MOLECULAR MECHANISM OF AUTOPHAGY-BASED UNCONVENTIONAL SECRETION OF IL-β

Shanya Jiang

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Shanya Jiang
Candidate
Molecular Genetics and Microbiology
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Vojo Deretic, Chairperson

Bridget Wilson

Eric Prossnitz

Robert Orlando
MOLECULAR MECHANISM OF AUTOPHAGY-BASED UNCONVENTIONAL SECRETION OF IL-1β

by

SHANYA JIANG

B.S., Biological Engineering (Medical), Jilin University, China, 2009

DISSertation

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At the end, glory to the Almighty one!
MOLECULAR MECHANISM OF AUTOPHAGY-BASED UNCONVENTIONAL SECRETION OF IL-1β

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ABSTRACT

Proteins without signal peptides can still be released into extracellular space via ER/Golgi-independent unconventional secretory pathway(s) that remain to be revealed. A class of leaderless proteins including the pro-inflammatory cytokine interleukin-1β (IL-1β) is processed in the cytosol by proteolytic machineries such as the inflammasome prior to secretion. Studies in yeast uncovered a new unconventional secretory pathway that is dependent on GRASP (Golgi Reassembly and Stacking Protein) and autophagy proteins. The purpose of this work was to determine whether this autophagy-based unconventional secretory pathway (secretory autophagy) is conserved in mammals, how this secretory autophagy differs from the traditional degradative autophagy, and how GRASP intersects with the autophagy pathways. Here, we provide the first evidence that autophagy mediates unconventional secretion of IL-1β in an inflammasome, GRASP55, and Rab8-dependent manner in mouse bone marrow-derived macrophages, a route which also applies to IL-18 and non-inflammasome substrate HMGB1. GRASP55 was also found to be mediating autophagosome formation possibly making secretory autophagosomes. We also give initial insights to the divergence between degradative
autophagy and this secretory autophagy pathway, which preferentially utilizes some of the autophagy-related proteins, LC3A, GABARAP, and p62, but not LC3B, NBR1, TBK1, and ULK1. We also discuss the way that GRASP55, the conserved player of unconventional secretion, negatively regulates removal of damaged mitochondria by mitophagy, which might be due to competition between secretory and degradative autophagy. Taken together, these data indicate there is a conserved, unconventional secretory pathway involving GRASP and autophagy. It also suggests that although secretory and degradative autophagy share some core components, secretory autophagy also involves specialized molecular machinery to execute its unconventional secretory function and balance with degradative autophagy.
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CHAPTER 1

INTRODUCTION
1.1 Unconventionally-secreted Proteins

Most eukaryotic proteins containing internal or N-terminal signal peptides are secreted by the ER/Golgi-dependent conventional secretory pathway. However, some cytosolic or nuclear proteins without such leader peptides can still be delivered into extracellular space by relatively elusive pathway(s) \(^1\). Pro-inflammatory cytokine interleukin-1β (IL-1β) is one of the earliest examples to lack a hydrophobic signal common in most secreted proteins \(^2\) and to be secreted unconventionally \(^3\). At the same time, some signal peptide-containing proteins are also shown to be delivered unconventionally in a COPII/Golgi-independent manner. Integral protein Cystic Fibrosis Transmembrane conductance Regulator (CFTR) \(^4\) and the *Drosophila melanogaster* α integrin subunit \(^5\) are the best examples of such proteins. These two processes are both called unconventional protein secretion.

A large number of proteins have been identified to be secreted/trafficked unconventionally (Table 1). These proteins are shown to be involved in wound healing, cancer angiogenesis, cancer metastasis, inflammation, cytoprotection, and neurodegeneration. Also, these proteins are often released during stress conditions and some of them have distinct but related intracellular and extracellular functions. Therefore, it is speculated that unconventional secretion might have developed early in the evolution before conventional secretion to rapidly connect diverse processes without going through extraordinary regulations \(^6\). Unconventional secretion might also be the mechanism to rapidly react to stress conditions.
<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Acb1/AcbA</td>
<td>Regulates spore viability</td>
<td>7, 8, 9, 10</td>
</tr>
<tr>
<td>α-integrin</td>
<td>Actin cytoskeleton organization and transduction of intracellular signals regulating cellular functions</td>
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<td>Ion channel which transports chloride and thiocyanate ions across epithelial cell membranes</td>
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<td>IL-1β</td>
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1.2 Known Factors for Unconventional Protein Secretion

Although an increasing number of unconventionally-secreted proteins were revealed, there are only a few factors discovered that play a role in unconventional protein secretion. Caspase-1 and GRASP are the most studied ones among those few factors.

1.2.1 Golgi Reassembly Stacking Proteins (GRASP)

Mammalian cells have two homologues of GRASP, GRASP65 and GRASP55, localizing in cis- and medial-Golgi respectively (Figure 1.1A)\(^{30}\). Both GRASPs have conserved N-terminal PDZ domains and less-conserved C-terminal Serin/Proline-Rich (SPR) domains. Both GRASPs are heavily phosphorylated at Serine/Proline-Rich domain during mitosis and are considered as tethering factors to mediate Golgi stacking and ribbon linking (Figure 1.1B)\(^{31}\). These functions are carried out by GRASPs via trans-oligomerization\(^{32,33}\). GRASPs are first anchored to a membrane via myristoylation at Gly2 and via PDZ2 domain binding to GM130 or Golgin45, enabling GRASPs to lie parallel to the membrane\(^{34,35,36}\). Then, an internal ligand in the PDZ2 domain within a GRASP on one membrane binds to the ligand binding pocket at the PDZ1 domain in another GRASP on the opposing membrane, bringing adjacent membranes to form Golgi stacks or to link Golgi ribbons\(^{37}\).

Surprisingly, the single homologue of GRASP in \textit{D. discoideum}, GrpA, was shown to be involved in ER/Golgi-independent, unconventional secretion of cytosolic protein AcbA, a factor required for differentiation of pre-spore cells\(^ {7}\). Also during epithelium remodeling in \textit{D. melanogaster}, dGRASP mediates unconventional transport of the transmembrane protein \(\alpha\) integrin to the plasma membrane\(^ {5}\).
Figure 1.1 Cellular localization and domain structure of GRASP65 and GRASP55

(A) Golgi localization of GRASP65 and GRASP5. (B) Functional domains of GRASP.
Figure 1.1 Cellular localization and domain structure of GRASP65 and GRASP55

GRASP65 and GRASP55 are localized on cis- and medial-Golgi respectively. Both GRASPs have conserved N-terminal PDZ domains and less-conserved C-terminal Serin/Proline-Rich (SPR) domains. Both GRASPs are heavily phosphorylated at the Serine/Proline-Rich domain during mitosis and are considered as tethering factors to mediate Golgi stacking and ribbon linking via trans-oligomerization. GRASPs are first anchored to a membrane via myristoylation at Gly2 and via the PDZ2 domain binding to GM130 or Golgin45, enabling GRASPs to lie parallel to the membrane. Then, an internal ligand at the PDZ2 domain within a GRASP on one membrane binds to the ligand binding pocket at the PDZ1 domain in another GRASP on the opposing membrane, bringing adjacent membranes to form Golgi stacks or to link Golgi ribbons.
However, whether GRASP is generally involved in the unconventional secretion/trafficking of other leaderless proteins and the mechanism by which a Golgi protein is involved in the ER/Golgi-independent unconventional secretion remains to be elucidated.

1.2.2 Caspase-1

Caspase-1 is a protease, which cleaves the precursor forms of IL-1β, IL-18 and IL-33 into active forms. Caspase-1 itself is usually activated by inflammasome components, which respond to various internal and external stimuli including endogenous danger signals (e.g. endogenous ATP released during cell death) \(^\text{27}\), exogenous toxic compounds (e.g. Alum and Silica) \(^\text{38}\), and pathogen-associated molecular patterns (PAMPs, e.g. microbial toxin Nigercin) \(^\text{39,40}\).

Interestingly, active Caspase-1 has been reported to regulate unconventional secretion of pro-IL-1α, FGF-2, Caspase-1 itself, and many other leaderless proteins like Galectin-3 and Annexin A2. Pro-IL-1α and FGF-2 even showed direct physical interactions with Caspase-1, although they are not catalytic substrates of Caspase-1 \(^\text{14}\). These findings suggest that Caspase-1 itself or some unknown substrates of Caspase-1 might be generally involved in the unconventional secretion.

However, whether Caspase-1 is serving as a general carrier, directly binding to all the proteins, that were discovered to be unconventionally secreted in Caspase-1-dependent manner, remains unknown. The relationship between Caspase-1 and GRASP55 during unconventional secretion also requires further investigation.
1.3 Unconventional Secretory Pathways

Unconventionally secreted/trafficked proteins can be divided into two general categories:
1) COPII/Golgi-bypassing unconventional trafficking of signal peptide-containing proteins, 2) ER/Golgi-independent, unconventional secretion of signal peptide-lacking cytosolic/nuclear proteins.

1.3.1 Golgi-independent unconventional trafficking of signal peptide-containing proteins

These proteins contain signal peptides and can be inserted into the ER lumen. However they are delivered later to the plasma membrane in a COPII/Golgi-independent manner. CFTR is the best-characterized example of unconventionally trafficked signal peptide-containing proteins. It is a cAMP-dependent chloride channel located at the apical membrane of various epithelia in lung, pancreas, kidney, and intestine. Normally, CFTR is transported unconventionally by COPII vesicles bypassing Golgi in a Syntaxin 5/ARF1/RAB1A/RAB2-independent manner, which is required for early conventional secretory pathway \(^4\). ΔF508, a deletion mutation in CFTR gene accounts for ~70% of the Cystic Fibrosis cases \(^41\). This deletion prevents proper folding of CFTR and its unconventional trafficking to the plasma membrane.

1.3.2 Unconventional secretion of signal peptide-lacking cytosolic/nuclear proteins

Five different kinds of unconventional secretory pathways have been proposed for signal peptide-lacking cytosolic/nuclear proteins and can be divided into two categories: vesicular and non-vesicular.
Non-vesicular pathways

Non-vesicular pathways involve two mechanisms: 1) Direct traverse across the plasma membrane, and 2) ABC transporter-dependent translocation.

1) Direct translocation across the plasma membrane

This type of pathway is well characterized through studies of the secretion of fibroblast growth factor 2 (FGF2), a pro-angiogenic factor \(^{42}\). FGF2 first interacts with the acidic membrane lipids phosphoinositide phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P\(_2\)) at the inner leaflet of the plasma membrane \(^{13}\). After being phosphorylated by Tec kinase at tyrosine residue 82, FGF2 is oligomerized by PtdIns(4,5)P\(_2\) and inserted into the plasma membrane \(^{43}\). Interaction with heparin sulfates at the outer leaflet of the plasma membrane then induces translocation of FGF2 across the plasma membrane \(^{12, 44}\). HIV-1 Tat and annexin A2 are also reported to be secreted in a similar way \(^{45, 46}\).

2) ABC transporter-dependent pathway

This pathway is responsible for translocating lipidated peptides and proteins across the plasma membrane. In yeast *Saccharomyces cerevisiae*, the secretion of signal peptide-lacking pheromone alpha-factor is mediated by Ste6 \(^{47}\), a member of the ATP-binding cassette (ABC) transporter family which regulates the translocation of peptides across the ER membrane and cholesterol across the plasma membrane. In *Drosophila melanogaster*, a geranyl-modified attractant of germ cell was also shown to be secreted by the ABC transporter Mdr49, a functional homologue of Ste6 \(^{48}\).
Vesicular pathways

These pathways involve vesicular intermediates and are best understood for IL-1β secretion. IL-1β is a pro-inflammatory cytokine and it belongs to IL-1 family cytokines, which also include IL-1α, IL-18 and IL-33. It is one of the earliest examples of signal peptide-lacking proteins that are secreted by ER/Golgi-independent unconventional secretory pathways. Upon induction by TLR ligands (e.g. LPS), it is synthesized as a 35kDa-precursor protein. Subsequently, it is processed into the 17kDa-active form by Caspase-1-containing in inflammasome complex, which is activated by DAMPs (Damage-associated molecular patterns, e.g. ATP, Monosodium urate crystals) and PAMPs (Pathogen-associated molecular patterns, e.g. bacterial toxin nigericin, double stranded-RNA) in the cytosol, then ultimately secreted into extracellular space. The exact mechanism of IL-1β secretion has been elusive. The following three pathways have been proposed to mediate unconventional secretion of IL-1β (Figure 1.2):

1) Endolysosome exocytosis-dependent

In LPS-activated human monocytes, a fraction of pro-IL-1β and pro-Caspase-1 were found to be contained within vesicles that co-fractionate with late endosomes and early lysosomes on Percoll density gradients. However, pro-IL-1β was only partially co-localizing with Cathepsin D (late endosomal/lysosomal marker) and Lamp1 (lysosomal marker), indicating that pro-IL-1β-containing vesicles belong to a specialized subset of endolysosomes. Subsequent stimulation by extracellular ATP triggered the efflux of K⁺
Figure 1.2 Proposed pathways for unconventional secretion of IL-1β
Figure 1.2  Proposed pathways for unconventional secretion of IL-1β

Three pathways have been proposed to mediate unconventional secretion of IL-1β.

1) Exosome release through multivesicular bodies; 2) Microvesicle shedding; 3) Exocytosis of secretory endolysosome. It is considered that the IL-1β-converting enzyme Caspase-1 is trans-located to the vesicles and secreted together with Caspase-1. However, the identity of the secretory vesicles remains elusive and no single player involved in this process has been identified.
from the cell, followed by Ca\(^{2+}\) influx and activation of three phospholipases: phosphatidylcholine-specific phospholipase C and calcium-independent and -dependent phospholipase A2. These ultimately resulted in secretory endolysosome exocytosis and the secretion of mature IL-1\(\beta\). Nevertheless, how pro-IL-1\(\beta\) might have been translocated into the secretory endolysosomes remains elusive.

2) Microvesicle-dependent

In THP-1 human monocytic cell lines, microvesicles of \(<0.5\ \mu\text{m}\) diameter were shown to be shed from their plasma membrane rapidly within 2–5 s after activation of P2X7 receptors (cation-permeable ligand gated ion channels that open in response to the binding of extracellular ATP) by ATP which is dependent on extracellular Ca\(^{2+}\). Within 2 min, bioactive IL-1\(\beta\) was present in the isolated microvesicles. The shed microvesicles then released the IL-1\(\beta\) contained inside, into the extracellular space \(^{26}\). However, later studies failed to reproduce the observation of microvesicle shedding \(^{50}\), implicating the secretion of mature IL-1\(\beta\) is dependent on Ca\(^{2+}\) release from intracellular stores, rather than an influx of extracellular Ca\(^{2+}\).\(^{51}\).

2) Exosome release-dependent

The formation of IL-1\(\beta\) containing-internal vesicles within multi-vesicular bodies (MVBs) and its subsequent fusion with the plasma membrane was also suggested to regulate IL-1\(\beta\) secretion. Because IL-1\(\beta\) secretion from ATP-stimulated BMMs was accompanied by the release of the MHC class II molecules (exosome markers), it therefore was proposed that exosomes derived from MVBs contained both IL-1\(\beta\) and
Caspase-1. However, MHC class II molecules are also a lysosomal marker and the authors failed to isolate exosomes containing mature IL-1 β, thus additional experiments were needed to support this mechanism.\(^{28}\)

To summarize, no solid conclusion of a single pathway for unconventional secretion of IL-1β was established and none of the factors directly involved in IL-1β secretion have been reported. Also, the steps of IL-1β processing and incorporation into vesicular intermediates is still unknown. Given that autophagy is a topological inverter to capture cytosolic proteins and components; and also considering autophagy’s close connection with multi-vesicular bodies and membrane trafficking, it suggests autophagy as a potential mediator for IL-1β secretion. Additionally, TLR signaling, which is important for IL-1β synthesis, processing, and secretion in human monocytes,\(^{52}\) induces autophagy in myelomonocytic cells.\(^{53}\) Actually, recent studies in yeast about unconventional secretion of Acb1 began to reveal the new secretory role of autophagy in unconventional secretion.\(^{8,10}\)

### 1.4 Autophagy-based Unconventional Protein Secretion

Macroautophagy (referred as autophagy hereafter) is typically considered as a degradative pathway for 1) digestion of cytoplasmic compartments to supply amino acids during starvation,\(^ {54}\); 2) destruction of long-lived protein aggregates\(^ {55}\) and damaged mitochondria;\(^ {56,57}\); 3) elimination of intracellular microbes like M. tuberculosis\(^ {58,59}\).
Recent studies in yeast about unconventional secretion of Acb1 shed light on a 4th important function for autophagy. In *S. cerevisiae*, starvation induced secretion of Acb1 and this process required Grh1 (yeast homologue of GRASP). Mutants defective for several Atg genes (Atg5, 7, 8, and 12) also failed to secrete Acb1 suggesting components in autophagy pathways are required for starvation-induced unconventional secretion Acb1. Vam3 (a syntaxin needed for the fusion of autophagosome with vacuole) and Ypt7 (yeast homologue of Rab7 required for the fusion of autophagosome with vacuole) were not involved in this process indicating that fusion of autophagosome-like vesicles containing Acb1 with the vacuole (lysosome-like structure in yeast) is not necessary for starvation-induced unconventional secretion of Acb1. Nevertheless, the involvement of Ypt6 (a Rab GTPase functioning in recycling of endosomal components), Tlg2 (endosomal t-SNARE which regulates membrane traffic through the yeast endocytic system), Vps4 (an ATPase responsible for ESCRT-III disassembly and required for MVB protein sorting), and Vps23 (a component of the ESCRT-I complex involved in ubiquitin-dependent sorting of proteins into the MVB sorting pathway) suggests that endosomal or MVB components play a role in the unconventional secretion of Acb1. The requirement of Sso1, a plasma membrane t-SNARE, also suggests that the fusion of autophagosome-like vesicles containing Acb1 with plasma membrane might have occurred during the Acb1 secretion process 8 *(Figure 1.3).*

Similarly in yeast *Pichia pastoris*, starvation and rapamycin treatments (which are often used to induce autophagy) both promoted unconventional secretion of Acb1, which required Grh1 and autophagy proteins Atg 1, 6, 8, 9, and 17. Interestingly, Atg11, which
is necessary for selective, receptor-dependent autophagy-related pathways, was also shown to be involved, indicating the requirement of some selectivity in this process. Vam7 and Ypt7, components required for fusion with the vacuole, were again shown not to be involved. Spo14 (encodes PLD which is important for exocytosis) and Sso1 were required for Acb1 secretion suggesting the fusion with plasma membrane is necessary for this process 10.

All these data suggest the existence of a conserved unconventional secretory pathway involving autophagy components and Grh1. This autophagy-based unconventional secretory pathway is plausible since autophagy is a bulk topological inverter for cytosolic proteins and components and provides a means to capture Acb1 into the lumen of autophagosome-like structures. Subsequently, these vesicles containing Acb1 might somehow avoid fusion with the vacuole for degradation, but rather are targeted to the plasma membrane for secretion.

Further proof is needed to show that this autophagy-based unconventional secretory pathway is conserved in higher eukaryotes. It is also unclear that if autophagosomes or MVBs transport unconventionally secreted cargoes directly to the cell surface and how autophagy recognizes these cargoes and avoids fusing with the lysosomes. Importantly, these studies have not isolated any autophagosome-like vesicles containing Acb1. Therefore, it is alternatively possible that the disturbance in organelle maturation affects unconventional secretion indirectly. In addition, the interplay between GRASP and autophagy during unconventional secretion needs to be further investigated.
Figure 1.3 Autophagy-based Unconventional Secretion of Acb1 in yeast
Figure 1.3 Autophagy-based Unconventional Secretion of Acb1 in yeast

In yeast, Acb1 is unconventionally secreted upon starvation and rapamycin treatment (conditions that usually induce autophagy). It is dependent on several Atg-related proteins: Atg1 and Atg17 (needed for autophagy initiation and autophagosome formation), Atg5/7/8/12/9 (required for autophagosome formation and elongation), Atg11 (involved in selective autophagy). It also requires endosomal proteins and multivesicular body components, Tlg2, Ypt6, Vps4, and Vps23, whereas Vamp3, Vamp7, and Ypt7, which are involved in the fusion with the vacuole (lysosome), are not needed. Sso1 (plasma membrane t-SNARE) and Spo14 (required for exocytosis) are involved. Grh1 (yeast GRASP) is also required, although its function is not clear. In summary, it suggests that cytosolic protein Acb1 might have been captured into the autophagosome-like vesicles, fused with multivesicular bodies and targeted to the plasma membrane for secretion by somehow avoiding fusion with the vacuole.
1.5 Autophagy Pathways

To better explore the autophagy-based unconventional secretion, it is necessary to take a closer look into the molecular pathways of autophagy. There is a set of autophagy-specific factors (Atgs) involved in the execution of autophagy. In mammals, autophagy initiating-ULK1 (Atg1, mammalian orthologue of yeast Atg1) complex responds to upstream cellular stress signals like starvation, hypoxia, ER stress, and microbe invasion. Then Class III PI(3)K Beclin1 (Atg6)/Vps34 complex initiates PI3P production, recruits PI3P effectors like DFCP1 and WIPIs (Atg18s) and mediates the formation of a pre-autophagosomal structure named isolation membrane. The Atg5/12 ubiquitin-like conjugation system conjugates PE to LC3 to elongate the isolation membrane into the autophagosome. The Atg8s are ubiquitin-like proteins that are suggested to play dual roles in the autophagosome formation by selectively incorporating autophagic cargoes through interacting with different autophagic adaptor proteins and promoting autophagosome elongation and closure. Autophagic receptor proteins such as p62 recruit ubiquitinated cargoes into the elongating autophagosome. Atg4B is also reported to mediate autophagosome closure. Proteins like UVRAG and TBK1 mediate autophagosome maturation by promoting its fusion with endosome and lysosome, forming autolysosomes. Finally, the substrates captured inside the autolysosomes, together with p62 and LC3 are degraded. (Figure 1.4)

1.6 Non-degradative Roles of Autophagy-related Proteins

The non-degradative roles of autophagy-related proteins are becoming increasingly appreciated. It has been shown that autophagy-related proteins additionally participate in
Figure 1.4 Overview of degradative autophagy pathway.
Figure 1.4  Overview of degradative autophagy pathway in mammals.

Upon upstream cellular stress signals like starvation, hypoxia, ER stress, and microbe invasion, autophagy initiating-ULK1 (mAtg1, mammalian orthologue of yeast Atg1) complex initiates autophagy. Then Class III PI(3)K Beclin1 (mAtg6)/Vps34 complex initiates PI3P production, recruits PI3P effectors like DFCP1 and WIPIs (mAtg18s), mediates formation of pre-autophagosomal structure named isolation membrane. Atg5/12 ubiquitin-like conjugation system conjugates PE to LC3/GABARAPs (mAtg8) to elongate the isolation membrane into the autophagosome. Autophagic receptor proteins like p62, NBR1, and NDP52, recruit specific ubiquitinated cargoes into the elongating autophagosome. Atg4B is also reported to mediate autophagosome closure. Proteins like UVRAG and TBK1 mediate autophagosome maturation by promoting its fusion with the endosome and lysosome, forming autolysosomes. Finally, the substrates captured inside the autolysosomes, together with p62 and LC3 are degraded.
various aspects of cell physiology including cell survival, apoptosis, protein sorting and secretion, cell signaling, DNA repair, gene regulation, and the immune response. These findings add extra strength to the hypothesis that autophagy is involved in unconventional secretion rather than merely in degradative processes. In order to investigate autophagy’s potential role in unconventional secretion, it is necessary to know the non-degradative functions of autophagy-related proteins.

1.6.1 Non-degradative roles of ubiquitin-like Atg proteins

Atg8 (LC3 in mammals) and Atg12 are ubiquitin-like Atg proteins shown to be involved in autophagosome formation. In human, there are six Atg8 homologues: LC3A/B/C, GABARAP, GABARAPL1, and GABARAPL2 (GATE-16). They interact with at least 67 other proteins through LC3-interacting regions (LIRs). Several more cryptic LIRs that are activated by the phosphorylation of serine and threonine residues in the vicinity of the LIR have been identified, enabling us to broaden the scope of LC3-interacting proteins. Among the non-autophagy related proteins that interact with mammalian Atg8s, they are mostly GTPases, GTPase- activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). Several GTPases have been discovered to regulate autophagy and LC3 has been shown to interact with a GAP protein TBC1D5 as a regulatory signaling platform. Also, LC3’s interaction with GEF impaired its ability to promote downstream signaling and non-lipidated LC3 was shown to regulate the viral replication. Additionally, Atg12–Atg5 conjugate was reported to suppress RIG-I or MDA5-mediated signals, thus negatively affecting the production of type I IFNs.
1.6.2 Non-degradative roles of other autophagy-related proteins

UNC-51, the *C. elegans* orthologue of human ULK1, was shown to regulate axon guidance \(^8^5\). Atg16 has been linked to regulated hormone secretion \(^8^6\) and secretion granule exocytosis \(^8^7\). In osteoclasts, Atg5, Atg7, Atg4B and LC3 participate in polarized Cathepsin K secretion \(^8^8\). Autophagic adaptor protein p62 is an important signaling hub for multiple signal transduction pathways, such as NF-κB signaling, apoptosis, and Nrf2 activation \(^8^9\). In addition, TBK1 which was shown to mediate autophagosome maturation has additional role to phosphorylate transcription factor IRF3 and upregulate NF-κB signaling \(^9^0\).

1.7 Complexity in the Regulation of Autophagy and Non-degradative Processes

Mediated by Autophagy-related Proteins

Notably in higher vertebrates, autophagy-related proteins have several homologues, isoforms or structurally-related proteins. For example, although yeast, *C. elegans* and *Drosophila* possess only one Atg1, in higher vertebrates there are at least five different kinases highly related to Atg1 (ULK1-ULK4 and STK36). Remarkably, the C-terminal domain in ULK1 and ULK2, which mediates the interaction with Atg13 and FIP200, is not conserved in ULK3, ULK4 and STK36. Only ULK1 and ULK2 have been found in a complex with Atg13, FIP200 and Atg101 \(^9^1, ^9^2, ^9^3\). Therefore, different mammalian Atg1 may play different role in regulating degradative autophagy and non-degradative processes mediated by autophagy-related proteins.

As mentioned before, mammalian Atg8 has six homologues: LC3A/B/C, GABARAP, GABARAPL1, GABARAPL2. LC3s are shown to be involved in elongation of the
phagophore membrane whereas the GABARAP/GATE-16 subfamily is essential for a later stage in autophagosome maturation \(^{68, 94}\). Correspondingly, there are several autophagic receptor proteins identified: p62, NDP52, NBR1, Optineurin, c-Cbl, Nix, and Smurf1 \(^{69, 95, 96, 97, 98, 99, 100, 101}\). These proteins are structurally related since they all possess LC3-interacting regions (LIRs) \(^{102}\). Each of them targets specific ubiquitinated cargoes for degradation. For example, selective binding of LC3C to autophagic receptor protein NDP52 is important for innate immunity in protection against *Salmonella* \(^{103}\).

Mice have four different isoforms (Atg4A-D), the protease that activates LC3 orthologues and also deconjugates LC3-II to free LC3-I. Atg4B has been associated with the secretion of otoconins by vestibular sensory cells of the inner ear \(^{104}\). Atg4 homologues also displayed selective preferences towards diverse Atg8 substrates \(^{105}\). The selective interactions between Atg4 isoforms and Atg8 homologues, displays the complexity in autophagy regulation and also suggests the potential involvement of autophagy-related proteins in non-degradative processes.

Mammalian Atg18 has four homologues (WIPI1-4) and WIPI2 has four isoforms (WIPI2A-D). They all belong to WIPI (WD-repeat protein interacting with phosphoinositides) family, which contain seven WD40 repeats that can fold into 7-bladed beta propeller proteins. WIPI1 and WIPI2 have been shown to be associated with autophagy \(^{106}\). The functions of other WIPI proteins remain to be defined.

To summarize, the expansion of the ATG8 family, autophagic receptor proteins, and other autophagy-related proteins in higher eukaryotes suggests that the specific
interactions between them may be involved in the complex regulation of specific autophagy processes and also the non-degradative processes (e.g. unconventional secretion) mediated by autophagy-related proteins.

1.8 Dissertation Objectives and Hypothesis

Studies in yeast revealed that autophagy, traditionally considered a degradative pathway, regulates the unconventional secretion of Acb1 upon nitrogen starvation or rapamycin treatment (conditions that induce autophagy). This autophagy-based unconventional secretory pathway, which we term as secretory autophagy, also involves a yeast homologue of Golgi Reassembly Stacking Protein (GRASP) that was shown to mediate the unconventional secretion of several other proteins. However, whether this secretory autophagy pathway is a generic process, which can be applied to other unconventionally secreted proteins in higher organisms, needs to be tested. The mechanism in which this secretory autophagy pathway differentiates from traditional degradative autophagy pathway requires further investigation. The exact role of a Golgi protein GRASP during the ER/Golgi-independent unconventional protein secretion remains elusive. In order to answer these questions, we utilized bone marrow-derived macrophages to study the unconventional secretion of IL-1β. I set out to test the hypothesis that there exists a specialized molecular and membrane machinery within the autophagy pathway which involves GRASP to carry out unconventional protein secretion. The objectives of this study were to determine: 1) whether this secretory autophagy pathway involving GRASP is conserved in mammalian cells; 2) how secretory autophagy pathway differs from degradative autophagy pathway; 3) whether GRASP is also involved in the degradative autophagy. Delineating this secretory autophagy pathway has both fundamental
biological and translational significance since the potential autophagy dependent-unconventionally secreted proteins are important in wound healing, cancer, neurodegeneration, and inflammation, etc.

The **specific aims** of this study were:

**SPECIFIC AIM 1: To test whether this secretory autophagy pathway is conserved in mammalian cells.**

SUB AIM 1.1 Test if the autophagic machinery is involved in the unconventional secretion of IL-1β.

SUB AIM 1.2 Determine if autophagic compartments intersect with IL-1β.

SUB AIM 1.3 Investigate whether the conserved player of unconventional secretion-GRASP has a role in starvation-induced unconventional secretion of IL-1β.

**SPECIFIC AIM 2: Examine the molecular divergence between secretory and degradative autophagy.**

**SPECIFIC AIM 3: Delineate whether GRASP is also involved in degradative autophagy.**

1.9 Chapter Summary

We tested the hypothesis and accomplished the proposed objectives by addressing the following three specific aims:

**Specific Aim 1 (Described in Chapter 2):** We discovered that, in mouse BMMs, induction of autophagy by starvation augmented the secretion of IL-1β and IL-18 in NALP3 inflammasome-dependent manner in response to various inflammasome stimuli.
While, Atg5 deficient-BMMs showed decreased level of IL-1β and IL-18 secretion. Bafilomycin A1 treatment reduced starvation-induced IL-1β secretion indicating autophagic maturation is required for starvation-induced IL-1β secretion. Also Rab8a, a regulator of polarized sorting to plasma membrane, co-localized with IL-1β and LC3 and its knockdown down-regulated IL-1β secretion. Importantly, the conserved regulator of unconventional secretion, GRASP55 was involved in the starvation-induced secretion of IL-1β and it was also required for autophagosome formation. This autophagy-based unconventional secretion was not restricted to inflammasome substrates but also applied to non-inflammasome substrate HMGB1. Altogether, these data suggest there might be a conserved molecular pathway in both lower organisms and mammals, which involve autophagy factors together with at least one of the two mammalian GRASPs to carry out the extracellular delivery role.

Specific Aim 2 (Discussed in Chapter 3): We found that autophagy-based unconventional secretion of IL-1β preferentially utilizes specific mammalian Atg8 isoforms, LC3A and GABARAP for the unconventional secretion of IL-1β. While not TBK1 and ULK1 that were shown to positively regulate degradative autophagy, were not required for this process. We further identified that less IL-1β is secreted from BMMs derived from transgenic mice lacking the autophagic adaptor Sequestosome 1/p62. Knock down of another adaptor protein NBR1 showed no effect. I also observed that GRASP55, the conserved regulator of unconventional secretion, selectively co-localized with WIPI2B and WIPI2D, but not with WIPI1. These findings strengthen the evidence for the existence of a specialized secretory autophagy pathway and provide first insights into the
divergence between degradative and secretory autophagy pathways.

**Specific Aim 3 (Covered in Chapter 4):** We showed that although GRASP55 knockdown increases mitophagy-dependent removal of damaged, superfluous mitochondria, whereas it does not affect the long-lived protein degradation and *M. tuberculosis* killing. GRASP55 appears to be involved in at least one aspect of degradative autophagy, mitophagy. GRASP55’s negative regulation on mitophagy might be beneficial to the autophagy-based unconventional secretion of IL-1β.

1.10 References


CHAPTER 2

AUTOPHAGY-BASED UNCONVENTIONAL SECRETORY PATHWAY FOR EXTRACELLULAR DELIVERY OF IL-1β
2.1 Authors Contributions

ND designed experiments, performed the experiments, analyzed the data, and helped write the manuscript. SJ performed the experiments and analyzed the data. MP, WO and DB performed the experiments and analyzed the data. VD conceived experiments, analyzed the data, and wrote the manuscript.
AUTOPHAGY-BASED UNCONVENTIONAL SECRETORY PATHWAY FOR EXTRACELLULAR DELIVERY OF IL-1β

Nicolas Dupont,1,* Shanya Jiang,1,* Manohar Pilli,1 Wojciech Ornatowski,1 Dhruva Bhattacharya,1 and Vojo Deretic a,1

1Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA
aDepartment of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, 915 Camino de Salud, NE, Albuquerque, NM 87131, USA. Tel.: +1 505 272 0291; Fax: +1 505 272 5309; E-mail: vderetic@salud.unm.edu

*These authors contributed equally to this work

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2.3 Abstract

Autophagy controls the quality and quantity of the eukaryotic cytoplasm while performing two evolutionarily highly conserved functions: cell-autonomous provision of energy and nutrients by cytosol autodigestion during starvation, and removal of defunct organelles and large aggregates exceeding the capacity of other cellular degradative systems. In contrast to these autodigestive processes, autophagy in yeast has additional, biogenesis functions. However, no equivalent biosynthetic roles have been described for autophagy in mammals. Here, we show that in mammalian cells, autophagy has a hitherto unappreciated positive contribution to the biogenesis and secretion of the proinflammatory cytokine IL-1β via an export pathway that depends on Atg5, inflammasome, at least one of the two mammalian Golgi reassembly stacking protein (GRASP) paralogues, GRASP55 (GORASP2) and Rab8a. This process, which is a type of unconventional secretion, expands the functional manifestations of autophagy beyond autodigestive and quality control roles in mammals. It enables a subset of cytosolic proteins devoid of signal peptide sequences, and thus unable to access the conventional pathway through the ER, to enter an autophagy-based secretory pathway facilitating their exit from the cytoplasm.
2.4 Introduction

Autophagy is a fundamental biological process in eukaryotic cells where it plays a number of roles associated with quality and quantity control of the cytoplasm \(^1,^2\). For the most part, autophagy enables cellular viability \(^3,^4\) by removing damaged organelles e.g. depolarized or leaky mitochondria \(^5\), digesting potentially toxic protein aggregates \(^6\), killing intracellular microbes \(^7\), and supplying energy and nutrients to the cell \(^8\) and the whole body \(^9\) by bulk autodigestion of the cytosol at times of starvation. Based on the known and on yet to be uncovered functions, autophagy has broad impact in fundamental biology \(^10\) and in human health \(^2\) including aging \(^11\), cancer \(^12\), neurodegeneration \(^6,^13\), myopathies \(^14\), metabolic disorders \(^15\), infections, immunity, and inflammatory diseases \(^16,^17\).

The *sensu stricto* autophagy, often referred to as macroautophagy, involves Atg factors-driven generation of intracellular membranes that sequester cytoplasmic targets earmarked for autophagic processing \(^1\). The principal components of the autophagic signaling and execution machinery are: (i) a protein kinase network including Tor, AMPK and Ulk1/2 \(^8,^18\) (ii) lipid kinases, principally the class III phosphatidylinositol 3-kinase hVPS34 \(^19\) and potentially class I p110\(\beta\) \(^20\), in cooperation with Beclin 1 and its multiple modifiers \(^21,^22\); (iii) a protein and protein-lipid conjugation Atg system resulting in a lipo-protein conjugate, LC3-II, which marks the autophagic membranes \(^1\); and (iv) Atg8- and LC3-interacting autophagic adaptors that recognize ubiquitinated targets destined for autophagic capture \(^23\) and may be associated, as in the case of p62/sequestosome 1, with autophagosomal nucleation sites \(^24\). The protein lipidation
system resulting in LC3-II is driven by the Atg5-Atg12/Atg16 complex acting as an E3 ligase equivalent that facilitates localized conversion of LC3-I into LC3-II, with the latter consisting of the LC3 moiety conjugated to phosphatidylethanolamine at its C-terminus. The cargo enveloped by autophagosomes normally follows what is considered to be its principal destiny, i.e. degradation of the captured material after conversion of autophagic organelles into degradative autolysosomes.

Although the central dogma of autophagy is that it is a degradative, disposal pathway, it also participates in several biogenesis processes documented in yeast, e.g. the Cvt pathway and the unconventional secretion of Acb1. The secretion of proteins devoid of leader peptides differs from the secretion of proteins endowed with this signal that brings them across the ER membrane into the canonical secretory pathway. The secretion of such leaderless peptides may involve unrelated pathways, but some common principles are beginning to emerge that are conserved at least for a subset of unconventional secretion substrates, in particular a dependence of their secretion on the Golgi reassembly stacking protein GRASP. The recent findings in yeast with Acb1, suggest that GRASP- and autophagy-dependent pathways may converge, but it is unknown whether these relationships extend to mammalian cells.

One of the promising systems to address the question of the potential role of autophagy in the unconventional secretion in mammalian cells, is the extracellular delivery of the proinflammatory cytokine IL-1β. IL-1 family members IL-1β and IL-18 (IL-1F4) are processed in the cytosol from their precursors upon inflammasome
activation and are secreted without transiting through the conventional secretory pathway. However, recent reports have indicated that autophagy plays an inhibitory role in inflammasome and IL-1β activation \(^ {32, 33, 34}\) thus potentially negating the idea that autophagy may contribute to the unconventional secretion of IL-1β. The mechanisms of autophagy-based suppression of inflammasome activation and IL-1β secretion may be indirect and seem to rely on autophagy-dependent mitochondrial homeostasis that normally suppresses release of reactive oxygen species (ROS) \(^ {32}\) and mitochondrial DNA \(^ {33}\), both of which can stimulate inflammasome. Alternatively, autophagic degradation of the inflammasome components has also been proposed \(^ {34}\) but this has not been experimentally tested.

Here, we have addressed the question of whether autophagy plays a direct role in inflammasome and IL-1β activation and secretion. We found that, whereas basal autophagy inhibits IL-1β secretion in concordance with the recent reports \(^ {32, 33}\), induced autophagy augments IL-1β secretion. We show that inflammasome and the autophagy apparatus synergize during IL-1β secretion in cells stimulated to undergo autophagy. We also show that autophagy induction cooperates with GRASP and Rab8a (a GTPase controlling post-Golgi polarized sorting and exocytosis) in driving IL-1β secretion. We thus define one of the first biogenesis functions of autophagy in mammalian cells and show that at least one type of unconventional secretion utilizes autphagic machinery in higher vertebrate cells.
2.5 Results

2.5.1 Induction of autophagy promotes inflammasome-dependent IL-1β secretion

Whereas it has been found that basal autophagy reduces extracellular release of the major proinflammatory cytokine IL-1β\textsuperscript{32,33}, we detected the opposite when autophagy was induced in primary murine bone marrow derived macrophages (BMM) (Fig. 2.1). Stimulation of autophagy by starvation strongly enhanced IL-1β secretion in response to conventional NLRP3 (NALP3) inflammasome agonist nigericin (Fig. 2.1A). This effect was also seen (Fig. 2.1B) in Western blots of caspase 1 and mature IL-1β of culture supernatants from cells grown in the absence of serum, as conventionally done when assessing IL-1β secretion by immunoblotting\textsuperscript{35}. A reduced secretion in BMMs from Atg5\textsuperscript{Fl/Fi} LyzM-Cre\textsuperscript{+} mice, compared to BMMs from their Cre\textsuperscript{-} littermates, was accompanied and contrasted by the higher level of cell-associated pro-IL-1β in Cre\textsuperscript{-} vs Cre\textsuperscript{+} BMMs (Fig. 2.1B). The BMM derived from Atg5\textsuperscript{Fl/Fi} LyzM-Cre\textsuperscript{+} mice for these and other experiments had, as expected, no detectable Atg5 (since the Atg5 gene is excised in Cre\textsuperscript{+} macrophages;\textsuperscript{36}) and LC3-II, a key marker of autophagy (Fig. A1A).

The effects of induced autophagy on secretion of inflammasome substrates described above were not limited to IL-1β, since secretion of another inflammasome-dependent cytokine from the IL-1 family, IL-18 (IL-1F4), was enhanced when autophagy was induced (Fig. 2.1C). Pharmacological induction of autophagy by mTOR inhibition with pp242 (Torkinib) increased secretion of IL-1β by BMM (Suppl. Fig. 2.1B).
Figure 2.1 Induction of autophagy enhances IL-1β secretion.
Figure 2.1  Induction of autophagy enhances IL-1β secretion.

(A) Atg5fl/fl Cre− and Atg5fl/fl Cre+ bone marrow-derived macrophages (BMMs), pretreated overnight with 100 ng/ml LPS, were stimulated for 1 h with the inflammasome agonist nigericin (20 μM) with (Starvation; EBSS) or without (Full; full medium) autophagic induction. Cell culture supernatants were assayed for murine IL-1β by ELISA. Data represent mean values±s.d. (n≥3); *P<0.05. (B) LPS-pretreated Atg5fl/fl Cre− and Atg5fl/fl Cre+ BMMs were stimulated with 20 μM nigericin for 1 h in OptiMEM and the release of active caspase-1 and IL-1β was determined by immunoblotting. (C) As in (A), assayed for IL-18. Data represent mean values±s.d. (n≥3); *P<0.05. (D) LPS-pretreated BMMs were exposed to alum (250 μg/ml) for 1 h with or without autophagic induction by starvation. Secreted IL-1β was measured as in (A). Data represent mean values±s.d. (n≥3); *P<0.05. (F) BMMs were transfected with scramble (Scr) control siRNA or siRNAs against ASC and NLRP3. After 48 h following transfection, cells were treated overnight with LPS and subjected to nigericin (20 μM) and starvation for 1 h. Data represent mean values±s.d. (n≥3); *P<0.05. (G) Immunoblot analysis of ASC and NLRP3 knockdowns. (H) BMMs were transfected with scramble (Scr) control siRNA or siRNAs against ASC and NLRP3. After 48 h following transfection, cells were treated overnight with LPS and subjected to silica (250 μg/ml) and starvation for 1 h. Data represent mean values±s.d. (n≥3); *P<0.05. (I) Colocalization of IL-1β with the basal autophagic machinery factor LC3. Fluorescence: LC3 (green, Alexa488); IL-1β (red, Alexa568). BMMs were from GFP–
LC3 knock-in mice, treated with LPS then prepared for immunofluorescence microscopy using fluorescently labelled antibodies against GFP and IL-1β. (J, K) A line fluorescence tracing from images in (I). (L) Pearson's colocalization coefficient for IL-1β and LC3. Pearson's coefficient was derived from three independent experiments with five fields per experiment, for a total of 15 fields contributing to the cumulative result.
An enhancement of IL-1β secretion upon induction of autophagy was also detected when particulate inflammasome agonists, alum \(37\) (Fig. 2.1D, Fig. A1C), silica (Fig. 2.1E) \(38\) and amyloid-β fibrils \(39\) (Fig. A1D), were used as inflammasome inducers. The enhancement of IL-1β secretion associated with autophagy induction was inflammasome dependent, as IL-1β activation was diminished by knockdowns of the inflammasome components ASC and NLRP3, regardless of whether the inflammasome agonist used was nigericin or silica (Fig. 2.1F-H). The knockdowns of ASC and NLRP3 did not change IL-1β expression (Fig. A1E, F). The increased secretion of IL-1β was not due to the increased cell death or non-specific membrane permeability as LDH release showed a kinetic lag behind release of IL-1β whether the inflammasome agonist used was nigericin or silica (Fig. A2A-D).

The stimulation of autophagy promoted IL-1β secretion in an Atg5-dependent manner, based on comparisons between BMM from Atg5\(^{Fl/Fl}\)-LyzM-Cre\(^+\) mice and BMM from their Atg5-competent (Atg5\(^{Fl/Fl}\) Cre\(^-\)) littermates (Fig. 2.1A). However, the loss was not complete in Cre\(^+\) BMMs (Fig. 2.1A). We interpret the incomplete reduction in IL-1β secretion in the absence of Atg5 as a net result of two opposing effects – one described here as a product of positive contribution of induced autophagy on extracellular delivery of IL-1β and the other being the recently reported negative regulation of IL-1β secretion by basal autophagy \(32, 33, 34\). In keeping with this interpretation and in contrast to the stimulatory effects of induced autophagy (Fig. 2.1A-E, Fig. A1B-D), basal autophagy negatively affected IL-1β and IL-18 secretion (Fig. A2E, F) in agreement with the recent reports in cells not induced for autophagy \(32, 33, 34\).
2.5.2 IL-1β and autophagic protein LC3 colocalize in the cytoplasm

How might induced autophagy enhance IL-1β secretion? We considered a model in which autophagy, as a process that can translocate cytosolic proteins and other targets (*en masse* or specific components) from the cytosol to the inside of vesicular compartments, brought IL-1β into the lumen of autophagic vacuoles followed by exocytosis. When we examined IL-1β and the key marker of autophagosomes LC3 by immunofluorescence confocal microscopy, LC3 and IL-1β colocalized and displayed major similarities in the overall intracellular organellar distribution (Fig. 2.1I-L). The overlap between IL-1β and LC3 remained detectable when cells were treated with nigericin (Fig. A3A-C). These observations indicate that autophagic organelles and IL-β intersect.

2.5.3 Inhibition of autophagy flux reduces IL-1β secretion

A question arose whether the LC3⁺ organelles containing IL-1β are on pathway to degradation or facilitated IL-1β secretion. We first tested the effects of bafilomycin A1, a conventional inhibitor of autophagic maturation, which acts as an antagonist of vacuolar H⁺ ATPase and prevents lumenal acidification and autophagosomal cargo degradation. If induction of autophagy acted to degrade IL-1β, bafilomycin A1 was expected to increase IL-1β levels. Instead, bafilomycin A1 decreased IL-1β secretion in cells stimulated for autophagy by starvation, whereas no change was observed with bafilomycin A1 in cells undergoing basal autophagy only (Fig. 2.2A). Thus, autophagy flux during autophagy induction was not inhibitory to IL-1β but was instead promoting IL-1β secretion. A similar trend was detected with another inflammasome substrate, IL-
Figure 2.2  Autophagic pathway progression promotes secretion of inflammasome substrates.
Figure 2.2 Autophagic pathway progression promotes secretion of inflammasome substrates.

(A, B) LPS-pretreated BMMs were treated with 20 µM nigericin (Nig) and 100 nM bafilomycin A1 (Baf) with (Starvation) or without (Full) autophagic induction for 1 h and secreted IL-1β (A) and IL-18 (B) were measured. Data represent mean values±s.d. (n≥3); *P<0.05. (C) LPS-pretreated BMMs were treated with 250 µg/ml of silica and 100 nM bafilomycin A1 (Baf) with (Starvation) or without (Full) autophagic induction for 1 h and secreted IL-1β were measured. Data represent mean values±s.d. (n≥3); *P<0.05. (D) Colocalization of cathepsin B with the basal autophagic machinery factor LC3 and IL-1β. Fluorescence; LC3 (green, Alexa488), IL-1β (red, Alexa568), and cathepsin B (blue, Alexa633). BMMs from GFP–LC3 knock-in mice were treated with LPS and then analysed for immunofluorescence. (E) Colocalization line tracing analysis from images in (D). (F) LPS-pretreated BMMs were treated with 20 µM nigericin and cathepsin B inhibitor CA-074 Me (10 µM), with (Starvation) or without (Full) autophagic induction, for 1 h and secreted IL-1β was measured. Data represent mean values±s.d. (n≥3); *P<0.05. (G) LPS-pretreated Atg5fl/fl Cre− and Atg5fl/fl Cre+ BMMs were stimulated with 20 µM nigericin for 1 h in OptiMEM and release of cathepsin B was determined by immunoblotting.
18 (Fig. 2.2B). Equivalent relationships have been observed for IL-1β secretion whether
nigericin (Fig. 2.2A) or silica (Fig. 2.2C) were used as inflammasome agonists. The
absence of IL-1β or IL-18 sparing effects of bafilomycin A1 is in keeping with the
interpretation that autophagy is not degrading inflammasome components but that an
unobstructed autophagy pathway is necessary for inflammasome-dependent IL-1 family
members secretion.

2.5.4 Lysosomal hydrolase cathepsin B is a positive factor in autophagy-driven IL-
1β secretion

Next we investigated the role of lysosomal hydrolases, focusing on cathepsin B. We
observed that IL-1β and LC3 colocalized with cathepsin B (Fig. 2.2D,E). However,
cathepsin B did not play an inhibitory role. Similarly to bafilomycin A1, cathepsin B
inhibitor CA-074 suppressed IL-1β production. Instead of protecting IL-1β from
potential degradation, CA-074 Me strongly inhibited IL-1β secretion in cells stimulated
for autophagy by starvation (Fig. 2.2F). No differences in expression of pro-IL-1β were
observed in cells treated with bafilomycin A1 or CA-074 (Fig. A3D). Of further interest
was that cathepsin B (mature form) was secreted along with the inflammasome substrates
in a manner dependent on an intact autophagic apparatus: loss of Atg5 in BMMs from
Cre+ mice (Atg5F/FiLyzM-Cre) diminished the levels of the secreted mature Cathepsin B
relative to BMMs from Cre− littermates (Fig. 2.2G). The findings with cathepsin B
inhibitor CA-074 indicate a positive role for cathepsin B in IL-1β activation and
autophagy-driven pathway of extracellular delivery of IL-1β. They can also help explain
in part the observations that a lysosomal hydrolase, cathepsin B, assists in inflammasome activation and IL-1β secretion in response to particulate inflammasome agonists 38, 39.

2.5.5 Rab8a, a regulator of polarized sorting to plasma membrane colocalizes with IL-1β and LC3 and controls IL-1β secretion

We next addressed the features of the compartment where LC3 and IL-1β colocalized. We observed an overlap between the LC3⁺ IL-1β⁺ profiles and Rab8a (Fig. 2.3A-C). Rab8a is a regulator of polarized membrane trafficking, constitutive biosynthetic trafficking, and plasma membrane fusion of insulin-responsive 40 and other vesicular carriers 41, 42, 43, 44. Rab8a also colocalized with LC3 and IL-1β in cells exposed to nigericin (Fig. A4A-C). Rab8a was required for enhanced IL-1β secretion caused by starvation-induced autophagy and inflammasome activation with nigericin, since siRNA knockdown of Rab8a diminished IL-1β secretion from BMMs under these conditions (Fig. 2.3D,E). Rab8a knockdown did not change pro-IL-1β mRNA levels (Fig. A4D). Overexpression of dominant negative Rab8a mutant (S22N) inhibited IL-1β secretion from RAW264.7 cells, employed in that experiment based on their high efficiency of transfection (Fig. 2.3F) (verified by flow cytometry of GFP-Rab8a for equal yields). Additionally, LC3⁺ IL-1β⁺ profiles were positive for subunits of the exocyst complex (Fig. A4E-J). Exocyst has been shown to cooperate with Rab8a in polarized plasma membrane delivery of vesicular carriers 44, 45. The presence of exocyst components on IL-1β⁺ autophagic organelles was also in keeping with a recent report implicating exocyst in autophagy 46. In summary, these experiments indicate that systems involved in vectorial vesicular transport to the plasma membrane participate in autophagy-based...
Figure 2.3 Rab8a is required for autophagy-activated IL-1β secretion.
Figure 2.3 Rab8a is required for autophagy-activated IL-1β secretion.

(A) Colocalization of Rab8a with the basal autophagic machinery factor LC3 and IL-1β. Fluorescence; LC3 (green, Alexa488), IL-1β (red, Alexa568), Rab8a (blue, Alexa633). BMMs from GFP–LC3 knock-in mice were pretreated with LPS and analysed by immunofluorescence microscopy. Arrows indicate triple colocalization. (B) Line tracing analysis of fluorescence signal intensity. (C) Pearson's colocalization coefficient for IL-1β and Rab8a. Pearson's coefficients were derived from three completely independent experiments with >5 fields per experiment, for a total of ≥15 fields contributing to the cumulative result. (D) BMMs were transfected with siRNAs against Rab8a or scramble (Scr) control. At 24 h after the first transfection, cells were transfected again with siRNA, treated with LPS and the day after subjected to nigericin in full medium for 1 h, and IL-1β secretion measured. (E) Immunoblot analysis of Rab8a knockdown in BMMs. (F) RAW 264.7 macrophages were transfected with GFP-tagged Rab8a constructs (WT, wild type; S22N, dominant-negative mutant), treated overnight with LPS and stimulated for 1 h with 20 µM nigericin along with induction of autophagy by starvation. IL-1β secretion was measured by ELISA. Data represent mean values±s.d. (n≥3); *P<0.05.
unconventional secretion and that Rab8a is required for efficient autophagy-dependent secretion of IL-1β.

2.5.6 GRASP55 controls secretion of IL-1β

Two studies in yeast 25, 26 have reported that autophagic machinery is required for unconventional secretion of the protein Acb1, and that this pathway depends on the yeast equivalent of a Golgi-associated protein GRASP in mammals 28, 47. Mammalian cells encode two GRASP paralogs, GRASP55 (GORASP2) and GRASP65 (GORASP1) 48, 49. We first tested whether any of the mammalian GRASPs were required for IL-1β secretion. We could not obtain a good knockdown of GRASP65 (GORASP1) and thus could not evaluate its involvement. However, a knockdown of GRASP55 diminished IL-1β secretion (Fig. 2.4A; Fig. A5A). A similar downregulation of IL-18 secretion was observed with GRASP55 knockdown (Fig. A5B). We next tested whether GRASP55 showed any detectable response to inflammasome stimulation. GRASP55 in resting cells is mostly localized aligned within the perinuclear Golgi (Fig. 2.4B; Fig. A6A). However, a fraction of it dispersed upon treatment of cells with the inflammasome agonist nigericin (Fig. A6B) and was found juxtaposed and partially overlapping with LC3 profiles (Fig. 2.4B,C). Thus, GRASP55 responds to inflammasome stimulation and is important for secretion of the inflammasome substrates IL-1β and IL-18.

2.5.7 GRASP55 controls autophagy initiation

In addition to being required for IL-1β secretion, GRASP55 showed functional effects on LC3 and autophagy, tested by employing two core assays 50: LC3-II lipidation and the
Figure 2.4  GRASP55 is required for autophagy-activated IL-1β secretion.
Figure 2.4  GRASP55 is required for autophagy-activated IL-1β secretion.

(A) BMM cells were transfected with scramble (Scr) control siRNA or siRNA against GRASP55. After 48 h of transfection, cells were treated with LPS and the day after subjected to 20 µM nigericin in EBSS, and secreted IL-1β was measured by ELISA. Data represent mean values±s.d. (n≥3); *P<0.05. Inset: Immunoblot analysis of GRASP55 knockdown. (B) Immunofluorescence confocal microscopy analysis of LC3 and GRASP55 distribution. LC3 (green, Alexa488), GRASP55 (red, Alexa568). BMMs were pretreated overnight with 100 ng/ml LPS and either not stimulated (Ctrl) or stimulated (Nig) for 30 min with the inflammasome agonist nigericin (20 µM) in full medium. (C) Line tracings, analysis of fluorescence signal intensity from images in (B). (D) Pearson's coefficients for LC3 and GRASP55 were quantified using SlideBook morphometric analysis software as a measure of adjacency between GRASP55 and LC3 profiles. Pearson's coefficients were derived from three independent experiments with five fields per experiment, for a total of 15 fields contributing to the cumulative result.
RFP-GFP-LC3 tandem probe. When GRASP55 was knocked down, autophagy initiation was negatively affected, as LC3-II levels were lower in both untreated and bafilomycin A1-treated cells (Fig. 2.5A, B). A partial downregulation of GRASP65 (to the extent that it could be achieved in BMMs) suggested a minor synergistic effect with GRASP55 on LC3-II levels upon induction of autophagy (Fig. A5C). Knocking down GRASP55 reduced the total number of autophagic puncta, and selectively reduced the formation of autophagosomes but not their maturation (Fig. 2.5C, D). This was apparent from the data obtained with the RFP-GFP-LC3 probe following published methods, which showed reduced GFP^RFP^ LC3 profiles (early autophagosome) and equal number of GFP^RFP^ LC3 profiles (mature autophagic organelles) in cells knocked down for GRASP55 (Fig. 2.5D). Thus, mammalian GRASP55, a paralog of GRASP from lower organisms that has thus far been the sole definitive molecular factor associated with unconventional secretion, displays important and previously unappreciated positive regulatory effects on autophagy induction. These findings strengthen the connections between autophagy and GRASPs in general, and specifically demonstrate the role of mammalian GRASP55 both in autophagy initiation and in the secretion of leaderless inflammasome substrates such as IL-1β and IL-18.

2.5.8 Autophagy-based unconventional secretion is not limited to proteolytically processed inflammasome substrates

We next wondered whether the unconventional process described above is limited to inflammasome substrates epitomized by IL-1β that are concomitantly with their secretion proteolytically processed from precursor pro-proteins into mature forms. We tested
Figure 2.5 GRASP55 controls autophagy initiation.
Figure 2.5  **GRASP55 controls autophagy initiation.**

(A, B) Effect of GRASP55 on autophagy induction by measuring LC3-II. BMM cells were transfected with GRASP55 siRNAs or scramble (Scr) control. At 72 h post transfection, cells were induced for autophagy, treated or not with Bafilomycin A1 (Baf) to inhibit autophagic degradation and LC3-II/actin ratios determined by immunoblotting (A) followed by densitometry (B). Data represent mean values±s.d. (n≥3); *P<0.05. (C, D) RAW 264.7 was transfected with GRASP55 siRNAs or scramble (Scr) siRNA control. Following 48 h of siRNA treatment, cells were transfected with RFP–GFP–LC3 plasmid (GFP is sensitive to acidification, whereas RFP is not), after 24 h induced for autophagy in EBSS for 1 h and autophagic induction and flux quantified (graph in D) by determining the number of early autophagic organelles (GFP+RFP+ puncta) and autolysosomal organelles (GFP–RFP+ puncta) per cell as illustrated in fluorescent images (yellow arrows, GFP+RFP+; red arrows, GFP–RFP+). Total, yellow+red puncta per cell. Data represent mean values±s.d. (n≥3); *P<0.05.
whether induction of autophagy affected other proteins not connected to proteolytic processing in the inflammasome, such as HMGB1 (high mobility group box 1 protein). HMGB1 is a major pro-inflammatory alarmin or DAMP (damage associated molecular pattern) normally present in the nucleus. This chromatin-associated nuclear protein (with additional intracellular and extracellular signaling roles), upon exposure to inputs including those that induce autophagy, undergoes a complex set of biochemical and localization changes. In the process, it first translocates from the nucleus into the cytoplasm and then is released from the cytoplasm to act in tissue remodeling signaling (when acting alone) or as an inflammatory mediator (when combined with bacterial agonists or other alarmins such as IL-1β). When tested, starvation and nigericin co-treatment caused HMGB1 extracellular release in an Atg5-dependent manner (Fig. 2.6A). HMGB1 band was detected by immunoblots in BMM culture supernatants upon stimulation of cells with nigericin, whereas HMGB1 was largely diminished when BMMs from Atg5<sup>Fl/Fl</sup> Cre-LyzM mice were tested (Fig. 2.6B). Nigericin was used in these experiments as an inflammasome agonist based on the reports that HMGB1, along with additional unconventional substrates, depends on inflammasome for secretion although the protein itself is not subjected to proteolytic processing by caspase 1. These experiments show that autophagy-based unconventional secretion affects release of HMGB1 in a manner similar to IL-1β. Our findings broaden the spectrum of autophagy-based unconventional secretion substrates, and establish this type of unconventional secretion as a more general process in extracellular delivery of cytosolic proteins.
Figure 2.6  HMGB1 is an autophagy-based alternative secretion substrate.
Figure 2.6   HMGB1 is an autophagy-based alternative secretion substrate.

(A) Atg5fl/fl Cre− and Atg5fl/fl Cre+ BMMs, pretreated overnight with 100 ng/ml LPS, were stimulated for 1 h with 20 µM nigericin (Nig; inflammasome agonist) while incubated in EBSS for induction of autophagy by starvation. Cell culture supernatants were assayed for murine HMGB1 by ELISA. Data (normalized to sample with maximum HMGB1 secretion in each experimental repeat; Cre− and Nig) represent mean values±s.d. (n≥3); *P<0.05. (B) LPS-pretreated Atg5fl/fl Cre− and Atg5fl/fl Cre+ BMMs were stimulated with 20 µM nigericin for 1 h in OptiMEM and the release of HMGB1 was determined by immunoblotting.
2.6 Discussion

The data presented in this work outline several elements of the autophagy-based unconventional secretory pathway in mammalian cells. This type of unconventional secretion is shown here to support the extracellular delivery of inflammasome substrates, in particular IL-1β and IL-18 and may potentially have a broader number of clients. A relevant aspect of the process described here is that induction of autophagy is required to observe the manifestations of this type of unconventional secretion. Since basal autophagy suppresses spurious induction of inflammasome \(^{32,33}\), autophagy provides both avoidance of unscheduled inflammasome activation and a platform for extracellular delivery of inflammasome substrates. Since a number of hormones, cytokines, pathogen associated molecular patterns, and danger associated molecular patterns \(^{16,53}\) are known to induce or inhibit autophagy, a link between autophagy and secretion of major immunomodulatory cytokines such as IL-1β could significantly influence the extent and duration of inflammation. Connections between metabolic syndrome, high fat diet and inflammasome activity are now beginning to be appreciated \(^{57,58}\), and we propose that autophagy-based unconventional secretion may be a key coupler between the metabolism and inflammation. Since a number of genetic links have been found between autophagy and idiopathic inflammatory diseases or infectious diseases with significant inflammatory components \(^{17}\) it is possible that at least in part the genetic associations between autophagy risk loci and disease states may stem from altered autophagy-based unconventional secretion of inflammatory cytokines.
The role of autophagy was represented here by the effects of induction of autophagy and by employing conditional knockout mice with a loss of Atg5 in macrophages. We interpret the incomplete inhibition of IL-1β secretion upon Cre-dependent \textit{Atg5}^{fl/fl} excision as a result of the composite effects of Atg5-dependent basal autophagy (inhibitory) \textsuperscript{32} and induced autophagy (stimulatory) although we cannot exclude the possibility of slight leakiness of the \textit{Atg5}^{fl/fl} \textit{LyzM-Cre} system or the existence of additional pathways. Importantly, blocking autophagic maturation has not salvaged IL-1β but rather inhibited its secretion. This appears to be in contrast to what has been reported for \textit{Acb1} in yeast \textsuperscript{25}. Moreover, cathepsin B activity was needed, suggesting that autophagic organelles here did not function as mere cargo carriers but provided a platform for activation of inflammasome and IL-1β. In keeping with these observations, cathepsin B has been implicated in inflammasome activation in response to particulate inflammasome agonists \textsuperscript{38,39}, such as those (alum, amyloid-β) used here in addition to nigericin, but how the substrate and cathepsin B meet has hitherto not been defined. Our data indicate that induction of autophagy enhances assembly of inflammasome-activating components and suggest that autophagic organelles may be a platform for concentration of components engaged in proteolytic activation of inflammasome components and inflammasome substrates. In keeping with this model of a muster station for inflammasome components, activation and subsequent extracellular delivery, is the translocation of pro-IL-1β to membranous organelles upon stimulation with the inflammasome agonist nigericin, as previously observed by R. Sitia and colleagues \textsuperscript{59} who have established early on that this process does not follow the conventional secretory pathway.
The autophagy-based unconventional secretion pathway in mammalian cells includes GRASP, one of the peripheral Golgi proteins involved in lateral organization of Golgi ribbons. Although the role of GRASP in alternative secretory pathway has been studied, its exact mechanism of action has not been elucidated. We observed here a potentially telling connection between GRASP and autophagy, by showing that GRASP affects autophagy induction, which places GRASP upstream of autophagy execution, including the conjugation systems involved in LC3 lipidation. The response of GRASP to nigericin stimulation in terms of its redistribution and juxtaposition to autophagic organelles, further links autophagy, inflammasome and GRASP, although alternative explanations are possible. The finding that Rab8a plays a functional role in autophagy-based unconventional secretion is of significance not just by assigning a trafficking regulator to this pathway but also by providing additional links with exocyst components, implicated to cooperate with Rab8a in other systems and to play a role in autophagy.

In summary, in this study we have uncovered the role of autophagy in the secretion of cytosolic pro-inflammatory factors that cannot enter the conventional biosynthetic pathway due to the absence of leader peptides that would bring them into the ER and the organelles of the canonical secretory pathway. Cytosolic IL-1β and IL-18 are both processed from their precursor proteins into their active forms via the inflammasome apparatus, however the process that delivers the proteolytically activated IL-1β and IL-18 to the extracellular environment has hitherto remained unclear. To be eligible for export outside of the cell without invoking a pore mechanism, cytosolic proteins first
need to be brought somehow into the lumen of vesicular carriers, as previously shown by others \(^5^9\). We have elsewhere noted \(^1^6,^6^0\) that autophagy is a bulk topological inverter for cytosolic proteins and other molecules, ferrying them from the cytosol into the organellar lumen. We now show that induction of autophagy does that with IL-1\(\beta\) in the process of its secretion. In doing so, autophagic machinery cooperates with the Golgi-associated factor GRASP and post-Golgi membrane trafficking and exocytosis regulator Rab8a. Autophagy captures cytosolic IL-1\(\beta\) and brings IL-1\(\beta\) into the organelles of a specialized unconventional secretory pathway.

Broadening the scope of autophagy-based alternative secretion pathway is the observation that it facilitates exit from cells of the alarmin HMGB1. HMGB1 is a DAMP that is actively released from immune cells unlike its passive release from several cell types secondary to cell death \(^5^2\). It has been recently shown that inflammasome, rather unexpectedly given that HMGB1 is not a known substrate for caspase-1 processing, plays a role in HMGB1 release \(^5^5\). One explanation that can be offered based on our studies is that a role of inflammasome may not be related solely to proteolytic substrate processing but that it may be hardwired into the secretory pathway studied here. This is in keeping with the requirement for NLRP3 and ASC and not the caspase 1 activity for HMGB1 release as a recently recognized noncanonical inflammasome client \(^5^6\). We propose here that autophagy-based unconventional secretion may be used for extracellular delivery of a spectrum of cytosolic proteins or processed cytoplasmic substrates, not restricted to the proteins explored here, and possibly including other biological mediators such as the recently discovered cryptides \(^6^0,^6^1\). A recent study that appeared while this work was in
revision suggests that an unconventional secretion process, also dependent on GRASP and autophagic machinery, may facilitate plasma membrane delivery of mutant CFTR, potentially expanding the range of substrates to integral membrane proteins. Given the capacity for either bulk transport or selectivity when coupled with autophagic adaptors, we predict that autophagy-based unconventional secretion serves a potentially broad spectrum of yet to be uncovered physiological functions.

2.7 Materials and Methods

2.7.1 Macrophages

Murine bone marrow-derived macrophages (BMM) cells were prepared from femurs of C57/BL6 mice, Atg5fl/fl LyzM-Cre mice and their Cre-negative Atg5fl/fl littermates, and GFP-LC3 transgene knock-in mice as previously described. RAW 264.7 macrophages were maintained and manipulated as previously described.

2.7.2 Pharmacological agonists, inhibitors, inflammasome and autophagy

To induce pro-IL-1β expression, cells were pretreated overnight with 100 ng/ml LPS (Sigma). Inflammasome was induced with 20 μM nigericin (Sigma) for 1 h or with 250 μg/ml Alum (Thermoscientific) for 1 h or with 250 μg/ml silica crystals (MIN-U-SIL-15, US Silica) for 1 h or with 5 μM Amyloid-β peptide (Aβ; American Peptide Company) fibrils prepared as described. Cells were treated with 100 nM bafilomycin A1 (LC Laboratories) or 10 μM Cathepsin B inhibitor (CA-074 Me) (Enzo Life Science). Autophagy was induced for 1 h by starvation in EBSS or with pp242 in full medium.
(Torkinib; Chemidea). Starvation and other treatments (except for macrophage priming with LPS done in advance) were carried out concurrently (i.e. initiated at the same time).

2.7.3 Transfections and siRNA knockdowns

BMM and RAW 264.7 cells were transfected by nucleoporation using Nucleofector Reagent Kit V or Kit Mouse Macrophage (Amaxa/Lonza biosystems). For murine NLRP3, ASC, Rab8a or GRASP knockdowns, cells were transfected with siGENOME SMARTpool reagents (Dharmacon).

Rab8a SMARTpool

(GAAUAAGUGUGAGUGAAU;GAAGACCUGUGUCCUGUUC;
GACCUACGAUUACCUGUUC;GAGCAGCCAUGGAGUCAAG),

ASC SMARTpool

(AUACAUCUCUACUUGGUGA;GCUUAGAGACUUGGCUUA;
GCAACUGCGAAGGCUUAU; CUGCAAACGACUAAAAGAAG),

NLRP3 SMARTpool

(GUUCUUCGCUAUGUAC; GCACCCAGGCUGUAACAUU;
UGAAGGACCCACAGUGUA; UCACAUUCUCUAUGGUAU),

GORASP1 SMARTpool
(CAUGAAGGUGCGCGAGGUA; CAGAGGACAUUGGUUCUAG; ACUCGAGGCUGAACAAGGA; GCUACGACCUCACAACUUA),

GORASP2 SMARTpool
(GAAGACCUGUUCAGCCUUA; UACCAAGUCUGAUGCCUUU; GUAAACCAGUCCCCUUGCUU; GAUCAUCACACACACUCU).
Non-targeting siRNA pool (Scrambled) was used as a control. Plasmid encoding tandem RFP-GFP-LC3 fusion for quantification of autophagic maturation was from T. Yoshimori (Osaka, Japan). Plasmids encoding Rab8a wt (wild type) and Rab8a S22N were from Johan Peranen (University of Helsinki, Finland).

2.7.4 Antibodies, immunoblotting, detection assays, microscopy

Cells extracts were analyzed by standard immunoblot techniques with antibodies to pro-IL-1β (Abcam), NLRP3 (AdipoGen), ASC (Enzo Life Sciences), LC3 (Sigma), GORASP1 (Novus), GORASP2 (Abcam), Rab8a (Abcam) and Actin (Sigma); staining was revealed with Super Signal West Dura chemiluminescent substrate (Pierce). For all conditions, cell-free supernatants were assayed by immunoblotting after TCA precipitation for mouse IL-1β p17 (R&D), caspase-1 p10 (Santa Cruz Biotechnology), HMGB1 (Abcam) and Cathepsin B (R&D) or by ELISA for mouse IL-1β (R&D), IL-18 (MBL) and HMGB1 (IBL). Immunofluorescence confocal microscopy was carried out using a Zeiss LSM 510 Meta microscope (laser wavelengths 488 nm, 543 nm and 633 nm). Antibodies against endogenous proteins IL-1β (AbCam), Sec6 (Shu C. Hsu, Rutgers University, NJ, USA), Cathepsin B (R&D), Rab8a (Abcam), GORASP1 (Novus),
GORASP2 (ProteinTech Group), GM130 (BD), LC3 (MBL) and GFP (Abcam; for BMMs from GFP-LC3 knock-in transgenic mice) were used for indirect immunofluorescence analysis. Pearson’s colocalization coefficients were derived using SLIDEBOOK 5.0 (Intelligent Imaging Innovations) applying the SLIDEBOOK 5 default algorithm command “AND”. All Pearson’s coefficients were derived from 3 completely independent experiments with 5 fields or more per experiment, for a total of ≥15 fields contributing to the cumulative result.

2.7.5 Statistics

All data were analysed using two-tailed unpaired Student’s t tests. All experiments were performed at least three times, with data representing means ± s.d. (standard deviation).

Supplementary data

Supplementary data are available at The EMBO Journal Online.

2.8 Acknowledgments

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2.9 Conflict of interest

The authors declare that they have no conflict of interest.
2.10 References


CHAPTER 3

AUTO PHAGY-BASED UNCONVENTIONAL SECRETION OF IL-1β
SPECIFICALLY UTILIZES PARTICULAR AUTO PHAGY-RELATED
PROTEINS
3.1 Abstract

Proteins without signal peptides can still be released into extracellular space via unconventional secretory pathway(s) that remain to be defined. A class of leaderless proteins, including the pro-inflammatory cytokine interleukin-1β (IL-1β), is processed in the cytosol by proteolytic machineries, such as the inflammasome prior to secretion. Previously, we found that autophagy, traditionally considered a degradative pathway, regulates the unconventional secretion of IL-1β. Here, we further test the hypothesis that there is a specialized molecular machinery for secretory autophagy pathway to be differentiated from degradative autophagy by testing the effects of knocking down known autophagy factors on the secretion of IL-1β from bone marrow-derived macrophages (BMMs). We found that autophagy-based unconventional secretion of IL-1β preferentially utilizes specific mammalian Atg8 isoforms, LC3A and GABARAP, but not TBK1 and ULK1. We further identified that less IL-1β is secreted from BMMs derived from transgenic mice lacking the autophagic adaptor Sequestosome 1/p62, while knocking down another adaptor protein NBR1 did not show any effect. We also observed that GRASP55, the conserved regulator of unconventional secretion, selectively co-localized with WIP12B and WIP12D but not with WIP11. These findings strengthen the evidence for the existence of a specialized secretory autophagy pathway and provide first insights into the divergence between degradative and secretory autophagy pathways.
3.2 Introduction

Most eukaryotic proteins containing N-terminal or internal signal peptides are secreted via the ER/Golgi-dependent conventional secretory pathway. However, leaderless proteins, like interleukin-1β (IL-1β) and acyl-CoA binding protein (Acb1), can still be released into the extracellular space via unconventional secretory pathway(s)\(^1,2,3,4\). The suggested routes for the unconventional secretion of IL-1β are: 1) microvesicle shedding\(^5,6\), 2) secretory endolysosome-dependent pathway\(^7,8\), and 3) exosome release\(^9\). Yet, these studies did not capture IL-1β inside the proposed secretory intermediates. Surprisingly, GrpA, the single homologue of GRASP (Golgi Re-assembly and Stacking Protein) in \textit{D. discoideum}, was shown to be involved in the ER/Golgi-independent unconventional secretion of AcbA (homologue of Acb1), a factor required for the differentiation of pre-spore cells\(^10\). Also, during epithelium remodeling in \textit{D. melanogaster}, GRASP mediates the unconventional transport of α integrin to the plasma membrane\(^11\). However, the exact molecular machinery remains elusive.

Recent studies in yeast and mammals shed the light on the new unconventional secretory aspect of autophagy, a pathway previously considered primarily as degradative. In yeast, several Atg proteins, Grh1 (yeast homologue of GRASP), proteins needed for fusion with plasma membrane and multi-vesicular body components were shown to mediate unconventional secretion of Acb1, upon starvation and rapamycin treatment (which are often used to induce autophagy)\(^12,13\). In mammalian cells, the unconventional secretion of IL-11β, IL-18, and HMGB1 depend on a pathway involving Atg5, inflammasome, at least one of the two mammalian Golgi reassembly stacking protein
(GRASP) paralogues, GRASP55 (GORASP2) and Rab8a. Also, the unconventional trafficking of the integral protein CFTR to the plasma membrane requires GRASP55 and Atg proteins needed for autophagosome formation. Autophagy is a bulk topological inverter for cytosolic proteins and components, thus cytosolic/integral proteins might be captured into the lumen of autophagosome-like structures, then targeted to the plasma membrane for secretion without fusion with the lysosome for degradation. A lot of unconventionally secreted proteins are released upon stress conditions and are involved in inflammation, development, tissue repair, and cytoprotection. Also considering autophagy as an important cellular stress-response mechanism, autophagy-based unconventional secretion might serve as an essential molecular pathway to mediate the protective and regenerative processes.

There is a set of autophagy-specific factors (Atgs) involved in the execution of degradative autophagy. Autophagy initiating-ULK1 (mammalian homologue of Atg1) complex responds to upstream cellular stress signals, like starvation, hypoxia, ER stress and microbe invasion. Then Beclin1/Vps34 complex initiates PI3P production, recruits PI3P effectors like DFCP1 and WIPIs (mAtg18s), mediates formation of pre-autophagosomal structure named isolation membrane. Atg5/12 ubiquitin-like conjugation system conjugates PE to LC3 (mAtg8) to elongate the isolation membrane into the autophagosome. The Atg8s are ubiquitin-like proteins that are suggested to play dual roles in autophagosome formation by selectively incorporating autophagic cargoes, through interacting with different autophagic adaptor proteins and promoting autophagosome elongation and closure. Autophagic adaptor proteins like p62,
recruit ubiquitinated cargoes into the elongating autophagosome. Proteins like UVRAG and TBK1 mediate autophagosome maturation by promoting its fusion with the endosome and lysosome, forming autolysosomes. Finally, the substrates captured inside the autolysosome together with p62 and LC3 are degraded.

Although the new secretory function of autophagy (which we term as secretory autophagy hereafter) is beginning to be appreciated, it is still unclear that if autophagosomes are directly delivering secretory cargoes to the cell surface since autophagy-related proteins also have non-autophagic roles in cell signaling, transcription, protein secretion, cell survival/apoptosis, and cellular transportation. For example, autophagic adaptor protein p62 is an important signaling hub for multiple signal transduction pathways, such as NF-κB signaling, apoptosis, and Nrf2 activation. Also, TBK1 which was shown to mediate autophagosome maturation has additional role to phosphorylate transcription factor IRF3 and upregulate NF-κB signaling. In addition, Atg proteins that are involved in secretory autophagy also regulate degradative autophagy. How do these two pathways interact with each other and make a balance? Since there are several homologues of Atg proteins and autophagic adaptors, we hypothesized that different homologues may carry out different roles in degradative and secretory autophagy to differentiate these two processes.

In order to answer these questions, we took the approach to knock down several known autophagy factors to test its effect on IL-1β secretion. Data show that secretory autophagy preferentially uses specific mammalian Atg8 homologues, LC3A and
GABARAP, together with the autophagic receptor protein p62. Positive regulators of degradative autophagy, ULK1 and TBK1, turned out to play inhibitory roles in IL-1β secretion. GRASP55 was observed to selectively co-localizes with autophagosome precursor WIPI2B and WIPI2D, but not with WIPI1. These findings suggest that secretory autophagy involves a specific molecular machinery to carry out its extracellular delivery role.

3.3 Results

3.3.1 Secretory autophagy preferentially utilizes LCA and GABARAP

There are only one Atg8 in yeast and several homologues in mammals: LC3A/B/C, GABARAP, GABARAPL1, and GABARAPL2 (GATE-16) 27, 28. However, the exact roles of different mammalian Atg8 remain largely unknown. In order to investigate the roles of different Atg8s in the autophagy-based unconventional secretion of IL-1β, we knocked down LC3A/B (mice do not have LC3C), GABARAP/L1/L2 in mouse BMMs, then stimulated for autophagy induction and IL-1β processing using starvation and NLRP3 inflammasome inducer Nigericin. We found significant reduction of IL-1β secretion in the supernatant of BMMs knocked down with LC3A and GABARAP while knocking down the other closely related homologues did not have any effect (Figure 3.1). This is still significant, considering Atg5 knockdown yielded about the same percentage (~30%) of decrease (Data shown in Chapter 2, Figure 2.1A). Surprisingly, LC3B, the most abundant and essential mammalian Atg8, was not required for the IL-1β secretion. This suggests that some mammalian Atg8s might be predominantly involved in secretory autophagy while others are used for degradation.
Figure 3.1 Autophagy induced-unconventional secretion of IL-1β favors specific LC3s/GABARAPs.
Figure 3.1 Autophagy induced-unconventional secretion of IL-1β favors specific LC3s/GABARAPs.

Mouse BMM cells were transfected with scramble (Scr) control siRNA or siRNAs against LC3A/LC3B/GABARAP/GABARAPL1/GABARAPL2. After 48 h of transfection, cells were pre-treated with LPS (100ng/ml) in full media and the day after subjected to 20 mM nigericin in EBSS for 1h, and secreted IL-1β was measured by ELISA. Data (normalized to the level of sample from scramble knockdown) represent mean values ±s.e.m (n=4); Statistical significance was calculated using student’s t-test. *p<0.05.
3.3.2 TBK1 knockdown increases IL-1β secretion

Previously, our group reported that Bafilomycin A1, which is an inhibitor of V-ATPase blocking autophagosome maturation, decreases IL-1β secretion \(^\text{14}\). This suggests that autophagy-based unconventional secretion requires proper acidification of secretory vesicles and autophagosome maturation. TBK1 is an atypical IKK that promotes autophagosome maturation and degradation by delivering lysosomal protease Cathepsin D to the autophagosome compartments \(^\text{30, 34}\). To address whether TBK1 is also involved in the secretory autophagy, TBK1 was knockdown in BMMs, then treated with starvation and nigericin. Knockdown of TBK1 caused 2-3 fold increase in starvation-induced IL-1β secretion (Figure 3.2A). Accordingly, there was less intracellular pro-IL-1β in the cell lysates of TBK1 knocked down cells (Figure 3.2B), which indicates that more pro-IL-1β have been processed and secreted. These data suggest that TBK1 inhibits the starvation induced-unconventional secretion of IL-1β and knocking down the positive regulator of degradative autophagy might up-regulate secretory autophagy.

3.3.3 p62 is involved in IL-1β secretion while NBR1 is not required

We next tested whether different autophagic receptor proteins are involved in the autophagy-based unconventional secretion of IL-1β. There are several autophagic receptor proteins identified: p62, NDP52, NBR1, Optineurin, c-Cbl, Nix, and Smurf1 \(^\text{29, 35, 36, 37, 38, 39, 40, 41}\). Each of them specifically targets certain substrates for degradative autophagy. Less IL-1β is secreted from BMMs derived from transgenic mice lacking the autophagic receptor Sequestosome 1/p62 (Figure 3.3A), while knocking down another
Figure 3.2  TBK1 inhibits autophagy-induced unconventional secretion of IL-1β.
Figure 3.2  TBK1 inhibits autophagy-induced unconventional secretion of IL-1β.

(A) BMM cells were transfected with scramble (Scr) control siRNA or siRNA against TBK1. After 48 h of transfection, cells were pre-treated with LPS (100ng/ml) in full media and the day after subjected to 20 mM nigericin in EBSS for 1h, and secreted IL-1beta was measured by ELISA. Data (normalized to the level of sample from scramble knockdown) represent mean values ±s.e.m (n=3); Statistical significance was calculated using student’s t-test. *p<0.05.

(B) Immunoblot analysis of TBK1 knockdown in BMMs.
closely related receptor protein NBR1 did not have any effect (Figure 3.3B). This indicates that some autophagic receptor proteins may be involved in the cargo selection for secretory autophagy or the unconventional secretion of IL-1β is affected by the signaling processes directed by such adaptors.

3.3.4 GRASP55 selectively intersect with WIPI2B and WIPI2D

GRASP (Golgi Re-assembly and Stacking Protein) is a conserved regulator of unconventional secretion in different organisms \(^4, 10, 12, 13, 14, 15\). However, it is still not clear that why a Golgi protein is required for the ER/Golgi-independent unconventional secretory pathway and what is the interaction between GRASP and autophagy pathway. Previously, the mammalian homologue GRASP55 was shown to be involved in the autophagosome formation, which can be either secretory or degradative (Figure 2.5) \(^{14}\). Considering GRASP55 as a tethering factor for Golgi ribbon linking and stacking, GRASP55 may be involved in tethering pre-autophagosomal structures to form secretory or degradative autophagosomes. So we tested if GRASP55 is co-localizing with some early autophagosomal markers-WIPIs (mAtg18s) \(^{21, 22}\) in U2OS-GFP-WIPI stable cell lines. In normal condition (Full), WIPI1/2B/2D all show diffuse distribution inside the cytosol. When autophagy was induced by starvation, WIPI2B and 2D form pre-autophagosome-like structures and localize in close proximity with GRASP55 (Figure 3.4). In contrast, WIPI1 forms punctated structures but does not co-localize with GRASP55. This indicates that there might be a functional separation in these WIPIs. Some WIPIs might be localized on degradative autophagosomes, whereas the others are tethered by GRASP55 to make secretory autophagosome.
Figure 3.3 p62 is required for autophagy-mediated unconventional secretion.
Figure 3.3  p62 is required for autophagy-mediated unconventional secretion.

(A) Wild type or p62 knock out BMM cells were pre-treated with LPS (100ng/ml) and the day after subjected to 20 mM nigericin (Ethanol as control) in EBSS for 1h, and secreted IL-1beta was measured by ELISA. Data represent mean values ±s.e.m (n=3); Statistical significance was calculated using student’s t-test. *p<0.05.

(B) Wild type BMM cells were transfected with scramble (Scr) control siRNA or siRNA against NBR1. After 48 h of transfection, cells were pre-treated with LPS (100ng/ml) in full media and the day after subjected to 20 mM nigericin in EBSS for 1h. Secreted IL-1beta was measured by ELISA. Data represent mean values ±s.e.m (n=3); Statistical significance was calculated using student’s t-test. n.s. stand for “not significant”.

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Figure 3.4 GRASP55 and WIPIs are localized in close proximity upon starvation.
Figure 3.4  GRASP55 and WIPIs are localized in close proximity upon starvation.

Immunofluorescence confocal microscopy analysis of WIPIs and GRASP55 distribution. U2OS cells stably expressing GFP-WIPI1 or GFP-WIPI2B or GFP-WIPI2D were cultured in DMEM (full) or EBSS (starvation) for 2h 30min, then stained for endogenous GRASP55. WIPIs (green, GFP), GRASP55 (red, Alexa568).
3.3.5 ULK1 knockdown up-regulates IL-1β secretion

Seeing the differentiation between secretory and degradative autophagy at almost every stage, we questioned that whether the fate of the autophagosome precursors are decided from the very beginning to be either degradative or secretory. In order to investigate this, we knocked down the autophagy-initiating kinase ULK1 (mAtg1) in BMMs and measured the IL-1β secretion in the supernatant by ELISA. Intriguingly, ULK1 knockdown increased IL-1β secretion rather than decreasing it (Figure 3.5), which is in correspondence with TBK1 knockdown. This is again suggesting that there might be a competition between degradative autophagy and secretory autophagy pathway. Thus, suppressing one pathway might cause up-regulation of the other one.

3.4 Discussion

In this study, we have identified additional players involved in secretory autophagy to support the existence of a specialized unconventional secretory pathway mediated by autophagy, which differs itself from conventional degradative autophagy at every step, including initiation, elongation, recognition, and degradation. Especially, there are several homologues or closely related proteins in some of the Atg-related proteins and they seem to be playing different roles in secretory and degradative autophagy.

LC3A and GABARAP knockdown significantly decreased IL-1β secretion, while the essential autophagy factor LC3B was not required. This is indicating that LC3A and GABARAP might be involved in making secretory autophagosomes while LC3B is mainly functioning during degradation. Similarly, p62-knockout BMMs showed reduced
Figure 3.5  ULK1 knockdown up-regulates autophagy-based unconventional secretion of IL-1β.
Figure 3.5  ULK1 knockdown up-regulates autophagy-based unconventional secretion of IL-1β.

BMM cells were transfected with scramble (Scr) control siRNA or siRNA against ULK1. After 48 h of transfection, cells were pre-treated with LPS (100ng/ml) in full media and the day after subjected to 20 mM nigericin in EBSS for 1h, and secreted IL-1beta was measured by ELISA. Data represent mean values ±s.e.m (n=3).
level of IL-1β secretion, whereas NBR1 knockdown did not affect this process. Although this might suggest that adaptor proteins are involved in the unconventional secretion of IL-1β, it is equally possible that p62-mediated signal transduction affects IL-1β secretion. Therefore, it is necessary to test whether the transcriptional level of IL-1β is altered upon p62 deficiency. Incomplete inhibition of IL-1β secretion is also noticed upon LC3A/GABARAP knockdown or p62 knockout, although the synergic effects of Atg8 proteins and the autophagic adaptor proteins or the existence of additional unconventional secretory pathways may explain this. It will be useful to check if some of the mammalian Atg8 or autophagic receptor proteins play redundant roles in secretory autophagy.

GRASP55 selectively co-localizes with early autophagosomal marker WIPI2B and WIPI2D but not with WIPI1 upon autophagy induction. Considering GRASP55’s role in mediating autophagosome formation, this may suggest that although WIPI1 and WIPI2 are both mammalian Atg18 homologues, their functions might be different, with WIPI2 being exclusively involved in secretory autophagosome formation.

The data about TBK1 and ULK1 was somehow unexpected. Previously, we showed that Bafilomycin A1 treatment decreased IL-1β secretion, suggesting that proper autophagosome maturation is required for secretory autophagy. Therefore, knockdown of TBK1, a protein mediating autophagosome maturation, was expected to show a similar effect. However, the effect was completely opposite that TBK1 knockdown significantly increased IL-1β secretion rather than decreasing it. Although TBK1 inhibition and
Bafilomycin A1 treatment both dampen autophagosome maturation, their effects on secretory autophagy are opposite, possibly because their mechanisms are different. TBK1 knockdown diminishes the delivery of lysosomal protease Cathepsin D to autophagolysosomal structures, thus it might suppress degradative autophagy and somehow up-regulate secretory autophagy. I originally thought about the alternative scenario for TBK1’s role in IL-1β secretion as a phosphorylating kinase for transcription factor IRF3. Then knocking down TBK1 should down-regulate NF-κB signaling and thus decrease IL-1β transcription and secretion. However, TBK1 knockdown is still accompanied with increased IL-1β secretion. Therefore, TBK1 is downregulating the unconventional secretion of IL-1β possibly in order to balance the two autophagy pathways, secretory and degradative autophagy.

ULK1 knockdown showed similar results. Since there are also several homologues (ULK1/2/3/4 and STK36) in mammalian Atg1 family, it is possible that ULK1 is exclusively used for degradative autophagy and others are involved in secretory autophagy. It will be useful to knock down all these homologues and see their effects on secretory autophagy. Taken together, these data suggest that there might be a balance between degradative autophagy and secretory autophagy, and knocking down a positive regulator of degradative autophagy may enhance the secretory autophagy pathway.

All together, these results demonstrate that although secretory autophagy and degradative autophagy share some components needed for autophagosome formation and cargo recognition, specific factors are required to determine their selectivity and the
balance between the two pathways. However, we have only focused on pro-inflammatory cytokine IL-1β. Therefore, it is necessary to test if these findings also apply to the unconventional secretion of other cargos.

3.5 Materials and Methods

3.5.1 Cells
Murine BMM cells were prepared from femurs of C57/BL6 mice and p62-deficient and proficient mice [44]. Human osteosarcoma (U2OS) cell line stably expressing GFP-WIPI-1, GFP-WIPI2B or GFP-WIPI2D were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Thermo Scientific), 100 U/mL penicillin/100 µg/mL streptomycin (Invitrogen), 0.6 mg/mL G418 (Invitrogen) at 37°C, 5% CO₂.

3.5.2 Pharmacological agonists, inhibitors, inflammasome, and autophagy
To induce pro-IL-1β expression, cells were pretreated overnight with 100ng/ml LPS (Sigma), induced with 20mM Nigericin (Invivogen) for 1h. Autophagy was induced for 1 h by starvation in EBSS (Sigma).

3.5.3 Antibodies, immunoblotting, detection assays and microscopy
Cells extracts were analysed by standard immunoblot techniques with antibodies to TBK1 (Abcam), pro-IL-1β (Abcam), Actin (Sigma); staining was revealed with Super Signal West Dura chemiluminescent substrate (Pierce). For all conditions, cell-free supernatants were assayed by ELISA for mouse IL-1β (R&D). Immunofluorescence confocal microscopy was carried out using a Zeiss LSM 510 Meta microscope (laser wavelengths 488, 543). Antibodies against
endogenous proteins GRASP55 (ProteinTech Group), GFP (Abcam) were used for indirect immunofluorescence analysis.

**3.5.4 Transfections and siRNA knock-downs**

BMM cells were transfected by nucleoporation using Nucleofector Reagent Kit Mouse Macrophage (Amaxa/Lonza Biosystems). For murine LC3A, LC3B, GABARAP, GABARAPL1, GABARAPL2, TBK1, NBR1, ULK1 knockdowns, BMM cells were transfected with siGENOME SMARTpool reagents (Dharmacon).

Murine LC3A SMARTpool

(GUAAGGAGGUGCAGCAGAU; ACCCAUCGCUGACAUCUAU; GCGAGUUGGUCAAGAUAU; CGACCCGGCCUUUCAAGCAG)

Murine LC3B SMARTpool

(CAGUGAUUAUAGAGCGAUA; CCGAAGUGUACGAGAGUGA; GCGCCGGGAGCUUUGAAACAA; GCUUGCAGCUAAUGCUAA)

Murine GABARAP SMARTpool

(GAAAGCGUCUAUGGUCUGU; GAAGCGAAUCAUCUCCGU; GGAGCAUCCGUUCGAGAAA; CCGCUCUGAGGGCGAGAAA)

Murine GABARAPL1 SMARTpool

(GGAAGUACCUGUGGCAGCUC; GGCCAGUUCUACUUCUAA;
GACCUGAGGACGCCCUUAUU; GAGGACGCCUUUUCUUCU)

Murine GABARAPL2 SMARTpool

(GAACACUUUUGGCuuUCUGA; UAGACAAAGAGGAAGUACUU;
GCGUGGAAUCCGCGAAGAU; GAUUCUUGUAUGUGGCuUA)

Murine ULK1 SMARTpool

(CCACUCAGGUGCACAAUUu; UCACAAAGCCCUCuUAUUG;
GCAUGGACUUUUGAUUGAAuU; UUACGGACCUGCUGCUuAA)

Murine ULK2 SMARTpool

(GUACGAAGGUCuAAuACCA; CGAAGGAAUGCGAUGUCA;
GCACGGUACCuACAuAGuA; GUAAuUAuCAGCGCAuAGA)

Murine TBK1 SMARTpool

(GAAGCCGUCUUGUGCAAAuA; UGACGGCGCAuAuAGAuUUu;
CUACGAAGGACGACGUuUA; GUuUGAAG- CGUUuAAuAGA)

Murine NBR1 SMARTpool

(GCGGAGACCUGUUGUGAUA; GAAAGGGAGCUGACAUuUC;
GUACAGCCACCGCUuUUGA; CGAGGGuACCAUGUGUA)

Non-targeting siRNA pool (Scrambled) was used as a control.
3.5.5 Statistics

All experiments were performed at least three times, with data representing mean values±s.e.m (standard error). Data were analyzed using two-tailed unpaired Student’s t-tests. *p<0.05; n.s. stands for “not significant”.

3.6 References


CHAPTER 4

THE ROLE OF GRASP55 IN DEGRADATIVE AUTOPHAGY
4.1 Abstract

GRASP (Golgi Reassembly and Stacking Protein) is a conserved regulator of unconventional secretion, which has been shown to be associated with autophagy-mediated unconventional secretion of cytosolic proteins (e.g. Acb1, IL-1β, IL-18, and HMGB1) and unconventional trafficking of integral proteins (e.g. CFTR) to the plasma membrane. One of the two mammalian GRASP homologues, GRASP55, is involved in autophagosome formation as well, which can be either secretory or degradative. However, whether GRASP55 is also mediating degradative autophagy remains unknown. Here, we report GRASP55’s involvement in at least one aspect of degradative autophagy, mitophagy. Unexpectedly, GRASP55 knockdown up-regulated autophagy-dependent removal of dysfunctional, superfluous mitochondria instead of dampening it. This suggests that GRASP55 might be a shared-component between secretory and degradative autophagy balancing these two processes. Thus, knocking down GRASP55 may result in abrogation in secretory autophagy while increasing the other autophagy pathway-degradative autophagy.
4.2 Introduction

GRASP55 is one of the two mammalian homologues of GRASP (Golgi Reassembly and Stacking Protein). It has two conserved N-terminal PDZ domains and less-conserved C-terminal Serin/Proline-Rich (SPR) domain. It is heavily phosphorylated at Serine/Proline-Rich domain during mitosis and are considered as tethering factors to mediate medial-Golgi stacking and ribbon linking ¹.

Surprisingly, the single homologue of GRASP in *D. discoideum*, GrpA, was shown to be involved in ER/Golgi-independent unconventional secretion of cytosolic protein AcbA, a factor required for differentiation of pre-spore cells ². Also during epithelium remodeling in *D. melanogaster*, dGRASP mediated unconventional transport of transmembrane protein α integrin to the plasma membrane ³. Studies in yeast further revealed that Grh1 (yeast homologue of GRASP), together with several Atg proteins, proteins needed for fusion with plasma membrane, and endosomal/multivesicular body components mediate unconventional secretion of Acb1 (yeast homologue of AcbA) upon starvation and rapamycin treatment (which are often used to induce autophagy) ⁴,⁵. In Chapter 2, we discussed about that starvation-induced autophagy together with GRASP55 contribute to the unconventional secretion of IL-1β, IL-18, and HMGB1 in mammalian cells ⁶. Another research group also reported that GRASP55 and autophagy proteins regulate the unconventional trafficking of mutant ΔF508-CFTR to the plasma membrane. Importantly, transgenic expression of GRASP55 in ΔF508-CFTR mice restores CFTR function and rescues mouse survival without apparent toxicity ⁷. We further discovered that autophagy-based unconventional secretion of IL-1β specifically
utilizes particular autophagy-related proteins LC3A, GABARAP, and p62 but not TBK1 and ULK1. These all imply the existence of a conserved specialized pathway for unconventional secretion/trafficking, which involve autophagy proteins and GRASP. It also suggests the potential application of autophagy and GRASP on the treatment of the diseases (inflammation, cystic fibrosis, etc.) involving unconventionally secreted/trafficked proteins. However, the potential interplay between GRASP and autophagy hasn’t been revealed fully.

We showed previously that GRASP55 controls autophagy initiation since its knockdown decreased the level of LC3BII, the important marker of autophagosome. In another assay, GRASP55 knockdown reduced the total number of autophagic puncta, especially reducing the formation of autophagosomes but not their maturation. Considering GRASP55 as a tethering factor for Golgi stacks, it is possible that GRASP55 tethers early autophagosomal structures to form autophagosomes needed for degradative autophagy as well as autophagosome-like vesicles needed for unconventional secretion. Thus, it was hypothesized that GRASP55 is involved in degradative autophagy as well. Investigating the potential involvement of GRASP55 in degradative autophagy is essential for us to determine whether it is an exclusive factor for secretory autophagy or it is a shared-component between secretory and degradative autophagy balancing these two processes. It is also important when applying GRASP55 to treat diseases involving unconventionally secreted/trafficked proteins without perturbing degradative autophagy.
In order to test the potential involvement of GRASP55 in degradative autophagy, GRASP55 was knocked down in wild-type BMMs and check its effect on degradative autophagy by the following standard autophagy assays since autophagy is responsible for the removal of intracellular pathogen\textsuperscript{8}, long-lived protein aggregates\textsuperscript{9} and damaged mitochondria\textsuperscript{10}. 1) Degradation of long-lived proteins by proteolysis; 2) removal of damaged mitochondria by mitotracker staining; 3) elimination of intracellular microbes by \textit{M. tuberculosis} killing.

Interestingly, GRASP55 knockdown resulted in increased removal of dysfunctional mitochondria under both basal and induced autophagy, suggesting GRASP55 is negatively regulating selective degradation of damaged, surplus mitochondria by mitophagy.

4.3 Results

4.3.1 GRASP55 knockdown up-regulates mitophagy

Appropriate control of mitochondrial turnover is essential for maintaining cellular energetics and homeostasis under both basal and stressed conditions. Selective degradation of damaged or superfluous mitochondria by autophagy (mitophagy) is induced upon stress conditions like hypoxia, starvation, and tissue damage in order to maintain cellular homeostasis and protect cells against accumulation of dysfunctional mitochondria. Mitophagy has been associated with development, cancer, aging, and neurodegeneration\textsuperscript{10,11,12,13}. Defects in mitophagy often result in enhanced Mitotracker
Green staining suggesting accumulation of depolarized mitochondria and increased mitochondrial mass (which can be labeled by Mitotracker Green FM dye).

In order to investigate the role of GRASP55 in mitochondria removal, GRASP55 was knocked down in BMMs, stimulated cells with starvation and stained for Mitotracker Green and Red to distinguish depolarized and polarized mitochondria. As reported before, autophagy induction by starvation triggered removal of depolarized, dysfunctional mitochondria (Mitotracker Green$_{\text{high}}$) and reduced mitochondrial mass as shown by decrease in the percentage of Mitotracker Green$_{\text{high}}$ cells. GRASP55 knockdown caused significant reduction in the percentage of Mitotracker Green$_{\text{high}}$ cells in full (basal) condition and even greater drop in starvation condition (autophagy induction), indicating more dysfunctional mitochondria are removed and mitochondrial mass is further decreased when GRASP55 is deficient upon autophagy induction (Figure 4.1 A-C). This suggests that GRASP55 knockdown is up-regulating mitophagy instead of inhibiting it.

4.3.2 GRASP55 knockdown doesn not affect the degradation of long-live proteins

While the ubiquitin-proteasome system primarily degrades short-lived proteins, autophagy regulates majority of degradation of long-lived proteins $^{14}$. Autophagy mediated-degradation of long-lived proteins is measured by proteolysis. Cells are usually incubated overnight with C14 or H3-valine or leucine to label total cellular proteins, then shortly incubated with excessive unlabeled valine or leucine in order to wash out the labeling of short-lived proteins, which causes exclusive radioactive labeling of long-lived
Figure 4.1  Dysfunctional mitochondria are cleared when GRASP55 is knocked down.

A

Scramble

Mitotracker Red CMXRos

Grasp55 KD

Full

Starve

Mitotracker Green

B

C

SiScr

SiGRASP55

SiScr

SiGRASP55

Starvation

% Mitotracker Green high Cells

% Mitotracker Green high Cells

Full

Starvation

GRASP55

LC3-I

LC3-II

Actin

-50kD

-15kD

-50kD
Figure 4.1 More dysfunctional mitochondria are cleared when GRASP55 is knocked down.

BMM cells were transfected with scramble (Scr) control siRNA or siRNA against GRASP55. After 72 h of transfection, cells were starved to induce autophagy for 1h and stained with 300nM Mitotracker green FM (depolarized, unfunctional) and Mitotracker red CMXRos (polarized, respiring). (A) Contour plots of FACS analysis are depicted. (B) Quantification of percentage of Mitotracker Green FM$^{\text{high}}$ cells. Data represent mean values ±s.e.m (n=3); Statistical significance was calculated using student's t-test. *p<0.05. (C) Immunoblot analysis of GRASP55 knockdown in BMMs.
proteins. Subsequently, cells are incubated again with cold valine or leucine that avoids re-utilization of the radiolabeled amino acids for protein synthesis. Trichloroacetic acid (TCA) precipitable radioactivity of the cell monolayers and the TCA soluble radioactivity in the media are determined. Leucine or valine release (a measure of degradation of long-lived proteins) is calculated as a ratio between TCA soluble supernatant and total cell-associated radioactivity.

So as to determine whether GRASP55 is involved in autophagy mediated-degradation of long-lived proteins, GRASP55 was knocked down and measured its affect on proteolysis in full media and Starvation media with or without autophagy flux inhibitor Bafilomycin A1, which inhibits autophagic degradation. As reported before, starvation treatment tends to increase proteolysis of long-lived proteins, while Bafilomycin A1 treatment inclines to decrease the clearance of long-lived proteins in both full and starvation conditions. However, GRASP55 knockdown did not affect this process (Figure 4.2).

4.3.3 GRASP55 knockdown doesn’t affect the in vitro *M. tuberculosis* killing

Besides mitochondrial removal and long-lived protein secretion, degradative autophagy is also involved in *M. tuberculosis* killing. Autophagic killing of ubiquitinated mycobacteria was first observed in vitro using *BCG* and *M. tuberculosis*-infected macrophages. It was also found that human IRGM (Immunity-related p47 guanosine triphosphatases) participates in IFN-γ/Rapamycin/Starvation–induced autophagy in human macrophages.
Figure 4.2 GRASP55 knockdown does not affect long-lived protein degradation.

![Graph showing GRASP55 knockdown and long-lived protein degradation](image-url)
Figure 4.2  GRASP55 knockdown does not affect long-lived protein degradation.

BMM cells were transfected with scramble (Scr) control siRNA or siRNA against GRASP55. After 48 h of transfection, cells were labeled with overnight with media containing 1uCi/ml H3 Leucine, the day after cells were incubated in full or starvation media with or without bafilomycin A1 for 90minutes. Trichloroacetic acid (TCA) precipitable radioactivity of the cell monolayers and the TCA soluble radioactivity in the media were determined. Leucine release (a measure of degradation of long-lived proteins) was calculated as a ratio between TCA soluble supernatant and total cell-associated radioactivity. Data represent mean values ±s.e.m (n=3); Statistical significance was calculated using student’s t-test.
to resist against *M. tuberculosis* infection. Autophagic receptor protein p62 delivers ribosomal proteins (rpS30) and ubiquitinated complexes (Fau, a fusion of rpS30 with a ubiquitin-like domain in its N-terminus) as inactive precursors to autophagosomes, which are processed later as neo-antimicrobial peptides upon digestion in lysosomes. Innate immunity regulator TBK-1 was shown to be required for IL-1β-induced autophagic killing of mycobacteria in macrophages by promoting autophagosome maturation and phosphorylating the autophagic adaptor p62 on Ser-403, a residue essential for its role in autophagic clearance. Recent studies also showed that extracellular bacterial DNA escaped through bacterial secretion system ESX1 can be recognized by the STING-dependent cytosolic pathway, which later ubiquitinates the bacteria. Delivery of ubiquitinated bacilli to autophagosomes was dependent on autophagic receptor p62, NDP52 and also TBK1. The ubiquitin ligase Parkin was shown to mediate K63-ubiquitination of *M. tuberculosis* and recruitment of autophagic receptor proteins. The mycobactericidal role of autophagy was also reported in vivo that autophagy not only controls tuberculosis infection and but also prevents excessive inflammatory reactions in the host.

To test the potential association of GRASP55 with autophagic killing of mycobacteria, GRASP55 was knocked down in BMMs, infected them with *M. tuberculosis* H37Rv strains and treated with starvation media to induce autophagic killing. Subsequently, cells were lysed and survived mycobacteria inside were quantified by colony forming unit (CFU) counting. As expected, autophagy induction by starvation
Figure 4.3  GRASP55 knockdown does not affect the in vitro *M. tuberculosis* killing.
Figure 4.3  GRASP55 knockdown does not affect the in vitro \textit{M. tuberculosis} killing.

After 72h of GRASP55 knockdown, BMMs was infected with \textit{M. tuberculosis} H37Rv (multiplicity of infection of 1/cell) for 1 hour, followed by washing, 4 hour in full and starvation media, lysis with water to harvest bacteria, then plated. Autophagic killing of \textit{M. tuberculosis} was quantified by colony forming unit (CFU) counting. Data represent mean values ±s.e.m (n=3); Statistical significance was calculated using student’s t-test. n.s. stand for “not significant”. ); *p<0.05.
significantly induced autophagic killing of *M. tuberculosis* as reflected by decreased number of CFU counting (**Figure 4.3**). However, GRASP55 knockdown did not affect *M. tuberculosis* killing either in Full (basal) or Starvation (autophagy induction) conditions.

### 4.4 Discussion

With the observation that GRASP55 is regulating autophagosome formation, I originally hypothesized that GRASP55 is also involved in degradative autophagy and knocking down GRASP55 will dampen autophagic degradation of damaged mitochondria, intracellular microbes, and long-lived protein. However, GRASP55 knockdown up-regulated removal of damaged mitochondria instead of inhibiting it while it did not show any effect on degradation of long-lived proteins and intracellular mycobacteria killing. These results show that GRASP55 is negatively regulating at least one aspect of degradative autophagy and it might a factor engaged in both secretory and degradative autophagy balancing these two processes.

The unexpected negative regulation of GRASP55 in mitophagy might be associated with the role of mitochondrial ROS in NLRP3 inflammasome activation. Inflammasome inducers typically damage cells and release lysosomal (e.g. Cathepsin B) and mitochondrial products (e.g. mitochondrial ROS) into the cytosol. Zhou *et al.* discovered that NLPR3 inflammasome is activated by mitochondrial ROS production triggered through NLRP3 inflammasome inducers and mitochondrial ROS production is inhibited by mitophagy-mediated clearance of damaged mitochondria. Therefore,
during autophagy-mediated unconventional secretion, GRASP55 might inhibit mitophagy to increase mitochondria ROS production and further trigger NLRP3 inflammasome activation and autophagy-dependent IL-1β secretion. Vice versa, decreased IL-1β secretion during GRASP55 knockdown might be related to decreased ROS production resulted from enhanced removal of damaged mitochondria by mitophagy. However, it is equally plausible that this effect is cell type-specific, since the experiment was exclusively done in macrophages where mitophagy is associated with inflammasome activation. It is necessary to test the involvement of GRASP55 in mitophagy in other cell types and also test GRASP55 knockdown effect on NLRP3 inflammasome activity.

To summarize, we found previously an unexpected role of GRASP55 in mitophagy-dependent removal of damaged mitochondria. This suggests that GRASP55 is a shared component fine-tuning secretory autophagy and degradative autophagy and GRASP55 might be engaged in autophagy-based unconventional secretion of IL-1β in multiple ways.

4.5 Materials and Methods

4.5.1 Cells

Murine BMM cells were prepared from femurs of C57/BL6 mice.

4.5.2 Transfections and siRNA knock-downs

BMM cells were transfected by nucleoporation using Nucleofector Reagent Kit Mouse Macrophage (Amaxa/Lonza Biosystems). For murine GRASP55 knockdown, BMM cells
were transfected with siGENOME SMARTpool reagents (Dharmacon). Non-targeting siRNA pool (Scrambled) was used as a control.

GRASP55 SMARTpool
(GAAGACCUGUUCAGCCUUA; UACCAAGUCUGAUGCCUUU; GUAAACCAGUCCCUUGCUU; GAUC AUCACACCAAAACUCU)

4.5.3 Antibodies and immunoblotting
Cells extracts were analysed by standard immunoblot techniques with antibodies to GRASP55 (ProteinTech Group), LC3B (Sigma) and Actin (Sigma); staining was revealed with Super Signal West Dura chemiluminescent substrate (Pierce).

4.5.4 Mitochondria removal assay
After 72h of GRASP55 knockdown, mouse bone marrow macrophage cells (BMMs) were starved for 1h to induce autophagy. Then cells were trypsinized and stained with 300nM of Mitotracker green (depolarized, dysfunctional) and 300nM of Mitotracker Red CMXRos (polarized, respiring) from Molecular Probes for 15 minutes at 37 degree. Flow cytometry was carried out on the LSRFortessa (BD Biosciences) and data was analyzed using FlowJo software (Tree Star).

4.5.5 Proteolysis
After 72h of GRASP55 knockdown, cells will be labeled with overnight with media containing 1uCi/ml H3 Leucine, the day after cells will be incubated in full or starvation
media with or without bafilomycin A1 for 90 minutes. Trichloroacetic acid (TCA) precipitable radioactivity of the cell monolayers and the TCA soluble radioactivity in the media will be determined. Leucine release (a measure of proteolysis of stable proteins) will be calculated as a ratio between TCA soluble supernatant and total cell-associated radioactivity.

4.5.6 M. Tuberculosis killing

After 72h of GRASP55 knockdown, BMMs was infected with M. tuberculosis H37Rv (multiplicity of infection of 1/cell) for 1 hour, followed by washing, 4 hour in full and starvation media, lysis with water to harvest bacteria, then plated. Autophagic killing of M. tuberculosis was quantified by colony forming unit (CFU) counting.

4.5.7 Statistics

All data were analysed using two-tailed unpaired Student’s t-tests. All experiments were performed at least three times, with data representing mean values±s.e. (standard error). n.s. stands for “not significant”; *p<0.05.

4.6 References


CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS
5.1 Introduction

The studies presented in this dissertation were designed and implemented to investigate the role of autophagy in the unconventional secretion of pro-inflammatory cytokine IL-1β. Although autophagy was primarily considered as a degradative pathway \(^1\), recent studies in yeast revealed the new role of autophagy in the unconventional secretion of Acb1 (a factor needed for differentiation of pre-spore cells), which engage several autophagy-related proteins, components of endosome/multi-vesicular bodies, and the conserved regulator of unconventional secretion Grh1 (yeast homologue of GRASP), upon nitrogen starvation or rapamycin treatment (conditions that induce autophagy) \(^2, 3\). The notion that autophagy might be involved in the unconventional secretion of signal-peptide-lacking cytosolic/nuclear proteins is conceivable since autophagy is a topological inverter which captures cytosolic proteins and components and it is also closely related to endosomes and multivesicular bodies. Defining the autophagy-based unconventional secretory pathway (which we term as secretory autophagy) has both fundamental biological and translational significance since the potential autophagy dependent-unconventionally secreted proteins usually have distinct but related intracellular and extracellular functions and are important in wound healing, cancer, neurodegeneration, and inflammation \(^4, 5, 6\).

However, since autophagy-related proteins are also involved in other non-autophagic (non-degradative) processes, it is equally possible that its effect on unconventional secretion in yeast is indirect \(^7\). Also, whether this secretory autophagy is a conserved process responsible for other unconventionally secreted proteins in higher organisms needs to be tested. Additionally, the mechanism that differentiates this secretory
autophagy pathway from traditional degradative autophagy pathway requires further investigation. Besides, the exact role of a Golgi protein GRASP during the ER/Golgi-independent unconventional protein secretion and its possible involvement in degradative autophagy remain unclear. In order to resolve these questions, bone marrow derived-macrophages were utilized to study the involvement of autophagy in the unconventional secretion of IL-1β, since autophagy has been implicated in the regulation of innate immunity \(^8,^9,^10\) and also TLR signaling that is important for IL-1β synthesis, processing, and secretion in human monocytes \(^11\), induces autophagy in myelomonocytic cells \(^12\). It was hypothesized that there is a specialized molecular and membrane machinery within the autophagy pathway which involves mammalian GRASP to carry out unconventional protein secretion of IL-1β. The objectives of this study were to determine: 1) whether this secretory autophagy pathway involving GRASP is conserved in mammalian cells as discussed in Chapter 2; 2) how secretory autophagy pathway differs from degradative autophagy pathway as reviewed in Chapter 3; 3) whether GRASP is also involved in the degradative autophagy as covered in Chapter 4.

### 5.2 Conclusions from Chapters

The main conclusions from chapters are presented in the following three sections. It also highlights a number of interesting questions raised by the studies presented in this dissertation that should be the focus of future work.
5.2.1 Is secretory autophagy pathway involving GRASP conserved in mammals?

Starvation-induced unconventional secretion of IL-1β is dependent on Atg5, inflammasome components, Cathepsin B, Rab8a, and at least one of the two mammalian GRASP paralogues, GRASP55 (Chapter 2).

Data presented in Chapter 2 shows that autophagy induction by starvation augments inflammasome-dependent unconventional secretion of IL-1β, IL-18, and HMGB1 while Atg5 deficiency (a protein needed for autophagosome formation) and Bafilomycin A1 treatment (autophagosome maturation inhibitor) dampens this process. We also found that IL-1β intersects with autophagosome marker LC3 and Rab8a (a GTPase involved in polarized membrane trafficking). These all suggest that starvation-induced autophagy is regulating unconventional secretion of IL-1β. This autophagy-based unconventional secretion of IL-1β was also dependent on inflammasome, Cathepsin B (NLRP3 inflammasome activator), Rab8a, and GRASP55, one of the mammalian homologues of GRASP. Interestingly, Caspase-1 and Cathepsin B secretion were also dependent on Atg5. Additionally, GRASP55 was regulating autophagosome formation, which was not seen in yeast studies. Thus, upon inflammasome and autophagy induction, IL-1β might be unconventionally secreted after being captured together with Caspase-1 and Cathepsin B into autophagosome-like vesicles (secretory autophagosomes) formed by GRASP55, processed inside and trafficked to the plasma membrane in Rab8a-dependent manner. This study reveals for the first time the new unconventional secretory role of autophagy in mammalian cells and the possible mechanism of unconventional secretion of IL-1β, which has been long unresolved. Later study also revealed GRASP55 and autophagy-
dependent unconventional trafficking of CFTR to the plasma membrane. Together with studies in yeast, it suggests the existence of an unconventional secretory pathway involving GRASP and autophagy-related proteins (Figure 5.1).

However, autophagy seems to play a dual role in IL-1β activation and secretion. Since Atg16L1 deficiency up-regulates IL-1β production in mice and autophagy inhibits IL-1β processing/activation indirectly by affecting inflammasome activation, since autophagy deficiency results in accumulation of damaged mitochondria that leads to release of mitochondria ROS and mtDNA, which are both NLRP3 inflammasome inducers. Additional studies also showed that pro-L-1β and ubiquitinated inflammasomes can be targeted for autophagic degradation. These studies reflect the housekeeping function of basal autophagy, which clears potentially harmful intracellular components and limits unnecessary inflammation. The induced-autophagy-based unconventional secretion of IL-1β early in the process to trigger necessary inflammatory response against intracellular and extracellular danger signals might be followed by the negative of regulation of inflammasome by autophagy. The latter becomes dominant at later time points to suppress excessive inflammasome, which can be destructive to the host. To summarize, autophagy negatively regulates inflammasome activation and positively controls IL-1β secretion, with the net result being the product of these two opposing processes depending on the time and extent of the triggers.

Although autophagy is shown to positively regulate unconventional secretion, autophagosome-like vesicular intermediates containing the unconventionally secreted
Figure 5.1 Autophagy-based Unconventional Secretion of IL-1β.
Figure 5.1 Autophagy-based Unconventional Secretion of IL-1β.

Upon inflammasome activation and autophagy induction, pro-inflammatory cytokine IL-1β, IL-18 and HMGB1 are unconventionally secreted in Atg5-dependent manner. Bafilomycin A1 treatment blocked IL-1β secretion, indicating acidification and maturation of autophagosome is required. IL-1β also intersects with LC3 and Rab8a, suggesting IL-1β might be captured in autophagosome-like secretory vesicles and delivered to the plasma membrane possibly mediated by Rab8a. Cathepsin B and Caspase-1 are also secreted together with IL-1β in Atg5-dependent manner suggesting the processing of pro-IL-1β might have occurred in the autophagosome-like secretory vesicles. Autophagy-based IL-1β secretion also involved GRASP55 and GRASP55 was found to mediate autophagosome formation suggesting GRASP55 might regulate tethering of early autophagosome structures to form secretory autophagosome.
cargoes have not been isolated yet. Since autophagy-related proteins are also regulating some non-autophagic (non-degradtive) processes, it is possible that autophagy’s effect on unconventional secretion is indirect. Therefore, it is important to identify topological features of the vesicles carrying unconventionally secreted cargoes either by visualization or biochemical fractionation in the future. Furthermore, it is necessary to dissect the autophagy-based unconventional secretory pathway to see how it distinguishes itself from the conventional degradative autophagy pathway, which further supports the existence of a specialized molecular pathway for secretory autophagy. Besides, since autophagy regulated unconventional secretion of non-inflammasome substrate HMGB1, it will be interesting to test if Caspase-1 is generally involved in autophagy-based unconventional secretion and how it works in addition to acting as IL-1β converting enzyme. Importantly, the role of GRASP55, the conserved player of unconventional secretion, has not been identified yet. The interplay between GRASP55 and autophagy (secretory and/or degradative) also remain elusive. In addition, more potential autophagy-dependent unconventionally secreted cargoes should be discovered using mass spectrometry. Thus, future studies should focus on these challenges.

5.2.2 How secretory autophagy differs from degradative autophagy?

Autophagy-based unconventional secretion of IL-1β specifically utilizes LC3A, GABARAP, and p62 but not NBR1, TBK1, and ULK1. GRASP55 selectively co-localizes with WIPI2B/2D but not with WIPI1 (Chapter 3).
Chapter 3 focused on identification of more autophagy-related proteins involved in starvation-induced unconventional secretion of IL-1β, which differentiates secretory autophagy from degradative autophagy and adds supporting evidence to the existence of a specialized molecular pathway for secretory autophagy. Data presented in Chapter 3 shows that LC3A and GABARAP are selectively consumed for autophagy-based unconventional secretion of IL-1β. While LC3B, the most studied mammalian homologue of Atg8 that mediates autophagosome elongation and closure, was not required. It suggests that some mammalian Atg8s can be used for making degradative autophagosomes whereas others are targeted for making secretory autophagosome-like vesicles. It is useful to check if LC3A and GABARAP show increased co-localization/co-fractionation with IL-1β upon inflammasome and autophagy induction compared to LC3B either by immunofluorescence or subcellular fractionation. Also, it is necessary to check if LC3A or GABARAP are secreted into the supernatant together with IL-1β.

The autophagic receptor protein p62, which targets ubiquitinated cargoes for degradation, is involved in autophagy-based unconventional secretion of IL-1β while the structurally related protein NBR1 was not. This suggests that p62 might recognize and target IL-1β into the secretory autophagosomes as well while other autophagic receptor proteins are exclusively targeting ubiquitinated cargoes for degradation. However, p62 knockout didn’t completely compromise the secretion of IL-1β, which suggests the potential involvement of other autophagic receptor proteins (Optineurin, Nix, c-cbl, etc.) or some unknown receptors for secretory cargoes. Vps23, ESCRT I complex subunit that regulates ubiquitinated cargo sorting, can be a candidate for such receptors since it has
been implicated in the unconventional secretion of Acb1 in yeast. It will be necessary to knockdown all the receptors and define their roles in the autophagy-based unconventional secretion of IL-1β. It will be also useful to check co-localization between the autophagic receptor proteins and IL-1β upon stimulation. Considering that autophagic receptor proteins usually target ubiquitinated substrates for degradation, it is possible that non-degradative ubiquitination of these secretory cargoes also serve as signals for them to be recognized by autophagic receptor proteins, incorporated into secretory autophagosomes. It is necessary to test whether these secretory cargos also undergo ubiquitination process that is secretory but not degradative. Considering that some mammalian Atg8 show preferential binding to the specific autophagic receptors to selectively target certain cargoes (e.g. LC3C selectively bound by NDP52 is required for antibacterial autophagy against Salmonella.), it is conceivable to hypothesize that specific combination of mammalian Atg8 and autophagic receptor protein is responsible for autophagy-based unconventional secretion of IL-1β. Thus, future studies should also identify the specific Atg8-Receptor combination required for unconventional secretion by immunoprecipitation.

Interestingly, autophagy-based unconventional secretion of IL-1β was promoted when TBK1, a kinase required for autophagosome maturation, was knocked down. TBK1 has been shown to interact with Rab8b, the closely related isoform of Rab8a that has been implicated in autophagy-based unconventional secretion of IL-1β. The negative regulation of TBK1 on IL-1β secretion might be explained by investigating the interaction between TBK1 and Rab8a. There might be competition between secretory and
degradative autophagy and TBK1 may exclusively bind with Rab8b being more important for degradative autophagy whereas it does not bind to Rab8a, thus suppress secretory autophagy.

ULK1 knockdown showed similar results. Since there are several mammalian Atg1 homologues, it is reasonable to hypothesize that different mAtg1s play different role in secretory and degradative pathways, with some involved more in degradative autophagy whereas others affect secretory autophagy. Therefore, blocking one pathway may promote the other pathway. It will be interesting to dissect the different roles of mAtg1s. Additionally, it is equally plausible that ULK1 acts as an inhibiting kinase of GRASP55 since GRASP55 phosphorylation was shown to be important for unconventional trafficking of CFTR.\textsuperscript{13}

In consistent with the findings listed above, GRASP55 showed selective co-localization with specific WIPIs, WIPI2B and WIPI2D, but not with WIPI1. Since WIPIs are markers of early autophagosomes, this result is consistent with my data that GRASP55 regulates autophagosome formation as discussed in Chapter 2, which was not observed in yeast studies. It also suggests that there might be functional separations among different WIPIs in secretory and degradative autophagy. To investigate their role in secretory autophagy, WIPI1/2B/2D should be knocked down and IL-1β secretion level should be measured. Since GRASP55 is a tethering factor during Golgi stacking\textsuperscript{21}, it might be involved in the tethering of WIPI puncta to make secretory autophagosomes. In order to test this,
GRASP55 should be knocked down and the size and number of WIPI puncta must be determined either by confocal microscopy or high-content microscopy.

5.2.3 Is GRASP55 involved in degradative autophagy as well?

*GRASP55 negatively regulates removal of damaged mitochondria by mitophagy* (Chapter 4).

Data presented in Chapter 4 shows that GRASP55 is involved in at least one aspect of degradative autophagy, mitophagy. Interestingly, GRASP55 knockdown promoted mitophagy-dependent removal of damaged, superfluous mitochondria, suggesting GRASP55 is negatively affecting mitophagy. Despite GRASP55’s potential role during secretory autophagosome formation, GRASP55 might have additional role to inhibit mitophagy, which causes accumulation of damaged mitochondria and increase of mitochondria ROS production and ultimately induces inflammasome activation. Thus, GRASP55 might promote unconventional secretion of IL-1β in multiple ways. It is also consistent with the arguments made in previous chapters that there might be a competition/balance between secretory autophagy and degradative autophagy, and inhibition of one pathway may result in up-regulation of the other.

However, further experiments should be performed to support this hypothesis. Mitochondrial ROS production should be measured when GRASP55 is knocked down or overexpressed and its effect on inflammasome activation should be examined upon inflammasome induction. Since whether GRASP55 is directly involved in mitophagy is
not clear, it is necessary to check GRASP55’s association with mitophagy regulators, such as Parkin, PINK1, and Nix by western blot and immunofluorescence. In addition, GRASP55’s potential involvement in other cell-types should be tested since the study was exclusively done in macrophages and it is possible that this effect is cell type-specific.

5.3 Implications for Current and Future studies

The studies presented in this dissertation defined one of the first biogenesis functions of autophagy in mammals, which mediates unconventional secretion of IL-1β secretion. This is consistent with studies in yeast, suggesting there is a conserved unconventional secretory pathway that require autophagy-related proteins and GRASP which we can term as secretory autophagy. The data presented here further supports the existence of a specialized molecular pathway for secretory autophagy, which specifically utilizes some of the autophagy-related proteins to carry out its unconventional secretory function. This work also provides first insights to the divergence between secretory and degradative autophagy (also reviewed in Ref. 22). Since there might be a competition/balance between these two pathways, these aspects should be taken into consideration when applying GRASP and autophagy into the treatment of diseases involving unconventionally secreted/trafficked proteins.
5.4 References


APPENDIX I

SUPPLEMENTARY MATERIALS FOR CHAPTER 2

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Fig. A1 Autophagy-based unconventional secretion of IL-1β upon different inflammasome inducers
Fig. A1 Autophagy-based unconventional secretion of IL-1β upon different inflammasome inducers

(A) Atg5fl/fl Cre- and Atg5fl/fl Cre+ BMM were induced for autophagy by starvation in EBSS (Starv) or not (Full) for 1 h. Total cell extracts were analyzed by immunobloting. (B) LPS-pretreated BMM were stimulated with 20 μM nigericin (Nig) for 1 h with or without co-treatment with the mTor catalytic inhibitor pp242. IL-1β secretion was measured by ELISA. (C) Bone marrow-derived macrophages (BMM), pretreated with 100 ng/ml LPS overnight to induce pro-IL-1β expression, were exposed to Alum (250 μg/ml) for 2 h with autophagic induction by starvation (Starv) in EBSS or were incubated in full medium (Full), and secreted IL-1β assayed. Data, means ± s.d. (n≥3); *, p<0.05. (D) LPS-pretreated BMM were stimulated with 1 or 5 μM amyloid-β peptide fibrils (Aβ) for 2 h with or without autophagic induction by starvation and IL-1β secretion measured. (E) BMM were transfected with scramble (Scr) control siRNA or siRNAs against ASC, NLRP3 or GRASP55. After 48 h following transfection, cells were treated overnight with LPS and IL-1β mRNA levels were quantified by real-time RT-PCR and internally normalized with respect to the HPRT1 gene. Data, means ± s.d. (n=3). †, p≥0.05. (F) BMM were transfected with scrambled control siRNA (Scr) or siRNAs against ASC, NLRP3 or GRASP55. After 48 h following transfection, cells were treated overnight with LPS and pro-IL1β level assayed by immunoblots.
Fig. A2  LDH release upon inflammasome and autophagy induction and negative regulation of basal autophagy on IL-1β secretion.
Fig. A2 LDH release upon inflammasome and autophagy induction and negative regulation of basal autophagy on IL-1β secretion.

(A-B) Bone marrow-derived macrophages (BMM), pretreated with 100 ng/ml LPS overnight to induce pro-IL-1β expression, were exposed to nigericin (20 µM) for 20, 30 or 60 minutes with autophagic induction by starvation (Starv) in EBSS or were incubated in full medium (Full). IL-1β secretion (A) and LDH released (B) was measured by ELISA and by a cytotoxicity detection kit respectively. Data, means ± s.e.m. (n≥3); *, p<0.05. (C-D) Bone marrow-derived macrophages (BMM), pretreated with 100 ng/ml LPS overnight to induce pro-IL-1β expression, were exposed to silica (250 µg/ml) for 20, 30 or 60 minutes with autophagic induction by starvation (Starv) in EBSS or were incubated in full medium (Full). IL-1β secretion (C) and LDH released (D) was measured by ELISA and by a cytotoxicity detection kit respectively. Data, means ± s.e.m. (n≥3); *, p<0.05; †, p≥0.05. E. BMM from Atg5fl/fl Cre- and Atg5fl/fl Cre+ mice were pretreated overnight with 100 ng/ml LPS, stimulated for 1 h with the inflammasome agonist nigericin (20 µM) in full medium (Full). Cell culture supernatants were assayed for murine IL-1β. Data, means ± s.d. (n≥3); *, p<0.05. F, as in E, assayed for IL-18. Data, means ± s.d. (n≥3); *, p<0.05.
Fig. A3  Co-localization between LC3 and IL-1β upon inflammasome activation.
**Fig. A3 Co-localization between LC3 and IL-1β upon inflammasome activation.**

**(A)** Colocalization analysis of IL-1β with the basal autophagic machinery factor LC3. Fluorescence: LC3 (green, Alexa 488); IL-1β (red, Alexa568). BMM from GFP-LC3 knock-in mice were pretreated with 100 ng/ml LPS overnight, stimulated for 30 min with the inflammasome agonist nigericin (20 µM) in full medium. BMM were prepared for immunofluorescence microscopy using fluorescently labeled antibodies against GFP and IL-1β. **(B)** A line fluorescence tracing from images in A. **(C)** Pearson’s colocalization coefficients for IL-1β and LC3. Data, means ± s.e.m. (n≥3). **(D)** LPS-pretreated BMM were starved (starv) for 1h with 20 µM nigericin and cathepsin B inhibitor CA-074 Me (10 µM) or with Bafilomycin A1 (Baf-100 nM). IL-1β mRNA levels were quantified by real-time RT-PCR, normalized with respect to the HPRT1 gene, and presented as fold increases. Data, means ± s.d. (n≥3); †, p≥0.05.
Fig. A4 IL-1β intersect with LC3, Rab8a and Sec6, exocyst component.
**Fig. A4  IL-1β intersect with LC3, Rab8a and Sec6, exocyst component.**

(A) Colocalization analysis of Rab8a with the basal autophagic machinery factor LC3 and IL-1β. Fluorescence; LC3 (green, Alexa488), IL-1β (red, Alexa568), Rab8a (blue, Alexa633). BMM from GFP-LC3 knocking mice were pre-treated with LPS and stimulated for 30 min with 20 µM nigericin in full medium. BMM were prepared for immunofluorescence microscopy. White arrows, triple colocalization. (B) A line fluorescence tracing from images in A. (C) Pearson’s colocalization coefficients for IL-1β and Rab8a. (D) BMM were transfected twice with scramble (Scr) control siRNA or siRNAs against Rab8a. After 48 h following transfection, cells were treated overnight with LPS and IL-1β mRNA levels were quantified by real-time RT-PCR, normalized with respect to the HPRT1 gene, and presented as fold increases. Data are means +/- SDV. (E) Colocalization of Sec6 with LC3 and IL-1β. BMM from GFP-LC3 knock-in mice were pre-treated with LPS and analyzed by immunofluorescence microscopy. White arrows, triple colocalization (Sec6-IL-1β-LC3; turquoise arrows, LC3-Sec6 colocalization. (F) Line tracing, fluorescence signal coincidence analysis. (G) Pearson’s colocalization coefficients for IL-1β and sec6. (H) Colocalization analysis of Sec6 with LC3 and IL-1β (as in A). White arrows, triple colocalization. (I) A line fluorescence tracing from images in H. J. Pearson’s colocalization coefficients for IL-1β and sec6. Pearson’s coefficients bars and error bars, means ± s.d. (n=3).
Fig. A5 GRASP55 is regulating unconventional secretion of IL-1β and IL-18 under basal autophagy condition.
Fig. A5 GRASP55 is regulating unconventional secretion of IL-1β and IL-18 under basal autophagy condition.

(A, B) BMM cells were transfected with siRNAs or scrambled (Scr) control siRNA. After 48 h of transfection, cells were treated with LPS and the day after subjected to 20 µM nigericin in full medium, and secreted IL-1β (A) and IL-18 (B) were measured. Data, means ± s.d. (n≥3); *, p<0.05. The number of cells in the experiment in panel A (2x10^5 cells) was 2.8 times higher than in Fig. 4A (0.7x10^5 cells). (C) Effect of GRASP65 on autophagy induction by measuring LC3-II. BMM cells were transfected with GRASP65 or with GRASP65 and GRASP55 siRNAs or scramble (Scr) control. At 72 h post-transfection, cells were induced for autophagy, treated or not with Bafilomycin A1 (Baf) to inhibit autophagic degradation. All lanes are from the same gel (one intervening lane was removed to avoid redundancy).
Fig. A6  Co-localization between GM130 and GRASP55.
Fig. A6 Co-localization between GM130 and GRASP55.

Fluorescent confocal microscopy images: GM130 (green, Alexa488), GRASP55 (red, Alexa568). (A) Control, resting BMM. (B) Nigericin, BMM treated with 20 µM nigericin.
Supplementary Methods

Quantitative real time RT-PCR
Total RNA was extracted from BMM and cDNA was synthetized using a Cells-to-Ct Kit (Applied Biosystems), according to the manufacturer’s instructions. Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems), and products were detected on a Prism 5300 detection system (SDS, ABI/Perkin-Elmer). The relative extent of IL-1β expression was calculated using the 2eΔΔC(t) method. The results presented herein are from three independent experiments, each of which was performed in duplicate. Conditions for real time PCR were: initial denaturation for 10 min at 95°C, followed by 40 amplification cycles with 15s at 95°C and 1 min at 60°C. The sequences of the primers (IDT DNA Technologies, MA) were as follows: HPRT1 forward 5- GGA GCG GTA GCA CCT CCT -3; HPRT1 reverse 5- CTG GTT CAT CAT CGCTAATCAC -3; IL-1β reverse 5- TCT TCT TTG GGT ATT GCTTGG -3; IL-1β forward 5- TGT AAT GAA AGA CGG CAC ACC -3

Extracellular LDH release assay
Lactate dehydrogenase (LDH) release was measured using a cytotoxicity detection kit (Promega, Madison, WI). All LDH data are means ± standard error of the mean (s.e.m).

Supplementary discussion
Some of the nuclear staining with IL-1β in BMMs (e.g. in Suppl. Figs. S3 and S4) may be nonspecific, but we cannot rule out functional roles (a topic beyond the scope of our
study). IL-1 family members such as IL-1α and IL-1β are known to be distributed between the cytoplasm and the nucleus, reflecting their dual functionality or to counter excessive inflammation\(^1\). Of further note is that IL-1β has been observed in the nucleus of microglia, a major macrophage cell type of the central nervous system\(^2\).

**Supplementary references**


APPENDIX II

MASS SPECTROMETRY RESULTS FOR AUTOPHAGY-BASED
UNCONVENTIONALLY SECRETED PROTEINS
Methods

Mass Spectrometry

Murine bone marrow macrophage (BMM) cells were prepared from femurs of C57/BL6 mice, Atg5fl/fl LyzM-Cre mice (Atg5 knock-out) and their Cre-negative Atg5fl/fl littermates (wild type). BMM cells were grown in 10cm dishes to 70% confluency. Cells (each one dish) were pretreated overnight with 100ng/ml LPS (Sigma). On the following day, cells were treated with 20µM inflammasome inducer nigericin (Invivogen) in EBSS (autophagy inducer, Sigma) for 1 h.

Supernatant was collected and centrifuged at 2,500rpm for 5 minutes at 4°C. After centrifugation, the supernatant was cleared by filtration (0.45 µm pore size). Then supernatant was concentrated to 1ml with Amicon ultrafiltration units (3kDa MWCO, Millipore). Protein concentrations were determined by BCA. 100µg protein of each sample was precipitated with acetone.

Samples were digested by trypsin and labeled with Tandem Mass Tag (TMT) reagents (Thermo Scientific). Peptides were then identified and quantified by tandem mass spectrometry. Total of 307 proteins were identified using Mascot Search. 153 proteins are likely to be secreted in Atg5 dependent manner since its secretion is decreased in the supernatant from Atg5 knockout BMMs. Among these, there were several unconventionally secreted proteins-Vimentin, Galectin-1, Galectin-3 and ASC and Thioredoxin.
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Identified Proteins (307) | Molecular Weight | Quant 2 |
GLOSSARY

**ABC transporter:** transmembrane proteins using ATP hydrolysis to translocates various substrates across membranes

**AcbA (Acb1):** Acyl-CoA binding protein, required for differentiation of pre-spore cells

**Atg5fl/fl LysM-Cre+:** Mice deficient of Atg5 in myeloid lineage cells

**Atg proteins:** Autophagy related proteins

**Bafilomycin A1:** A specific inhibitor of vacuolar type H+-ATPase (V-ATPase), also blocks autophagosome maturation

**BMMs:** bone marrow-derived macrophages

**Caspase-1:** Interleukin-1 converting enzyme, that proteolytically cleaves the precursor forms of the inflammatory cytokines IL-1β and IL-18 into active mature peptides

**Cathepsin D:** a ubiquitously expressed lysosomal aspartyl protease involved in the normal degradation of proteins

**Cathepsin K:** a cystine protease involved in bone resorption

**CFTR:** Cystic Fibrosis Transmembrane conductance Regulator

**COPII:** coat protein complex II

**CUPS:** Compartment for Unconventional Protein Secretion

**DAMPs:** Damage Associated Molecular Patterns

**DFCP1:** Double FYVE-containing protein 1, the FYVE domain mediates the recruitment of proteins involved in membrane trafficking and cell signaling to phosphatidylinositol 3-phosphate (PtdIns(3)P)-containing membranes

**ESCRT:** Endosomal Sorting Complex Required for Transport
FGF2: Fibroblast Growth Factor 2

GABARAP: γ-aminobutyric acid receptor-associated protein

Galectins: a family of proteins defined by their binding specificity for β-galactoside sugars

GAP: GTPase- Activating Proteins

GEF: Guanine-nucleotide Exchange Factors

GATE-16 (GABARAPL2): Golgi-associated ATPase enhancer of 16 kDa

GRASP55: Golgi Reassembly And Stacking Protein 55

GrpA: Golgi-associated protein GRASP

Grh1: Grasp homology 1

IL-1: Interleukin-1

LC3: light-chain 3, mammalian homologue of Atg8

MVB: MultiVesicular Body

NF-κB: nuclear factor kappa B

Nrf2: NF-E2 related factor 2

PAMPs: Pathogen Associated Molecular Patterns

P2X7 receptors: cation-permeable ligand gated ion channels that open in response to the binding of extracellular ATP

Rab8a: a GTPase required for polarized membrane trafficking, constitutive biosynthetic trafficking, and plasma membrane fusion of insulin-responsive and other vesicular carriers

RIG-I: retinoic acid-inducible gene 1

SNARE: soluble NSF-attachment protein receptor
**Sso1**: plasma membrane specific t-SNARE

**Tlg2**: endosomal t-SNARE which regulates membrane traffic through the yeast endocytic system

**TLR**: Toll-like receptor

**ULK1**: Unc-51-like kinase 1

**Vamp7**: vesicle-associated membrane protein7

**Vps4**: an ATPase responsible for ESCRT-III disassembly and required for MVB protein sorting

**Vps23**: a component of the ESCRT-I complex involved in ubiquitin-dependent sorting of proteins into the MVB sorting pathway