Rab7 on endosomal membranes is highly dependent upon the formation of PI3P on membranes.

Transmission of materials between two distinct vesicles within the cell occurs through a process of tethering and fusion of membranes. Coordination of fusion events during endocytic vesicle trafficking is mediated by the class C core vacuole/endosome tethering factor (CORVET) complex for endocytic vesicle to early endosome fusion and homotypic fusion and protein sorting (HOPS) complex for late endosome to lysosome fusion [34-36]. Either complex facilitates recognition of vesicle specific soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [34, 36](Fig. 1.5). The SNARE proteins are anchored to membranes by the c-terminal tail and, upon tethering with another SNARE, fuse the vesicle membranes together by “zippering” from the amino terminal tail to the c-terminal tail [35]. Both CORVET and HOPS consist of the same core proteins, VPS11, VPS16, VPS18, VPS33 (A, B), and differ by two proteins which ultimately dictate the Rab and SNARE membrane tethering specificity [35, 36]. The CORVET complex interacts with active Rab5 through VPS3 and VPS8, while Rab7 is recognized by the VPS39 and VPS41 proteins in the HOPS complex [35, 37]. During endosome maturation the HOPS complex recognizes Rab7 but also the Rab7 effector Rab interacting lysosomal protein (RILP) that can interact with the dynein-dynactin motors responsible for the transport of late endosomes to lysosomes [37]. Interestingly, the class-III PI3K complex containing UVRAG interacts with the HOPS complex to effect Rab7 activation [3, 23, 24]. Altogether, the Rab proteins affect sorting, fusion, and trafficking through interactions with downstream effectors (Fig. 1.5).

Although not the focus of this dissertation, the recycling of endosomes nonetheless plays an essential role in cellular homeostasis. Recycling endosomes are governed by the activity of the Rab4 protein for fast recycling directly from early endosomes, or by Rab11 in the classical recycling pathway, while Rab9 mediates Golgi retrograde trafficking [2]. As mentioned previously M6P recycling is essential for properly functioning lysosomes through the role it has in delivering acid hydrolases to early endosomes.
Endosomal acidification and lysosomal maintenance

In addition to the membrane alterations due to effector proteins, the endosome lumen is increasingly acidified during endosome trafficking towards lysosomes by the vacuolar-ATPase (v-ATPase) (Fig. 1.5) [3]. As the least acidified compartment, the early endosome lumen is observed to have a pH of 6.8-6.1, while late endosomes are between 6.0-4.8 and lysosomes having the most acidic lumen at a pH as low as 4.5 [3]. The acidification and transport of v-ATPase towards lysosomes serves two purposes: 1) to release the ligands from the receptors, and 2) activate acid hydrolases necessary for breaking down biomolecules in the late endosome/lysosomes [3, 38, 39]. In addition, the endosomes carry ion transporters towards the lysosome which work in conjunction with v-ATPase during acidification [39, 40]. Although it has been proposed that fusion of endosomes are in part regulated by endosome acidification [39, 41], recent studies suggest that acidification may not be important for vesicle fusion [42, 43]. Regardless, endosomal trafficking of v-ATPase is necessary for the degradative capacity of lysosomes.

In addition to v-ATPase trafficking towards lysosomes, endosomal maturation is responsible for delivering lysosomal associated membrane protein (LAMP1, LAMP2) and more than fifty acid hydrolases [44]. Of the proteins related to the lysosome membranes LAMP1 and LAMP2 make up the majority (~50%) [44]. The LAMP proteins are highly glycosylated in order to protect the integrity of the lysosomal membrane and the lysosome itself from the lipid and acid hydrolases present within the lysosome [44]. Trafficking of proteins to the lysosome can occur either through the trans-Golgi to endosome pathway by binding to M6P receptors, or by being processed and delivered from the trans-Golgi to the plasma membrane only to be internalized and processed through the endosomal maturation pathway [44]. As such, endosome maturation is necessary to provide lysosomes with the proteins and complexes necessary for function and formation.
Figure 1.5: General overview of endosome maturation
Tethering of incoming endocytic vesicles occurs through the CORVET complex recognition of active Rab5 and accompanying SNAREs contained on the early endosome and incoming endocytic vesicle. Active Rab5 also helps to recruit the class-III PI3K to produce PI3P on membranes. Upon fusion receptors can be sorted by the ESCRT machinery to make intralumenal vesicles (ILV), or recycled back to the plasma membrane by the retromer and SNX-BAR machinery. Along with receptor internalization, endocytosis delivers essential components for lysosomal function such as the vacuolar ATPase, which increasingly acidifies the lumen of endosomes as maturation progresses. Progression of maturation from early to late endosomes requires the switch of Rab5 to Rab7 on the endosome through the use of the Rab7 guanine exchange factor (GEF) SAND1/MON1-CCZ1. Rab7 can then aid in the movement of the late endosomes towards the lysosome through the Dynein-dynactin motors by interaction with Rab interacting lysosomal protein (RILP). In addition Rab7, along with RILP, can then interact with the HOPS complex to tether and fuse late endosome with lysosomes. Figure adapted from review authored by Solinger, J. A. and Spang, A. [37].
Autophagy

Autophagy comes in many forms such as macroautophagy, microautophagy, and chaperone-mediated autophagy [45]. For this dissertation, the term autophagy refers to macroautophagy, which entails the sequestration of small portions of the cytosol and organelles within the cytosol through the creation of the double membrane organelle known as the autophagosome [45]. Although autophagy has distinct machinery necessary for function, the process of autophagosome maturation can crosstalk with the endosome maturation proteins and complexes. Regardless, autophagy is another highly membrane dependent process that requires protein interactions and modifications at the membranes involved in order to function.

Basic functions and regulation by mTORC1

Typically autophagy functions as two states within the cell, a basal state under nutrient rich conditions and an “induced” state under nutrient scarce conditions [45]. Basal autophagy is necessary for the constitutive turnover of cytosolic components, while induced autophagy produces amino acids necessary for survival due to the dearth of nutrients [45, 46]. The serine/threonine kinases mechanistic (formally mammalian) target of rapamycin (mTOR) exist as two complexes (mTORC1, mTORC2) and are central in regulating cell growth and cycle [46]. The functions of either complex differ based upon the scaffolding proteins that interact with the core mTOR protein, as such mTORC1 affects growth, cell metabolism, and cell cycle, while mTORC2 affects cell survival, cytoskeletal organization, and metabolism [46]. Canonically, autophagy is inhibited by the active form of mTORC1, while inhibition of mTORC1 activity results in the activation of autophagy [47]. Activity of mTORC1 is dictated by the recognition of amino-acids and nutrients directly, by the levels of energy rich substrates within the cell, or by growth receptor signaling. Sensing the dwindling levels of the energy rich adenosine tri-phosphate (ATP) and the increases in energy depleted form adenosine mono-phosphate (AMP), AMP protein kinase (AMPK) can phosphorylate mTORC1 to inhibit activity thereby activating autophagy to help produce ATP. Conversely, growth
hormone signaling through the class-I phosphatidylinositol 3-kinase (class-I PI3K)-protein kinase B (PKB, AKT) growth signaling axis, upon growth receptor signaling of nutrient availability by a ligand like insulin, can activate mTORC1 signaling shutting down autophagy (Fig. 1.7) [46, 48].

**Autophagosome maturation**

**Initiation**

Upon mTORC1 inactivation, the autophagy related proteins (ATG), and the resultant protein complexes, carry out the process of making the autophagosome and transportation towards lysosomal degradation. The dynamic process of autophagosome formation in mammalian systems is typically defined in several steps which include: initiation and formation of the isolation membrane/phagophore, elongation of the isolation membrane and subsequent engulfment of cytoplasmic material, closure of the isolation membrane thus creating the autophagosome, and the final step of autophagosomes fusion with, and subsequent degradation by, lysosomes (Fig. 1.7) [45, 49]. Creation of the autophagosome requires membranes from various sources within the cell such as mitochondria, endosomes, endoplasmic reticulum (ER), plasma membrane, and the Golgi apparatus [47, 50, 51]. Although the source of autophagosome membranes includes almost every membrane within the cell [47], the site at which autophagosomes are initiated, known as the omegasome, appears to be localized to ER-mitochondria contact sites [52].

In mammalian cells, the most upstream ATG protein after autophagy induction is the uncoordinated 51-like kinases 1 (ULK1), or the closely redundant homologue ULK2, which contains a serine-threonine kinase domain [53]. ULK1 is observed to be in a constitutively formed complex with focal adhesion kinase family kinase-interacting protein of 200 kDa (FIP200) and ATG13. The activity of ULK1 is believed to be dependent upon ATG13; however, this has been recently challenged by the observation that autophagy was still viable after the use of an ATG13 binding mutant [54]. Regardless, the ULK1 complex, upon dissociation from mTORC1 [55], can
phosphorylate Beclin-1 at serine 14 to enhance PI3P formation through the class-III PI3K [53, 56]. Overall, the location of activity of the class-III PI3K depends upon the accessory proteins associated with Beclin-1, as seen in class-III PI3K (complex-II) on endosomes through UVRAG. During isolation membrane initiation and elongation, the production of PI3P occurs through the association of ATG14-like (ATG14L) protein with Beclin-1 and the class-III PI3K to form complex-I [57]. Recruitment of ATG14L to nascent isolation membranes appears to be dependent upon the formation and recognition of PI(4,5)P2 on membranes by PIPKIγi5, which can interact with ATG14, but not the VPS34 complex [58]. Nevertheless, the formation of PI3P by VPS34 on the nascent isolation membranes recruits the WD-repeat protein interacting with phosphoinositides (WIPI) proteins which are essential for elongation of the nascent autophagosomes [59].

**Elongation and closure of autophagosomes**

In mammalian systems the WIPI protein family consist of four homologues (WIPI(s) 1-4), and subsequent isoforms, which contain an FRRG motif essential for the preferential binding to PI3P [59-62]. This preferential binding brings the WIPI proteins, WIPI1 or WIPI2B or D, to the isolation membranes [60-63]. Subsequently WIPI1 is localized to, and WIPI2 can interact with, ATG16L which is a part of the downstream protein complex necessary for the creation of the lipidated form of microtubule-associated protein 1 light chain 3 (MAPLC3/ LC3) [60, 63-65].

Conjugation of LC3 on membranes, viewed as an essential step in the elongation of isolation membranes, requires two ubiquitin-like reactions. The ubiquitin-like reactions consist of the processing and loading of LC3 to the E3-like ligase for conjugation onto phosphatidylethanolamine (PE) in membranes, and the processing of ATG12 to be loaded onto ATG5 [66]. Initially, upon translation LC3 contains an arginine amino acid residue on the carboxyl-terminus (pro-LC3), which is subsequently cleaved by the cysteine protease ATG4 to expose the glycine residue necessary for ligation reactions and consequently creates the non lipidated form of LC3 (LC3-I) (Fig. 1.6) [67]. The E1 like modifying-activating enzyme for both LC3-I and ATG12 is ATG7. Conjugation of ATG12 or LC3-I onto ATG7 is achieved, with the consumption of ATP,
by forming a thioester bond with the catalytic cysteine residue on ATG7 and the glycine residue of either LC3-I or ATG12 [66]. LC3-I is transferred from ATG7 to the E2 like conjugating enzyme ATG3, while ATG12 is transferred to another, but mutually exclusive, E2 like conjugating enzyme ATG10 (Fig. 1.6). The formation of the LC3 lipidation E3-like ligase occurs when ATG10 transfers ATG12 to ATG5, the subsequent E3-like ligase (ATG12-ATG5) can then catalyze the reaction to create an amide bond between the glycine of LC3 and the amine head group of PE [66, 68]. The ATG12-ATG5 conjugate can then form a non-covalent bond with ATG16L in a 2:2:2 heterohexamer, which is not essential for the E3-like ligase activity, but nonetheless localizes the ATG12-ATG5 E3-like ligase to isolation membranes (Fig. 1.6)[69]. Although LC3 is well established to play a role in selective autophagy, where LC3 can interact with proteins that contain LC3 interacting region (LIR) domains such as p62, BCL-2 interacting protein 3 (bNIP3), and FUN 14 domain containing 1 (FUNDC1) [70] to degrade specific cargo [71], the molecular mechanisms responsible for autophagosome expansion or closure are not as well understood [49]. Interestingly, a recently paper by Tsuboyama et al. has demonstrated that, indeed, the LC3 conjugation system is not necessary for formation of autophagosomes, but for efficient closure and delivery to lysosomes [72].
Figure 1.6: LC3 lipidation ubiquitin-like reaction
Schematic representation of LC3 lipidation. The process begins with the removal of Arginine (Arg) by cleavage performed by ATG4 which exposes the glycine (Gly) carboxyl residue to produce LC3-I. ATG12 and LC3-I are both conjugated to ATG7 in a mutually independent manner through forming a thioester bond with the cysteine residue (Cys) on ATG7. LC3-I is then transferred to the E2 conjugating enzyme ATG3, while ATG12 is transferred to the E2 conjugating enzyme ATG10. The formation of the E3-like ligase occurs through transfer from ATG10 to ATG5 forming the ATG12-5 complex, which can then localize to membranes by interactions with ATG16L. The E3-like ligase ATG12-5 by associating with ATG16L can then conjugate LC3 onto the phosphatidylethanolamine (PE) lipids on membranes through forming an amide bond creating LC3-II. Recycling of LC3 from the outer membrane of the mature autophagosome occurs through cleavage of the amide bond by ATG4 to reconstitute LC3-I. Figure adapted from the review authored by Nakatogawa, H. [66].

Another protein involved in isolation membrane expansion, but not as well understood, is the six-transmembrane protein ATG9 which is localized to the trans-Golgi network and endosomal networks [73-75]. Expansion of isolation membranes requires the use of ATG9 in mammalian systems and is believed to shuttle between membrane rich regions to deliver lipid membranes essential for elongation (Fig. 1.7) [49, 65, 76]. The trafficking of ATG9 between the isolation membrane and subsequent recycling has been an area of intense study. It has been shown that ATG9 requires the use of the UVRAG-VPS34 complex-1, WIPI2, ATG2, ULK1 for successful trafficking to the autophagosomes or recycling [77].
Isolation membranes also acquire ATG2A and ATG2B proteins at WIPI, ATG16L, ATG9A, ATG5, ULK1, and LC3 positive structures [78]. Temporally, ATG2A/B is the last structure believed to be recruited to the isolation membrane since deletion does not impair the localization of other proteins to the isolation membrane [78, 79]. Moreover, ATG2A/B localization to membranes are dependent upon PI3P but not ATG12-ATG5-ATG16L activity[78]. In yeast, ATG2 and ATG18 (WIPI1 in mammals) control the recycling of ATG9 back from the autophagosome [79]; however, in mammalian cells it has been only hypothesized that ATG2A/B has a similar function, even though WIPI1/2 can interact with ATG2A/B [78]. Regardless, ATG2A/B in mammalian cells is an essential component in the closure and creation of autophagosomes [78]. Finally, ATG4 can cleave and recycle LC3-II back to LC3-I from the outer autophagosome membrane to be utilized again in another round of autophagosome formation (Figs. 1.6 and 1.7) [49, 67].

The autophagosome is an amalgam of the various membranes within the cell; as such the membrane SNARE proteins and effector proteins associated with the vesicles that comprise the autophagosome ultimately affect fusion during biogenesis and trafficking towards lysosomes [80]. During biogenesis, the Rab proteins that mediate endosome trafficking or post-translational trafficking from the ER to the Golgi are important for autophagosome elongation. For example, Rab1 is important for ER to Golgi trafficking, and is integral for autophagosome biogenesis [80]. Additionally, the early endosome associated Rab5, upon growth factor withdrawal, is stabilized by a subunit of the class1-PI3K p110β which enhances VPS34 activity during autophagosome biogenesis [81]. The list of Rab proteins continues with Rab32 and Rab33B, both of which can affect autophagy by their unique contributions and interactions. Overall, autophagy biogenesis relies not only on unique autophagy proteins, but resident vesicle trafficking effectors and SNARES to produce a mature autophagosome.

**Fusion and trafficking of autophagosomes**

Upon closure, the autophagosome is trafficked towards the lysosome, or in some instances fused to the plasma membrane to release contents [80, 82]. Regardless of the
final destination, the vesicles travel along the microtubule system through the interactions with Dynein-Dynactin molecular motors [82]. In contrast, autophagosome elongation and formation, but not autophagosome movement, relies upon F-actin; however, actin plays a role in autophagosome-lysosome fusion via the myosin VI motor [82].

The SNARE proteins are highly involved in the fusion events of autophagosomes with either the lysosome, late endosome, or plasma membrane [82]. In particular, the Q-SNARE syntaxin17 (SNX17) interacts with ATG14L during autophagosome biogenesis and after autophagosome maturation to help stabilize the interaction between SNX17 and fellow SNARE synaptosome associated protein 29 (SNAP29) [83]. Complete autophagosomes can fuse with late endosomes to form structures known as amphisomes [84]. The function of this fusion is believed to help share late endosome trafficking proteins for the delivery of autophagosome materials to lysosomes, although this is not entirely clear since autophagosomes can fuse directly with lysosomes [85]. Regardless, the late endosome protein Rab7 is important for the trafficking and fusion events of autophagosomes/amphisomes to lysosomes. Although Rab7 can associate with the HOPS complex, Rab7 can also interact with the pleckstrin homology and RUN domain containing M1 (PLEKHM1) adaptor protein [82]. By interacting with PLEKHM1, Rab7 can interact with LC3, and the HOPS and SNARES effectively linking the autophagosome and endosome systems [82].
Figure 1.7: Autophagy overview
Schematic of autophagy during nutrient starvation. Upon activation from mTORC1 inhibition due to nutrient deprivation, nucleation of the isolation membranes at the omegasome located at the endoplasmic reticulum is initiated by the ULK1 complex. ULK1 can then activate the PI3P complex 1 to provide PI3P on nascent isolation membranes that recruit WIPI1 or 2 proteins that interact and recruit other membrane proteins such as the LC3 conjugation systems and ATG9 positive vesicles for expansion of the isolation membrane. Closure of the isolation membrane produces the mature autophagosome and is believed to include ATG2 and ESCRT proteins, although the exact mechanisms have not been fully elucidated [78]. The mature autophagosome can either fuse with late endosomes to create amphisomes and then lysosomes, or directly to lysosomes which results in the degradation of internalized material through proteasomal degradation.

Apoptosis
Programmed cell death via apoptosis is carried out by initiator and effector cysteine proteases known as caspases [86, 87]. Subsequent cleavage of executioner caspase 3/7 by caspase 8/9 ultimately leads to cell death through cleavage of DNA repair proteins and other critical proteins necessary for cellular functions [87]. Initiation of apoptosis, and subsequent activation of the caspase cascade, occurs either through an extrinsic pathway by signaling through cell death receptors, intrinsically through mitochondrial outer membrane permeabilization (MOMP), or by a combination of both depending on the cell type [86].
**Extrinsic pathway**

Extrinsic cell death is initiated by the activation of death receptors (e.g. Tumor necrosis factor receptor 1 (TNFR1), cluster of differentiation 95 receptor (CD95), death receptor(s) (DR) DR3, DR4, DR5) by death ligands (e.g. tumor necrosis factor-α (TNF-α), first-apoptosis signal ligand (FasL)) [87]. Dimerization of CD95 upon FasL binding initiates the recruitment of Fas-associated death domain (FADD), which then recruits caspase 8 to form the death inducing signaling complex (DISC). Subsequent dimerization of caspase 8 causes self-activation through cleavage [88, 89]. Cleaved caspase 8, being an initiator caspase, then subsequently activates the effector or “executioner” caspase 3/7, which ultimately leads to the cells eventual demise (Fig. 1.8A) [87].

**Intrinsic pathway**

Mitochondria contain several pro-apoptotic factors within the intermembrane space, which when released into the cytosol lead to apoptosis [87]. Initiation of mitochondrial apoptosis occurs from cell stresses such as: overproduction of reactive oxygen species (ROS), DNA damage, cell cycle arrest, ultra-violet radiation (UV) damage, toxins from various pathogens, withdrawal of growth factors, and cell detachment from the basal lamina (anoikis) [87]. Although intrinsic initiation can originate from various sources and require differing pathways and proteins, they all converge at the pore forming proteins of the B-cell lymphoma-2 (BCL-2) family of proteins to achieve MOMP (Fig. 1.9) [90].

The BCL-2 family consists of the pro-apoptotic pore forming, anti-apoptotic, and pro-apoptotic BCL-2 homology-3 (BH-3) domain only proteins [90, 91]. Permeabilization of the outer mitochondrial membrane is prevented by the anti-apoptotic proteins (e.g. BCL-2, BCL-XL, and MCL-1) through sequestration of both the pro-apoptotic and BH3-only proteins (Fig. 1.9). Activation of the pro-apoptotic pore forming members BCL-2 associated X (BAX) and BCL-2 antagonist/killer (BAK) occurs upon interaction with the BH3-only members. Subsequently, the BH3-only members can be further divided into activators and sensitizers (Fig. 1.9). The BH3 only activators are
more effective in activation of the pro-apoptotic BAX or BAK [91]. On the other hand, the BH3 only sensitizers, while less potent in directly activating the pro-apoptotic BAX or BAK [92], dislodge the activators or release the already activated pro-apoptotic pore forming proteins from the anti-apoptotic proteins to induce MOMP (Fig. 1.9) [90]. The pro-apoptotic effectors released during MOMP include cytochrome c, second mitochondria-derived activator of caspase/ direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), and high temperature requirement protein A2 (HtrA2\OMI). Cytochrome c release and subsequent interaction with the scaffolding protein apoptotic protease-activating factor 1(APAF1) induces the formation of the caspase 9 activation complex in the cytosol, the apoptosome (Fig. 1.8B) [86]. Caspase 9 is an initiator caspase, which when activated can cleave and activate the executioner caspase 3/7 [86]. Although some cells (type 1 cells) rely solely upon the extrinsic pathway for apoptosis, others (type 2 cells) rely upon both signaling pathways to execute apoptosis. The extrinsic and intrinsic pathways are able to crosstalk through the BCL-2 protein BH3 interacting-domain death agonist (BID), which upon cleavage by caspase 8 becomes truncated-BID (t-BID) that acts as an BH3-only activator protein (Fig. 1.8B) [87].
Figure 1.8: Methods of apoptosis induction

Schematic representations of the (A) extrinsic pathway and (B) intrinsic apoptosis pathways. (A) Extrinsic apoptosis is initiated by a death ligand binding to death receptors (CD95, TNFR1) to initiate the caspase cascade through assembly of the Death Inducing Signaling Complex (DISC) to activate caspase 8. Subsequent cleavage of executioner caspase 3 by caspase 8 ultimately leads to cell death through cleavage of DNA repair proteins and other critical proteins necessary for cellular functions. B) Intrinsic cell death pathway, within darker colored oval, can cross talk with extrinsic cell death initiation occurs through the activity of active caspase 8 by cleaving BH3 domain containing BID to create t-BID. The cleavage subsequently allows t-BID to interact and activate pro-apoptotic proteins BAX, which resides mainly in the cytosol, and BAK at mitochondrial membranes in order to oligomerize and permeabilize the outer mitochondrial membrane. Initiation of intrinsic apoptosis can occur through other methods, including ROS overproduction, and cell detachment (anoikis). Following mitochondrial outer membrane permeabilization (MOMP), release of mitochondrial contents leads to the activation of the apoptosome and activation of caspase 9 which subsequently cleaves caspase 3.
**Figure 1.9: Interactions of the BCL-2 family of proteins in apoptosis induction**

Schematic representation of the interactions between the BCL-2 family members and the influences each protein has on apoptosis induction through mitochondrial outer membrane permeabilization (MOMP). The anti-apoptotic proteins (middle column) can inhibit MOMP by binding and inhibiting the functions of the pro-apoptotic BAX and BAK (purple boxes) from inducing mitochondrial apoptosis. In addition, the BCL-2 anti-apoptotic proteins can prevent the direct activation of the pro-apoptotic BAX and BAK by sequestering the direct BH3 only activators (left column). The BH3 sensitizers (right column) can induce apoptosis via the indirect pathway whereby the BH3 sensitizer can bind to and release an already activated pro-apoptotic protein or BH3 activator from the anti-apoptotic protein. The interactions between the BH3 sensitizers and anti-apoptotic proteins are fairly selective as observed with BCL-2 associated death promoter (BAD) only interacting with BCL-2, BCL-XL and BCL-W, while NOXA preferentially interacts with MCL-1 and BFL-1/A1. On the other hand, the more promiscuous Bcl-2 modifying factor (BMF) or BCL-2 interacting killer (BIK) can interact with almost all anti-apoptotic proteins with varying affinities. Figure adapted from the review authored by Liu, Q and Wang, H.G. [93].

**Mitochondrial dynamics**

Mitochondria exist as a highly dynamic network, which is continually modified through membrane fission and fusion events in order to respond to various noxious stresses, nutrient levels, and inter/intra-cellular signals [94-96]. Cellular and organism function rely upon mitochondria since these double membrane bound organelles provide a suitable environment for ATP production via the tricarboxylic acid (TCA) cycle and
oxidative phosphorylation pathway to occur. However, the importance of mitochondria expands beyond energy and metabolite production since these organelles are critical regulators of apoptosis, calcium homeostasis, and ROS reduction [96-100]. To maintain cellular homeostasis, mitochondrial networks are subjected to dynamic regulation by fission, fusion, and selective autophagic clearance of damaged mitochondria (mitophagy); disruptions of which are implicated in various disease states.

**Cellular role**

Mitochondrial dynamics refer to the continuous balanced cycle of fission and fusion events that occur in order to maintain mitochondrial and cellular homeostasis. Morphologically, fission results in the fragmentation of mitochondria into two distinct daughter mitochondria, while fusion results in elongation and condensing of mitochondrial fragments to form mitochondrial networks (Fig. 1.10A). This dynamic balance between these two opposing forces allow mitochondria to preserve the mitochondrial membrane potential (ΔΨₘ) necessary for a robust oxidative phosphorylation respiratory chain. Mitochondria are sources of ROS, which can damage the proteins and lipids within the mitochondria reducing the effectiveness of the TCA cycle. Mitochondrial dynamics helps to clear these dysfunctional proteins and lipids through fission in order to protect the TCA cycle and maintain the ΔΨₘ. A shift in mitochondrial dynamics towards fission results in an assortment of mitochondria with various ΔΨₘ that have a reduced capacity to produce ATP through decreased oxygen consumption (Fig. 1.10A) [101]. Conversely, increases in fusion are believed to rescue the mitochondrial respiratory functions and allow for dissipation of ΔΨₘ to partially damaged portions of the mitochondrial network to restore a more homogeneous network [102, 103]. Furthermore, fusion of mitochondria is important for maintaining the mitochondrial genome (mtDNA) as a damaged portion of mtDNA can be complemented by the functional mtDNA (Fig. 1.10B) [101, 104-106]. Although fusion can rescue defective mitochondria, excessive fusion results in lowered respiration and eventual loss of mtDNA further emphasizing the necessity of the dynamic balance between fusion and fission in mitochondrial homeostasis [102]. Mitochondrial fission is also important for
transport of mitochondria within cells, a function essential in neurons due to the energy needs in synapses and dendrites which exist relatively far away from the cell body [107-110]. Defective fission can also decrease inheritance of mitochondria into daughter cells during the cell cycle [111, 112], while defective fusion alters cell cycles due to the bioenergetic role it plays in G1-S phases [102, 113-115]. Fission is also involved in the process of apoptosis, which is essential during development and a protective response to cytotoxic stress (Fig. 1.10B) [116, 117].

**Figure 1.10: Mitochondrial dynamics effects on cell physiology**
A) Mitochondria undergo a constant cycle of fission and fusion in order to maintain a healthy mitochondrial network. Fission results in the division of mitochondria into heterogeneous populations based on membrane potential (ΔΨm). Damaged mitochondria, those with a decrease in ΔΨm, can be selectively degraded by autophagy in a process known as mitophagy. B) Fusion of mitochondria within the cell helps to reclaim parts of mitochondria through dissipation of the ΔΨm or to rescue the mitochondrial genome (mtDNA). Fission on the other hand is necessary for the transportation of mitochondria within the cell, transmission of the mitochondria to daughter cells during cell division, clearance of damaged portions through mitophagy, or makes apoptosis induction more efficient through BAX insertion.
**Molecular mechanisms: fission, fusion, and mitophagy**

The fission and fusion events are governed by several proteins [118, 119]. Scission of mitochondria during fission is controlled by dynamin-related/-like protein 1 (Drp1) and ultimately Dnm2 upon recruitment to scission sites by Drp1 [119, 120]. Accumulation and recruitment of Drp1 and Dnm2 to fission sites occurs through interactions with the N-terminally anchored mitochondrial dynamics proteins of 49 and 51 kDa respectively (MiD49 and MiD51), or mitochondrial fission factor (Mff) (Fig. 1.11A) [102, 119-122]. Mitochondrial fusion within mammalian cells is mediated by three large GTPases, the mitofusins (Mfn1, Mfn2) and optic atrophy 1 protein (Opa1) [119]. The mitofusins regulate outer membrane tethering through hydrolysis while Opa1 is responsible for inner membrane fusion and organization (Fig. 1.11B) [102, 103, 117-119, 123, 124].

Regulation of cellular homeostasis through the effects of mitochondrial dynamics is most pronounced during apoptosis or mitophagy. Components within the apoptosis pathway can effect, or be affected by, mitochondrial dynamics. For example, Mfn proteins interact with the cytosolic form of pro-apoptotic BAX at scission sites to regulate fusion [125], or the activity of DRP1 during fission enhances mitochondrial apoptosis through BAX insertion [126, 127].

Under basal conditions, resultant fission of mitochondria usually creates two distinct daughter mitochondria with a disparity in the ΔΨm (Fig. 1.10A) [101, 128]. To help maintain the health of the mitochondrial network, the damaged portions are culled through mitophagy (Fig. 1.10A) [129, 130]. The degradative autophagic machinery, mitochondrial dynamics, and other supporting mitophagy inducing proteins need to be functional for mitophagy to occur. Stresses, such as hypoxia and starvation, cause massive alterations to mitochondrial dynamics. As such, cells deficient in fission, or that undergo extensive fusion when challenged with either hypoxic or starvation conditions, are believed to delay or be unable to perform mitophagy due to mitochondrial size [131-133]. The steps involved in mitophagy involve: fission of damaged mitochondria, ubiquitination and proteasomal degradation of outer membrane proteins involved in
fusion, resultant recognition and engulfment of damaged mitochondria by autophagosomes, and subsequent degradation in auto-lysosomes [129, 134].

The process of ubiquitination of the outer membrane proteins is mediated by PTEN-induced kinase-1 (PINK1) stimulation of the E3-ubiquitin ligase Parkin through phosphorylation [130]. Parkin can subsequently target and degrade the proteins responsible for fusion, Mfn1 and Mfn2, rendering the damaged portion unable to rejoin the mitochondrial network [135]. Subsequent ubiquitin ligation on damaged mitochondria allows for proteasome degradation and recognition by the autophagy isolation membranes through adaptor proteins [129]. Ubiquitin-binding protein p62 is one such adaptor since it can bind to ubiquitinated proteins/organelles as well as interacting with autophagosomes through the membrane-bound autophagy protein LC3-II. In addition to p62, mitochondria have other specialized proteins that do not need ubiquitin, but serve in the same capacity in the recognition of damaged mitochondria such as; FUNDC1 which can aid mitophagy by inducing fission and directly interacting with LC3-II, or through bNIP3 [70, 129, 136].
Figure 1.11: Mitochondrial fission and fusion proteins

A) Mitochondrial fission is mediated by the proteins mitochondrial fission factor (Mff), or mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 or Mid51), which coalesce at fission sites to recruit dynamin-related/-like protein 1 (Drp1) which can then interact with and recruit dynamin 2 (Dnm2) for fission. Subsequent fission results in two daughter mitochondria with different levels of (ΔΨm).

B) Mitochondrial fusion utilizes the mitofusins (Mfn1, Mfn2), which can form hetero or homodimers, for tethering and fusion of the outer mitochondrial membrane. Optic atrophy 1 protein (Opa1) maintains mitochondrial inner membrane morphology by fusing the inner membranes.

The endophilin family of proteins

Endophilin protein structure

Within the large heterogeneous superfamily of BAR domain-containing proteins, which are known for membrane trafficking within cells, there exists the endophilin family of proteins [9]. The endophilin proteins contain an amino-terminal-BAR (N-BAR)
domain which can induce membrane invagination upon dimerization [9, 137, 138]. Membrane curvature is aided by two amphipathic membrane insertion segments, Helix 0 (H0) and Helix 1(H1), within the N-BAR domain (Fig. 1.12A) [137]. The H0 and H1 insertion regions induce membrane asymmetry by wedging into the membranes aiding in membrane bending along with the N-BAR dimer shape [9]. Additionally, the N-BAR domain-containing proteins can form lattices on membranes through the H0 insertion region in a rather specific manner that can allow for the recruitment and interactions of various other membrane altering proteins such as dynamin (Fig. 1.12B) [139]. In general, most BAR-containing proteins are dependent upon dimerization to interact with negatively charged membranes [137], particularly with the amphipathic helixes within the N-BAR proteins [140]. Furthermore, the endophilin proteins act as membrane scaffolding protein due to the carboxyl-terminal Src-homology 3 (SH3) domain that can interact with proline-rich proteins [9]. Overall, the endophilin families of proteins are highly involved in the processes of membrane re-arrangements that can affect cellular and organism homeostasis.
Figure 1.12: N-BAR proteins structure induces membrane bending

(A) The endophilin N-BAR domain is comprised of three kinked, antiparallel alpha-helices that can interact and insert themselves into membranes containing phospholipids. Insertion into the lipid bilayer occurs through the Helix-0 (H0) and Helix 1 (H1) amphipathic regions. Figure adapted from paper published by Gallop, J.L. et al. [140].

(B) Membrane bending of lipid bilayers occurs through the insertion of the amphipathic regions within the endophilin proteins, which can then tubulate membranes and form scaffolds along the membrane. Scaffolding is stabilized by interactions between the H0 regions. Figure adapted from the paper published by Mim et al. [139].
**Endophilin A family and functions**

The endophilin A family of proteins is highly associated with synaptic vesicle endocytosis in neurons, although all tissues express some isoform of endophilin A [9]. In particular, endophilin A1 is highly expressed in the brain, endophilin A2 is expressed in all tissues, and endophilin A3 has higher expression in brain and testes [141, 142]. At the subcellular level, the endophilin A family of proteins is observed to localize with synaptic vesicles and throughout the cytosol [14, 143, 144]. Upon synaptic stimulation, the endophilin A proteins are observed to accumulate at the presynaptic membrane in both neurons and non-neuronal cells [9]. Studies using subcellular fractionation localize the endophilin A proteins to the light membrane fraction, which consists of endosomes, ER/Golgi, and lysosomes, as well as the cytosolic, and mitochondria fractions [145, 146]. Along with membrane curvature induction, the endophilin A proteins can interact with proteins involved in endocytic internalization such as the clathrin un-coating protein synaptotagmin, the membrane scission protein dynamin I, and the membrane interacting-dynamin recruiting protein amphiphysin [15, 143, 147]. Overall, the endophilin A family is closely associated with the processes surrounding endocytosis rather than endosome trafficking and intracellular membrane dynamics [9].

**Endophilin B family and functions**

**Discovery, expression profile, and localization**

The endophilin B family of proteins was first discovered by our lab and another independent group using a yeast-two hybrid screening for proteins that interact with pro-apoptotic BAX [148, 149]. Of the endophilin B proteins identified, only endophilin B1 (SH3GLB1/BIF-1) was shown to interact with BAX, while endophilin B2 (SH3GLB2) was only identified to bind to endophilin B1 and not BAX [148]. In particular, the first eleven amino acids on the amino-terminus of endophilin B1 are responsible for the interactions of endophilin B1 to BAX [148]. The cellular distribution of endophilin B1 is mainly cytosolic and human tissue expression is observed in brain, pancreas, prostate, ovaries, colon, thymus, spleen, kidney, liver, lung, small intestines, placenta, peripheral
blood leukocytes, heart, and skeletal muscle [148-154]. The subcellular distribution of endophilin B1, determined through fractionation experiments, is observed to localize with early endosomes, Golgi, lysosomes, ER, and slightly with mitochondrial fractions within the cell [145, 154-156]. A more recent study identifies that endophilin B1 localizes to small cytoplasmic vesicles, especially those consisting of caveolin or clathrin, but only upon EGF stimulated EGFR internalization [157]. Unlike the endophilin A proteins, the endophilin B proteins appear to mainly interact with intracellular membranes [154]. Several human isoforms of endophilin B1 have been described and are tissue specific. In particular endophilin B1a tends to be expressed in most tissue, while endophilin B1b and B1c are localized and expressed in the brain [153] and endophilin B1t is expressed specifically in the testis [158].

On the other hand, in human tissues endophilin B2 has been observed to be expressed in brain, prostate, mammary glands, skeletal muscles, adipocytes, lung, and colon tissues [148, 159-162]. The subcellular distribution of endophilin B2 is not as well defined as endophilin B1; however, the few observations regarding subcellular localization indicate that endophilin B2 appears to have a cytosolic distribution that tends to form aggregates around the nucleus upon overexpression [148, 163].

**Endophilin B1 in mitochondrial dynamics and apoptosis**

Subsequent studies revealed endophilin B1 as being a factor in maintaining normal mitochondrial morphology as depletion of endophilin B1 resulted in an increased dissociation between the inner and outer membranes of mitochondria [156]. Although mitochondrial membrane structures appear to be disrupted, endophilin B1 depletion had no effect on the rates of mitochondrial fission and fusion [156]. Furthermore, it has been observed that endophilin B1 acts downstream of Drp-1, suggesting that endophilin B1 has a marginal effect on mitochondrial morphology dynamics [156]. Nevertheless, endophilin B1 was identified to interact with mitochondrial outer membranes under basal conditions.

As a BAX interacting protein, endophilin B1 was initially characterized as an enhancer of the activation and oligomerization of BAX necessary for insertion and pore
formation in mitochondrial apoptosis, respectively [164]. Under basal conditions, endophilin B1 is observed in the cytosol, whereas upon apoptotic stimulation endophilin B1 can localize to mitochondrial membranes with BAX [156]. Subsequently, this interaction of endophilin B1 with BAX at mitochondrial membranes increases the release of cytochrome C and eventually mitochondrial apoptosis [149, 164]. On the other hand, reduction or deficiency of endophilin B1 expression reduces the release of cytochrome C thus delaying apoptosis and increases the tumorigenic properties of cells [164]. In addition to BAX activation, endophilin B1 also enhances the other pro-apoptotic BCL2 member BAK on mitochondrial membranes [164]. Given the role of endophilin B1 with BAX activation and interactions at the mitochondrial membrane, it has been proposed that endophilin B1 increases the activation of BAX and BAK due to the nature of membrane interactions and curving properties of endophilin B1 [138, 156, 164].

In addition to affecting mitochondrial apoptosis induced by cytotoxic stressors, endophilin B1 can be post-translationally modified to reduce BAX activation and activity upon anoikis (cell detachment-induced apoptosis). Specifically, endophilin B1 was observed to be phosphorylated at tyrosine 80 by the proto-oncogene c-SRC to suppress the binding of and subsequent activation of BAX to endophilin B1 [165]. This phosphorylation site is located within the H1 amphipathic membrane insertion region and is suspected to disrupt membrane interactions of endophilin B1, which is assumed to play a role in the activation and oligomerization of BAX in mitochondrial membranes [156, 165]. Overall, endophilin B1 can enhance the activation of apoptosis through the activation of BAX/BAK and release of inter-membrane space proteins responsible for apoptotic induction.

**Endophilin B1 in endosomal maturation**

As the Latin suffix implies, the endophilin family have an “affinity for endocytosis”, or in the case of endophilin B proteins, endocytic trafficking. Unlike endophilin A proteins, endophilin B does not affect endocytic internalization [153, 166]. However, endophilin B1 interacts with UVRAG through the SH3 domain and affects the activity of the class-III PI3K complex II to produce PI3P on membranes [23, 167].
Specifically, endophilin B1, through interactions with UVRAG and subsequently the class-III PI3K complex II, can enhance degradation and acidification of endosomes during EGF-stimulated EGFR degradation [23]. In another study, endophilin B1 has been observed to be in a complex with HIV-1 tat interactive protein 2, 30 kDa (TIP30) and acyl-CoA synthetase long chain family member 4 (ACSL4) at early endosomes to aid in the recruitment of v-ATPase to early endosomes in liver [168]. Subsequently, loss of expression of endophilin B1 has been observed to suppress the degradative endocytic trafficking of EGFR [23, 166]. Tropomyosin-related kinase A receptor (TrkA) bound by nerve growth factor (NGF) [169], and nicotinic acetylcholine receptors [170, 171]. Regardless of the proteins observed to be involved in the trafficking of receptors, or the receptors being degraded, all reports indicate that endophilin B1 deficiency decreased acidification and trafficking of receptors towards late endosomes and subsequently lysosomal degradation.

**Endophilin B1 in autophagy**

Previous studies from our lab revealed that deficiency of endophilin B1 reduced caspase-independent cell death under starvation, or autophagy-mediated cell death [167]. The group identified that endophilin B1, through the N-BAR domain binding to membranes and SH3 domain-mediated interaction with UVRAG, acts as an enhancer of the activity of the class-III PI3K kinase complex II for autophagic flux [167]. In addition, endophilin B1 was observed to localize to nascent autophagosomes during nutrient deprivation and loss of endophilin B1 expression ultimately increased tumorigenesis in mice [167].

In addition, endophilin B1 also affects autophagy through the trafficking of ATG9 [73, 74, 155]. Specifically, the H0 and H1 domains in the N-BAR domain of endophilin B1 are necessary for the formation of ATG9 puncta in cells [155]. Endophilin B1 was also localized with nascent autophagosomes with ATG9 signals near the trans Golgi network, a potential source of autophagosome membranes [155]. Moreover, class-III PI3K complex II affected this trafficking through interactions with endophilin B1 [155]. Furthermore, a more recent study identified that endophilin B1 can interact with Dnm2
through the SH3 domain and affect ATG9 vesicle trafficking out of Rab11 positive recycling endosome-like vesicles [73]. These data suggest that endophilin B1 can not only recruit UVRAG, but the membrane scission protein Dnm2 to assist in autophagosome formation through ATG9 trafficking [73].

The effects of endophilin B1 in autophagy extend into mitophagy and the development of cancer. In a mouse model of lymphoma driven by the Eμ-MYC oncogene, endophilin B1 was identified as a haploinsufficient tumor suppressor as mice lacking one or both alleles of endophilin B1 accelerated disease onset and mortality [172]. Along with defective mitophagy, mice lacking both alleles of endophilin B1 were not born at the expected Mendelian ratio due to embryonic re-absorption, while loss of one or both alleles increased the chromosomal instability within cells [172]. In particular, cells lacking endophilin B1, or the haploinsufficient prelymphomatous lymphocytes, retained an abundance of dysfunctional mitochondria that were surrounded by incomplete autophagosome structures [172].

**Endophilin B2**

Although the subcellular localization and functions of the endophilin B2 protein are not as well understood, recent studies have begun to reveal some functionality and localization of endophilin B2. The structure of endophilin B2 exhibits a 58% homology in amino acids and 67% homology in nucleotide sequence to endophilin B1 in humans, although endophilin B2 does not bind with BAX (Fig. 1.13)[148]. The cellular localization of endophilin B2 appears to be cytosolic and form aggregates upon over-expression near the nuclei of cells [163]. Although endophilin B2 does not appear to interact with BAX, endophilin B2 has been reported to interact with the Vimentin network through interactions with the cytoskeletal linker protein Plectin 1 [163]. Subsequent depletion of endophilin B2 disrupts the Vimentin cytoskeletal network and the localization of the nucleus within the cell [163].

Another study, using neuronal cells as a model, identified endophilin B2 interacting with candidate plasticity gene 2 (CPG2) protein which effects endocytic internalization through interactions with F-Actin in an receptor activation dependent
manner [173]. Using deletion mutants, Loebrich et al. mapped the interaction between endophilin B2 and CPG2 to the second coiled coil domain within CPG2 and the second coiled coil of endophilin B2 with the last thirty four amino acids of endophilin B2 being essential (amino acids 152-186) [173]. Overall, the interaction between endophilin B2 and activated receptors helps in recruiting endocytic machinery (e.g., clathrin) to activated glutamate receptors, whereas CPG2 interaction helps with the subsequent trafficking via F-actin [173].

More recently, another group identified endophilin B2 and endophilin B1 dimers as enhancing mitophagy [174]. Using deletion mutants, Wang et al. [174] identified the dimer domain (amino acids 153-185 in endophilin B2, and 156-188 in endophilin B1) and subsequent hetero-dimerization of endophilin B2 to B1 as being necessary for clearance of depolarized mitochondria. Interestingly, they identified that the dimer domain for endophilin B2 is necessary for both hetero and homo-dimerization, whereas endophilin B1 dimer domain is only necessary for homodimerization [174]. Overall, endophilin B2 heterodimerization with endophilin B1 is necessary for clearance of damaged mitochondria.
Figure 1.13: Endophilin family protein alignment

Alignment of human endophilin A1 (accession EAW58661.1), A2 (EAW69229.1), A3 (EAW62422.1), B1 (EAW73181.1), and B2 (CAI12365.1). Alpha-helices (H1a and b H2, and H3) in black comprise the backbone of the N-BAR domain. Amphipathic insertion regions Helix 0 (H0) insertion in light blue coil, and Helix1 labeled as CAH for central amphipathic helix in red. Major domains are outlined below the alignment for N-Bar, and SH3 domains. Figure adapted from review authored by Kjaerulff, O., Brodin, L, and Jung, A. [9].
Summary

Although endophilin B1 is involved in many facets of cellular homeostasis, the role of endophilin B2 is less well understood. Recent reports identify endophilin B2 as interacting with cytoskeletal networks [163], enhancing activated glutamate receptor endocytosis in neurons [173], and affecting mitophagy through endophilin B1 heterodimerization [174], yet more remains to be elucidated. Whereas endophilin B1 is an enhancer of apoptosis, the role of endophilin B2 is unknown. It has been proposed that endophilin B2 may act as a negative regulator of endophilin B1 homodimers, or even could be redundant to endophilin B1 [175], but the cellular and subcellular localization of endogenous endophilin B2 is not completely known.

Of the few studies identifying endophilin B2 in disease, most attribute it having similar function to endophilin B1 [159-162], including work from my lab [175]. In this dissertation, I describe the cellular and subcellular distribution of endophilin B2, and explore the role of endophilin B2 in mitochondrial morphology, apoptosis, endocytosis, and autophagy. I show that endophilin B2 is dispensable for apoptosis, and does not disrupt either endocytosis or embryonic development in mice. The work contained within this dissertation identifies endophilin B2 as a positive regulator of endosomal acidification and subsequent maturation as well as autophagic flux. The significance of the effects of endophilin B2 is exemplified by the disruption of nuclear viral entry of influenza A virus in endophilin B2 deficient cells.
Chapter 2: **Materials and Methods**

**Antibodies and plasmids**

The following antibodies were used for immunoblotting (IB), immunofluorescence (IF), and immunoprecipitation (IP): mouse anti-β-Actin monoclonal (Sigma, #A5441; 1:20,000 for IB); mouse anti-flag tag monoclonal antibody (Sigma, # F1804; 1:2,000 for IB, 1:500 for IP); rabbit anti-calnexin polyclonal (Thermo Fisher Scientific, #MA3027; 1:2,000 for IB); rabbit anti-endophilin B2 polyclonal (Proteintech Group, #15897-1-AP; 1:5,000 for IB, 1:1,000 for IF); rabbit anit-TGN46 polyclonal (Novus, #NBP1-49643, 1:400 for IB); mouse anti-endophilin B1 monoclonal (Novus, #NBP2-24733, 1:500 for IB, 1:800 for IF); goat anti-endophilin B1 polyclonal (GeneTex, #GTX89961, 1:500 for IB); rabbit anti-LC3 polyclonal (Novus, #NB100-2220, 1:500 for IB); rabbit anti-LC3 monoclonal (Cell Signaling Technology, # 3868S, 1:2000 for IB); rabbit anti-EGF receptor monoclonal (Cell Signaling Technology, #4267S, 1:1000 for IB); rabbit anti-Rab5 monoclonal (Cell Signaling Technology, #3547, 1:700 for IB); rabbit anti-Rab7 monoclonal (Cell Signaling Technology, #9367, 1:700 for IB); rabbit anti-myc tag monoclonal (Cell Signaling Technology, #2278S, 1:1000 for IB); mouse anti-human-CD107A (Lamp1) monoclonal (BD-Biosciences, #555798, 1:200 for IF); guinea pig anti-P62 polyclonal (American Research Products, #03-GP62-C, 1:5000 for IB); rabbit anti-Tom20 polyclonal (Santa Cruz Biotechnology, #SC-11415, 1:500 for IB); mouse anti-Influenza A-nucleoprotein monoclonal (EMD Millipore, #MAB825, 1:1000 for IF).

The pCDH1-3xFlag-IRES-GFP plasmid was generated by subcloning PCR-amplified 3xFlag cDNA (Xba I-EcoR I site) from p3xFlag-CMV-10 (Sigma, #E7658) and the IRES-GFP sequence (Eco RI-Sal I site) from pBMN-I-GFP (Addgene, #1736) into pCDH1-MCS1-EF1-Puro vector (System Biosciences, #CD510A-1). The cDNA encoding human BMF (Addgene, #24264) was amplified by PCR and subcloned into the EcoR I-Xho I site of pCDH1-3xFlag-BMF-IRES-GFP. The human endophilin B2 was
obtained from Invitrogen (#FL1002), amplified by PCR and subcloned into the Xba I-EcoR I site of pCDH1-MCS1-EF1-Puro vector. The primer sequences used to amplify 3xFlag, human BMF and human endophilin B2 cDNAs were: 3xFlag, 5’-GCTCTAGAGCCACCAGACTACAAAGACCAGTAC-3’ and 5’-GGAATTCCTTGTCATCGTCATCCTTG-3’; human BMF, 5’-GGGAATTCTGAGCCACCATCTCAGTG TGT-3’ and 5’-GGTGTCGACTCACCTAGGGCCTGCC-3’; human endophilin B2, 5’-CCGCCAGTTCTAGAGCCACCAGTTCAAA-3’ and 5’-ATGCATGGAATTCC TAGCTGAGCAGTTCC-3’. The guide sequence targeting exon 1 of human endophilin B2 (5’-AAGAAGCTGGCGTCGGACGC-3’) was designed and subcloned into lentiCRISPRv2 plasmid (Addgene, # 52961) according to the Dr. Feng Zhang lab’s protocol [176]. The mRFP-GFP-LC3 (tFLC3) cDNA was obtained from Addgene (#21074) and subcloned into the Nhe I-Eco RI site of pCDH1-MCS1-EF1-Puro vector. The DsRED2-mito fusion protein encoded in the pDsRED2-mito expression plasmid originally from Clontech (clontech, # 632421) was subcloned into the pCDH1 vector by Nhe-I and Not-I restriction sites. In addition, the GFP-mito plasmid, targeted to the mitochondria through by expressing the mitochondrial ATPase subunit-9 (a gift from Dr. Richard Youle), was subcloned into the pCDH1 plasmid by EcoRI and NotI restriction sites. The vectors of pEF1-Flag-UVRAG and pEF1-Flag were gifts from Dr. Chengyu Liang, pBW-Myc-endophilin B1 and endophilin B2 were created by subcloning endophilin B1 and endophilin B2 into pBW-myc vector by Eco RI and Xho I restriction sites.

**Generation of endophilin B2 knockout mice**

Endophilin B2 deficient mice with a 129/SvEv and C57BL/6 mixed genetic background were generated by the Texas A&M Institute for Genomic Medicine (TIGM, Houston, TX) by the gene trap strategy using the OMNIBANK ESC clone OST224737. This gene trap cassette includes a splice adaptor at the 5’ end with neomycin selection and poly-A termination signals followed by another ES only promotor that expresses the first exon of Bruton’s Tyrosine Kinase (BTK) gene with multiple termination codons to prevent expression of downstream fusion transcripts. The resultant mice were back
crossed with C57BL/6 mice (Jackson Labs, Bar Harbor, Maine) more than 10 times to obtain endophilin B2KO mice with the genetic background used in this study. Genotyping was performed using standard PCR methods with the following primers: genomic forward primer (B2-F) 5’-GTTG GTGCTAATGGTTGCATCC-3’, genomic reverse primer (B2-R) 5’-AGATCCTAG CTTCTGACATCC-3’, and LTR-reverse (LTR-R) 5’-ATAAACCCTCTTGCAGTT GCATC-3’. All mice were maintained in accordance with federal guidelines and studies were approved by the Pennsylvania State University Animal Care and Use Committee.

Semi quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from tissues using Trizol reagent (Thermo-Fisher Scientific, #15596026) according to manufacturer’s protocol. Quality of RNA was tested using the BioAnalyzer RNA 6000 nanochip (Agilent Technologies). cDNA was created from RNA using a High Capacity cDNA reverse transcriptase kit (Thermo-Fisher Scientific, #4368814) according to manufacturer’s protocol. PCR amplification of endophilin B2 isoforms was achieved by using 2.5 ng of each cDNA using the primer pairs found on table 2.1. Semi-quantitative PCR was performed using the GoTaq Colorless Master Mix (Promega, #M713) according to manufacturer’s protocol using the scheme and protocol outlined in figure 3.4A and table 2.1, respectively. Isoforms of endophilin B2 were quantified by the BioAnalyzer High Sensitivity chip (Agilent Technologies) and relative mRNA values of each isoform were calculated based on the ratios and differences of the PCR1 and 2 products.
**Table 2.1: Semi-quantitative PCR protocol**

PCR primers along with PCR temperatures and cycles are outlined in the tables for PCR1 and PCR2. Resultant products pairs including predicted BP sizes included at the bottom of each table.

**Cell culture, viral transduction and generation of stable cell lines**

HeLa (CCL-2) and HEK293T/17 cells were obtained from ATCC. SV40 large T antigen-immortalized mouse embryonic fibroblasts (MEFs) were generated as described previously [172]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% of Antibiotic-Antimycotic Solution (Corning, # 30-004-CI). Recombinant lentiviruses were produced and transduced to targeted cells as described previously [178]. Lentiviral multiplicity of infection (MOI) was calculated from infectious viral units/mL (IFU/mL) and determined using Lenti-X q-rtPCR kit (Clontech, #631235). To generate endophilin B2 knockout HeLa cells, cells transduced with lentiviruses encoding Cas9 and endophilin B2 sgRNA were selected with 1.5 μg/mL puromycin for 5 days followed by single clone isolation by serial dilution. The resultant single clones (three individual clones) were pooled together and used for the experiments.

**Immunoblotting**

Tissue homogenates and total cell lysates were prepared in radio-immunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% SDS,
1% Triton X-100, 1% Deoxycholate, 5 mM EDTA, pH 8.0) containing protease and phosphatase inhibitors and subjected to immunoblotting as described previously [73, 172]. The signals were measured and quantified using a LI-COR Odyssey CLx image scanner with the Image Studio v5 software (LI-COR Biotechnology).

Fluorescence microscopy

Cells were fixed in 4% paraformaldehyde-PBS for 10 min, permeabilized with 100 μg/mL digitonin for 10 min and stained for the indicated antibodies unless otherwise noted. Fluorescence images were obtained using an OLYMPUS IX81 deconvolution microscope (60x PLAN S-APO oil immersion objective, NA=1.35; 40x PLAN FLUORITE oil immersion objective, NA=1.30) equipped with a Hamamatsu ORCA-R2 Digital CCD camera (#C10600-10B) or a Leica AOBS SP8 laser-scanning confocal microscope (40x PLAN-APOCHROMAT, NA=1.3 oil immersion objective), deconvolved using SlideBook software (Intelligent Imaging Innovations) or Huygens deconvolution software (Scientific Volume Imaging), and analyzed using SlideBook software or and Imaris software (Bitplane).

Mitochondrial morphology and dynamics assay

To measure mitochondrial fission and fusion as well as visualize the mitochondrial morphology of cells, lentiviral plasmids encoding DsRED2-mito and GFP-mito were transduced into MEFs. Polyethylene glycol (PEG) was then used to chemically induce cell fusion as described by Legros et al. and Davidson et al. [179, 180]. Briefly, cells were seeded at equal densities into the same well after pre-mixing in a test tube and allowed to attach overnight. The following day cells were pre-treated with 20μG/mL cyclohexamide 1h before treatment PEG to inhibit protein synthesis. Cells were then with a solution of pre-warmed (37°C) 50% PEG and PBS for 1 min before thoroughly rinsing with pre-warmed media with FBS. Cells were then incubated in a humidified cell incubator at 37°C and 5% CO2 and subsequently fixed and mounted at indicated time points and subjected to fluorescence microscopy. Pearson’s correlation coefficient was determined using SlideBook software.
**Cell viability and apoptosis assays**

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, #G3582), Sulforhodamine B (SRB) (Sigma, #S1402) and PrestoBlue (Thermo-Fisher Scientific, #A-13261) assays according to manufacturer’s protocols. Spectrophotometry and fluorometry was performed using a Clariostar plate reader with MARS data management software (BMG-labtech). To determine apoptosis by flow cytometry, cells were stained with Annexin V-PE (BD Biosciences, #559763) and analyzed by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo v10 data analysis software (Tree Star). Dose response curve analysis was performed using non-linear regression in GraphPad Prism 7.0.

**Endocytosis assays**

To measure fluid phase endocytosis and endosome acidification, cells were incubated in Live Cell Imaging Solution (LCIS) (Thermo-Fisher Scientific, #A14291DJ) containing 50 μg/mL FITC-dextran MW 10,000Da (Sigma, #FD10S-100MG) or pHrodo Red-dextran MW 10,000Da (Thermo-Fisher Scientific, #P10361), respectively, at 37°C for 5 min, rinsed once or twice with LCIS, and analyzed by spectrofluorometry using a CLARIOstar plate reader with the MARS data management software (BMG Labtech). Fluorescence signals were normalized according to cell viability determined by PrestoBlue staining as described above.

EGF receptor-mediated endocytosis and endocytic trafficking were determined as described previously [166]. Briefly, cells were serum-starved for 16 h, incubated in DMEM containing 20 mM Hepes (pH7.5), 0.1% bovine serum albumin (BSA) (EMD Millipore, #126575), and 100 ng/ml EGF (Thermo-Fisher Scientific, #PHG0311) at 37°C for the indicated periods of time, and subjected to immunoblotting to measure EGFR degradation. To monitor receptor-mediated endocytosis, serum-starved cells were rinsed once with ice-cold PBS and then incubated in uptake medium (DMEM, 2% BSA, 20 mM Hepes, pH7.5) containing 1 μg/mL Alexa Fluor 488-EGF (Thermo-Fisher Scientific,
on ice for 1 h. After washing three times with ice-cold PBS to remove unbound ligands, cells were incubated at 37°C for the indicated periods of time, fixed in 4% PFA-PBS for 10 min, and subjected to immunofluorescence microscopy. Colocalization reported as Manders’ overlap coefficient was determined using the colocalization module in the Imaris 8.1 software by manual thresholding.

**Autophagic flux assays**

To determine lysosomal turnover of LC3-II, cells were incubated in serum and amino acid-free Dulbecco’s modified Eagle’s medium (SM) (custom-ordered from Invitrogen) or control complete medium (CM) in the presence or absence of 100nM Bafilomycin-A1 (Baf A1) (Enzo Life Sciences, #BML-CM110-0100) for 2hr, and subjected to immunoblotting as described above. The levels of LC3-II were normalized to β-actin, and autophagic flux of LC3-II was calculated as follows: basal flux = (CM with Baf A1)-CM; induced flux = (SM with Baf A1)-SM as defined in [181].

For the mRFP-GFP-LC3 (tfLC3) assay, cells were transduced with lentiviruses encoding tfLC3 for 3 days, starved for 2 h, fixed in 4% PFA for 10 min, and subjected to fluorescence microscopy. Pearson’s correlation coefficient was determined using SlideBook software.

**Virus entry assay**

The mouse adapted influenza A virus H1N1 strain A/PR 8/34 (PR8) was produced in 10 day old embryonated chicken eggs, purified by sucrose density gradient centrifugation, and quantitated by a fluorescence focus assay as previously described [182]. To monitor viral entry, trafficking and replication, MEFs seeded on coverslips at 150,000 cells/well of a 24-well plate were rinsed once with PBS and incubated in serum-free DMEM containing PR8 viruses for 1.5 h at 4°C. After rinsing twice with PBS, cells were further incubated in DMEM supplemented with 5% FBS at 37°C for the indicated periods time, rinsed twice with PBS, fixed in 4% paraformaldehyde-PBS containing 0.1% Triton X-100 for 15 min, blocked in 1% normal goat serum for 60 min, stained for endophilin B2 and/or NP, and subjected to fluorescence deconvolution or confocal
microscopy. Nuclei were stained with DAPI to generate nuclear masks and NP fluorescence signals in the nucleus were quantified using SlideBook software.

**Immunoprecipitation**

Hek293T/17 cells were transfected with 4.5 µg of DNA of either pEF1-Flag-UVRAG or pEF1-Flag empty vector, and 4.5 µg of either pBW-myc-endophilin B1 or pBW-myc-endophilin B2. Cells were then lysed in a 1% CHAPS solution containing 150mM NaCl and 10mM HEPES buffer at a pH of 7.5. Immunoprecipitation procedure performed as described previously [167]. Briefly, equal amounts of protein lysates for each transfection type were pre-cleared with protein G conjugated sepharose beads (GE healthcare, # 17-0618-01) for an hour at 4°C while rotating. Cleared supernatants were then transferred to protein G-conjugated sepharose beads that were pre-loaded with anti-flag antibody and incubated at 4°C overnight while rotating. The following morning, supernatants were collected and the beads rinsed three times with lysis buffer, and subsequently prepared for immunoblotting and probed with indicated antibodies (anti-flag and anti-myc).

**Statistical analyses**

Multiple independent experiments were tested for statistical significance using Graph Pad Prism 7.0. The statistical tests used were Student’s T test or ANOVA with appropriate post hoc tests as appropriate and noted in each figure legend. Threshold for statistical significance for each test was set at α=0.05 confidence level.
Chapter 3: Results

Preface

Despite endophilin B2 having high sequence and structural similarity to endophilin B1 [175], the cellular function remains unclear. Here, using genetic approaches, we show that endophilin B2 plays an important role in endosome acidification, maturation, and autophagy while being dispensable for apoptosis and endocytosis.

This section was completed with the following contributions: J.M.S., Y.T., LL, and Z.Z. performed the experiments. Y.I., Y.L., Z.T., L.Y., J.M.A. and M.M.Y. assisted with the experiments and manuscript preparation. J.M.S., Y.T., Z.C.C., and H-G.W. designed the experiments, analyzed data, prepared the figures and wrote the manuscript.
Endophilin B2 is dispensable for embryonic development:

To determine the importance of endophilin B2 in vivo, endophilin B2-deficient mice were generated from an embryonic stem cell clone containing a gene trap cassette in the first intron of the Sh3glb2 gene (Fig. 3.1A). Genotyping of the resultant mice demonstrated successful insertion of the cassette, which disrupted endophilin B2 protein expression in isolated mouse embryonic fibroblasts (MEFs) (Figs. 3.1B and C). Similar to endophilin B1 [164], loss of endophilin B2 does not affect embryonic development, as pups were born with normal ratios of Mendelian inheritance and were indistinguishable from wild-type littermates. Moreover, endophilin B2 demonstrates a similar tissue expression profile to endophilin B1, as indicated by co-expression of endophilins B1 and B2 were detected in the majority of tissues examined (Fig. 3.1D). Interestingly, as endophilin B2 is known to assemble heterodimers with endophilin B1 [148], we found that loss of endophilin B2 resulted in decreased endophilin B1 expression in several tissues including the heart, stomach, spleen, lymph node, bladder, prostate, skeletal muscle and brown adipose tissue, indicating that an epistatic interaction between endophilin B1 and B2 genes may regulate the stability of endophilin B1 in these tissues.

Furthermore, several tissue-specific isoforms of endophilin B1 have been implicated in alternative cellular functions in brain and testis [158, 183]. Since at least three distinct bands for endophilin B2 were detected in brain tissue lysates (Figs. 3.1D and 3.2), we sought to identify and characterize the isoforms of endophilin B2 in mice. The mouse endophilin B2 gene located on chromosome 2 and contains 13 exons that are predicted by the Ensembl database to encode five isoforms varying from 374 to 404 amino acids in length (Figs. 3.1E and 3.3) [177]. To assess the tissue expression profile of the endophilin B2 isoforms, we performed semi-quantitative reverse transcription PCR (RT-PCR) (Fig. 3.4A and materials and methods Table 2.1). We observed that isoform 1 of endophilin B2 was the most ubiquitously expressed, while isoforms 2 and 4 were increased in the brain, and isoforms 3 and 5 were undetectable in the tissues that we examined (Figs. 3.1F and 3.4B). Collectively, these results show that, while endophilin B2 is dispensable for embryonic development, it shares a similar tissue distribution
profile with endophilin B1 and exists as three predominant isoforms including the ubiquitously-expressed isoform 1 and the brain-enriched isoforms 2 and 4 in mice.

Figure 3.1: Endophilin B1 and B2 share similar expression profiles in mice. (A) Schematic of gene trap cassette insertion into endophilin B2 (B2) gene intron, genotyping primer design (described in methods) and cassette scheme (long terminal repeat =LTR, OV=Omnibank Vector). WT, wild-type; KO, knockout. (B) Genomic DNA prepared from mouse tail was subjected to PCR analysis using the primer sets shown in A. (C) Total cell lysates were prepared from MEFs with indicated genotypes and subjected to immunoblotting using the indicated antibodies. (D) Tissue homogenates were prepared from 10-week old endophilin B2 KO and wild-type mice and subjected to immunoblotting using the indicated antibodies. Sk. Muscle, skeletal muscle; WAT, white adipose tissue; BAT, brown adipose tissue.
(E) Schematic of predicted murine endophilin B2 isoforms with predicted splicing and amino acid length.

(F) The mRNA expression profile for each endophilin B2 isoform was determined by semi-quantitative RT-PCR as described in Fig.S3. Relative expression of each isoform in each tissue was normalized to isoform 1 expression.

Figure 3.2: Multiple isoforms of endophilin B2 exist in brain tissue
Western blot image from blot of figure 3.1 using a lower exposure to show multiple bands of endophilin B2 in Brain tissue lysates.
Figure 3.3: Endophilin B2 isoforms protein alignment
Alignments of proteins from predicted endophilin B2 isoforms in Mice according to ensembl database [177].
Figure 3.4: Semi-quantitative PCR for Endophilin B2

A) Schematic of the protocol for semi-quantitative RT-PCR amplification used to determine the amount of isoforms within the indicated tissues using primers at indicated areas on isoforms. B) Semi-quantitative RT-PCR amplification results normalized to isoform 1 containing products. *Isoforms 3 and 5 were not detected (ND) in this method of amplification.
Endophilin B2 is dispensable for mitochondrial apoptosis and mitochondrial dynamics:

Endophilin B1 was discovered as a pro-apoptotic BAX-interacting protein that promotes mitochondrial apoptosis [148, 149, 164]. Since endophilin B2 can dimerize with endophilin B1 [148] and displays a similar tissue expression profile, we first aimed to examine whether endophilin B2 plays an important role in mitochondrial apoptosis. To this end, endophilin B2-deficient (KO) and control wild-type (WT) MEFs were transduced with lentivirus encoding the BH3-only protein BCL-2 Modifying Factor (BMF) at an equal multiplicity of infection (MOI). BMF sensitizes cells to apoptosis by directly antagonizing anti-apoptotic BCL-2 family proteins (BCL-2, BCL-XL, and MCL-1) to allow for Bax and Bak activation [184]. Unexpectedly, we observed a similar decrease of cell viability in WT and endophilin B2-deficient MEFs after transduction with BMF lentivirus (Fig. 3.5A). Likewise, no statistical difference in cell death was detected between WT and endophilin B2-deficient MEFs exposed to the potent apoptosis inducers actinomycin D (ActD) and staurosporine (STS) (Fig. 3.5B). To further confirm the results, we generated endophilin B2-deficient HeLa cells using the CRISPR-Cas9 gene editing system (Fig. 3.5C). Consistently, depletion of endophilin B2 in HeLa cells did not alter the induction of mitochondrial apoptosis in response to BMF or ActD (Fig. 3.5, D and E). Notably, as observed in MEFs, the expression level of endophilin B1 in HeLa cells was not altered by the loss of endophilin B2 (Figs. 3.1C and 3.5C). Taken together, we conclude that endophilin B2 is not required for intrinsic apoptosis.
Figure 3.5: Endophilin B2 is dispensable for apoptosis induction in MEFs and HeLa cells.

(A, B) MEFs were transduced with lentiviruses encoding BMF at a multiplicity of infection (MOI) of 3 for 48 h and subjected to Sulforhodamine B (SRB) cell viability assay (A) or treated with 5 μg/mL actinomycin D (ActD) or 1 μM staurosporine (STS) for 48 h and subjected to MTS cell viability assay (B). Data from two independent experiments performed in triplicate are shown. (C) WT and endophilin B2 KO (CRISPR B2) HeLa cells were subjected to immunoblotting using the indicated antibodies. (D) HeLa cells were treated with various doses of ActD for 24 h and subjected to PrestoBlue cell viability assay. A representative dose response curve is shown from one of four independent experiments performed in triplicate. (E) HeLa cells were transduced with lentiviruses encoding BMF at a MOI of 3 for 24 h, stained with Annexin V, and analyzed by flow cytometry. Data represent two independent experiments (n = 6; duplicates from three performed in triplicate. Statistical significance was determined by unpaired Student’s T test with Holm-Sidak multiple comparison correction (A, B, and E), and non-paired two-tailed Student’s T test (D). All values are mean ± SD. ns, not significant.
In addition to reducing the insertion and oligomerization of Bax in mitochondrial membranes, endophilin B1 loss results in abnormally shaped mitochondria [156]. To further identify endophilin B2 as not affecting mitochondrial morphology or dynamics, MEFs were made to express either a DsRED or GFP fluorescent protein that localizes in the mitochondrial. To measure mitochondrial dynamics MEFs were fused together using a solution of poly-ethylene glycol to observe mixing of the fluorescent proteins within mitochondria through fission and fusion processes [179]. We observed that knockout of either endophilin B protein does not appear to greatly affect mitochondrial morphology in MEFs (Fig. 3.6A). In addition, the mitochondrial fusion assay reveals that neither endophilin B protein appears to affect the mixing of mitochondrial contents (Figs. 3.6, A and B). This result is consistent for endophilin B1 where mitochondrial fission and fusion appeared to be unaffected by expression [156]. However, the alterations to mitochondrial morphology upon loss of endophilin B1 expression is inconsistent since we did not observe any obvious defects as compared to previous results [156]. These data suggest that endophilin B2 is not a factor in mitochondrial dynamics.
Endophilin B2 plays a critical role in endosome maturation:

To further explore the cellular function of endophilin B2, we examined the subcellular localization of endophilin B2. Immunofluorescence microscopy revealed that endophilin B2 resides in the cytoplasm where it colocalizes with endophilin B1 (Fig. 3.7A). Moreover, we did not observe major changes in the localization of endophilin B2...
upon endophilin B1 deficiency (and vice versa), indicating that both proteins are able to sustain subcellular localization independent of heterodimerization. Subcellular fractionation analysis further revealed that endophilin B2 is enriched in the early (Rab5) and late (Rab7) endosomal fractions as well as in the LC3-II-enriched autophagosomal fractions (Fig. 3.7B) to suggest that endophilin B2 may regulate endocytic trafficking and/or autophagy.
Figure 3.7. Endophilin B2 colocalizes with endophilin B1 and is enriched in endosomal and autophagosomal compartments.

(A) MEFs were stained for endophilin B1 and B2 and analyzed by fluorescence deconvolution microscopy. Nuclei were stained with DAPI. Magnified images of boxed areas are shown on the right. Scale bars represent 10 μm. (B) Post-nuclear supernatant (PNS) prepared from HeLa cells was subjected to subcellular fractionation using a continuous 10–40% OptiPrep gradient and analyzed by immunoblotting using the indicated antibodies.
To determine if endophilin B2 plays a role in the endocytic pathway, we first examined fluid-phase endocytosis using fluorescein (FITC)-labeled dextran [185]. We did not observe any difference in the uptake of FITC-dextran between WT and endophilin B2-deficient HeLa cells (Fig. 3.8A) or MEFs (Fig. 3.8B) to indicate that endophilin B2 is not required for plasma membrane internalization during fluid phase endocytosis. To assess whether endophilin B2 regulates the endocytic trafficking of internalized vesicles, we utilized dextran conjugated to the pH-sensitive pHrodo-Red dye. As the fluorescence intensity of this dye increases in response to decreasing pH, pHrodo-Red dextran monitors the acidification and maturation of endosomes [3]. As expected, a time-dependent increase in the fluorescence intensity of pHrodo-Red dextran was observed upon pulse-chase of WT HeLa cells (Fig. 3.8C), indicating the progressive acidification of endosomes that reaches a plateau approximately 5 min after addition of the dye. Interestingly, while pulse-chase of endophilin B2-deficient cells with pHrodo-Red dextran resulted in a time-dependent increase in fluorescence intensity, the maximum fluorescence intensity was significantly reduced compared to WT cells (Figs. 3.8, C and D; \( p = 0.0261 \)). Similar results were obtained in MEFs (Figs. 3.8, E and F; \( p = 0.0322 \)) to suggest that loss of endophilin B2 impairs the acidification of endosomes.
Endosome acidification is a critical part of endosome maturation that allows for the activation of acid hydrolases along the endosomal-lysosomal pathway. Therefore, we sought to determine the effect of endophilin B2 deficiency on lysosomal degradation of EGFR, which is internalized from the plasma membrane by receptor-mediated endocytosis. To this end, cells were serum starved overnight to accumulate EGFR at the plasma membrane. Upon stimulation with EGF, EGFR is internalized and trafficked to the lysosome for degradation with minimal receptor recycling to the plasma membrane [1, 185]. Our results reveal that the loss of endophilin B2 significantly delayed the
degradation of EGFR upon EGF stimulation (Figs. 3.9, A and B; \( p<0.0001 \)). Lysosomes are dynamic organelles that are re-formed after fusion events and require an acidic lumen for the acid hydrolases to properly degrade material [44]. To determine if endophilin B2 is important for the biogenesis and/or acidification of lysosomes, HeLa cells were stained with the pH-insensitive lysosomal dye LysoTracker and the pH-sensitive dye LysoSensor, respectively. We observed that both LysoTracker and LysoSensor signals were not decreased but rather slightly increased in endophilin B2-deficient cells as compared to WT (Fig. 3.9C; \( p= 0.0001 \)), indicating that suppressed EGFR degradation observed in endophilin B2 deficient cells is not indirectly caused by lysosome impairment.
Figure 3.9. Loss of endophilin B2 suppresses EGFR degradation.

(A) HeLa cells were serum starved for 16 h, incubated with 100 ng/mL EGF for the indicated durations and subjected to immunoblotting using the indicated antibodies. Representative immunoblot from one of three independent experiments is shown. (B) EGFR levels in A were quantified and normalized to β-actin. (C) HeLa cells were co-stained with 50 nM LysoTracker Deep Red and 1 μM LysoSensor Green DND-189 for 30 min and analyzed by flow cytometry (n = 12; three independent experiments performed in quadruplicate). Data are presented relative to the WT mean. Statistical significance was determined by two-way ANOVA with Tukey’s multiple comparison test (B, C). All values are mean ± SD. B, p < 0.0001; C, p=0.0001.

Since endophilin B2 deficiency does not appear to affect lysosomes, we sought to further assess whether EGFR trafficking towards lysosomes is responsible for the delay
in degradation observed by fluorescence microscopy. Utilizing fluorescently tagged-EGF, endophilin B2 deficiency was demonstrated to significantly suppress the delivery of internalized EGFR to Lamp1-positive lysosomal structures (Figs. 3.10 A and B; $p<0.0001$). Importantly, and in accordance with our results for fluid phase endocytosis, the plasma membrane internalization of EGFR is independent of endophilin B2 (Fig. 3.10A). Taken together, these results indicate that endophilin B2 facilitates endosome maturation in both fluid-phase and receptor-mediated endocytic pathways.
Figure 3.10. Endophilin B2 deficiency impairs the lysosomal delivery of internalized EGF.

(A) HeLa cells were serum starved for 16 h, incubated with 1 μg/mL Alexa Fluor 488-conjugated EGF (AF-EGF) for the indicated periods of time, stained for Lamp1, and subjected to confocal microscopy. Images shown are representative of two independent experiments. Magnified images of boxed areas are shown on the right. AF-EGF and Lamp1 colocalization was determined as described in Materials and Methods. Scale bars represent 10 μm. (B) Mander’s overlap coefficient for AF-EGF with Lamp1 in A (n > 20 from two independent experiments). Statistical significance was determined by two-way ANOVA with Tukey’s multiple comparison test. All values are mean ± SD. ****, p < 0.0001.

Endophilin B2 deficiency decreases autophagic flux upon nutrient starvation:

During autophagy, autophagosomes mature into degradative autophagic vacuoles by fusing with late endosomes and/or lysosomes (reviewed in: [186, 187]). To determine
if the impairment in endosome maturation observed in endophilin B2-deficient cells affects autophagy, we performed an autophagic flux assay. During autophagic stimulation, such as nutrient starvation, cytosolic LC3-I is lipidated to form LC3-II bound to the autophagosome membrane [45]. Autophagosome maturation occurs upon fusion with lysosomes and degradation of lumenal contents; thus, measurement of the lysosomal turnover of LC3-II has been used as a reliable method to monitor autophagic flux [181, 188]. As increases in LC3-II can indicate either an increase in autophagosome biogenesis or a block in lysosomal degradation, autophagic flux assays were performed in the presence or absence of the lysosomal inhibitor, Bafilomycin A1 (Baf A1). We found that the starvation-induced lysosomal turnover of LC3-II was significantly suppressed in endophilin B2-deficient HeLa cells (Fig. 3.11; lane 8 minus lane 7) compared to WT (lane 4 minus lane 3). Quantification analysis further revealed that, indeed, starvation-induced autophagic flux is significantly suppressed by loss of endophilin B2 (Fig. 3.11B; \( p=0.0007 \)). Although endophilin B2-deficient cells also appear to have a decrease in basal autophagic flux under normal culture conditions (Fig.3.11A; compare lane 6 minus lane 5 versus lane 2 minus lane 1), statistical significance was not obtained (Fig. 3.11B). Similar experiments were performed in MEFs. Consistently, we observed that lack of endophilin B2 expression significantly suppressed starvation-induced autophagic flux (Fig. 3.11, C and D; \( p=0.0317 \)).

To further demonstrate the importance of endophilin B2 in autophagic flux, we performed the tandem-fluorescent LC3 (tf-LC3; mRFP-GFP-LC3) assay. The tf-LC3 assay discriminates between autophagosomes from autolysosomes (autophagosomes fused with lysosomes) due to the differences in pKa values between RFP and GFP. Upon autophagosome and lysosome fusion, the GFP moiety of tf-LC3 becomes quenched by the acidic environment to result in RFP\(^+\)GFP\(^-\) puncta [189]. We found that nutrient starvation of HeLa cells increased RFP\(^+\)GFP\(^+\) puncta (autophagosomes) regardless of endophilin B2 expression, indicating that endophilin B2 is not required for autophagosome formation (Fig. 3.11E). In contrast, the generation of RFP\(^+\)GFP\(^-\) puncta (autolysosomes) was reduced in nutrient starved endophilin B2-deficient cells compared to WT (Fig. 3.11E), suggesting impaired delivery of autophagosomes to acidified late
endosomes and/or lysosomes. Indeed, RFP\textsuperscript{+}GFP\textsuperscript{+} signals significantly accumulated in endophilin B2-deficient cells during nutrient starvation compared to WT (Fig. 3.11F; \( p < 0.0001 \)). As Baf A1 slightly but significantly enhanced the abundance of RFP\textsuperscript{+}GFP\textsuperscript{+} puncta in nutrient starved endophilin B2-deficient cells (Fig. 3.11F; \( \ddagger\ddagger\ddagger \ p < 0.0001 \)), a small degree of autophagic flux is maintained in the absence of endophilin B2. Nonetheless, these results clearly demonstrate an important role of endophilin B2 in the promotion of autophagosome maturation.
Figure 3.11. Loss of endophilin B2 suppresses autophagic flux.

HeLa cells (A, B) and MEFs (C, D) were incubated in starvation medium (serum and amino acid-free DMEM; SM) or control complete medium in the presence or absence of 100 nM bafilomycin A1 (Baf A1) for 2 h. (A, C) Total cell lysates were subjected to immunoblotting using the indicated antibodies. Representative immunoblots from five (A) and three (B) independent experiments are shown. (B, D) Autophagic flux under non-starved (basal flux) and starved (induced flux) conditions in A and C were calculated as described in the Materials and Methods. All values are mean ± SD. (E) HeLa cells were transduced with lentiviruses encoding mRFP-GFP-LC3 (tf-LC3) for 72 h, starved for 2 h and analyzed by fluorescence deconvolution microscopy. Nuclei were stained with DAPI. Scale bars represent 10 μm. (F) Pearson's correlation coefficient for mRFP and GFP in E (n > 70; duplicates from two independent experiments). The lines, boxes, and error bars represent median values, 25-75 percentiles, and 5-95 percentiles, respectively. Statistical significance was determined by two-way ANOVA with Tukey’s multiple comparison test. F, **** and ††††, p < 0.0001; B, p = 0.0007; D, p = 0.0317.
Loss of endophilin B2 delays nuclear translocation and replication of influenza A viruses:

To address the biological significance of the above results, we monitored influenza A virus infection in WT and endophilin B2-deficient cells. Influenza A virus is a member of the Orthomyxoviridae family of enveloped viruses. Influenza A virus is internalized through receptor-mediated endocytosis and trafficked to late endosomes. The acidic environment (~pH 5.0) of late endosomes triggers conformational changes in the viral envelope hemagglutinin that result in the fusion of the endosomal and viral membranes, release and transport of viral ribonucleoprotein particle (vRNP) to the nucleus, and replication of the viral RNA genome (vRNA) in the nucleus [190-195]. Notably, influenza A is a pathogen that requires both endosomal acidification and deployment of autophagy to efficiently replicate in host cells [196]. As endophilin B2-deficient cells were observed to have a defect in endosome acidification, we hypothesized that endophilin B2 may regulate viral trafficking. To this end, we monitored the subcellular localization of endophilin B2 in MEFs during influenza A virus infection. Indeed, endophilin B2 is observed to colocalize with the influenza A vRNA-binding nucleoprotein (NP) in the cytoplasm at approximately 1 to 1.5 hours post-infection (Fig. 3.12A), suggesting a role for endophilin B2 in viral trafficking. We next determined the effect of endophilin B2 depletion on vRNA trafficking and replication. In WT cells, we observed a time-dependent increase of NP in the nucleus and then in the cytoplasm, indicating replication of the viral genome and generation of new virions (Fig. 3.12B). Similar to fluid phase and receptor-mediated endocytosis, endophilin B2 was dispensable for the internalization of influenza A virus into host cells (Fig. 3.12B). However, both the nuclear and cytoplasmic accumulation of NP were significantly delayed by loss of endophilin B2 (Fig. 3.12C; \( p<0.0001 \)), suggesting that endophilin B2 is important for the efficient trafficking of vRNAs to the nucleus. Taken together, these data suggest that endophilin B2 deficiency effectively impairs the pH-dependent escape of influenza A virus from endosomes to suppress vRNA translocation into the nucleus for replication, a result consistent with impaired endosomal acidification upon the loss of endophilin B2.
Figure 3.12: Endophilin B2 deficiency attenuates nuclear trafficking of influenza A viral nucleoprotein.

WT or endophilin B2 KO MEFs were infected with influenza A virus H1N1 PR8 strain at a MOI of 5, incubated for the indicated durations, and co-stained for viral nucleoprotein (NP) and endophilin B2 (A) or stained for NP alone (B). (A) Fluorescence images were analyzed by confocal microscopy. Magnified images of boxed areas are shown on the right. Arrows indicate colocalization of endophilin B2 with NP. Scale bars represent 10 μm. (B) Cells were analyzed by fluorescence microscopy. (C) Mean NP fluorescence signals in the nucleus at the indicated time points in B were quantified using Slidebook 5.0 software (n > 30 from two independent experiments). The lines, boxes, and error bars represent median values, 25-75 percentiles, and 5-95 percentiles, respectively. Statistical significance was determined by two-way ANOVA with Tukey’s multiple comparison test. ns, not significant; ****, p < 0.0001.
**Endophilin B2 interacts with UVRAG**

Endophilin B1 interacts with UVRAG aiding in the production of PI3P on membranes during endosome and autophagosome maturation [23, 24, 167]. In an attempt to explain how endophilin B2 affects endosome and autophagosome maturation, we sought to observe whether endophilin B2 can interact with class III PI3K complex II binding partner UVRAG. To observe these interactions, we performed co-immunoprecipitation assays co-expressing flag-UVRAG with myc-tagged endophilin B1 or endophilin B2. Upon immunoprecipitation of flag-UVRAG we observed that over-expressed endophilin B1 and B2 do indeed interact with UVRAG (Fig. 3.13). Although the interaction between UVRAG and the endophilin B proteins is observed, these interactions appear to be a small portion of the total of endophilin B proteins being expressed. This is not surprising since, as demonstrated by another group, the interaction between endophilin B1 and UVRAG is enhanced upon stimulation such as nutrient deprivation, while this assay was performed under basal culture conditions [74]. Overall, these data help to identify future potential targets of how endophilin B2 can affect endosome and autophagosome maturation.

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**Figure 3.13: Endophilin B2 interacts with UVRAG without stimulation**

Representative immunoblot from three independent assays of HEK293 transfected with either flag-UVRAG or flag tag alone (empty flag), and either Myc-endophilin B1 (Myc-B1), or Myc-endophilin B2 (Myc-B2). Cells were subjected to co-immunoprecipitation (IP) as described in methods and materials and immunoblotted (IB) for anti-Flag tag and anti-Myc tag antibodies. All cells were harvested under normal culture conditions without stimulation by starvation or EGFR ligand stimulation by EGF. *B2 is the expected molecular weight for endophilin B2.
Chapter 4: **Discussion**

My study demonstrated novel roles for endophilin B2 in endocytic membrane trafficking. Notably, ablation of endophilin B2 impaired endosomal maturation as shown by suppressed endosomal acidification during fluid phase endocytosis, decreased lysosomal degradation of internalized EGFR, impaired starvation-induced autophagic flux, and delayed nuclear translocation of influenza A vRNA. Furthermore, upon removal of endophilin B2 expression, delivery of EGFR upon receptor-mediated endocytosis and endocytic trafficking to Lamp1-positive compartments is delayed. Additionally, deficiency of endophilin B2 expression abrogated fusion of newly generated autophagosomes to acidified late endosomes and/or lysosomes upon nutrient starvation. As the loss of endophilin B2 did not impair internalization from the plasma membrane or lysosomal function, endophilin B2 appears to regulate endocytic membrane trafficking along the endosomal-lysosomal pathway.

In contrast, endophilin B2 has recently been reported to be indispensable for the internalization of ligand-stimulated glutamate receptors in cultured neuronal cells [173]. The discrepancy in results may be due to the differential expression of endophilin B2 isoforms in neuronal and non-neuronal cells. Indeed, this study has identified and characterized the murine endophilin B2 isoforms and revealed a tissue-specific increase of isoforms 2 and 4 in neuronal tissues compared non-neuronal tissues that predominantly express isoform 1. Interestingly, while endophilin B1 promotes apoptosis in non-neuronal cells, brain-specific isoforms of endophilin B1 have been reported to have anti-apoptotic functions during ischemic injury [183]. Thus future studies are warranted to elucidate the importance of the differential expression, or the function, of endophilin B2 isoforms. Introducing the various isoforms back into knock out cells of the appropriate tissue type should help to identify any functional differences. Additionally, since the long form (isoform 4) of endophilin B2 has been reported to interact with CPG2 in neuronal cells, it may be prudent to establish the binding partners of the various isoforms within tissues.
Doing so will help further the understanding of how endophilin B2 affects endosome and autophagosome maturation and whether endophilin B2 functions differ between tissues or cell-types.

Although endophilin B1 and endophilin B2 assemble heterodimers and have a similar subcellular localization in endothelial and fibroblast cells, this dissertation provides insight into unique functions of each family member. Most notably, we revealed that endophilin B2 is dispensable for mitochondrial apoptosis. While this result is not entirely surprising given that endophilin B2 is not a direct interactor of Bax [148], the results suggest that endophilin B1 homodimers may be critical for its pro-apoptotic function. In contrast, the novel roles of endophilin B2 in endocytic trafficking, endosomal maturation, and autophagy are similar to previously defined functions of endophilin B1 in EGFR trafficking and degradation [166] and autophagic flux [167, 172], suggesting that heterodimers of endophilin B proteins may regulate endocytic membrane dynamics. In support of this hypothesis, endophilin B2 was observed to co-fractionate with endophilin B1 in endosome and autophagosome-enriched fractions. Moreover, loss of endophilin B2 led to impairment of starvation-induced autophagic flux. Consistently, a similar effect on autophagic flux is observed in endophilin B1-deficient models [167, 172] and a recent report has shown that endophilin B2 promoted mitophagy through dimerization with endophilin B1 [174]. Moreover, this finding is in agreement with the importance of endosome maturation for autophagic flux [187]. Further studies are warranted to elucidate the interaction between Endophilin B family homodimers and heterodimers in the regulation of intracellular membrane dynamics.

The precise molecular mechanism behind the regulation of endosome maturation by endophilin B2 is yet to be determined. As discussed above, endophilin B2 may heterodimerize with endophilin B1 to promote endosome maturation through the activation of phosphatidylinositol 3-kinase Vps34 and/or the recruitment of the UVRAG-Hops complex to early endosomes [24, 167]. In fact, the preliminary data show that UVRAG can be co-precipitated not only with endophilin B1 but also with endophilin B2. Further efforts are necessary to confirm the interaction of endophilin B2 with UVRAG, and whether this interaction can affect PI3P production on membranes.
Furthermore, defining which domains within the endophilin B2 protein are necessary, how the interactions take place, or whether the dimerization of endophilin B2 with endophilin B1 is necessary for these interactions need to be addressed. Alternatively, or in addition, we propose that endophilin B2 may regulate endosomal maturation through its effects on the cytoskeletal network. Recently, endophilin B2 has been identified as a regulator of the vimentin cytoskeletal network via its SH3-mediated interaction with the cytoskeletal linker protein Plectin 1 [163]. As the vimentin network controls late endosome and lysosome positioning as well as the acidification of endosomes and lysosomes [197], we propose that this interaction may be critical for the phenotype reported here. Moreover, vimentin has also been identified as a vital regulator for the trafficking and endosomal escape of influenza A vRNP [198]. As the phenotype of vimentin-null cells infected with influenza A virus is similar to that observed in endophilin B2-deficient cells, we hypothesize that endophilin B2 may cooperate with vimentin to regulate endosome acidification and/or the positioning of endosomes during maturation. Therefore, future studies should include observations into the state of the vimentin network and the state of the lysosomal and endosomal acidification.

The endocytic degradation of EGFR plays an important role in tumorigenesis. EGFR, being an oncogene, is observed to be overexpressed or mutated in many cancer types [201-205]. Upon activation of EGFR, an array of signaling cascades are initiated, which include: growth signaling through mitogen-activated protein kinase (MAPK), protein transcription through nuclear transcription factor-kappa B (NF-κB) upon activation through class-1-PI3K stimulation of AKT/PKB, or intracellular calcium modulation by phospholipase C-γ (PLC-γ) [206]. In glioblastoma, EGFR is overexpressed in approximately 50% of cases [207] typically in the form of the truncated mutant of EGFR (EGFRvIII)[205]. Although EGFRvIII does not bind ligands due to the truncation resulting in an improperly formed ligand-binding pocket, the tyrosine kinase activity in the intracellular portion of the protein remains functional [208, 209]. In addition to constitutive activation, EGFRvIII is preferentially recycled rather than processed towards degradation exacerbating signaling from, and over-expression of, the receptor [209]. Interestingly, the tumor suppressor phosphatase and tensin homologue
deleted on chromosome 10 (PTEN), one of the 5-phosphatases that can dephosphorylate PI(3, 4, 5)P3 at the plasma membrane, has recently been identified to be important in trafficking EGFR to late endosomes through dephosphorylation of Rab7 at the amino acid residues serine 72 and tyrosine 183 [214]. In addition, PTEN activity helps to bring GDP bound Rab7 to early endosomes since PTEN can associate with PI3P positive membranes [214, 215]. Therefore, dysregulation of PTEN not only results in the unfettered growth signaling through the PI3K-AKT signaling axis, but also the disruption of EGFR degradation through endosome maturation.

In humans, Endophilin B1 is found within the chromosome region 1p22, which is frequently deleted in various cancer types [216-218]. Moreover, endophilin B1 expression has been noted to be lower in several cancers including liver, prostate, pancreas, colon, and gastric cancers [151, 152, 219-222]. Decreased expression of endophilin B1 has been correlated with a poor prognosis in both early stage colorectal cancer, pancreatic cancers, and liver cancer [220, 222, 223]. As mentioned previously, EGFR degradation is impaired upon loss of endophilin B1 expression in breast cancer cells thereby increasing the metastatic potential of the cells [166]. Thus, decreases in endophilin B1 may increase metastatic potential through disruption of endosome maturation and receptor degradation.

Similarly, autophagy has a pathological impact on tumorigenesis. Although controversy surrounds whether autophagy acts as a driver of cancer development, since the majority of the core autophagy proteins do not harbor mutations, autophagy nonetheless serves as a survival mechanism by providing metabolites and energy substrates necessary for cancer cells undergoing selective pressures [226, 227]. Inhibition of autophagy, by use of small-molecule inhibitors, is being actively explored in several clinical trials as a means of therapy in addition to conventional cancer chemotherapies. However, due to the duality of autophagy, being either a pro-survival or pro-death mechanism, there is concern over the effectiveness and outcomes of these trials [227-229]. Regardless, dysregulation of autophagy is implicated in cancer development due to the increased ROS generated by damaged mitochondria. Indeed, this phenomenon was observed by our group in an endophilin B1 deficient mouse model of lymphoma [172].
Although endophilin B2-deficient mice in this study were born at expected Mendelian ratios and indistinguishable from wild-type littermates, evidence of endophilin B2 in the pathogenesis of disease is currently lacking. However, high expression of endophilin B2 has been reported in aggressive human prostate tumors as well as primary tumors from a transgenic mouse model of prostate cancer to suggest that endophilin B2 could serve as a potential biomarker [159-161]. Furthermore, I identified endophilin B2 expression affecting relapse free survival in breast cancer (Fig. 4A), and overall survival in gastric cancers (Fig. 4.1B) through a meta-analysis using a publicly available database that assesses gene expression and patient survival [224, 225]. Although high levels of expression in breast cancers identifies with a better prognosis in relapse free survival \((p=2.5\times10^{-8})\), high expression in gastric cancers leads to a poorer prognosis overall survival \((p=0.0078)\). With the understanding that relapse free survival and overall survival is not comparable due to the differences between the definitions of either, this finding does provide evidence that endophilin B2 expression can affect cancer progression. Further studies are warranted to determine whether endophilin B2 expression may become dysregulated in cancers, and whether or not this dysregulation ultimately has impact on patient survival. In addition, studies into how the expression of endophilin B2 affects cancer metastasis should be performed given how our data indicates decreased receptor degradation and autophagic flux upon loss of endophilin B2. The potential outcome of this study would likely show loss of endophilin B2 increasing the metastatic potential of cells since decreased receptor degradation results in increased signaling from receptors. Additionally, it may show endophilin B2 deficiency increases the genetic damage of cells due to ROS generation from lack of autophagic clearance of damaged mitochondria. On the other hand, overexpression of endophilin B2 in cancers poses an intriguing contradiction since it may actually increase the endosome trafficking and autophagic flux. In addition, while never explored in this dissertation, the possibility exists that receptor recycling may be impacted by the expression of endophilin B2.

Collectively, our studies provide a foundation to understand the physiological and pathological roles of Endophilin B2 in human health and disease.
Figure 4.1: Endophilin B2 expression in breast and gastric cancers affects patient survival

Kaplan-Meier curve of either (A) breast cancer patients showing the probability of relapse free survival (RFS), or (B) gastric cancer patients showing the probability of overall survival (OS), based on the expression of endophilin B2. Statistical analysis performed by log rank test (A) \( p=2.5 \times 10^{-8} \), hazard ratio (HR) =0.64 with a standard deviation= (0.55-0.75), (B) \( p=0.0078 \), HR=1.39 with a standard deviation = (1.09-1.77). Data obtained from [224, 225].
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90


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