ATG9A and ATG13 Cooperate to Drive Basal Autophagy

Daniel Morgan Poole
Brigham Young University

Follow this and additional works at: https://scholarsarchive.byu.edu/etd
Part of the Physical Sciences and Mathematics Commons

BYU ScholarsArchive Citation
Poole, Daniel Morgan, "ATG9A and ATG13 Cooperate to Drive Basal Autophagy" (2022). Theses and Dissertations. 9421.
https://scholarsarchive.byu.edu/etd/9421

This Dissertation is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact ellen_amatangelo@byu.edu.
ABSTRACT

ATG9A and ATG13 Cooperate to Drive Basal Autophagy

Daniel Morgan Poole
Department of Chemistry and Biochemistry BYU
Doctor of Philosophy

Autophagy, as the name suggests, is a cellular process of self-eating in which cytoplasmic debris is engulfed by a double membrane vesicle dubbed the autophagosome and is ultimately degraded and recycled by proteases in the lysosome. The process is initiated by a group of core ATG proteins, including a multi-pass transmembrane protein called ATG9A. Although ATG9A has been shown to be essential for both stress induced and basal autophagy, its mechanism and interaction network remain largely illusive. Our current study employs BioID proteomics to identify a network of interactors, including regulators of membrane fusion and vesicle trafficking, such as TRAPP, EARP, GARP, exocyst, AP-1 and AP-4 complexes, as well as members of the ULK1 autophagy kinase complex. Further investigations confirm that two components of the ULK1 complex, ATG13 and ATG101, directly interact with ATG9A. Using CRISPR, we show that deletion of ATG13 or ATG101 disrupts ATG9A trafficking and causes an accumulation of ATG9A at p62/SQSTM1-positive ubiquitin clusters. Lentivirus reconstitution and split-mVenus approaches using an ULK1 binding deficient mutant of ATG13 reveal that ATG9A interacts with ATG13 and ATG101 in an ULK1-independent manner. Together, these data reveal ATG9A interactions in vesicle trafficking and autophagy pathways, including a role for an ULK1-independent ATG13 complex in regulating ATG9A.

Keywords: ATG9A, ATG13, ATG101, ULK1, p62, ubiquitin, macroautophagy, BioID, subcomplex
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my Ph.D. advisor, Dr. Joshua L. Andersen, for his continuous support, guidance, motivation and immense knowledge throughout my research project and my Ph.D. experience. I would like to also express my thanks to my committee members, Dr. John C. Price, Dr. James D. Moody, Dr. K. Scott Weber and Dr. Ken A. Christensen (temporary member) who greatly contributed to my project, growth and learning. I thank the Fritz B. Burns foundation, Simmons Center for Cancer Research, American Cancer Society Research Scholar (Grant 133550-RSG-19-006-01-CCG) and National Cancer Institute/National Institutes of Health (Grant 2R15CA202618-02) for the funding that made this project possible. I thank the Duke University School of Medicine for use of the Proteomics and Metabolomics Shared resource and assistance with proteomics. I also thank the BYU College of Physical and Mathematical Sciences and the Department of Chemistry and Biochemistry for accepting me into the Biochemistry program and for their support during my time here at BYU.

I am grateful for Ashari Kannangara for her excellent contribution of data, support, and training in this project and Vajira Weerasekara for his ideas and data, which provided an excellent foundation for this project. I’m also thankful for Colten McEwan, Chrissy Egbert, Roshan Balasooriya and all the former and current members of the Andersen lab for providing input, support, motivation and friendship during my Ph.D.

Finally, a special thanks goes to my family. First, to my mother, Rebecca Poole, for her love, care, sacrifice and support throughout my life. Second, to my wife, Grace Poole, for her love, encouragement and sacrifice, and for being the glue that holds our little family together. Last, for my sons, Maverick and Thatcher, for being my motivation, strength and drive.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
</tbody>
</table>

## 1 INTRODUCTION

1.1 *AUTOPHAGY-RELATED PROTEIN 9 (ATG9)* ................................................................. 1
   1.1.1 Yeast Atg9 ........................................................................................................ 1
   1.1.2 Mammalian ATG9/ATG9A ............................................................................. 3

## 2 BIOID REVEALS AN ATG9A INTERACTION WITH ATG13-ATG101 IN THE DEGRADATION OF P62/SQSTM1-UBIQUITIN CLUSTERS ................................................................. 8

2.1 ABSTRACT .................................................................................................................. 8
2.2 INTRODUCTION .......................................................................................................... 8
2.3 RESULTS ..................................................................................................................... 12
   2.3.1 BioID reveals proximity-based interactions between the ATG9A C-terminus and a network of trafficking proteins and complexes. ....................................................... 12
   2.3.2 ATG9A interacts with an ULK1-independent ATG13 subcomplex that includes ATG101. ....................................................................................................................... 16
   2.3.3 The loss of ATG13 and ATG101 result in an accumulation of ATG9A at large clusters of p62/SQSTM1 ....................................................................................................... 19
   2.3.4 The loss of ATG13 results in increased ATG9A recruitment to stalled clusters of p62/SQSTM1 ................................................................................................................... 23
   2.3.5 A split-mVenus approach captures an ATG13-ATG101 interaction with ATG9A. 25
2.4 DISCUSSION .............................................................................................................. 29
2.5 ACKNOWLEDGEMENTS ........................................................................................... 34
2.6 SUPPLEMENTAL FIGURES ....................................................................................... 35
2.7 MATERIAL AND METHODS .................................................................................... 48
   2.7.1 Cell culture, transfection and viral transduction .................................................. 48
   2.7.2 Antibodies and chemicals .................................................................................. 49
   2.7.3 Plasmids ............................................................................................................ 50
   2.7.4 CRISPR-Cas9 ................................................................................................... 52
   2.7.5 Immunoprecipitation and immunoblotting ......................................................... 53
   2.7.6 Gel filtration assay ............................................................................................. 55
   2.7.7 Bio-ID coupled Mass spectrometry ................................................................... 55
   2.7.8 Quantitative LC-MS/MS data analysis ............................................................. 56
   2.7.9 Statistical analysis of LC-MS/MS data ............................................................. 57
LIST OF FIGURES

Figure 1-1. ATG9A is recruited to the initiation membrane early and independently of other proteins................................................................. 3
Figure 1-2. C-terminus of ATG9A bears hallmarks of a signaling hub .............................................. 4
Figure 2-1. Bio-ID reveals a network of ATG9A interactors, including multiple trafficking regulators and members of the ULK1 complex................................. 13
Figure 2-2. ATG9A interacts with an ULK1-independent ATG13 complex that includes ATG101 ................................................................. 17
Figure 2-3. The loss of ATG13 causes a shift in colocalization of ATG9A with organelle and autophagy markers and triggers an accumulation of ATG9A with p62/SQSTM1........... 20
Figure 2-4. The loss of ATG13 and ATG101 result in an accumulation of ATG9A at large clusters of p62/SQSTM1.................................................................................. 22
Figure 2-5. ATG13-mediated rescue of ATG9A accumulation at p62/SQSTM1 clusters requires the ATG13 HORMA domain but is independent of ULK1................................. 24
Figure 2-6. Loss of ATG13 results in increased ATG9A recruitment to stalled clusters of p62/SQSTM1 ........................................................................................................... 26
Figure 2-7. A split-mVenus approach captures an ATG13-ATG101 interaction with ATG9A .. 28
Figure 2-8. An ULK1-independent ATG13-ATG101 complex regulates basal ATG9A function and p62/SQSTM1 turnover.................................................................................. 30
Supplimentary Figure 2-1. The C terminus of ATG9A harbors hallmarks of a signaling hub and is involved in self association, and ATG9A trafficking. ..................................................... 35
Supplimentary Figure 2-2. Functional validation of HA ATG9A-BirA constructs......................... 37
Supplimentary Figure 2-3. ATG9A interacts with an ULK1-independent ATG13-ATG101 subcomplex via the ATG13 HORMA domain. ................................................................. 39
Supplimentary Figure 2-4. ATG13 colocalization with ATG9A is independent of FIP200/ULK1 complex........................................................................................................... 41
Supplimentary Figure 2-5. ATG13 colocalization with ATG9A requires the ATG13 HORMA domain, but occurs independently of ULK1 binding.................................................. 42
Supplimentary Figure 2-6. BioID identifies a network of proximity-based ATG9A interactions.................................................................................. 43
Supplementary Figure 2-7. Validation of the HCT-116 ATG9A-HA KI cell line.................................. 44
Supplementary Figure 2-8. Loss of ATG13 and ATG101, but not FIP200, lead to the accumulation of ATG9A in large puncta........................................................................................................ 45
Supplementary Figure 2-9. Evaluation of a split mVenus system to capture the ATG13-ATG101 dimer. .................................................................................................................................................. 46
1 INTRODUCTION

Deregulated autophagy underlies the pathophysiology of many human diseases, including a variety of degenerative disorders, autoimmunity, infectious disease and cancer. The central event in autophagy is the formation of the autophagosome, which begins as a double-membrane cisterna that expands and captures (via random and selective mechanisms) portions of the cytosol and ultimately closes to form a sealed vesicle. The sealed vesicle (called an autophagosome) fuses with the lysosome, which allows its contents to be degraded and recycled to the cell. The flux of autophagy substrates through this degradative pathway occurs at a low level under basal conditions and increases in response to a variety of stresses, including nutrient deprivation and misfolded protein accumulation. However, the regulation and dynamic cooperation of proteins that govern both basal and upregulated autophagy is still poorly understood.

1.1 AUTOPHAGY-RELATED PROTEIN 9 (ATG9)

Autophagy-related protein 9 (ATG9) is a multi-pass transmembrane protein involved in the initiation of both basal and upregulated autophagy. ATG9 resides in lipid membranes such as the ER, Golgi, endosomes, small vesicles termed ATG9 vesicles, and large lipid deposits termed ATG9 reservoirs or compartments. Although the role of ATG9 in autophagy and cellular biology more broadly remains a mystery, its necessity is not disputed; genetic deletion of ATG9 in cell culture abolishes autophagy, mice lacking ATG9 do not survive beyond the neonatal weening period and the bulk of ATG9 is highly conserved from yeast to mammals (Young et al., 2006; Yamamoto et al., 2012).

1.1.1 Yeast Atg9

In yeast, the vast majority of Atg9 is found in small cytoplasmic mobile vesicles around 30-60 nm
in diameter. Most of these mobile vesicles come from the Trans-Golgi Network, but peripheral Atg9 reservoirs also provide lipids and Atg9 molecules for the formation of Atg9 vesicles. Each of these vesicles contains between 24 and 32 Atg9 molecules, depending on the status of nutrients, with more Atg9 per vesicle when the cell is nutrient deprived. Upon induction of autophagy by nutrient deprivation, Atg9 is one of the first proteins recruited to the pre-autophagosomal structure (PAS), where it is incorporated into the budding isolation membrane (IM) (Yamamoto et al., 2012).

Atg9 recruitment and incorporation into the IM is dependent on Atg17, which acts as a membrane tether holding Atg9 to the Atg1 kinase complex upon starvation (Sekito et al., 2009, Matscheko et al., 2019). In basal conditions (no nutrient deprivation), selective autophagy adaptors bind to ubiquitinated cargo and activate Atg11. Atg11 then dimerizes and tethers Atg9 to the marked cargo, which initiates the formation of the autophagosomal membrane selectively around the ubiquitinated cargo (Matscheko et al., 2019). Although Atg9 is incorporated in the autophagosomal membrane in both starvation induced and basal autophagy, it is not seen in mature autophasomes upon fusion with the vacuole and must, therefore, be recycled from the autophagosome shortly after incorporation (Reggiori et al., 2004). Retrieval of Atg9 from the forming autophagosome is dependent on recruitment of several downstream complexes. First, the Atg1 complex phosphorylates the PI3K complex, which in turn phosphorylates phosphatidylinositol-3-phosphate (PI3P), which finally recruits Atg18 and Atg2 (Nickerson et al., 2009). The interaction of Atg18 and Atg2 in complex with Atg9 allows Atg9 to leave the autophagosome (Reggiori et al., 2004). The early recruitment and subsequent retrieval of Atg9 suggests that Atg9 is critical only for the initiation phase of autophagy, most likely for the
expansion of the growing autophagosome or the nucleation of the autophagosome in selective autophagy.

1.1.2 Mammalian ATG9/ATG9A

In mammals, ATG9 is encoded by two paralogs: ATG9A and ATG9B. The function and cellular localization of these two paralogs overlaps somewhat, but ATG9B is only highly expressed in esophagus mucosa, the cervix and pituitary glands, and under hypoxic stress, while ATG9A is ubiquitously expressed. Although genetically isolated on two different chromosomes, the center transmembrane region of ATG9A and B are well conserved in humans, but the N and C terminus diverge in sequence, suggesting a similar function but different regulation.

ATG9A localizes to the perinuclear region, the Trans-Golgi Network (TGN), late endosomes, recycling endosomes, mitochondria and autophagosomal membrane (Young et al., 2006; Yan et al., 2021). As in yeast, ATG9A can be found in large vesicular compartments (reservoirs in yeast) but mostly resides in small mobile vesicles (Orsi et al., 2012). Mammalian ATG9A is also one of the earliest recruited proteins to the IM but seems to do so independently of any other proteins (Figure 1-1). Unlike in yeast, mammalian ATG9A only transiently interacts with the initiation membrane and is not integrated into the membrane, whereas yeast Atg9 remains in the growing membrane until closure (Ungermann and Reggiori, 2018).

![Figure 1-1. ATG9A is recruited to the initiation membrane early and independently of other proteins.](image-url)
Graphic of autophagy pathway showing ULK1 complex, ATG9A, LC3II and cargo each recruited in respective autophagy stage.

ATG9A has a short N-terminus and a long C-terminus compared to ATG9B or Yeast Atg9. The C-terminus of ATG9A makes up nearly half of its length and bears hallmarks of a signaling hub for interactors, including high intrinsic disorder and high density of phosphorylation sites (Figure 1-2). Four of these phosphorylation sites (S735, S738, S741 and S828) have been independently identified in more than 20 PTM mass spectrometry analyses, and one (S761) was identified by our group as a 14-3-3 ζ docking site regulated by AMPK activation during stress (Weerasekara et al., 2014).

1.1.2.1 Human ATG9A cryo-EM structure reveals molecular mechanism

Mammalian ATG9 was first determined to be an autophagy protein homologous to yeast Atg9 in 2005, and since then, its structure has been predicted and tested only in identifying, counting and roughly locating transmembrane domains (Yamada et al., 2005; Reggiori et al., 2005). Proteinase K and fluorescence-quenching experiments provide evidence that both the N-terminus and C-terminus are on the cytoplasmic side of the vesicles and that 6 regions of ATG9A are protected within membranes (Young et al. 2006). It was assumed that these protected regions were in fact transmembrane domains, an assumption reinforced when the first eukaryotic ATG9 structure from the plant, Arabidopsis thaliana, was finally determined at 7.8 Å, predicting 6 helices fully

Figure 1-2. C-terminus of ATG9A bears hallmarks of a signaling hub

Left y-axis measures ANCHOR score with higher scores for residues with high predicted disorder. Right y-axis measures the number of independent studies identifying each phosphorylation within ATG9A. (predicted by ANCHOR web server at http://anchor.elte.hu) (top). Schematic of ATG9A domain structure (bottom)
crossing the membrane (Lai et al., 2020). Then, a groundbreaking study in June 2020 brought us the first human ATG9A structure, putting all speculation to rest (Guardia et al., 2020).

Guardia et al. resolved the structure of human ATG9A to 2.9 Å and found that ATG9A, in humans, does not have six transmembrane domains but rather four transmembrane domains and two alpha helices that are embedded in the membrane on the cytosolic side but do not cross it to the luminal side. Guardia also found, in agreement with Lai, that ATG9A exists as a homotrimer with the trimer interfaces protected inside the membrane layer. Guardia also confirmed that the C-terminus of ATG9A is exposed on the outer surface of the protein and would, therefore, be perfectly situated to act as a signaling hub. Lastly, this study used computational analysis to predict that multiple ATG9A trimers coalescing on the same membrane could warp its surface and force the membrane to bubble outward. This characteristic explains the localization pattern of ATG9A to mostly small vesicles and may also provide insight into the function of ATG9A in autophagosome formation.

The conclusions of this study were verified just four months later in October when another structural paper provided lower resolution (3.4 Å) structures of human ATG9A (Maeda et al., 2020). This later study was also able to determine that mammalian ATG9A acts as a scramblase which moves lipids from the cytosolic side of bilayers to the lumen side. They found that ATG9A has two pores, the Lateral Pore (LP) and the Vertical Pore (VP), which are used for the movement of a variety of lipid types through to the luminal side. Mutations in these pores hinder the expansion of liposomes in vitro and the transport of PI3P lipids across bilayers in cell assays and reduce autophagosome size.

1.1.2.2 Growing literature on the function of ATG9A
At the same time that Maeda et al. published on the scramblase activity of human ATG9A, Matoba et al. published a structure for budding yeast at 3.0 Å with the same conclusion (Matoba et al., 2020). In addition, this study found that yeast Atg9 colocalizes on the ER with Atg2, which has a recently discovered lipid transfer function in both yeast and humans (Osawa et al., 2019; Maeda et al., 2019; Valverde et al., 2019; Osawa et al., 2020). Atg2 is tethered to the ER by Atg18 (WIPI4 in humans), where it transfers lipids from the ER to the growing autophagosome. However, the luminal leaflet of the autophagosome could not expand without transferring a large proportion of lipids from the cytosolic leaflet inwards. Matoba shows that Atg9 interacts with Atg2 and predicts that they work in concert to transfer lipids from the ER to the cytosolic leaflet of the autophagosome and then to the inner luminal leaflet for even distribution of lipids as the autophagosome expands. Although ATG9A has not been shown to integrate into the autophagosome itself in mammals, the scramblase and lipid transfer functions of ATG9A and ATG2 have been confirmed. Thus, a similar lipid pathway in mammals seems likely.

Functions of ATG9A outside of autophagy have also been determined in the last two years. The scramblase is important for mitochondrial health and the mobility of lipid droplets in the cell. Depletion of ATG9A increases the size/number of lipid droplets in the cell and blocks the transfer of fatty acids to the mitochondria for use in mitochondrial respiration. (Mailler et al., 2021). ATG9A is also necessary for cell mobility, and depletion of ATG9A alters the formation of actin filaments used in movement (Campisi et al., 2022). Our recent study (chapter 2) also shows that ATG9A colocalizes with large clusters of p62/SQSTM1, which are likely phase-separated condensates. It seems, therefore, that ATG9A can form autophagosomes de novo at sites of p62/SQSTM1 positive condensates. An in vitro study in yeast lends credence to this hypothesis by
showing that Atg9 vesicles can, in fact, be the seeds for autophagosome nucleation and the hub for autophagy protein recruitment (Sawa-Makarska et al., 2020).

1.1.2.3 Regulation of ATG9A trafficking

Regulation of ATG9A trafficking as it cycles through the ER, Golgi, TGN, endosomal networks and autophagy is one of the most elusive questions left to be answered in the autophagy field. Post translational modifications, such as the glycosylation of N99, the acetylation of the N-terminus and the plethora of phosphorylations have been shown to affect the trafficking patterns of ATG9A. Disruption of these trafficking patterns, such as abolishing an interaction with AP-4, hinders the formation of autophagosomes (Mattera et al., 2017).

Our recent study uses BioID mass spectrometry, which verifies an interaction between ATG9A and the AP-4 complex, as well as several other vesicle trafficking complexes, including AP-2, EARP, GARP, Retromer, TRAP and SNARE complexes. As these complexes are heavily involved in progression of the endosomal pathway, it stands to reason that ATG9A vesicles are also regulated by these complexes. A few studies have already shown this to be the case. AP-4 is required for ATG9A trafficking away from the TGN (Mattera et al., 2017), TRAPPC8 is required for export of ATG9A from recycling endosomes (Imai et al., 2016) and depletion of AP-2 causes the formation of large stalled ATG9A deposits (Popovic and Dikic, 2014). It therefore follows that understanding ATG9A vesicle formation and deployment to sites of autophagy initiation will require further investigation of the interaction of ATG9A with these complexes.
2 BIOID REVEALS AN ATG9A INTERACTION WITH ATG13-ATG101 IN THE DEGRADATION OF P62/SQSTM1-UBIQUITIN CLUSTERS

First author work published by EMBO Reports Journal in October 2021. I hereby confirm that the use of this article is compliant with all publishing agreements.

2.1 ABSTRACT

ATG9A, the only multi-pass transmembrane protein among core ATG proteins, is an essential regulator of autophagy, yet its regulatory mechanisms and network of interactions are poorly understood. Through quantitative BioID proteomics, we identify a network of ATG9A interactions that includes members of the ULK1 complex and regulators of membrane fusion and vesicle trafficking, including the TRAPP, EARp, GARP, exocyst, AP-1 and AP-4 complexes. These interactions mark pathways of ATG9A trafficking through ER, Golgi and endosomal systems. In exploring these data, we find that ATG9A interacts with components of the ULK1 complex, particularly ATG13 and ATG101. Using knock-out/reconstitution and split-mVenus approaches to capture the ATG13-ATG101 dimer, we find that ATG9A interacts with ATG13-ATG101 independently of ULK1. Deletion of ATG13 or ATG101 causes a shift in ATG9A distribution, resulting in an aberrant accumulation of ATG9A at stalled clusters of p62/SQSTM1 and ubiquitin, which can be rescued by an ULK1 binding deficient mutant of ATG13. Together, these data reveal ATG9A interactions in vesicle trafficking and autophagy pathways, including a role for an ULK1-independent ATG13 complex in regulating ATG9A.

2.2 INTRODUCTION

The recycling of misfolded proteins, dysfunctional organelles and other molecules through macroautophagy (referred to here as autophagy) is critical for maintaining cellular homeostasis
and promoting cell survival during stress. Deregulated autophagy underlies the pathophysiology of many human diseases, including a variety of degenerative disorders, cancer, autoimmunity, and infectious disease. The central event in autophagy is the formation of the autophagosome, which begins as a double-membrane cisterna that expands and captures portions of the cytosol/cell and ultimately closes to form a sealed vesicle. The autophagosome then fuses with the lysosome for degradation and recycling of the autophagosome contents. The flux of autophagy substrates through this degradative pathway increases in breadth and rate under nutrient deprivation. In contrast, under nutrient replete conditions, a more selective, lower level of autophagy (referred to here as ‘basal autophagy’) maintains organelle and protein homeostasis (Antonucci et al, 2015; Hara et al, 2006; Komatsu et al, 2006; Komatsu et al, 2005). Defects in basal autophagy can lead to the accumulation of defective mitochondria and toxic protein aggregates that underlie a variety of degenerative diseases (Dikic & Elazar, 2018; Hara et al., 2006; Komatsu et al., 2006).

Our understanding of the upstream signaling that controls autophagy mainly derives from studies on nutrient deprivation, in which the inhibition of mTORC1 results in the activation of the ULK1 kinase complex that includes FIP200, ATG101 and ATG13 (Egan et al, 2011; Hosokawa et al, 2009a; Kim et al, 2011; Lee et al, 2010; Shang et al, 2011). Active ULK1 complex then coordinates a variety of autophagy events, such as recruitment of VPS34 lipid kinase complex, that stimulates formation of the membrane precursor to the autophagosome, referred to as the isolation membrane (IM) (Zachari & Ganley, 2017). The location of this emergent autophagosome is also called the phagophore assembly site (PAS). Additional autophagy regulatory proteins are recruited to the IM/PAS, including ATG5-12-16 conjugation systems that attach the ubiquitin-like protein LC3 to autophagosomes.
In contrast to starvation-induced autophagy, basal autophagy is primarily driven by a variety of autophagy adaptors, including p62/SQSTM1, Optineurin and TAX1BP1, that selectively deliver cargo to the autophagosome. For example, p62/SQSTM1 interacts with poly-ubiquitinated cargo via its ubiquitin association domain and then tethers these cargo to the LC3-decorated autophagosomes via its LC3-interacting region (Pankiv et al, 2007; Seibenhener et al, 2004). Transition of these p62/SQSTM1-poly-ubiquitinated protein complexes into phase-separated droplets appears to be a precursor to cargo degradation (Cloer et al, 2018; Jakobi et al, 2020; Sun et al, 2018). However, given that basal autophagy occurs under conditions in which ULK1 activity is low (and MTORCl activity is high), the hierarchy of signaling that governs basal autophagy, including how core autophagy machinery (e.g., ATG9A) is engaged and regulated, is not yet clear.

ATG9A is essential for the formation of autophagosomes (Kuma et al, 2004; Saitoh et al, 2009; Yamamoto et al, 2012), but is one of the least understood of the core ATG proteins. Studies from yeast and mammalian cells suggest that ATG9A (referred to as Atg9 in yeast) traffics on small membrane vesicles and accumulates at several sites within vesicular trafficking pathways, including the Golgi, endosomes, and ER where it co-localizes with IM/PAS markers (Imai et al, 2016; Kakuta et al, 2017; Mari et al, 2010; Nishimura et al, 2017; Orsi et al, 2012; Takahashi et al, 2016; Young et al, 2006). A few proteins have been identified as regulators of ATG9A trafficking, including the coat adaptors AP-1, AP-2 and AP-4, components of the ULK1 complex, BIF-1 and p38IP (Davies et al, 2018; Guo et al, 2012; Ktistakis & Tooze, 2016; Mattera et al, 2017; Orsi et al., 2012; Popovic & Dikic, 2014; Takahashi et al, 2011; Tang et al, 2011; Young et al., 2006). The trafficking/mobilization of ATG9A to the IM/PAS is considered an apical step in autophagy (Itakura et al, 2012; Karanasios et al, 2016; Kishi-Itakura et al, 2014). While at the
IM/PAS, ATG9A is thought to supply membrane to growing autophagosomes, although the mechanism by which this may occur is still unclear (Judith et al, 2019; Yamamoto et al., 2012).

Several recent studies indicate that, in addition to the role of ATG9A in starvation-induced autophagy, ATG9A is essential for basal autophagy—potentially in ways that do not easily fit within current autophagy paradigms. Although ATG9A KO MEFs still display LC3B puncta (suggesting that autophagosomes still form in the absence of ATG9A) (Saitoh et al., 2009), studies focused on the basal lysosomal turnover of autophagy adaptors demonstrate a strong requirement for ATG9A. For example, degradative flux of the autophagy adaptor NBR1 is largely independent of ULK1 and ATG factors required for LC3 lipidation but is entirely dependent on ATG9A. Similarly, ATG9A emerged as a top hit in a genome-wide CRISPR/Cas9 screen for proteins required for basal lysosomal degradation of p62/SQSTM1, while a variety of core ATG proteins were notably not essential (Goodwin et al, 2017). In addition, the tyrosine kinase Src phosphorylates ATG9A at Tyr8 to maintain active ATG9A trafficking under basal conditions (Zhou et al, 2017). Furthermore, defective ATG9A trafficking (or genetic loss of ATG9A) is associated with impaired clearance of protein aggregates (De Pace et al, 2018; Winslow et al, 2010; Yamaguchi et al, 2018). Together, these data support a central role for ATG9A in basal autophagy. However, the general mechanisms that control basal autophagy are poorly understood, including how ATG9A may interact with autophagy machinery to promote the constitutive turnover of basal autophagy cargo/adaptors.

Here we take advantage of BioID and quantitative LC-MS/MS to identify a network of proximity-based ATG9A interactions that include a variety of vesicular trafficking complexes and autophagy regulators. In exploring these interactions further, we discover that ATG9A interacts with an ULK1-independent ATG13 ‘subcomplex’ that is essential for proper ATG9A trafficking
and basal turnover of p62/SQSTM1. Together, our data elucidate a diverse array of novel ATG9A interactions and reveal, to our knowledge, the first ULK1-independent role for ATG13 in regulating ATG9A function.

2.3 RESULTS

2.3.1 BioID reveals proximity-based interactions between the ATG9A C-terminus and a network of trafficking proteins and complexes.

With the ultimate goal of elucidating the interactome of ATG9A, we first assessed potential protein-protein docking regions along the putative ATG9A structure. The long C-terminus of ATG9A bears some hallmarks of a signaling hub, including a high degree of predicted intrinsic disorder and a concentration of phosphorylation sites that are repeatedly identified in global PTM mass spectrometry studies (Supplementary Figure 2-1A). These include several phosphorylations with over 20 independent mass spectrometry identifications (S735, S738, S741, S828) and an AMPK-mediated phosphorylation at S761 that we identified as a 14-3-3\(\xi\) docking site (Weerasekara et al, 2014). In addition, there is evidence from structural and molecular studies that ATG9A self-associates via its C-termini, which might further expand its ability to act as a protein docking site or signaling hub (He et al, 2008; Lai et al, 2020; Staudt et al, 2016). In support of this idea, we found that ATG9A fused to split mVenus molecules at its C-termini produced robust BiFC signal in a perinuclear pattern (Supplementary Figure 2-1B), consistent with known localization patterns of ATG9A (Orsi et al., 2012; Young et al., 2006). In addition, we found that a C-terminally truncated ATG9A was unable to fully rescue defective LC3 processing in an ATG9A KO line (Supplementary Figure 2-1C). These data
suggest that the ATG9A C-terminus is critical for ATG9A function and likely a hub of multiple protein-protein interactions.

Our efforts to probe ATG9A protein-protein interactions by co-IP proteomics had limited success (unpublished). The multi-pass transmembrane nature of ATG9A presents challenges to co-IP proteomics, most notably the difficulty of extracting ATG9A from intracellular membranes while maintaining protein-protein interactions. On the other hand, these same qualities make ATG9A a good candidate for BioID (Roux et al, 2012), in which promiscuous interactions are relatively limited by ATG9A being fixed in membrane. Thus, we fused the modified bacterial biotin ligase BirA (R118G—denoted with an asterisk) to the C-terminus of hemagglutinin (HA)-tagged ATG9A (HA-ATG9A-BirA*) (Rees et al, 2015; Roux et al., 2012). We verified that fusion of BirA* to the ATG9A C-terminus did not impair the function of ATG9A, as the HA-ATG9A-
BirA* construct was able to fully rescue the defects in p62/SQSTM1 degradation and LC3B lipidation in ATG9A KO cells and showed the same cellular localization patterns as endogenous ATG9A (Figure 2-1A, Supplementary Figure 2-2A-C). We also verified that biotin signal overlaps with HA-ATG9A-BirA* (Supplementary Figure 2-2D).

To pursue BioID proteomics, we generated cell lines stably expressing either HA-ATG9A-BirA* or, as a control, HA-BirA* alone. These cells were supplemented with biotin, followed by detergent lysis and capture of biotinylated proteins on streptavidin resin. An initial evaluation of captured proteins by coomassie staining suggested an overall lower level of biotinylation by HA-ATG9A-BirA* compared to HA-BirA* alone, as perhaps expected given the anchored, transmembrane nature of ATG9A (Figure 2-1B). Therefore, we proceeded with BioID proteomics following the experimental schematic outlined in Figure 2-1C. LC-MS/MS data from these experiments are available in table S1. Quantitative LC-MS/MS of biological triplicates of the experiment in Figure 2-1C revealed 283 proteins that were significantly enriched (≥2-fold increase, ≤0.05 p-value; Dataset EV1) in the HA-ATG9A-BirA* samples versus HA-BirA* alone (see volcano plot, Figure 2-1D, Supplementary Figure 2-6A-B). These spanned an array of autophagy and trafficking regulators, including multiple components of EARP/GARP, AP-1, AP-3, AP-4, Retromer, TRAPP and SNARE complexes and all components of the canonical ULK1 complex (Figure 2-1D), a subset of which were validated by immunoblot (Figure 2-1E). Several of these HA-ATG9A-BirA*-biotinylated proteins are already known to interact with ATG9A, including STX16, Arfaptin-1, TBC1D5, AP-1, AP-2 and AP-4, which increased our confidence in the BioID data (Aoyagi et al., 2018; Davies et al., 2018; Imai et al., 2016; Judith et al., 2019; Lamb et al., 2016; Mattera et al., 2017; Orsi et al., 2012; Popovic & Dikic, 2014; Soreng et al., 2018; Zhou et al., 2017). Furthermore, this proximity-based ATG9A interactome was highly enriched for
proteins associated with the organelles where ATG9A is known to reside, including the ER, TGN, ERGIC and endosomal systems (Figure 2-1F-G).

2.3.2 ATG9A interacts with an ULK1-independent ATG13 subcomplex that includes ATG101.

Among the BioID proteomics data (Dataset EV1), our attention was drawn to members of the ULK1 complex, which emerged as top hits (Figure 2-1D). Of the ULK1 complex proteins, ATG13 showed the highest fold-change increase in signal across all of the HA-ATG9A-BirA* replicates and we had previously observed interaction between ATG9A with ATG13 by co-IP (Kannangara and Andersen, unpublished).

To investigate the interaction between ATG9A and ATG13 further, we generated ATG13 KO cells and then stably reconstituted them with WT ATG13 or one of two mutants of ATG13: ATG13Δ2AA, which lacks a C-terminal 2-amino acid segment required for ULK1 binding (Alers et al, 2011; Hieke et al, 2015); or ATG13ΔHORMA, which lacks the HORMA domain required for interaction with ATG101 and reported in yeast to be essential for recruiting Atg9 vesicles to the PAS (Figure 2-2A) (Jao et al, 2013; Qi et al, 2015; Suzuki et al, 2015). We verified that the ATG13Δ2AA indeed fails to interact with ULK1 (Supplementary Figure 2-3A). Likewise, we found that the ATG13ΔHORMA mutant is defective in interacting with endogenous ATG9A in mammalian cells (Supplementary Figure 2-3B).

Importantly, in HA-ATG9A-BirA*-expressing cells, the loss of ATG13 had no effect on streptavidin capture of ULK1, but completely abrogated the capture of ATG101 (Figure 2-2B), suggesting that ATG13 is required for the interaction of ATG9A with ATG101, but not ULK1. Also, the streptavidin capture of ATG101 was rescued by reconstituting the ATG13 KO cells with WT ATG13, while reconstitution with ATG13ΔHORMA did not recover ATG101 binding (Figure 2-2B), which is consistent with a model wherein ATG9A interacts with ATG101 via
(A) Schematic representation of ATG13 mutations used in the study. (B) HA-ATG9A-BirA* was expressed in HCT-116 ATG13 WT, ATG13 KO or ATG13 KO cells reconstituted with WT ATG13 or ATG13 ΔHORMA. Cells were grown in full DMEM media, treated with 50 µM biotin for 12 hrs, followed by detergent lysis and incubation with streptavidin resin. The graph on right shows quantification of normalized ATG101 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Fisher’s LSD tests. (C) Cells were treated as in panel B but included reconstitution with ATG13 Δ2AA mutant. The graph on right shows quantification of normalized ATG101 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Fisher’s LSD tests. (right). (D) HA-ATG9A-BirA* was overexpressed in WT and ULK1/2 Double KO MEFs. Cells were subjected to streptavidin pulldown and immunoblotting with indicated antibodies. The graph on right shows quantification of normalized ATG13 infrared signal. Mean ± SEM. n=3 (biological replicates). Significance measured using one-sample t-test compared to hypothetical mean of 1 (right). ns=p>0.05, *=p≤0.05, **= p≤0.01, ***=p≤0.001, ****= p≤0.0001.

ATG13. In contrast to ATG13 ΔHORMA, the reconstitution of ATG13 KO cells with the ULK1 binding-defective ATG13 Δ2AA completely restored HA-ATG9A-BirA*-mediated biotinylation of ATG101 (Figure 2-2C). Furthermore, in mouse embryonic fibroblasts, HA-ATG9A-BirA* biotinylates ATG13 regardless of the presence or absence of ULK1/2 (Figure 2-2D). Reciprocal co-IP experiments also demonstrated that ATG9A-ULK1 binding was not affected by loss of ATG13 (Supplementary Figure 2-3C-D).
To confirm our biochemical observations of the ATG9A-ATG13 interaction, we used CRISPR/Cas9 to knock-in an HA affinity tag in-frame on the C-terminal end of genomic ATG9A in HCT-116 cells (Supplementary Figure 2-7A). Deep sequencing of multiple clones verified that the HA sequence was inserted correctly. To further validate the knock-in, we detected an HA signal at the predicted molecular weight of ATG9A. We then verified that this signal was indeed ATG9A-HA by knocking out the ATG9A locus with CRISPR/Cas9 and measuring the corresponding loss of HA signal (Supplementary Figure 2-7B). We were also able detect a strong ATG9A-specific signal by immunostaining for HA and confocal imaging, which was lost upon CRISPR/Cas9 targeting of ATG9A (Supplementary Figure 2-7C).

Using these HA KI cells (ATG9A-HA KI), we found that endogenous ATG13 and ATG9A colocalize in discrete semi-perinuclear puncta. Importantly, the colocalization of ATG9A and ATG13 is abrogated by loss of ATG101 but only marginally affected by loss of FIP200 (Supplementary Figure 2-4), which we also confirmed by direct co-IP of ATG9A-HA and ATG13 (Supplementary Figure 2-3E-F). Conversely, as we suspected, the interaction between ATG13 and ATG101 did not require ATG9A (Supplementary Figure 2-3G), suggesting that ATG9A is not an integral part of the ATG13-ATG101 complex and that they may only transiently interact. Furthermore, in ATG13 KO reconstitution experiments, ATG9A showed increased colocalization with the ULK1 binding-defective ATG13 Δ2AA and decreased colocalization with ATG13 ΔHORMA compared to WT ATG13 (Supplementary Figure 2-5). Taken together with the co-IP and BirA* experiments in Figure 2-2, these data suggest that, aside from the canonical ULK1 complex, ATG9A interacts with an ULK1-independent ATG13 subcomplex that includes ATG101.
2.3.3 The loss of ATG13 and ATG101 result in an accumulation of ATG9A at large clusters of p62/SQSTM1

To understand what role ATG13 may play in regulating ATG9A under basal conditions, we analyzed the effect of ATG13 KO on endogenous ATG9A in the ATG9A-HA KI cells. Confocal imaging of these cells revealed a striking accumulation of ATG9A in large spherical puncta. We then questioned whether these large accumulations of ATG9A in ATG13 KO cells were a result of a defective ULK1 complex or could be attributed to an ULK1-independent function of ATG13 and potentially ATG101. Thus, in addition to ATG13 KO lines, we used our ATG101 and FIP200 KO versions of the ATG9A-HA KI cell line as well as an additional panel of HEK-293T lines. Of note, our attempts to disrupt ULK1 by CRISPR/Cas9 were unsuccessful (no viable cells recovered), despite using multiple sgRNAs and cell lines, so we relied on FIP200 as a surrogate for ULK1 complex KO, given that loss of FIP200 has been shown to disrupt the ULK1 complex (Gammoh et al, 2013; Hara et al, 2008). We found that loss of ATG101 resulted in the same distinctly large ATG9A puncta (Supplementary Figure 2-8A), while FIP200 KOs showed no significant increase in ATG9A puncta size by confocal imaging. Furthermore, the increase in ATG9A puncta size in ATG13 KOs was rescued to a normal ATG9A distribution by reconstitution with WT or ATG13 Δ2AA, but not ATG13 ΔHORMA (Supplementary Figure 2-8B).

To identify where in the cell these large ATG9A structures reside, we co-stained the cells for markers of various organelles or proteins known to colocalize with ATG9A, including the ER, golgi, endosomes, lysosomes, p62/SQSTM1, and IM/PAS. While the majority of organelle or protein markers decreased or showed no significant change in colocalization with ATG9A, we found that the large accumulations of ATG9A in ATG13 KO cells were almost entirely colocalized with p62/SQSTM1 (Figure 2-3A-G, quantification in panel H).
Figure 2-3. The loss of ATG13 causes a shift in colocalization of ATG9A with organelle and autophagy markers and triggers an accumulation of ATG9A with p62/SQSTM1.

(A)-(G) Representative images of ATG9A colocalization with different organelle and autophagy markers. HCT-116 ATG9A-HA KI-ATG13 WT or ATG13 KO cells were grown in full DMEM media, fixed and labeled with antibodies for HA and indicated organelle/cellular markers and imaged (Scaler bar=10 µm). (H) Quantification of ATG9A colocalization with indicated organelle
markers of golgi (GOLGIN97), mitochondria (TUFM), ER (PDIA3), endosomal system (VPS26A), early endosome (EEA1), lysosome (LAMP1), autophagy adaptor (p62), and autophagosome markers (ATG16L1, WIPI4, ATG2A, LC3II, WIPI2) respectively. Mean ± SEM, n=1 independent experiment with 30 technical replicates. Images for p62 colocalization (G) are from the same single independent experiment as Figures 4C and 5C.

By immunoblot, we found that p62/SQSTM1 levels were significantly elevated in the ATG13, ATG101 and ATG9A KO lines, while FIP200 KOs showed a marginal increase in p62/SQSTM1, depending on the cell type (Figure 2-4A-B). In agreement with these data, confocal imaging revealed a high level of p62/SQSTM1 accumulation in ATG13, ATG9A, and ATG101 KO cells (Figure 2-4C-D). Similarly, loss of ATG101 phenocopied the effect of ATG13 KO (Figure 2-4C, D) by inducing a large accumulation of ATG9A at the p62/SQSTM1 puncta. Interestingly, although FIP200 KO cells showed no significant increase in ATG9A puncta size overall, we did see increased ATG9A-p62/SQSTM1 colocalization in these cells, perhaps reflecting a subtle p62/SQSTM1 build-up that we were unable to detect by imaging, but could see by immunoblot (Figure 2-4C, E).

Next, we used our panel of ATG13 mutants to more definitively assess the ULK1-independence of ATG13 in these experiments. We found that reconstitution of ATG13 KO cells with the ULK1 binding-defective ATG13 Δ2AA mutant completely rescued the accumulation of p62/SQSTM1 and restored a more normal distribution of ATG9A in the cell (Figure 2-5A-E; Supplementary Figure 2-8B). The ATG13 ΔHORMA mutant failed to rescue the defect in ATG9A accumulation at large p62/SQSTM1 puncta (Figure 2-5A-E; Supplementary Figure 2-8B). We did not see any exacerbation of these p62/SQSTM1 puncta in starved conditions (Figure 2-5B). These data support the idea that interaction with ATG101, but not ULK1, is essential to promote the basal autophagy function of ATG13.
Figure 2-4. The loss of ATG13 and ATG101 result in an accumulation of ATG9A at large clusters of p62/SQSTM1.

(A) Endogenous p62/SQSTM1 level in HCT-116 ATG9A-HA KI-ATG13 WT, ATG13 KO, ATG101 KO, ATG9A KO and FIP200 KO clones was measured by immunoblotting with indicated antibodies. Cells were grown in full DMEM media, treated with or without 100 nM Bafilomycin for 24 hrs and whole cell lysates were subjected to immunoblotting (left). The graph on right shows quantification of normalized p62 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Fisher’s LSD tests (right). (B) Endogenous p62/SQSTM1 level in HEK-293T ATG13 WT, ATG13 KO, ATG101 KO, ATG9A KO and FIP200 KO clones was measured by immunoblotting with indicated proteins. Cells were grown in full DMEM media, treated with or without 100 nM Bafilomycin for 24 hrs and whole cell lysates were subjected to immunoblotting. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Fisher’s LSD tests (right). (C) Confocal images of ATG9A colocalization with p62/SQSTM1. HCT-116 ATG9-HA KI-ATG13 WT, ATG13 KO, ATG101 KO, ATG9A KO and FIP200 KO cells were grown in full DMEM media, fixed, labeled with antibodies for HA and p62/SQSTM1 and imaged (Scale bar 10 µm). ATG101 KO was stained and quantified separately. Images are from the same single independent experiment as Figures 3G and 5C. (D) Quantification of average surface area of p62/SQSTM1 puncta in C. Mean ± SEM, n=1 independent experiment with 30 technical replicates. ATG101 KO was stained and quantified separately. Mean ± SEM, n=1 independent experiment with 30 technical replicates. A break was inserted in the Y axis to accommodate the wide range of p62/SQSTM1 puncta sizes. (E) Quantification of ATG9A colocalization with p62/SQSTM1. Mean ± SEM, n=1 independent experiment with 30 technical replicates. ATG101 KO was stained and quantified separately. Mean ± SEM, n=1 independent experiment with 30 technical replicates.

2.3.4 The loss of ATG13 results in increased ATG9A recruitment to stalled clusters of p62/SQSTM1.

To explore more fully the mechanism of the accumulation of ATG9A in ATG13 KO, we observed the dynamics of ATG9A and p62/SQSTM1 in live cells using EGFP-p62/SQSTM1 and mRuby2-ATG9A stably expressed in ATG13 KO cells. We found that trafficking of ATG9A seemed to increase with the size of the p62/SQSTM1 cluster, which was particularly evident in the ATG13 KO cells (Figure 2-6A and supplemental video files published in online version). We also found that these stalled p62/SQSTM1 clusters colocalized almost entirely with ubiquitin, consistent with these clusters being accumulations of ubiquitinated cellular material (Figure 2-6B). To confirm that these clusters of p62/SQSTM1 and ubiquitin were a result of stalled degradation in the ATG13
Figure 2-5. ATG13-mediated rescue of ATG9A accumulation at p62/SQSTM1 clusters requires the ATG13 HORMA domain but is independent of ULK1.
(A) Endogenous p62/SQSTM1 level was measured in HCT-116 ATG9A-HA KI-ATG13 WT, ATG13 KO OR ATG13 KO cells reconstituted with ATG13 WT, ATG13 ΔHORMA and ATG13 Δ2AA by immunoblotting with indicated proteins (left). Cells were grown in full DMEM media and whole cell lysates were subjected to immunoblotting (left). Quantification of normalized p62 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Fisher’s LSD tests (right). (B) HCT-116 cells in A were incubated in EBSS for 4 hours then processed and analyzed as in A. (C) Confocal images of ATG9A colocalization with p62/SQSTM1 in HCT-116 ATG9A-HA KI- ATG13 WT, ATG13 KO or ATG13 KO cells reconstituted with ATG13 WT, ATG13 Δ2AA and ATG13 ΔHORMA. Cells were grown in full DMEM media, fixed, labelled with antibodies for HA and p62/SQSTM1 and imaged (Scale bar=10 µm). Images are from the same single independent experiment as Figures 3G and 4C. (D) Quantification of average surface area of p62/SQSTM1 puncta in C. Mean ± SEM, n=1 independent experiment with 30 technical replicates. A break was inserted in the Y axis to accommodate the wide range of p62/SQSTM1 puncta sizes. (E) Quantification of ATG9A colocalization with p62/SQSTM1 in C. Mean ± SEM, n=1 independent experiment with 30 technical replicates.

KO, we performed a modified pulse chase by using HA-ATG9A-BirA* to pulse-label (using a pulse of biotin) p62/SQSTM1, which we could then track over time by purifying any residual biotinylated p62/SQSTM1 on streptavidin resin. As expected, the loss of ATG13 results in delayed degradation of the biotinylated p62/SQSTM1 (Figure 2-6C).

2.3.5 A split-mVenus approach captures an ATG13-ATG101 interaction with ATG9A.

To characterize the function and cellular localization of the ULK1-independent ATG13-ATG101 complex more fully, we created a bimolecular fluorescence complementation (BiFC) assay with split mVenus halves fused to ATG13 and ATG101 (Figure 2-7A). We found that the ATG13- and ATG101-fused split mVenus halves expressed well, were able to dimerize/reconstitute as measured by FACS, and that the ATG13 ΔHORMA mutant abrogated this reconstitution (Supplementary Figure 2-9A-B). We also verified that the split mVenus-fused ATG13 and ATG101 were functional, because when expressed together, they were able to rescue defective autophagy in an ATG13-ATG101 double KO cell line (Supplementary Figure 2-9C). We then took advantage of a
Figure 2-6. Loss of ATG13 results in increased ATG9A recruitment to stalled clusters of p62/SQSTM1.

(A) Live cell imaging of HCT116 ATG13 WT and HCT116 ATG13 KO cells stably expressing mRuby2–ATG9A and EGFP-p62/SQSTM1. Representative montages show mRuby2–ATG9A recruitment to EGFP-p62/SQSTM1 puncta (Scale bar=5 µm). (B) Confocal images of Ubiquitin colocalization with p62/SQSTM1 in HCT116 ATG13 WT and HCT116 ATG13 KO cells. Cells were grown in full DMEM media, fixed, labelled with antibodies for Ubiquitin and p62/SQSTM1 and imaged (Scale bar=10 µm) (left). Quantification of Ubiquitin colocalization with p62/SQSTM1 (right). Mean ± SEM, n=1 independent experiment with 30 technical replicates. (C) Modified pulse chase experiment. HEK293T ATG13 WT and HEK293T ATG13 KO cells stably expressing HA ATG9A-BirA* were pulse labeled with biotin for 24 hours, media was replaced with full DMEM and streptavidin pulldown was performed at indicated time points. Quantification of normalized p62 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using Student’s t test (bottom). ns=p>0.05, *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.
GFP-TRAP nanobody resin to capture the intact ATG13-ATG101 dimer for co-IP immunoblotting (Figure 2-7A-B). We found that both WT and Δ2AA mutant ATG13-ATG101 dimers interact with ATG9A, supporting the idea AT9A interacts with an ULK1 independent ATG13-ATG101 dimer. We also found that FIP200 coimmunoprecipitates with the ATG13 Δ2AA mutant, suggesting that ATG13 can also interact with FIP200 in an ULK1-independent manner (Shi et al, 2020).

We then took advantage of the ATG13-ATG101 BiFC signal to see the cellular localization of the intact ATG13-ATG101 dimer. In support of the mVenus-capture coIP in Figure 2-7B, we found that ATG9A interacts with the ATG13-ATG101 dimer in small puncta that do not include ULK1 (Figure 2-7C). Interestingly, we also found that the WT and Δ2AA mutant ATG13-ATG101-ATG9A complexes show colocalization with ULK1 in the largest clusters, but some of these clusters only partially overlapped with ULK1 or contained no ULK1 at all (Figure 2-7C), supporting the idea that ATG13 and ULK1 arrive independently at structures destined for degradation (Shi et al., 2020). Immunoblotting for phospho-S318 of ATG13 confirmed the lack of functional interaction between ULK1 and ATG13 Δ2AA (Figure 2-7D), despite their occasional colocalization.

To ask whether the interaction between ATG9A and endogenous ATG13 occurs at stalled clusters of p62/SQSTM1, we used the PI3K inhibitor Wortmannin to force the accumulation of p62/SQSTM1. Indeed, ATG13 and ATG9A colocalize at large p62/SQSTM1 clusters (Figure 2-7E). Taken together, these data, along with the protein-protein interaction data in previous figures, support the model that the ATG13-ATG101 complex interacts with ATG9A, independently of ULK1, at p62/SQSTM1 clusters, likely in an effort to dispose of these clusters through basal autophagy. All together, these data support a model in which the ATG13-ATG101 complex, independent of its physical interaction with ULK1, interacts with ATG9A to promote the basal
A. Venus halves reconstitute with ATG13-ATG101 interaction

Incubate with GFP-TRAP silicon beads to purify reconstituted Venus

B. blotting experiment

C. Confocal images of WT and Δ2AA of ATG13

Overlap of mVenus, ATG9A, and ULK1

D. Western Blot of FLAG-ATG13

E. Immunofluorescence images of HCT116 WT

28
2.4 DISCUSSION

In this study, we began with an interest in mapping the proximity-based interactome of ATG9A under conditions of basal autophagy (Dataset EV1). These data revealed an ATG9A interactome heavily weighted toward vesicle trafficking pathways, including entire (or nearly entire) complexes of trafficking regulators such as the EARP, GARP, TRAPP, AP-1, AP-4, AP-3, SNARE and Retromer complexes (Figure 2-1G). In addition, all components of the ULK1 complex were prominent in the ATG9A BioID interactome (Figure 2-1D). We initially focused on two members of the ULK1 complex, ATG13 and ATG101, given that their interaction with ATG9A seemed the most robust and direct. The prevailing model for ATG13 and ATG101, based primarily on studies done from the perspective of ULK1 during nutrient stress, attributes their function in autophagy to supporting the ULK1 complex. Indeed, ATG13 is required for nutrient stress-induced ULK1 kinase activity, which, in turn, is necessary to activate downstream turnover of p62/SQSTM1-associated clusters of ubiquitinated cellular material (Figure 2-8).
Figure 2-8. An ULK1-independent ATG13-ATG101 complex regulates basal ATG9A function and p62/SQSTM1 turnover.
During adaptor-mediated basal autophagy, an ULK1-independent ATG13-ATG101 complex interacts with ATG9A at p62/SQSTM1 clusters and promotes the ATG9A-mediated turnover of these clusters. Upon loss of the ULK1-independent ATG13-ATG101 complex, or disruption of the ATG13-ATG101-ATG9A interaction, the p62/SQSTM1 clusters accumulate, resulting in an accumulation of ATG9A at p62/SQSTM1 clusters.

Autophagic machinery and inhibit mTORC1 (Cheong et al, 2008; Ganley et al, 2009; Hosokawa et al., 2009a; Jung et al, 2009; Kawamata et al, 2008; Yamamoto et al, 2016). However, the role of ATG13 and ATG101 in basal autophagy, whether strictly within or outside the ULK1 complex, is poorly understood.

Some evidence suggests that ATG13 can act, at least to some degree, independently of ULK1. First, ATG13 and ULK1 do not overlap completely in phenotype. Mice lacking ULK1, and its semi-redundant homologue ULK2, succumb to a neonatal lethality during the weening period, similar to the loss of other core autophagy genes (Chan et al, 2007; Cheong et al, 2011; Kundu et al, 2008; Lee & Tournier, 2011). In contrast, mice lacking ATG13 die in utero, suggesting that ATG13 may have additional functions outside the ULK1 complex (Kaizuka & Mizushima, 2016). In addition, Hurley and colleagues demonstrated that in order for Atg1 (yeast homologue of ULK1) to promote phagophore expansion via its early autophagy targeting and tethering (EAT) domain, it must exist in an Atg13-free state (Lin et al, 2018). Furthermore, super resolution microscopy in yeast demonstrated markedly different stoichiometry and localization patterns for Atg1 and Atg13 at the PAS, as well as Atg13-independent localization of Atg1 to the PAS (Lin et al., 2018). Moreover, while ATG13 and ULK1 both associate with membrane via their own lipid binding motifs, they show different biochemical properties, with ULK1, but not ATG13, showing tight, detergent-resistant association with membrane fractions (Chan et al, 2009).

Previous studies on ATG13 mutants that fail to bind ULK1 also suggest a function for ATG13 that doesn’t require physical interaction with ULK1. Stork and colleagues demonstrated,
via a knockout-reconstitution approach in MEFs, that cells expressing ULK1-binding deficient ATG13 mutants have near-WT levels of autophagy. In contrast, cells expressing HORMA domain mutants of ATG13, which are incapable of binding ATG101, are severely autophagy impaired (Alers et al., 2011; Hieke et al., 2015; Wallot-Hieke et al, 2018), suggesting that ATG13 and ATG101 cooperate to promote autophagy independently of ULK1. In addition, recent work suggests that ATG13 functions independently of ULK1 in mitophagy (Zachari et al, 2019). Our results build on these data by showing an ULK1-independent ATG13-ATG101 complex interacts with ATG9A in basal autophagy and is sufficient to promote the basal autophagic turnover of p62/SQSTM1.

Work by Ohsumi and colleagues demonstrated that the HORMA domain of yeast Atg13 interacts with Atg9 (Suzuki et al., 2015). We were able to support this observation by showing that deletion of the HORMA domain from ATG13 impairs its interaction with ATG9A by co-IP in mammalian cells (Supplementary Figure 2-3B), and also results in the same defects in basal autophagy that we observed with ATG101 KO or ATG9A KO cells (p62/SQSTM1 accumulation) (Figures 4 and 5). We also found that ATG13 was required for the interaction between ATG9A and ATG101; while vice versa, ATG101 was required for the interaction between ATG9A and ATG13 (Figures 2B-C, S5E). These data support the model that an intact ATG13-ATG101 subcomplex interacts with ATG9A to promote basal ATG9A function in autophagy.

Our data raise additional questions about the dynamics of interactions between the ATG13-ATG101 dimer and ATG9A and what additional roles ATG13-ATG101 plays outside the ULK1 complex. Ktistakis and colleagues found that ATG13 translocates, independently of ULK1/2, to mitophagy structures and that ATG13 also colocalizes with a pool of ATG9A (Karanasios et al., 2016; Zachari et al., 2019). This pool of ATG13 was presumed to mark intact ULK1 complex.
However, using the split-mVenus system, we were able to visualize an ULK1-independent ATG13-ATG101 dimer in complex with ATG9A, which often occurred in small vesicles, suggesting that this ATG13-ATG101 dimer may traffic with ATG9A. In addition, the ATG13-ATG101-ATG9A complex accumulated at large clusters of p62/SQSTM1 and ubiquitin (Figure 2-7). Interestingly, ULK1 also appeared at some of these large clusters, even in cells expressing the ULK1 binding-defective ATG13, supporting the idea that components of the ULK1 complex arrive independently at pre-autophagosomal structures (Itakura et al., 2012; Shi et al., 2020). This idea is also supported by an ULK1-independent interaction between the ATG13-ATG101 dimer and FIP200 (Figure 2-7B) and our binary co-IP data showing that ATG13 doesn’t require FIP200 or ULK1 to interact with ATG9A; and vice versa, ULK1 doesn’t require ATG13 to interact with ATG9A (Figures 2D, S5C-D and S5F).

Another intriguing question relates to how ATG9A gets recruited to clusters of ubiquitin and p62/SQSTM1. Perhaps in the absence of ATG13 or ATG101, the accumulation of ubiquitin and/or p62/SQSTM1 sends a persistent, interminable signal to recruit ATG9A. Indeed, the concept of p62/SQSTM1 recruiting active signaling molecules to autophagy structures has been demonstrated previously (Duran et al, 2011; Goodall et al, 2016; Komatsu et al, 2010) and could help explain why the accumulation of p62/SQSTM1, caused by loss of ATG13 or other core autophagy regulators, resulted in an apparent ‘dead-end’ recruitment of ATG9A to these structures. Alternatively, ATG9A itself may somehow act as a sensor of ubiquitin build-up. This would be an elegant way for a cell to calibrate its basal autophagic response to whatever level of cellular debris needs recycling (Yamasaki et al, 2020).

Lastly, what additional proteins associate with the ULK1-independent ATG13-ATG101 complex in basal autophagy? Gel filtration studies show co-elution of ATG13 and ATG101 below
the 3-4 MDa ULK1 complex, but within a range that suggests other components of the complex and/or higher order oligomerization of ATG13/ATG101 (Hosokawa et al., 2009a; Hosokawa et al, 2009b). ULK1-binding deficient mutants of ATG13 and the split-mVenus ATG13-ATG101 system will be useful tools to answer this question.

In conclusion, our study uncovers the first BioID-based interactome for ATG9A, which includes an array of vesicle-trafficking complexes along the ER-Golgi-endosomal axis, and autophagy regulatory proteins that include ATG13 and ATG101. From these data, we discovered an ULK1-independent role for the ATG13-ATG101 in regulating ATG9A-mediated turnover of p62/SQSTM1-tethered clusters of ubiquitin. These data expand our understanding of basal autophagy and have implications for the development of therapeutic strategies aimed at degenerative diseases in which defective basal autophagy plays a critical role.

2.5 ACKNOWLEDGEMENTS

We thank the Fritz B. Burns Foundation for student fellowships and critical instrumentation. We thank the Duke University School of Medicine for use of the Proteomics and Metabolomics Shared resource and for generous guidance and assistance with proteomics. We thank the Simmons Center for Cancer Research for graduate fellowships to ARK, DMP, and CMM. JLA is supported by a generous gift from the Fritz B. Burns Foundation, an American Cancer Society Research Scholar Grant (133550-RSG-19-006-01-CCG) and a National Cancer Institute/National Institutes of Health grant (2R15CA202618-02).
Supplementary Figure 2-1. The C terminus of ATG9A harbors hallmarks of a signaling hub and is involved in self association, and ATG9A trafficking.

(A) Graph showing disorder tendency of ATG9A across its amino acid sequence (predicted by ANCHOR web server at http://anchor.elte.hu/) and PTM distribution across the protein quantified
by High Throughput Paper (HTP) observations (generated by PhosphoSitePlus® at https://www.phosphosite.org/psrSearchAction) (top). Schematic of ATG9A domain structure (bottom) B. Schematic representation showing N or C terminal halves of mVenus fused to C terminus of ATG9A (top left). HCT-116 cells expressing ATG9A C-terminally labeled N mVenus or N+C mVenus constructs were grown in full DMEM media, fixed and imaged (Scale bar=10 µm) (right). Fluorescence intensity of cells expressing ATG9A C-terminally labelled mVenus constructs was measured via flow cytometry. Mean ± SEM, n=1 (bottom left). (C) Hela ATG9A WT, ATG9A KO or ATG9A KO cells reconstituted with ATG9A WT and ATG9A ΔC mutant stably expressing GFP-LC3 were grown in full media and whole cell lysates followed by immunoblotting with indicated antibodies (left). Quantification of normalized GFP and normalized LCII/LC3I ratio. Mean ± SEM, n=3 (biological replicates). Significance determined by RM one-way ANOVA test followed by Šidák's multiple comparisons tests. ns=p>0.05, *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****= p≤0.0001.
Supplementary Figure 2-2. Functional validation of HA ATG9A-BirA constructs.

(A) HEK293T WT, ATG9A KO, and ATG9A KO cells reconstituted with HA-ATG9A and HA ATG9A-BL were grown in full DMEM media, lysed and immunoblotted with indicated proteins (left). Quantification of normalized LC3B infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Šidák's multiple comparisons tests (right).

(B) HEK293T WT, ATG9A KO and ATG9A KO cells reconstituted with HA-ATG9A and HA ATG9A-BL were grown in full DMEM media with or without 100nM Bafilomycine for 24 hrs, lysed and immunoblotted with indicated proteins (left). Quantification of normalized p62 infrared signal. Mean ± SEM, n=3 (biological replicates). Significance measured using RM one-way ANOVA test followed by Šidák's multiple comparisons tests (right).

(C) Confocal images of p62/SQSTM1 colocalization with ATG9A-BirA* in HEK293T ATG13 WT and ATG13 KO cells stably expressing ATG9A-BirA*. Cells were grown in full DMEM media, fixed and labelled with antibodies for p62/SQSTM1 and HA (Scale bar=10 µm).

(D) Confocal images of biotinylated proteins colocalized with HA-ATG9A-BirA*. HA-ATG9A-BirA*-expressing cells were grown in full DMEM media with or without biotin incubation (12 h), fixed and labelled with Alexafluor488-conjugated streptavidin and HA antibody (Scale bar=10 µm).

ns=p>0.05, *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****= p≤0.0001.
Supplementary Figure 2-3. ATG9A interacts with an ULK1-independent ATG13-ATG101 subcomplex via the ATG13 HORMA domain.

(A) The ULK1 binding deficient mutant of ATG13 (ATG13 Δ2AA) was validated by immunoprecipitating HA-ATG13 or HA-ATG13 Δ2AA and immunoblotting for Myc-ULK1 in HCT-116 ATG13 KO cells. (B) HCT-116 parental or HCT-116 ATG9A-HA KI ATG13 KO cells reconstituted with 3X FLAG-ATG13 WT or 3X FLAG-ATG13 ΔHORMA were grown in full DMEM media and subjected to size exclusion chromatography. Fractions with high levels of ATG9A-HA were pooled to facilitate immunoprecipitation. ATG9A-HA was
Supplementary Figure 2-4. ATG13 colocalization with ATG9A is independent of FIP200/ULK1 complex.

HCT-116 ATG9A-HA KI ATG13 WT, ATG13 KO, ATG101 KO, ATG9A KO and FIP200 KO cells were grown in full DMEM media, fixed, labeled with antibodies for HA and ATG13 and imaged (Scale bar=10 µm) (left). Quantification of ATG9A colocalization with ATG13. Mean ± SEM, n=1 independent experiment with 30 technical replicates. Images are derived from the same experiment as Supplementary Figure 2-5 and Supplementary Figure 2-8.
Supplementary Figure 2-5. ATG13 colocalization with ATG9A requires the ATG13 HORMA domain, but occurs independently of ULK1 binding.

HCT-116 ATG9A-HA KI ATG13 WT, ATG13 KO or ATG13 KO cells reconstituted with ATG13 WT, ATG13 Δ2AA, and ATG13 ΔHORMA. Cells were grown in full DMEM media, fixed, labelled with antibodies for HA and ATG13 and imaged. (Scale bar=10 µm). (C) Quantification of ATG9A colocalization with ATG13. Mean ± SEM, n=1 independent experiment with 30 technical replicates. Images are derived from the same experiment as Supplementary Figure 2-4 and Supplementary Figure 2-8.
Supplementary Figure 2-6. BioID identifies a network of proximity-based ATG9A interactions.

(A) A heat map generated for all putative interactors from the BioID proteomics data for HA-BirA* vs HA-ATG9A BirA* streptavidin pull-downs. (B) A heat map of the top 50 putative interactors from the HA-ATG9A-BirA* BioID proteomics data with the highest p-value and fold-change. Normalized protein levels of HA-BirA* and HA-ATG9A-BirA* were utilized to calculate z-score.
Supplementary Figure 2-7. Validation of the HCT-116 ATG9A-HA KI cell line.

(A) Schematic representation of the CRISPR/Cas9-mediated insertion of a 1x HA tag sequence at the C-terminal end of genomic atg9a (HCT-116 ATG9A-HA KI). Single cell-derived clones were validated by targeted deep sequencing. (B) The HCT-116 ATG9A-HA KI 2E6 clone was validated by knocking out the ATG9A locus with CRISPR/Cas9 (clones 3, 6 and 8 were successful). Clones were validated by measuring the loss of HA signal by immunoblotting with indicated antibodies. (C) Confocal images of HCT-116 ATG9A-HA KI parental cell line or ATG9A KO cells generated in HCT-116 ATG9A-KI KI cell line by CRISPR/Cas9. Cells were grown in full DMEM media, fixed, labelled with HA and imaged (Scale bar, 10 μm).
Supplementary Figure 2-8. Loss of ATG13 and ATG101, but not FIP200, lead to the accumulation of ATG9A in large puncta.

(A) Confocal images of ATG9A puncta in HCT-116 ATG9A-HA KI-ATG13 WT, ATG101 KO, FIP200 KO and ATG13 KO cells. Cells were grown in full DMEM media, fixed, labelled with HA and imaged (Scale bar=10 μm) (left). Quantification of average ATG9A puncta surface area. Mean ± SEM, n=1 independent experiments with 30 technical replicates. (B) Confocal images of ATG9A puncta in HCT-116 ATG9A-HA KI-ATG13 WT, ATG13 KO or ATG13 KO cells reconstitutes with ATG13 Δ2AA and ATG13 ΔHORMA. Cells were grown in full DMEM media, fixed, labelled with HA and imaged (Scale bar=10 μm) (left). Quantification of average ATG9A puncta surface area. Mean ± SEM, n=1 independent experiment with 30 technical replicates. Confocal images for A and B were obtained from the same single independent experiment as Supplementary Figures 2-4 and 2-5.
A

No Transfection Control

ATG13 VenusN Only

ATG101 VenusC Only

WT ATG13-ATG101 Venus

ATG13Δ2AA-ATG101 Venus

ATG13ΔHORMA-ATG101 Venus

B

- - - - - - + VenusN-ATG13ΔHORMA-Myc
- - - - - + - VenusN-ATG13Δ2AA-Myc
- + - - + - VenusN-ATG13 WT-Myc
- - + + + + VenusC-ATG101-3xFLAG

ATG13

ΔHORMA

ATG101

Actin

C

Split Venus

p62/SQSTM1

Merged

Detail

ATG101 DKO

ATG13Δ2AA-ATG101 Venus

DKO Wt ATG101 Venus

p62 puncta

ATG101 DKO

DKO Δ2AA Venus

DKO ΔHORMA Venus

46
Supplementary Figure 2-9. Evaluation of a split mVenus system to capture the ATG13-ATG101 dimer.

(A) HEK293T cells were overexpressed with VenusC-ATG101-3X FLAG only, VenusN-ATG13-Myc only, VenusN-ATG13 Δ2AA only, VenusN-ATG13 ΔHORMA or both Venus N and C halves together as indicated. Cells were grown in full DMEM media, trypsinized, separated in a cell strainer, and analyzed by flow cytometry without fixation. Gates, laser power, and detector were kept constant between samples. 10,000 cells displayed per histogram. (B) HEK29T cells in A were also collected and analyzed by western blot to show overall expression. (C) Confocal images of HCT116 ATG9A-HA KI ATG13-ATG101 double KO cells with or without stable reconstitution with VenusC-ATG101-3X FLAG and VenusN-ATG13-Myc or VenusN-ATG13 Δ2AA. Cells were grown in full DMEM media, fixed, and stained with p62/SQSTM1 antibody as indicated (Scale bar=10 μm). Quantification of average ATG9A puncta surface area. Mean ± SEM, n=1 independent experiments with 30 technical replicates.
2.7 MATERIAL AND METHODS

2.7.1 Cell culture, transfection and viral transduction.

HEK-293T cells, HCT 116 cells and their derivatives were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, 11965-092) supplemented with 10% fetal bovine serum (FBS; Genesee Scientific, 25-514) at 37°C in a 5% CO2 incubator. ULK1/2 knock-out Mouse Embryonic Fibroblasts (MEFs), kindly provided by Sharon Tooze, London Research Institute, United Kingdom were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO2 incubator. HEK-293T and HCT 116 cells were transiently transfected using polyethylenimine (PEI) (Polyscience, Inc.) or transporter 5 (Polysciences, 2600-8-5) according to the manufacturer’s protocols. MEF cells were transiently transfected using Lonza AmaxaTM NucleofectorTM kit (VPD1004) with Nucleofector II device (Amaxa biosystems) Original and manipulated plasmids used for transient transfection listed in plasmids section.

To stably express 3X FLAG-ATG13 WT, 3X FLAG-ATG13 Δ2AA and 3X FLAG-ATG13 ΔHORMA in ATG13 KO HCT 116 cell line (HCT 116 ATG9A-HA KI ATG13 KO), cDNA constructs were cloned into pLenti puro (addgene, 39481) vector backbone (primer sequences are mentioned below in plasmids section). For virus generation, LentiX-293T cells were plated to 20% confluency the day before transfection in 15cm tissue culture dishes. The next day, cells were transfected with pLenti puro vector containing ATG13 constructs, viral packaging (psPAX2), viral envelope (pMD2.G) at 4:2:1 DNA ratio with 14 ug total DNA, 600 µL of serum free media and 42 µL PEI. Supernatant was removed from LentiX-293T cells after 72 hrs., centrifuged at 2000 rpm for 5 min and then syringe filtered using a 0.45 um filter (Millipore). Polybrene was then added to a final concentration of 8 ug/ml and HCT 116 ATG9A-HA KI ATG13 KO
KO cells were infected overnight. Cells were then allowed to recover for 24 hr in DMEM/10% FBS before being selected with 2 ug/ml puromycin for 72 hr.

HA-BirA* stably expressing HEK-293T cell line was generated by using pLEX-uORF-HA-birA*-STOP-IRES-Puro plasmid according to above protocol. To generate HA-ATG9A-BirA* stably expressing HEK293T cell line, HA-ATG9A-BirA* was cloned to pLenti puro plasmid and introduced with viral transduction according to above protocol. ATG9A WT and ΔC were also cloned onto pLenti plasmid for generation of stable expression in ATG9A KO HeLa cells according to the above protocol. Cell lines used for live-cell imaging were derived from HCT 116 ATG9A-HA KI and HCT 116 ATG9A-HA KI ATG13 KO cells. EGFP-p62/SQSTM1-3xFLAG and HA-mRuby2-ATG9A were cloned into pLenti puro vector backbone and induced with viral transduction according to above protocol. Cell lines used for split mVenus BiFC confocal images were derived from HCT 116 ATG9A-HA KI and HCT 116 ATG9A-HA KI ATG13-ATG101 double KO cells. VN173-ATG13-Myc (N-term mVenus half) and VC155-ATG101-3xFLAG (C-term mVenus half) were cloned into pLenti puro vector backbone and induced with viral transduction according to above protocol. Cells used for BiFC histograms, expression blot, and immunoprecipitation were transiently transfected using the same plasmids with PEI transfection.

2.7.2 Antibodies and chemicals.

The following antibodies and chemicals were used. HA-Tag Mouse monoclonal (microscopy dilution-1:500, Cell Signaling Technology, 2367S), HA-Tag Rabbit monoclonal (microscopy dilution-1:500, Cell Signaling Technology, 3724S), ATG101 Rabbit monoclonal (Cell Signaling Technology, 13492S), FIP200 Rabbit monoclonal (Cell Signaling Technology, 12436S), ATG13 Rabbit monoclonal (Abcam, ab201467), ULK1 Rabbit monoclonal (Abcam, ab128859), ULK1

2.7.3 Plasmids.

HA-ATG9A plasmid was kindly provided by Sharon Tooze, London Research Institute, United Kingdom. HA-ATG9A-BirA* plasmid was kindly provided by Dr. Daniel Simmons, Brigham Young University. pLEX-uORF-HA-birA*-STOP-IRES-Puro plasmid was a gift from Paul Khavari (Addgene Plasmid #120558). HA-hATG13 plasmid was a gift from Do-Hyung Kim.
(Addgene Plasmid #31967). p3xFLAG-CMV10-hAtg101 plasmid was a gift from Noboru Mizushima (Addgene Plasmid 22877). HA-62 plasmid was a gift from Qing Zhong (Addgene Plasmid 28027). pEGFP-N1-FLAG plasmid was a gift from Patrick Calsou (Addgene Plasmid). pLenti-puro plasmid was a gift from Ie-Ming Shih (Addgene Plasmid 39481). pCE-BiFC-VN173, pCE-BiFC-VC155, pBiFC-VN173, and pBiFC-VC155 plasmids were a gift from Chang-Deng Hu (Addgene Plasmids # 22019, 22020, 22010, and 22011 respectively). Viral assembly plasmids were psPAX2 and pMD2.G, a gift from Didier Trono (Addgene Plasmids # 12260 and 12259 respectively)

For generation of ATG9A and ATG13 stable addback plasmids, we used Twist Bioscience to clone into the pLenti-puro plasmid. We inserted WT, C-terminus truncated, and mRuby2 fusion ATG9A into the plasmid between MluI and AgeI restriction sites. ATG13 WT was also inserted between MluI and AgeI. ATG13 WT was then further processed into ATG13 Δ2AA using primers TAAGCGGCCGCTAAGTAAG (forward) and GGTTTCCACAAAGGCATCAAAC (reverse) and into ATG13 ΔHORMA using primers ATTAACCTGGCATTCAATGTC (forward) and CTTTCTGTCTGGAATTG (reverse).

Mutant ATG9A and ATG13 DNA constructs were created using Q5 reagents and protocol from New England Biolabs (NEB, E0554). Primer sets used for mutagenesis are: ATG9A ΔC TACGTCTATCTAGTCTTACACATC (forward) and AAGAGGGCATTTTCAGGG (reverse), ATG13 ΔHORMA ATTAACCTGGCATTCAATGTC (forward) CTTTCTGTCTGGAATTG (reverse), and ATG13 Δ2AA TAAGCGGCCGCTAAGTAAG (forward) GGTTTCCACAAAGGCATCAAAC (reverse). HA-ATG9-mVenus constructs were created by insertion of EcoRV and KpnI restriction enzyme cut-sites onto the HA-ATG9A plasmid using Q5 mutagenesis and primer set: AAGATATCAGACAAGGCGCTGAGC (forward) (EcoRV) and
GGTACCATACTTGTGCACCTGAG (reverse) (Kpn1). Both HA-ATG9A and BiFC-mVenus plasmids were then digested and ligated using T4 ligase (NEB, M0202) to create HA-ATG9A-VC155 and HA-ATG9A-VN173. ATG13 and ATG101 mVenus constructs were created by NEB HiFi Assembly reagents and protocol from New England Biolabs (NEB, E2621) combining pLenti ATG13 with pBiFC-VN173 and pLenti puro with p3xFLAG-CMV10-hAtg101 and pBiFC-VC155 respectively. pLenti EGFP-p62/SQSTM1-3xFLAG was created by NEB HiFi Assembly combining HA-p62, pEGFP-N1-FLAG, and pLenti-puro plasmids.

2.7.4 CRISPR-Cas9.

ATG9A C-terminal 1xHA-tagged HCT 116 cells were generated at the Genome Engineering and iPSC Center at Washington University School of Medicine (St. Louis, MO). The 1xHA tag was introduced using CRISPR-Cas9 ribonucleoproteins (RNPs) using following guide RNA and a single-stranded oligonucleotide with 60 bp homology arms.

5’-TCTCCCCACAGGTATAGACA-3’
5’gcaggtatacagcagctgaagactatctccattaaccctttctccccacaggtaTACCCATACGACGTACCAG ATTACGCTtagacaaggctgagcagggttcctgtggcccaggatggaggccaccgctgccctgccatc-3’.

Targeted deep-sequencing was used to validate reagents and genotype single cell derived clones as previously described (Sentmanat et al., 2018) with following tailed PCR primers

Fwd 5’-TCAGGTGCACAAGGTAAGGGCCCC-3’
Rev 5’-GCCAGGGAACACTCAGAGGAGCCGT-3’.

Cells were maintained according to ATCC guidelines with McCoy's 5a Modified Medium (Cat. No. 16600108) and 10% fetal bovine serum supplemented with GlutaMax (Gibco, Cat. No. 35050061) and penicillin-streptomycin (Gibco, Cat. No.15070063).
To generate ATG13 knock-out cells, two independent single-guide RNAs #1: 5’-GGACAGCTGCCTGCAGTCGGG-3’, #2: 5’-ACACGGTGTACAACAGACTG-3’, were designed against human ATG13 (ENSG00000175224). CRISPR design tools available at www.atum.bio and crispr.mit.edu were used. The gRNAs were cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid. PX459 was a gift from Feng Zhang (Addgene, 48139). Cells expressing the gRNA constructs were separated by serial dilution and monoclonal lines were isolated manually under puromycin selection. Knockout efficiency was measured by western blotting.

ATG9A knock-out cells were generated using single-guide RNA 5’-CTGTTGGTGCACGTCGCCGAG-3’ against human ATG9A (ENSG00000198925). ATG101 knock-out cells were generated using single-guide RNA 5’-ACCTACTCCATTTGGCACCAG-3’ against human ATG101 (ENSG00000123395). FIP200 knock-out cells were generated using single-guide RNA 5’-CAGGTGCATCTAGAAGACCC-3’ against human FIP200 (ENSG00000023287). All gRNA’s were designed at crispr.mit.edu or www.atum.bio and cloned/propagated according to above protocol.

2.7.5 Immunoprecipitation and immunoblotting.

To prepare whole-cell extracts, cells were washed twice and harvested with ice-cold phosphate – buffered saline (PBS). Cell pellets were resuspended in RIPA lysis buffer (25 mM Tris-HCl [pH 7.5], 75 mM NaCl, 0.5% [wt/vol] Triton X-100, 2.5 mM EDTA, 0.05% [wt/vol] SDS, and 0.25% [wt/vol] Deoxycholate) supplemented with protease and phosphatase inhibitors and incubated for 15 min on ice or at 4°C with gentle rotation. Lysates were syringed through a 25-gauge needle 10 times and centrifuged at 21,000 rpm for 10 min at 4°C.

For co-immunoprecipitation, cells were transfected with HA-ATG9A or HA ATG13 for 48 hours or HCT 116 ATG9A-HA KI cells expressing endogenous ATG9A-HA were used. Cells
were lysed with ATG9A lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.3% [wt/vol] Triton X-100, and 5 mM EDTA) supplemented with protease and phosphatase inhibitors and lysates were incubated with anti-HA–agarose beads for 1h at 4°C with gentle rotation. The beads were then washed once with lysis buffer and three times with cold PBS. The co-immunoprecipitated proteins were eluted with modified Laemmli sample buffer by boiling at 100°C for 5 min. The proteins were analyzed, followed by immunoblotting using infrared fluorescent secondary antibodies and a Li-Cor Odyssey imaging system.

For proximity-dependent biotin ligase assay-based protein co-precipitation; cells that were transiently transfected with biotin ligase constructs for 48 hours or stably expressing biotin ligase constructs, treated with 5 μM Biotin for 12 hours and lysed in ATG9A lysis buffer as mentioned above. Cleared lysates were incubated with streptavidin agarose resin for 1 hour at 4°C with gentle rotation. The resin was then washed twice with lysis buffer and three times with cold PBS. The precipitated proteins were eluted with modified Laemmli sample buffer by boiling at 100°C for 5 min. The proteins were analyzed, followed by immunoblotting using infrared fluorescent secondary antibodies and a LI-COR Odyssey imaging system.

For the quantification of immunoblots, infrared fluorescent signal was normalized to a reference control to obtain fold-change ratios for all other lanes. The fold change differences were then compared using repeated measures (paired) one-way ANOVA followed by uncorrected Fisher’s LSD tests excluding the reference control (no standard deviation). Significance is indicated by asterisks according to the following scale: ns=p>0.05, *=p≤0.05, **= p≤0.01, ***=p≤0.001, ****= p≤0.0001. Immunoblots with only two samples (reference control and one other) were normalized the same way and significance was determined using a one-sample t-test.
compared to a hypothetical mean of 1 (reference control fold-change). Significance indicated by the same scale. Graphs and statistics were completed using GraphPad Prism 9 software.

2.7.6 Gel filtration assay.

Cells were lysed in ATG9A lysis buffer as mentioned above. Lysates were centrifuged three times at 4°C for 15 min at 13,200 rpm, and the protein concentration of the supernatant was determined by using the Bio-Rad DC protein assay kit (Bio Rad, 5000116). Five hundred microliters of 8 mg/ml of lysates was loaded onto a Superose6 10/300GL column at a flow rate of 0.25 ml/min to collect fractions.

2.7.7 Bio-ID coupled Mass spectrometry.

HEK-293T cells stably expressing HA-BirA* or HA-ATG9A-BirA* cells were lysed in ATG9A lysis buffer and proteins were co-precipitated as mentioned above. Precipitated proteins by Streptavidin resin were eluted with modified Laemmli sample buffer by boiling at 100°C for 5 min. The following steps were performed at Duke Proteomics Core Facility. Samples in loading buffer were supplemented with SDS for a final concentration of 5% for digestion and spiked with undigested casein at a total of either 200 and 400 fmol. Samples were then reduced with 10 mM dithiothreitol for 30 min at 80°C and alkylated with 25 mM iodoacetamide for 30 min at room temperature. Next, they were supplemented with a final concentration of 1.2% phosphoric acid and 765μL of S-Trap (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins were trapped on the S-Trap, digested using 20 ng/ul sequencing grade trypsin (Promega) for 1 hr at 47C, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All samples were then lyophilized to dryness and resuspended in 240 μL 1%TFA/2% acetonitrile containing 12.5 fmol/μL yeast alcohol dehydrogenase (ADH_YEAST). A QC Pool was created by taking 3 μL from each sample, which was run periodically throughout the acquisition period.
2.7.8 Quantitative LC-MS/MS data analysis.

The MS/MS data was searched against the SwissProt homo sapiens database (downloaded in Nov 2019) appended with a contaminant database, and an equal number of reversed-sequence “decoys” for false discovery rate determination. Mascot Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion spectra and to perform the database searches. Database search parameters included fixed modification on Cys (carbamidomethyl) and variable modifications on Meth (oxidation) and Asn and Gln (deamidation). Peptide Validator and Protein FDR Validator nodes in Proteome Discoverer were used to annotate the data at a maximum 1% protein false discovery rate.

Additional data filtering was accomplished using the following strategy: missing values were imputed after sample loading and total intensity normalization in the following manner. If a peptide had less than two quantitated values across all of the samples, the entire peptide entry was removed. If less than half of the values are missing in a treatment group, values are imputed with an intensity derived from a normal distribution defined by measured values within the same intensity range (20 bins). If greater than half values are missing for a peptide in a group and a peptide intensity is > 5e6, then it was concluded that peptide was misaligned and its measured intensity is set to 0. All remaining missing values are imputed with the lowest 5% of all detected values. Please note that all subsequent analyses were from these normalized protein levels.

The overall dataset had 38,797 peptide matches. Additionally, 740,677 MS/MS spectra were acquired for peptide sequencing by database searching. Following database searching and peptide scoring using Proteome Discoverer validation, the data was annotated at a 1% protein false discovery rate, resulting in identification of 38,797 peptides and 4014 proteins. After data
filtering and normalization, 38,472 peptides and 3994 proteins were quantitated (the processed list of interactors is in dataset EV1).

### 2.7.9 Statistical analysis of LC-MS/MS data.

BirA\* vs HA-ATG9A-BirA\* were compared for statistical analysis. Fold-changes and a two-tailed heteroscedastic t-test on log2-transformed data for each of these comparisons were calculated. Briefly, proteins were filtered to include those with a greater than 2-fold expression and a p-value of <0.05. A total of 283 proteins passed this filter, including ATG9A. For each of these proteins, the log2 values of the normalized proteins were plotted against the -log10 of the p value. Relevant GO categories were projected onto the significant 283 proteins for illustration purposes.

### 2.7.10 Confocal Microscopy.

HCT 116 and derivative cell lines were used for confocal microscopy colocalization experiments. Cells were seeded onto acid-etched coverslips and cultured for 36 hours before fixation. Cells were fixed for 20 minutes with 2% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100/PBS for 5 minutes (HEK 293 cells were permeabilized for 15 minutes to increase immunostaining). Samples were then blocked with 10% FBS/SEA BLOCK Blocking Buffer (Thermo Scientific) and incubated with indicated antibodies (microscopy dilutions listed in antibodies and chemicals section) at 4°C overnight. Cells were washed with 0.1% Tween/PBS (PBS-T) and incubated with Alexa fluor-conjugated secondary antibodies (Abcam ab150105, Thermo Scientific A21206 and A10034, or Millipore Sigma SAB4600238 for Alexa fluor 488) (Thermo Scientific A10037 or A11036 for Alexa fluor 568) (Thermo Scientific A21071 or Invitrogen A-21126 for Alexa fluor 633) at 1:500 dilution in SEA BLOCK blocking buffer. Cells were washed with PBS-T and counter-stained with 1.43 µM DAPI for 5 minutes. Coverslips
were then mounted with Prolong Diamond Antifade Mountant (Thermo Scientific P36961). Images were acquired on a LEICA TCS SP8 confocal microscope fitted with a HC PL APO 63X/1.40 Oil CS2 objective and a HyD detection system (Leica Microsystems).

Images taken for mVenus ATG13-ATG101 and EGFP-p62 were of cells stably expressing respective fluorescent fusion proteins and processed as described above with the indicated antibodies. mVenus constructs were expressed in an ATG13-ATG101 double KO background to remove any endogenous dimerization while the p62/SQSTM1 construct was expressed with endogenous p62/SQSTM1 still intact.

ATG9A-BirA* validation confocal experiments were performed in HEK293T cells stably expressing the ATG9A-BirA* construct in a WT HEK293T background (endogenous ATG9A still intact). ATG9A-BirA* was visualized using the HA mouse antibody previously described, p62/SQSTM1 was visualized using endogenous antibody, and biotinylated proteins were visualized using Alexa fluor 488-conjugated streptavidin.

Live-cell microscopy was performed in HCT 116 WT and ATG13 KO cells stably expressing EGFP-p62/SQSTM1-3xFLAG and HA-mRuby2-ATG9A. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum until seeded onto glass bottom microwell dishes (MatTek P35GC-1.5-10-C) at which time cells were transferred to FluoroBrite DMEM (Fisher Scientific A1896701) supplemented with 10% fetal bovine serum. Cells were allowed to adhere and grow for 48 hours prior to imaging. Images were taken every 1.524 seconds for 100 frames totaling 2.54 minutes. Frames were zoomed into p62 puncta and cropped for still-frame images while videos show all frames full-sized and then repeated zoomed into p62 puncta and a HyD detection system (Leica Microsystems).

2.7.11 Confocal microscopy data analysis.
Samples for each set were seeded, fixed, and stained on the same day with identical antibody concentrations, laser power, magnification, and image resolution by set. Global analysis sets were prepared and analyzed individually, but all parameters were maintained for each WT/KO pair and laser power adjustments were only made to maintain equal fluorescence between pairs.

All images were processed using Huygens Essential express deconvolution tool and Pearson’s coefficient was calculated using the colocalization analyzer tool in the same software. Threshold intensity values for all sets were determined by multiplying the average intensity for each channel by the same factor to maintain consistency within each set. Puncta surface area was calculated using Leica 3D analysis software with a minimum threshold of 0.5 \( \mu \text{m}^2 \). All puncta beneath this threshold were omitted in our analysis to remove background signal. Mean and standard error of the mean were calculated for both Pearson’s coefficient and puncta surface area by GraphPad Prism 9 software.

The ATG101 KO set in p62/SQSTM1 analysis was prepared and analyzed separately from other conditions in the same set and was therefore analyzed against a replicate wild-type rather than being integrated into the same data set. Antibody concentrations, magnification, and image resolution were maintained between ATG101 KO and the remaining p62/SQSTM1 analysis, but laser power was adjusted to maintain equal ATG9A-p62/SQSTM1 staining between preparations. All other preparation and analysis protocols remained the same.

2.7.12 Flow cytometry.

HCT 116 cells were transfected with HA-Atg9-mVenus155 and HA-Atg9-mVenus173 or HA-Atg9 as a control and allowed to incubate 48 hours. Cells were harvested with trypsin and fixed with 2% PFA for 20 minutes. Samples were then washed three times with PBS-T and resuspended in PBS. Cytometry data was attained on a Beckman Coulter Cytoflex Cytometer using 488 nm
laser excitation and detection on 525/40 BP fluorescence channel. Positive and negative fluorescent cells were gated based on negative control fluorescence peak using Flowjo analysis software. Mean intensity of positive and negative populations was determined using all cells within respective gate.

For split-mVenus flow data, HCT 116 cells were transfected as previously described with pLenti VN173-ATG13-Myc WT/Δ2AA and pLenti VC155-ATG101-3xFLAG (overexpression not stably transduced) and incubated for 48 hours. Cells were then washed with PBS and harvested with trypsin. Live cells were resuspended in PBS and separated using a sterile cell strainer (Thermo Scientific 22-363-548). Cytometry data was attained on a BD FACSariaTM Fusion Cell Sorter using 488 nm laser excitation and detection on 525/50 FITC channel. Positive and negative fluorescent cells were gated based on negative control fluorescence peak.

### 2.7.13 Modified Pulse Chase Experiment.

HCT 116 cells were transfected with HA-Atg9-mVenus155 and HA-Atg9-mVenus173 or HA-Atg9 as a control and allowed to incubate 48 hours. Cells were harvested with trypsin and fixed with 2% PFA for 20 minutes. Samples were then washed three times with PBS-T and resuspended in PBS. Cytometry data was attained on a Beckman Coulter Cytoflex Cytometer using 488 nm laser excitation and detection on 525/40 BP fluorescence channel. Positive and negative fluorescent cells were gated based on negative control fluorescence peak using Flowjo analysis software. Mean intensity of positive and negative populations was determined using all cells within respective gate.
2.8 REFERENCES


17. Egan D, Kim J, Shaw RJ, Guan KL. 2011. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy. 7:643-644


variant can partially restore autophagic activity in ATG13-deficient cells. *Autophagy.* 11:1471-1483


52. Lee EJ, Tournier C. 2011. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. Autophagy. 7:689-695


BIOTECH METHOD

Mechanistic discoveries and simulation-guided assay optimization of portable hormone biosensors with cell-free protein synthesis

John Porter Hunt1 | Jackelyn Galiardi2 | Tyler J. Free1 | Seung Ook Yang1 | Daniel Poole3 | Emily Long Zhao1 | Joshua L. Andersen3 | David W. Wood2 | Bradley C. Bundy1

1 Department of Chemical Engineering, Brigham Young University, Provo, UT, USA
2 Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA
3 Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, USA

Correspondence
Bradley C. Bundy, Department of Chemical Engineering, Brigham Young University, 330 N Engineering Building, Provo, UT 84602.
Email: bundy@byu.edu

Funding information
National Science Foundation, Grant/Award Number: 1254148; Simmons Center for Cancer Research

Abstract
Nuclear receptors (NRs) influence nearly every system of the body and our lives depend on correct NR signaling. Thus, a key environmental and pharmaceutical quest is to identify and detect chemicals which interact with nuclear hormone receptors, including endocrine disrupting chemicals (EDCs), therapeutic receptor modulators, and natural hormones. Previously reported biosensors of nuclear hormone receptor ligands facilitated rapid detection of NR ligands using cell-free protein synthesis (CFPS). In this work, the advantages of CFPS are further leveraged and combined with kinetic analysis, autoradiography, and western blot to elucidate the molecular mechanism of this biosensor. Additionally, mathematical simulations of enzyme kinetics are used to optimize the biosensor assay, ultimately lengthening its readable window by five-fold and improving sensor signal strength by two-fold. This approach enabled the creation of an on-demand thyroid hormone biosensor with an observable color-change readout. This mathematical and experimental approach provides insight for engineering rapid and field-deployable CFPS biosensors and promises to improve methods for detecting natural hormones, therapeutic receptor modulators, and EDCs.

KEYWORDS
cell-free protein synthesis, endocrine disrupting chemical, enzyme kinetics, estrogen receptor, hormone assay

1 | INTRODUCTION

Nuclear receptors (NRs) influence numerous metabolic processes critical for growth, homeostasis, and reproduction.[1] Chemicals which bind to NRs and activate their cellular functions include natural hormones, endocrine disrupting chemicals (EDCs),[2] and pharmaceuticals. In fact, drugs which specifically target NRs treat disorders from diabetes, cancer, and atherosclerosis to emphysema, nausea, and osteoporosis.[3,4] Additionally, industrial and commercial chemicals have been identified as NR ligands and are classified among EDCs, which interfere with correct NR function. Many chemicals currently in use have not been screened for EDC activity[5,6] and it is estimated that the societal cost of EDC exposure is high.[7,8] Thus, detection of NR ligands is a public health interest.

Traditional methods to identify NR ligands include animal studies, mammalian cell cultures, microbial sensors,[9–11] and competitive binding assays. However, these methods are not ideal for high-throughput
screening and portable detection because of their equipment requirements, protracted assay durations, expensive preparations, and/or potential confounding toxicity effects.\[12–15\]

An especially promising class of biosensors has recently leveraged the advantages of cell-free protein synthesis (CFPS)\[16\] to create a Rapid Adaptable Portable In vitro Detection (RAPID) assay for NR ligands.\[17,18\] At the heart of the assay is a chimeric fusion protein construct comprising four domains: maltose binding protein, an engineered intein from Mycobacterium tuberculosis, a human or animal nuclear hormone receptor ligand-binding domain (LBD), and β-lactamase (βLac) as a reporter protein (Figure 1A).\[17,18\] The assay first uses CFPS to express the sensor construct in the presence of sample and then measures the resulting βLac enzymatic activity – greater βLac activity results from higher hormone concentration. Both expression and enzymatic readout can be completed in a few as 40 min and can be deployed from lyophilized reagents.\[17,19\] These assays are liberated from many toxic matrix effects which plague cell-based assays, are noninfective and even potentially endotoxin-free,\[20\] and have demonstrated ligand detection in raw sewage, urine, and whole blood.\[17,18\] The versatility of this construct to integrate various LBDs suggests potential for rapid detection and screening of ligands for many different NRs, including orphan receptors.\[21,22\] However, the molecular mechanism of this sensor has not been elucidated, and additional insight could facilitate enhanced assay sensitivity. Furthermore, while the potential for a portable hormone biosensor assay has been demonstrated with lyophilization, the final embodiment has not yet been achieved – critical questions regarding the dilution factor, substrate concentration, and readable window have not yet been addressed.

In this work, sensor constructs comprising either the human thyroid receptor β (hTRβ) or the human estrogen receptor β (hERβ) LBD are studied using CFPS, autoradiography, and western blotting, revealing that intein cleaving is hormone-dependent. Next, mathematical simulations of enzyme kinetics indicate that this cleaving is integral to the biosensor molecular mechanism for ligand-dependent signal generation. Then, additional simulations of enzyme kinetics facilitate optimization of the biosensor assay according to dilution factor, substrate concentration, and assay duration. This approach enables more than three-fold improvement in the assay readable window and two-fold improvement in assay signal strength and thus naked-eye limit of detection. These discoveries facilitate the creation of a portable hormone detection assay and highlight the utility of CFPS for studying complex protein activities. We anticipate that this work will (1) facilitate rapid and portable detection of natural hormones, therapeutic receptor modulators, and EDCs, and (2) inform the optimization of other future portable biosensor assays which employ reporter enzymes.

2 | EXPERIMENTAL SECTION

17β-estradiol (E2; human estrogen receptor agonist), 3,3′,5-triiodothyroacetic acid (TRIAC; human thyroid receptor agonist), and amino acids were purchased from Millipore Sigma (Billerica, MA). Tris Buffer was purchased from VWR (Radnor, PA). Phosphoenolpyruvate and nitrocefin were purchased from Cayman Chemical (Ann Arbor, MI). Dithiothreitol was purchased from GoldBio (St. Louis, MO).

Preparation of Escherichia coli cell extract was performed as previously reported.\[23\] Briefly, BL21 Star DE3 E. coli cells were cultured at 37°C in 2xYT medium, induced with 1 mM IPTG at OD₆₀₀ = 0.6, and harvested during the mid-log growth phase. Cells were recovered
by centrifugation, washed, then suspended in Buffer A (10 mM Tris-acetate, 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol, pH 8.2), and homogenized with an Avestin Emulsiflex B15 French Press Homogenizer (Ottawa, Ontario, CA). Lysate was clarified by centrifugation at 12k RCF, incubated at 37°C for 30 min, flash frozen into aliquots, and stored at −80°C.

Biosensor expression was performed as previously reported, and saturation of the assay response was previously observed above about 5000 nM hormone. Lyophilized CFPS was created by assembling cell extract and 12 nM plasmid DNA coding for the biosensor constructs under T7 promoter with the following concentrations after rehydration: 15 mM magnesium glutamate, 1 mM 1.4-diaminobutane, 1.5 mM spermidine, 33.33 mM phosphoenolpyruvate, 10 mM ammonium glutamate, 175 mM potassium glutamate, 2.7 mM potassium oxalate, 0.33 mM nicotinamide adenine dinucleotide, 0.27 mM coenzyme A, 1.2 mM ATP, 0.86 mM CTP, 0.86 mM GTP, 0.86 mM UTP, 0.17 mM folic acid, and 2 mM of the other canonical amino acids. This mixture was aliquoted into test tubes and lyophilized using the Labconco FreeZone 2.5 L freeze-drier (Labconco Corp., Kansas City, KS) for 6 h. Hormone dissolved in DMSO was combined with aqueous sample to rehydrate the lyophilized CFPS reaction. The final DMSO concentration in all CFPS reactions was 5% v/v. CFPS reactions were incubated at 37°C for 1 or 3 h, diluted with PBS, and finally with nitrocefin in PBS at the indicated concentrations to the desired final dilution. The on-demand sensor result shown in Figure 1 was achieved with a 500x final dilution and a final nitrocefin concentration of 160 μM. Fresh CFPS reactions were assembled and incubated as above without lyophilization.

The extent of enzymatic nitrocefin hydrolysis was measured by OD490 using a SynergyMx plate reader (Biotek, Winookski, VT). OD490 reads are first normalized by the OD490 at the completion of enzymatic activity for each reaction before conversion to hydrolyzed product concentration. The final product concentration was confirmed with final OD values for each well and converted to nitrocefin hydrolysis product concentration for analysis. Mathematical simulations were programmed with Python and parameter fits were calculated using least-squares regression.

3 | RESULTS AND DISCUSSION

Previous efforts to elucidate the molecular mechanism of the protein biosensor construct in bacteria have postulated a potential allosteric activation mechanism but have been complicated by microbial viability (Figure 1A). CFPS of the biosensor decouples construct expression and ligand-dependence from microbial metabolism and liberates the sensor protein for direct biochemical analysis following protein expression. Greater mechanistic understanding could lead to enhanced sensor performance, and a study of the sensor mechanism was undertaken using CFPS. Constructs employing hERB and hTRβ LBD as the sensing element and extended spectrum β-lactamase (βLac) as the reporter protein were chosen for this investigation (Figure 1A).

3.1 | Role of intein domain in cell-free biosensor expression

In their native environment, NRs function via ligand-induced allosteric conformational changes which originate in the LBD and result in repositioning of helix 12. This sensor construct is designed such that helix 12 is linked directly to the N-terminus of the C-terminal intein segment, with the reporter protein then linked directly to the C-terminus of the intein. It has been hypothesized that the intein domain stabilizes the construct and transduces the ligand-binding conformational change from the NR LBD to the reporter protein. To test this hypothesis using CFPS, constructs containing the maltose binding
Figure 2 (A) Western blot for βLac with biosensor constructs. White indicates the βLac protein. Lane 1 = No-plasmid control reaction. Lanes 2–6 analyze hTRβ construct expressed with the following TRIAC concentrations: 2 = 0 nM; 3 = 10 nM; 4 = 100 nM; 5 = 300 nM; and 6 = 10,000 nM. Lanes 7–11 analyze hERβ construct expressed with the following E2 concentrations: 7 = 0 nM; 8 = 10 nM; 9 = 100 nM; 10 = 300 nM; and 11 = 10,000 nM. Kinetic enzymatic analysis of hormone biosensor readout. (B) Data and equation fit to the results of the hTRβ hormone biosensor assay. Hydrolyzed nitrocefin concentration was measured as OD490. Each data trace represents one CPFS reaction with the indicated hormone concentration, with values given in the accompanying table. The ratio of enzyme concentration parameter estimate ([E]x) between 10,000 nM TRIAC and 0 nM TRIAC is 6.7. (C) Data and equation fit to the results of the hERβ hormone biosensor performed with the same methods as B. (D) Performance parameters of the hTRβ and hERβ biosensors, including the LOD, SNR, and Michaelis–Menten kinetic parameters. The closed-form solution to the Michaelis–Menten equation was used, where [P]x(t) is the concentration of enzymatic product as a function of assay duration and hormone concentration, t the time, [S]0 the initial substrate concentration, Km the Michaelis–Menten constant, kcat the catalytic rate constant, and [E]x is the putative active βLac concentration as a function of hormone concentration. W designates the real branch of the lambert w function for the argument. The given kinetic parameters were obtained by the fit represented in B and C. (E) Biosensor βLac enzymatic assay readout represented as sensor signal. Sensor signal is calculated as the difference between the enzymatic activity of biosensor CFPS in the presence of hormone and the enzymatic activity of CFPS in the absence of hormone. (F) Enzymatic activity of βLac with enzyme concentrations differing by exactly 10-fold. The same kinetic parameters Km and kcat were obtained from a fit to both data series with independent [E]x values fit to each series. These values are given in the accompanying table. Data traces represent duplicate reactions. The true concentration of assayed βLac was 5.3 and 0.53 nM as determined by radiolabeling of βLac. CFPS, cell-free protein synthesis; hERβ, human estrogen receptor β; hTRβ, human thyroid receptor β; βLac, β-lactamase; LOD, limit of detection; SNR, signal-to-noise ratio.

3.2 Allosteric investigation of biosensor construct

Next, the allosteric character of the biosensor protein mechanism was investigated. Full-length sensor construct was first expressed in CFPS without ligand. Next, ligand was added to the constructs, incubated to allow binding, and reporter activity was assessed. In contrast to biosensor expressed in the presence of ligand, these constructs did not show ligand-dependent reporter activity (Supplementary Figure S1). Interestingly, the fraction of protein that expresses in soluble form is not affected by the concentration of ligand present in the reaction.[17,18] These results uncover an important component of the sensor’s ligand response: the biosensor protein must be expressed in the presence of ligand for ligand-dependent reporter activity. This suggests that, during translation, ligand-bound constructs may fold into enzymatically active conformations, while the apo form of the construct may traverse an alternative folding pathway, thereafter, energetically prohibited from forming a more-active conformation. The fact that the construct must be expressed in the presence of the protein, NR LBD, and βLac, but omitting the intein domain, were expressed and then assayed for βLac activity using nitrocefin. These constructs resulted in low soluble expression yield and reduced ligand-dependent reporter activity (Supplementary Figure S1), significantly limiting utility like previous cell-based results.[10] These observations confirm the necessary role of the intein for construct performance.
sample highlights the critical role of CFPS for on-demand sensing; pre-expressing and lyophilizing biosensor construct will apparently not enable ligand detection; only by lyophilizing poised CFPS translation components can this sensor be deployed on-demand. CFPS also enables synthesis of the construct in complex sample matrices, including some which are potentially toxic to microbe growth. Although these results also suggest that the construct is not allosterically activated like the native NR, previous experiments confirm that the affinity of the ligand for the LBD determines the strength of the biosensor response, and correct distinction between NR agonists and antagonists has even been reported. This assay therefore retains remarkably high fidelity with native NR signaling.

### 3.3 Catalytic cleaving of sensor reporter domain

Although *M. tuberculosis* RecA mini-inteins, similar to that employed by this construct, exhibit catalytic cleaving activity, the role of catalytic cleaving in the mechanism of this fusion biosensor, a functionality that might be engineered to enhance ligand sensitivity, has not previously been established. To investigate the role of intein cleaving in sensor performance, the hTRβ and hERβ constructs were expressed in the presence of radioactive C\(^{14}\) leucine at increasing ligand concentrations then analyzed on denaturing agarose gel. All reactions yielded approximately 4 µm biosensor fusion protein, regardless of ligand concentration, which is consistent with previous results. Bands containing radioactive leucine were then visualized with autoradiography (Supplementary Figure S3A). Although the results indicate an abundance of full-length, intact sensor constructs (120 kDa), they also clearly demonstrate an appreciable degree of construct cleaving. Significantly, a radioactive band appears at ≈30 kDa. For reference, the molecular weight of the reporter protein, βLac, is 29.9 kDa. To confirm the identity of this ligand-dependent band, samples of these constructs were analyzed using western blot with primary antibody specific to βLac (Figure 2A). Western blot results confirm that this band is the cleaved βLac reporter domain and that its intensity increases with ligand concentration (Supplementary Figure S3B). Importantly, ligand-saturated reactions (10,000 nM TRIAC and E2) yielded about 10 times the amount of cleaved beta-lactamase reporter when compared with the no-ligand reactions, as analyzed by densitometry (Supplementary Figure S3B). Densitometry further suggests that the cleaved βLac domain likely represents between 0.2% and 2.0% of the βLac still covalently attached to intact, full-length construct (Supplementary Figure S3B). Overall, the blot indicates that constructs expressed in the presence of hormone are more likely to cleave than those expressed without hormone.

### 3.4 Kinetic analysis elucidates sensor molecular mechanism

The results of the western blot experiment suggest that catalytic cleaving of the reporter domain could be significant to the molecular mechanism of hormone dependence. To further explore the extent to which cleaved βLac contributes to the enzymatic activity of the biosensor, free βLac was expressed in CFPS then assayed for enzymatic activity alongside hTRβ and hERβ constructs expressed with CFPS in the presence of 10,000 nM TRIAC and 10,000 nM E2, respectively. The results of this analysis are consistent with a sensor mechanism wherein enzymatic activity is generated by cleaved reporter domain (Supplementary Figure S4). If 2% of the biosensor construct cleaved the reporter domain, the concentration of free βLac in the hTRβ and hERβ constructs samples would have yielded 11.3 and 9.5 pg μL\(^{-1}\) βLac, respectively. By comparison, the concentration of free βLac which yielded similar enzymatic activity curves was 14.0 pg μL\(^{-1}\). The residual enzymatic activity could come from constructs with less active reporter domain than cleaved counterparts (Figure 1A).

To further investigate the significance of cleaving with kinetic analysis, a closed-form solution to the Michaelis–Menten equation (Supplementary Figure S5) was employed with βLac activity data from biosensor construct in parallel with free βLac alone (Figure 2). For mathematical confirmation, simulations were also performed with a pseudo-first-order rate equation (Supplementary Figure S6). The best-fit equations to enzymatic activity data indicate a 6.7-fold increase in the reporter enzyme concentration parameter \([E]_x\) when comparing the 10,000 nM TRIAC reactions with the no-hormone reactions (Figure 2). By comparison, free βLac at a 10-fold difference in enzyme concentration resulted in a 10-fold difference in the best-fit enzyme concentration parameter \([E]_x\), thus validating the fidelity of the chosen mathematical model. These latter results suggest that a 10-fold difference in enzyme concentration parameter \([E]_x\), would be observed if cleaved reporter were entirely responsible for the sensor’s hormone-dependent enzymatic response. However, this ratio is less than 10, suggesting that the true mechanism is less hormone-responsive than a purely cleaving-mediated mechanism. These results confirm that catalytic cleaving is likely responsible for a major portion, though not the entirety, of construct hormone-dependent enzymatic activity (Figure 1A), consistent with observed enzymatic activity of biosensor construct which omits the intein domain (Supplementary Figure S1).

Overall, the results of these studies suggest that catalytic cleaving of the reporter domain is a critical part of the intein-mediated, hormone-dependent biosensor enzymatic signal generation. It is hypothesized that the biosensor construct generates a hormone-dependent enzymatic response through co-translational hormone-stabilized folding of the intein into conformations which promote an active reporter domain and favor catalytic cleaving of the polypeptide backbone. Cleaved βLac is expected to be more enzymatically active than uncleaved βLac because of the reduction in steric hindrance to substrate diffusion and potential destabilizing effects of the other construct domains. These discoveries hold promise for expanded and improved biosensing of hormones and EDCs. For example, the consistency between the mechanistic observations of hTRβ and hERβ constructs shows potential for screening ligands of orphan NRs in the future. Furthermore, protein engineering techniques may increase ligand-dependent activity by enhancing the rate of ligand-induced
cleaving of the βLac reporter enzyme. It is therefore anticipated that this work will enable improved sensing and screening of natural hormones, therapeutic receptor modulators, and EDCs.

3.5 | Optimization of the two-step sensor assay

An exciting feature of cell-free biosensors is that the reporter protein is not contained within cells and thus is amenable to straightforward optimization of the assay readout.[29] Such optimization is necessary for a field-deployable sensor that would be sensitive to ambient conditions and a number of other important operating parameters. Factors selected in this study to optimize visual readout include the volume and concentration of enzyme substrate added to the assay (or CFPS dilution factor) and the time after initiation of the assay that the signal is obtained. Discovering an excellent mathematical representation of the βLac sensor readout (Figure 2), this work next sought to demonstrate a global optimization of the hTRβ biosensor across CFPS dilution factor, substrate concentration, and readout time in order to maximize assay usability and provide practical operating parameters for a field-deployable format. The prohibitive sample space required by a purely experimental optimization approach highlighted the advantages of a mathematical approach confirmed by select experiments. Importantly, the closed-form solution to the Michaelis–Menten equation already showed excellent agreement with the results of the βLac enzymatic assay (Figure 2).

First, biosensor signal was defined as the difference in enzymatic product concentration (hydrolyzed nitrocefin in this case) between ligand and no-ligand sensor reactions (Figure 2B). The optical density obtained by spectrophotometry and an absorbance standard curve provide a quantitatively rigorous readout. Alternatively, an observable color change provides a simpler, more qualitative readout. Notably, these data show that the intensity of the readout signal reaches a short-lived maximum, after which the less active construct expressed without ligand eventually hydrolyzes all of the substrate and the signal disappears (Figure 2E). This observation highlights the significance of the assay read time in obtaining the strongest possible signal for a target ligand concentration. In the case of an on-demand, point-of-care embodiment, the volume and concentration of nitrocefin to add to the CFPS reaction, and the time at which to record an observation, are important assay parameters.

Next, the effect of initial substrate concentration on the assay signal strength was explored, assuming that the amount of cleaved reporter protein between ligand and no-ligand sensor reactions remains fixed. These results indicate that the expected maximum achievable signal strength increases with increasing substrate concentration (Supplementary Figure S7A). Because naked-eye limit of detection (LOD) depends on discernable color differences, greater signal strength yields an improved limit of detection. Accordingly, the reported optimization procedure is expected to improve portable detection by two-fold.

The expected time at which the maximum signal is achieved as a function of both enzyme dilution and substrate concentration was also investigated (Supplementary Figure S7B). Finally, the expected signal strength as a function of time and initial substrate concentration at three different enzyme dilutions was represented experimentally and mathematically. Enzymatic activity of thyroid biosensor expressed in the presence of 0 and 10 μM TRIAC was assessed with either 170 or 340 μM nitrocefin at 50-, 100-, and 200-fold dilution (Figure 3A). Experimental results are consistent with simulated biosensor signal results (Figure 3B). Importantly, increasing nitrocefin concentration from 170 to 340 μM increased the biosensor reporter signal by two-fold.

These results clearly indicate that a higher reporter substrate concentration results in a higher assay signal strength, a two-fold increase in sensor signal strength when 340 μM nitrocefin is used instead of 170 μM. This advantage is only expected to be limited by substrate solubility. Next, these results show that an increased dilution factor increases the time window during which a strong assay signal is readable. A dilution factor of 200× was the most advantageous of those tested experimentally, increasing the readable time window by at least five-fold while only delaying the onset of maximum signal readout by about 5 min. This result indicates that the reaction which expresses the biosensor construct requires only a very small sample size because of the large dilution factors which are required to generate a discernable
readout within a reasonable time window. This feature would be especially advantageous for detection in patient samples at point-of-care.

Guided by these results, an on-demand hTRβ hormone assay was created in which CFPS machinery and energetic molecules were first lyophilized into a test tube. Next, the CFPS reaction was reconstituted with aqueous sample with and without 12.5 μM TRIAC, a concentration selected to achieve maximum signal, and incubated for 1 h. Finally, the CFPS reaction was diluted, and the visible color change was observed after 5 min (Figure 3C). More precise quantification of biosensor signal can be obtained with a portable spectrophotometer.

This approach enabled the creation of an on-demand hormone assay, leveraging the high-throughput nature of CFPS systems to provide rapid readouts. These results may be usefully generalized to other portable cell-free biosensors which rely on conversion of substrate to product for signal readouts of a variety of different analytes in many different sample types. A larger reporter dilution factor yields a longer-lived signal which appears later in the assay but does not improve maximum signal strength. Instead, the highest possible substrate concentration should be used to maximize the sensor signal strength, subject to solubility and instrument or observable saturation constraints. The assay optimization and mechanistic discoveries presented here represent important progress toward RAPID detection of thyroid and estrogen hormones at clinical and environmental concentrations, and further efforts are ongoing.

4 | CONCLUSION

This work highlights the utility of CFPS for studying and optimizing complex protein activities. CFPS, autoradiography, and western blots of the biosensor construct show greater cleaving incidence with higher hormone concentrations in construct expressed with CFPS. This observation suggests that intein catalytic cleaving activity is critical to the construct’s hormone response. Mathematical simulations of enzyme kinetics confirm that cleaved reporter protein contributes significantly to biosensor enzymatic signal. Taken together, these data indicate that a catalytically active intein conformation is critical for hormone-dependent signal generation in both the estrogen and thyroid sensor constructs. Conversely, cell-free biosensor constructs expressed in the absence of ligand lose ligand-dependent signal generation. Similarly, constructs omitting the intein domain yield significantly reduced hormone-dependent signal generation. Additionally, mathematical simulations predict, and experiments confirm that increased substrate concentration and dilution factor improve assay signal strength by two-fold and increase assay signal duration by five-fold, thus increasing naked-eye limit of detection. An on-demand hTRβ hormone assay was created using these design insights. Importantly, these assay optimization principles may be usefully generalized to other portable biosensors, which rely on conversion of substrate to product for signal readout. Furthermore, the on-demand test reported herein is an important step toward rapid and field-deployable biosensors of EDCs, therapeutic receptor modulators, and natural hormones.

ACKNOWLEDGEMENTS

The authors thank Professor Dario Mizrachi for the kind gift of primary antibody for western blot. This research was funded by the National Science Foundation (grant number: 1254148) and by the Simmons Center for Cancer Research at Brigham Young University.

CONFLICTS OF INTEREST

J.P.H., D.W.W., and B.C.B. have filed a patent application regarding cell-free methods for detecting bioactive ligands. All other authors have no other conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Bradley C. Bundy https://orcid.org/0000-0003-4438-183X

REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.