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Honors Thesis

THE REGULATION OF ATG9A-MEDIATED
AGGREPHAGY BY AN ULK1-INDEPENDENT ATG13-
ATG101 COMPLEX

by
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Submitted to Brigham Young University in partial fulfillment of
graduation requirements for University Honors

The Department of Chemistry and Biochemistry
Brigham Young University
June 2022

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ABSTRACT

THE REGULATION OF ATG9A-MEDIATED AGGREPHAGY BY AN ULK1- INDEPENDENT ATG13-ATG101 COMPLEX

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Department of Chemistry and Biochemistry

Bachelor of Science

Aggrephagy, a type of autophagy, is an essential cellular process by which protein aggregates are collected and broken down in the lysosome. Protein aggregates are implicated in several diseases including Alzheimer's disease, diabetes, and cancer. Here, we investigate the ATG13-ATG101 protein complex, a sub-complex of the canonical ULK1 complex whose regulatory role in aggrephagy is not completely understood. We also develop a protein fragment complementation (PFC) assay using the biotin ligase TurboID to study the functions of the ATG13-ATG101 complex with increased specificity. We demonstrate that ATG13 is required for optimal degradation of p62-ubiquitin condensates. We also show that a lack of ATG13 expression causes a deficiency in autophagic flux as indicated by the accumulation of ATG9A positive p62-ubiquitin condensates. This function is mediated by ATG13 and requires ATG101, suggesting that this is a phenomenon of ATG13 and ATG101 in complex. Lastly, we use the split TurboID PFC assay to observe the interaction between the whole ATG13-ATG101 complex and ATG9A using an ULK1 non-binding mutant of ATG13, validating the existence of an ATG13-ATG101 complex with functions independent of the ULK1 complex.

ACKNOWLEDGMENTS

I would first like to thank my advisor Dr. Josh Andersen for his invaluable guidance, support, and trust. Dr. Andersen has allowed me to work on impactful projects that have taught me valuable skills both in the lab and in life. I am confident that any amount of success I attain in the scientific community will ultimately be the product of training in his lab. In addition, I would like to acknowledge the contributions of my thesis committee members Dr. J.C. Price and Dr. Wally Paxton.

I would like to acknowledge the graduate students with which I have spent hours learning, practicing, and deliberating about projects, Dr. Ashari Kanannagara and Dr. Daniel Poole. They have proven to be indispensable in my training as a scientist, and I am forever grateful for the bench skills they have taught to me. Additionally, I would like to acknowledge the undergraduates who have both contributed much to this project and have proved to be life-long friends, Riley Lewis and Brian Lewis.

Lastly, I would like to thank my wife Emily and my parents Craig and Tammy for all the support they have provided in the completion of this thesis. From emotional support to picking me up late at night from the lab to being a listening ear when things go wrong, they have been an integral part of the completion of this work, and I am indebted to them and grateful for them.

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INTRODUCTION

The aggregation of proteins is, to one extent, essential, and to another extent, greatly deleterious to the viability of the cell. In recent years, it has been shown that the localized concentration of proteins into phase separated condensates through a process termed Liquid-Liquid Phase Separation (LLPS) is of functional value to the cell.¹ Such condensates as the nucleolus, P bodies, and stress granules act to localize and transiently compartmentalize functions necessary to the cell.^{2, 3} Aberrant protein aggregation and condensation is harmful to the cell and is a primary cause of many diseases including Alzheimer's disease, diabetes, sickle cell anemia, and, as is being increasingly discovered, certain types of cancer.^{1, 4}

One method by which aberrant protein aggregates are degraded is selective autophagy or aggrephagy, a specific type of the cellular recycling pathway autophagy. This role of aggrephagy implicates this complex pathway in the menagerie of protein aggregate diseases, principle of which in this investigation is cancer. The relationship of aggrephagy with cancer is nuanced, neither being wholly supportive of nor wholly deleterious to oncogenesis or tumor proliferation. Understanding the mechanism of aggrephagy will afford researchers and clinicians a better knowledge of how to target and treat cancers in which aggrephagy is implicated.

Aggrephagy begins with the ubiquitination of protein aggregates, closely followed by binding of the adaptor protein p62 to those ubiquitin chains and the

formation of p62 condensates through LLPS.⁵ Autophagy machinery is then recruited to this site of p62 condensation to form and elongate a double-layered lipid structure known as the autophagosome. After the autophagosome has fully encapsulated the original debris, it is trafficked to the lysosome for degradation.

Autophagy has traditionally been studied in the context of metabolic stress, and more specifically in the context of energy deprivation, which can arise from both hypoxia and nutrient deprivation. As ADP and AMP levels rise in the cytosol, AMP-activated protein kinase (AMPK) is activated by phosphorylation.⁶ Activated AMPK performs two compound functions in the activation of autophagy. First, AMPK inhibits the protein complex mTORC1. mTORC1 inhibits autophagy by phosphorylating ULK1/2. Second, AMPK directly phosphorylates ULK1/2, activating it.⁷

Activated ULK1/2 phosphorylates several substrates. These include all three members of the ULK1 initiatory complex: FIP200, ATG13, and ATG101. In addition to ULK1 complex members, ULK1 phosphorylates BECLIN-1, VPS34, ATG14L, and ATG4B, increasing lipidation and integration of LC3-II into the elongating autophagosome.^{8, 9}

LC3 is a protein responsible for anchoring the autophagosome to cargo by binding to adaptor proteins such as p62. LC3 must go through two major changes to be incorporated into the membrane of the autophagosome. First, ATG4 cleaves the N-terminus of LC3, generating LC3-I. Next, in a manner similar to the conjugation of ubiquitin to proteins, LC3-I is conjugated to PI3P by several proteins including ATG7, ATG3, and the heterotrimer ATG12/ATG16L/ATG5.

An additional substrate of ULK1 is ATG9A, which is a transmembrane vesicle protein implicated in the elongation of the autophagosome and regulation of autophagy.¹⁰ Guardia et al. found that ATG9A exists primarily as a trimer, the protomers of which exist in a dynamic confirmation.¹¹ Matoba et al. discovered that ATG9A has lipid scramblase activity which is mediated by a domain-dependent conformational change in the ATG9A trimer. Additionally, they discovered that ATG9A positive liposomes contain phosphorylated lipids on both leaflets compared to ATG9A-negative liposomes, which only contain phospholipids on the outer leaflet.¹² Because PI3P is required for LC3 lipidation and cargo anchoring, ATG9A may play an essential role in providing PI3P to the inner leaflet of the autophagosome during elongation. These data strengthen the assertion that ATG9A is an essential regulator of autophagy.

The relationship between ATG9A and the ULK1 complex is poorly understood. Early studies by Zhou et al. show that ULK1 directly phosphorylates ATG9A at Serine 14. This phosphorylation event is required for ULK1-mediated redistribution of ATG9A to endosomes under conditions of metabolic stress.¹⁰ Thus, under conditions of metabolic stress, direct interaction between ULK1 and ATG9A is required for the initiation of autophagy. These data suggest that ULK1 has an important role in initiating ATG9A-mediated autophagy; however, little is known about the function of the other members of the ULK1 complex.

FIP200, the largest protein of the ULK1 complex by molecular weight, has multiple reported functions. It was first characterized as a regulator of retinoblastoma 1 (Rb1), hence its original name, Rb1-inducible Coiled-Coil 1

(RB1CC1). Chano et al., the group that discovered this protein, found that RB1CC1 localized to the nucleus and that expression levels of Rb1 in a variety of cell types are positively correlated to RB1CC1 expression levels.¹³ This same group later confirmed through ChIP-Seq and DNA foot-printing that RB1CC1 binds the Rb1 promoter to drive Rb1 expression.¹⁴ The Focal Adhesion Kinase Interacting Protein of 200kD (FIP200) was discovered independently of the discovery of RB1CC1, and in the context of autophagy instead of transcription regulation.¹⁵

FIP200 is an obligate interactor of ULK1 in the induction of autophagy. Hara et al. discovered that, in FIP200^{-/-} MEF cells, LC3-II formation, a marker of autophagic flux, is markedly decreased after starvation compared to WT MEF cells.¹⁵ Additionally, Wei et al. were able to suppress autophagy by knocking out FIP200 expression in mice, though they do not delineate whether this autophagy is initiated by the ULK1 complex.¹⁶

The other two members of the ULK1 complex are ATG13 and ATG101. ULK1 phosphorylation of FIP200 is mediated by ATG13, and LC3-II formation is stalled in cells lacking either ATG13 or ATG101 expression. This suggests that both ATG13 and ATG101 play an important, though unknown role in the progression of autophagy. Recent studies by Kananngara and Poole et al. show that ATG9A interacts with all four members of the ULK1 complex, but that ATG9A also interacts with ATG13 and ATG101 when a ULK1 non-binding mutant of ATG13 is introduced.¹⁷ These data suggest that ATG13 and ATG101 may interact with ATG9A in a ULK1-independent and ATG13-mediated manner,

which suggests an ATG13-ATG101 complex that interacts with ATG9A. The role of a putative ATG13-ATG101 complex remains unknown.

In the specific context of aggrephagy, the ULK1 complex has not been implicated except in the context of metabolic stress.¹⁸ Otherwise, the induction and regulation of aggrephagy is not well understood. Kanannagara and Poole et al. have shown that colocalization of ATG9A with p62 dramatically increases when ATG13 expression is knocked out.¹⁷ This suggests some function of ATG13 in the recruitment of autophagy machinery to the site of autophagophore formation, which is not well characterized. This project aims to understand the molecular mechanism of aggrephagy and is centered on characterizing the function of ATG13 and ATG101 in ATG9A-mediated aggrephagy.

Here we demonstrate that ATG13 regulates ATG9A-mediated aggrephagy in an ATG101-dependent manner. We also demonstrate that this regulatory function is carried out independent of the ULK1 complex, suggesting that aggrephagy may be stress signal-independent in some cases. Additionally, we show that ATG13 is required for p62 degradation at a basal level. Lastly, we have developed a novel protein fragment complementation (PFC) assay using the biotin ligase TurboID to study the active ATG13-ATG101 complex directly and distinguish its functions from those of ATG13 or ATG101 independently. Using this assay, we have shown that ATG9A interacts with ATG13 and ATG101 in a complete complex, independent of ULK1 and FIP200.

METHODS

Cell Culture

Hct116 cells were split down to 20% confluency every 2-3 days with 0.05% trypsin and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/Streptomycin solution, hereafter called complete DMEM.

Lentiviral Transduction

Lentivirus was generated with LentiX-293T cells. First, LentiX-293T cells were seeded at 20% confluency, then transfected with the appropriate transgene, viral packaging genes (psPAX2), and viral envelope genes (pMD2.G) in a 4:2:1 DNA ratio. DNA was added to 300 μ L of serum-free DMEM while 56 μ g of the transfection reagent PEI Max was added to an additional 300 μ L of serum-free DMEM. These solutions were combined, vortexed, and allowed to incubate at room temperature for 10 minutes. After incubation, the transfection solution was diluted in 5mL of complete DMEM and added to cells for 8-12 hours in an incubator at 37°C and 5% CO₂. After incubation, the transfection media was replaced with 10mL of complete DMEM and allowed to incubate for an additional 24 hours. Virus was collected at 24- and 48-hours post-transfection. Virus was collected by filtering the cellular supernatant through a Steriflip 0.22 μ m vacuum filter. To produce cells stably expressing the Split TurboID

system, lentiviral transductions were performed on Hct116 cells in which ATG13 and ATG101 expression had been abolished and in which an HA tag had been added to endogenous ATG9A via the CRISPR-Cas9 system. Hct116 HA KI ATG13/ATG101 Double KO cells were infected for 8-16 hours with 4-5mL of TurboID(C)-ATG13-Myc and TurboID(N)-ATG101-FLAG lentivirus solution supplemented with polybrene to a final concentration of 10µg/mL. After infection, cells were allowed to recover for 24 hours before selection. Cells were selected with 3µg/mL of puromycin for at least 7 days before isolating monoclonal populations. After transduction, monoclonal populations were isolated via limited dilution methods.

Split TurboID Protein Fragment Complementation Assay

To perform the protein fragment complementation assay with TurboID, cells expressing ATG13/ATG101 Split TurboID were incubated with 50µM biotin in 5mL of complete DMEM for 10 minutes at 37°C and 5% CO₂. Cells were then harvested and lysed with ATG9 lysis buffer. Cell lysates were then used in immunoblot and immunoprecipitation experiments.

Immunoblotting and Immunoprecipitation

To perform immunoprecipitation of biotinylated proteins, protein levels of cell lysates in which proteins had been labeled with biotin were quantified using the

Bio-Rad DC protein assay. These lysates were then incubated with streptavidin-conjugated agarose beads at 4°C with gentle rotation for 1-2 hours. Beads were washed three times with ATG9A lysis buffer before being incubated for 5 minutes at 95°C in 1X SDS running buffer. After pelleting the beads, the supernatant, containing biotinylated proteins, was loaded in a well of an SDS-PAGE gel. SDS-PAGE was then performed at 150V for 1 hour.

After SDS-PAGE, proteins were transferred to a membrane using the iBlot Dry-transfer system. The membrane was then blocked in a 5% (w/v) fat-free milk solution in PBS-Tween for 1 hour. After blocking, the membrane was then washed twice with PBS-T and once with PBS before being incubated overnight with either the appropriate primary antibody or with a solution of fluorescently labeled streptavidin at 4°C with gentle rocking. The next day, the membrane was again washed twice with PBS-T and once with PBS before incubating with appropriate infrared fluorescent secondary antibody for 1 hour at room temperature. If blotting with streptavidin, blots were immediately imaged after overnight incubation. Finally, the membrane was washed once in PBS-T and twice with PBS and then imaged using the Li-Cor Odyssey imaging system.

RESULTS AND DISCUSSION

The loss of ATG13 or ATG101 results in an accumulation of p62 into large condensates that colocalize with ATG9A

To determine the function of the ATG13 and ATG101 complex in autophagic flux, we knocked out expression of ATG13, ATG101, ATG9A, and FIP200 in Hct116 cells in which an HA affinity tag was knocked in via CRISPR on the C-terminal end of ATG9A (hereafter called Hct116 HA KI cells). This experiment was done with and without bafilomycin treatment. Bafilomycin is a V-ATPase inhibitor that inhibits autophagic flux. Samples in which cells were treated with bafilomycin represent a positive control for comparison of deficiencies in autophagic flux. Autophagic flux was then evaluated by observing levels of p62 by Western blot.

We observed that p62 builds up in untreated cells at levels similar to those of the bafilomycin treated cells when ATG13 is knocked out. Additionally, when ATG101 is knocked out of untreated cells, p62 also builds up significantly compared to wild-type levels. This buildup of p62 is significantly reduced in untreated cells in which either ATG9A or FIP200 are not expressed (Figure 1D). Additionally, p62 levels are significantly higher in ATG13KO cells and ATG101 KO cells that have been treated with bafilomycin when compared to treated WT cells. This suggests that ATG13 and ATG101 exacerbate the effects of bafilomycin, indicating a possible role of these proteins in the regulation of late-stage autophagy including docking of the autophagosome and eventual degradation of cargo.

Imaging these cells by confocal microscopy, we observed that ATG9A colocalizes with these accumulations of p62 in ATG13 KO and ATG101 KO cells. In these images, p62 accumulates in distinct, large puncta in ATG13 and ATG101 KO cells, while remaining diffuse and in comparatively small puncta in WT cells. Additionally, ATG9A does not colocalize with p62 in FIP200KO cells significantly more than in wild-type cells (Figure 1A-C). These data suggest that ATG13 and ATG101 play a significant role in promoting autophagic flux, whereas FIP200 has little observable effect.

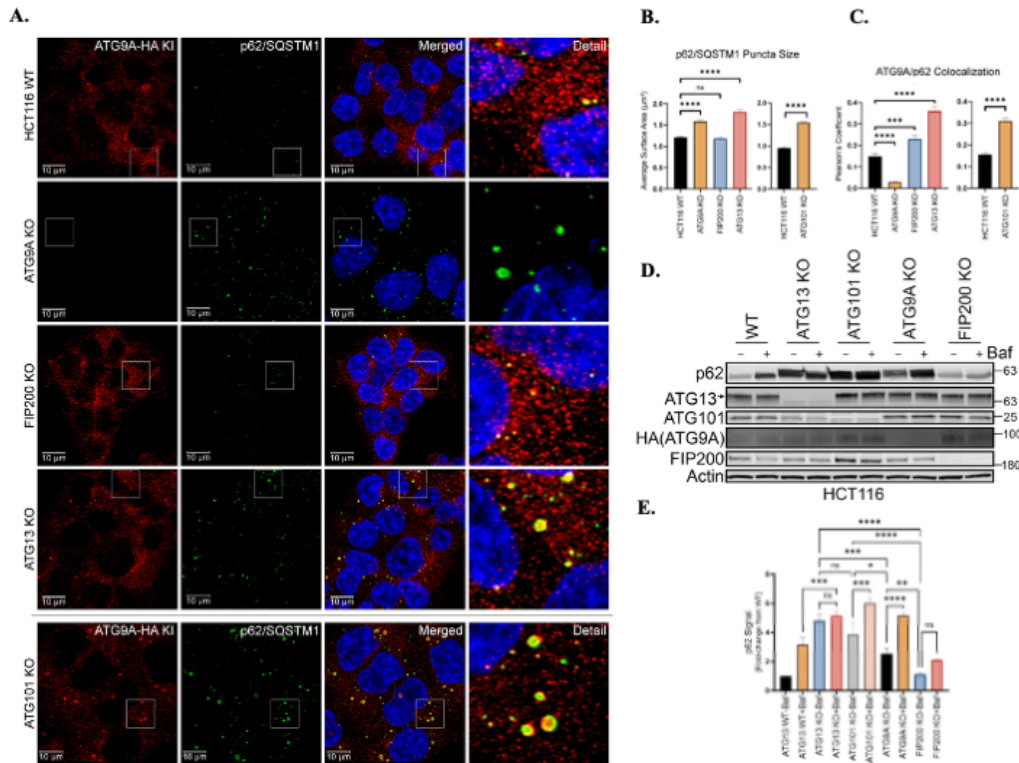


FIGURE 1. The loss of ATG13 or ATG101 results in an accumulation of p62 into large condensates that colocalize with ATG9A. (A) Confocal images of ATG9A colocalization with p62 in indicated CRISPR KO cells. (B) Quantification of average surface area of p62 puncta. (C) Quantification of ATG9A colocalization with p62. (D) Western blot showing endogenous p62 levels in CRISPR KO clones treated with or without 100 nM Bafilomycin for 24 hrs. (E) Quantification of p62 signal in D.

ATG13-mediated rescue of ATG9A accumulation at p62 condensates requires the ATG13 HORMA domain but is independent of ULK1 binding domain

To confirm that the observed colocalization of ATG9A with large p62 condensates is affected by ATG13 and ATG101 independent of ULK1, we generated non-binding mutations of ATG13. We generated a ULK1 non-binding mutant of ATG13 called ATG13 Δ 2AA and an ATG101 non-binding mutant of ATG13 called ATG13 Δ HORMA. ATG13 expression was abolished in Hct116 HA KI cells. These cells were then transduced with lentivirus containing either WT ATG13, ATG13 Δ 2AA, or ATG13 Δ HORMA transgenes. We then analyzed these cells by confocal microscopy.

We observed a similar pattern in both ATG9A colocalization with p62 puncta and p62 puncta size. Depleting cells of ATG13 expression causes a significant increase in colocalization between ATG9A and p62 puncta. The addition of either wild-type ATG13 or ATG13 Δ 2AA brings both ATG9A colocalization with p62 puncta and p62 puncta size down to wild-type levels. When ATG13 Δ HORMA is stably added back to ATG13 KO cells, ATG9A colocalization with p62 and p62 puncta size return to levels similar to those of ATG13 KO cells. With these data we show that ATG13-mediated promotion of autophagic flux is independent of ULK1 and requires the interaction of ATG13 and ATG101 (Figure 2).

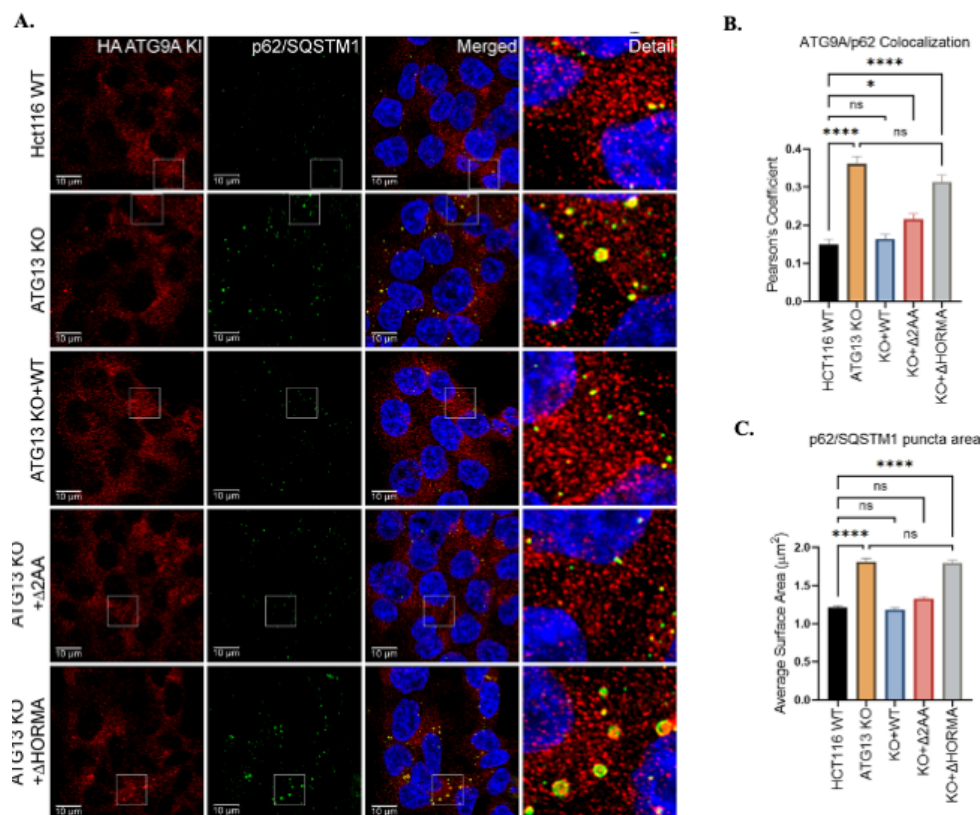


FIGURE 2. ATG13-mediated rescue of ATG9A accumulation at p62 condensates requires the ATG13 HORMA domain but is independent of the ULK1 binding domain. (A) Confocal images of ATG9A colocalization with p62 in HCT-116 ATG9A-HA KI- ATG13 WT, ATG13 KO or ATG13 KO cells reconstituted with ATG13 WT, ATG13 Δ2AA and ATG13 ΔHORMA. (B) Quantification of ATG9A colocalization with p62 in A. (C) Quantification of p62 puncta surface area in A.

The loss of ATG13 causes impaired degradation of p62 and an accumulation of ubiquitin at p62 condensates

To address the question of how ATG13 affects p62 stability, we performed a pulse chase experiment using ATG9A-BirA-HA. In this experiment, cells stably expressing ATG9A-BirA-HA either expressed (ATG13 WT) or did not express (ATG13 KO) ATG13. These cells were pulsed with biotin for 12 hours, harvested at six-hour intervals, and analyzed by Western Blot. We observed that p62 initially accumulates in ATG13 KO cells compared to ATG13

WT cells. Following this initial accumulation, degradation of p62 is slowed compared to ATG13 WT cells. These data show that ATG13 plays a role in promoting the degradation of p62, consistent with previously shown data that suggests a role of ATG13 in late-stage aggrephagy.

To ensure that these p62 accumulations are in fact condensates targeted by aggrephagy, we analyzed ATG13 WT and ATG13 KO cells by confocal microscopy. We found that p62 colocalizes significantly more with ubiquitin in ATG13 KO cells compared to wild-type cells. This indicates that the lack of ATG13 expression impairs p62 degradation and causes increased concentration of ubiquitin at p62 condensates.

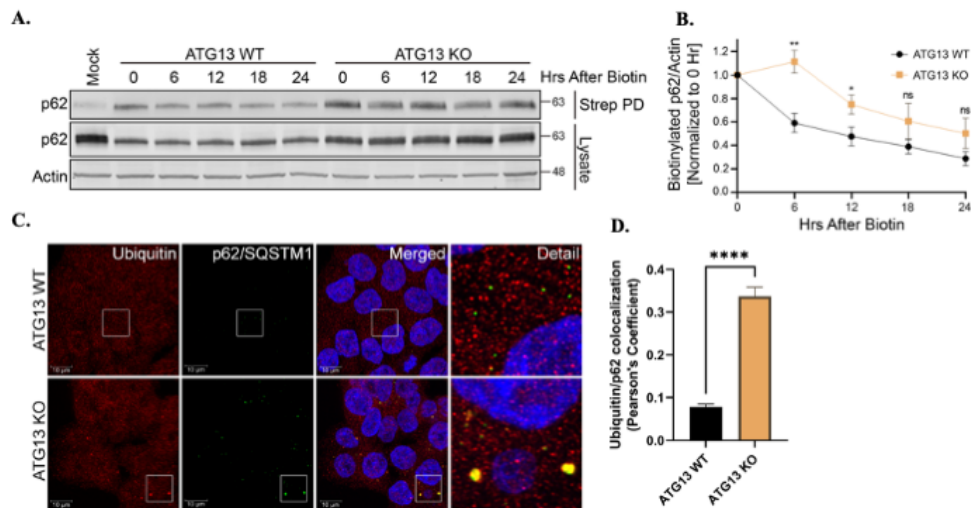


FIGURE 3. The loss of ATG13 causes an impaired degradation of p62 and an accumulation of ubiquitin at p62 condensates. (A) HA-ATG9A-BirA was expressed in HCT-116 ATG13 WT and KO cells. Cells were grown in full DMEM media, treated with 50 μ M biotin for 12 hours, washed in PBS, and incubated in full media for time indicated. Cells were then lysed and incubated with streptavidin resin before immunoblotting with indicated antibodies. (B) Quantification of biotinylated p62 in A. (C) Representative images of p62 colocalization with ubiquitin. (D) Quantification of Ubiquitin/p62 colocalization.

Split TurboID is a functional protein fragment complementation assay which can be used to study the ATG13-ATG101 complex

While it is relatively straightforward to study the behavior of single proteins through conventional means such as Western blot and confocal microscopy, it is quite difficult to study the behavior and functions of a heterodimer. One major limitation in our current data is the inability to confidently assign function to the ATG13-ATG101 complex when we only study the effects of manipulation to one or the other protein. It is known that ATG9A interacts with ATG13 and ATG101 through ATG13; however, the interaction of ATG9A with an ATG13-ATG101 complex is impossible to observe explicitly using current methods. Additionally, it is impossible to distinguish the phenotype of p62 accumulation or ATG9A colocalization with p62 condensates as being regulated by ATG13 alone or in active complex with ATG101. Data gathered from cells in which the ATG13 Δ HORMA mutant has been stably added back only indicate that interaction between ATG13 and ATG101 is required at some point for p62 accumulation and ATG9A-p62 colocalization to return to wild-type levels. It is difficult for these data to distinguish an active ATG13-ATG101 complex from a transient, though obligate ATG13-ATG101 interaction.

To explicitly study the ATG13-ATG101 complex, we developed the split TurboID protein fragment complementation assay for ATG13 and ATG101. This assay, based on the split TurboID protein developed by the Dr. Alice Ting lab at Sandford University takes advantage of the ability of TurboID, a biotin ligase, to

regain functional tertiary structure after being recombinantly split in half.¹⁹ In this assay, the C-terminal half of TurboID is cloned onto the N-terminus of ATG13-Myc, while the N-terminal half of TurboID is cloned onto the N-terminus of ATG101-FLAG. When these two constructs are co-expressed in cells, ATG13 and ATG101 are free to interact in their complex. Upon interaction, the two free halves of TurboID come together and assume native tertiary structure and TurboID regains its biotin ligase activity. Proteins that are in the proximity of the ATG13-ATG101 complex are biotinylated and can be analyzed with a variety of methods including Western blot and mass spectrometry. The great advantage of this system is its specificity. Each half of TurboID on its own lacks biotin ligase activity, meaning that proteins are only biotinylated when they interact with the active ATG13-ATG101 complex. Additionally, the two halves of split TurboID do not have enough affinity for each other to reconstitute on their own. They require two interacting chaperones such as ATG13 and ATG101. Using this assay, we can now distinguish between the active complex and transient interaction (Figure 4A).

To validate this assay, we transiently co-expressed TurboID(C)-ATG13-Myc with TurboID(N)-ATG101-FLAG as well as TurboID(C)-ATG13 Δ 2AA-Myc with TurboID(N)-ATG101-FLAG in HEK293 cells. Before harvesting, these cells were spiked with 5 mL of 50 μ M biotin in complete DMEM and then allowed to incubate for 10 minutes. These cells were then analyzed by Western blot using a solution of fluorescently labeled streptavidin. We observed a streak of biotinylated proteins across all molecular weights similar to that found in a full

length TurboID control (Figure 4B). This is evidence that the split TurboID PFC assay is functional.

The ATG13-ATG101 complex is directly observed to interact with ATG9A

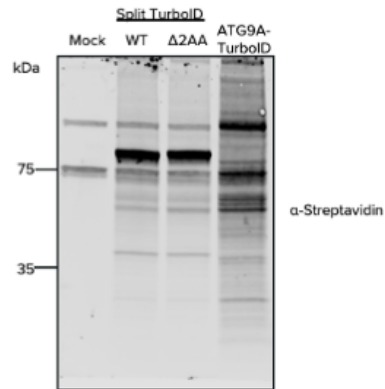
To observe the interaction of the independent ATG13-ATG101 complex, a panel of TurboID(C)-ATG13 mutants were co-expressed with TurboID(N)-ATG101-FLAG in Hct116 HA KI cells. We then performed a streptavidin immunoprecipitation to isolate biotinylated proteins and analyzed these proteins by Western blot.

ATG9A is biotinylated by the split TurboID ATG13-ATG101 system independent of both ULK1 and FIP200. Additionally, ATG9A is not biotinylated when an ATG9A non-binding mutant of ATG13 (ATG13 Δ ATG9A BD) is expressed (Figure 4C). This suggests that the interaction between ATG9A and the ATG13-ATG101 complex is completely independent of the canonical ULK1 complex and carried out through the ATG9 binding domain housed within the HORMA domain of ATG13.

A. Identifying interacting partners of the ATG13-ATG101 dimer



B.



C.

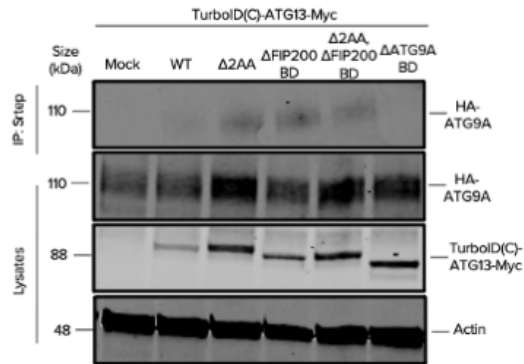


FIGURE 4. Use of the split TurboID protein fragment complementation assay to show that ATG9A interacts with the ATG13-ATG101 complex independent of ULK1 and FIP200. (A) Split TurboID allows for the purification and subsequent analysis of proteins biotinylated by the ATG13-ATG101 complex and not by ATG13 or ATG101 alone. (B) Validation of the Split TurboID system via Western Blot. HEK293T cells were transfected with either the Split TurboID system or ATG9A-TurboID and treated with 50 μ M biotin for 10 minutes. (C) Split TurboID system was expressed in Hct116 HA KI cells using indicated mutants of ATG13 (BD - binding domain). ATG9A interacts with the ATG13-ATG101 subcomplex independent of ULK1 and/or FIP200 as indicated by biotinylation of ATG9A. Binding between the ATG13-ATG101 complex and ATG9A requires the ATG9A binding domain of ATG13.

CONCLUSIONS AND FUTURE WORK

Our data support a model in which ATG13 and ATG101 act together in a complex independent of the canonical ULK1 initiatory complex. We show that this independent ATG13-ATG101 complex interacts with ATG9A (Figure 4C). We also show that the absence of ATG13 expression causes a build-up of p62-ubiquitin condensates that colocalize with ATG9A (Figure 3C-D). Lastly, we show that ATG13 is required for optimal degradation of p62 (Figure 3A-B). These data suggest a role of the ATG13-ATG101 complex in which the ATG13-ATG101 complex interacts with ATG9A to promote ATG9A-mediated aggrephagy.

The mechanism by which the ATG13-ATG101 complex regulates aggrephagy remains uncharacterized and represents a fascinating avenue for future investigation. We show that the ATG13-ATG101 complex has a role in promoting the degradation of p62 (Figure 3A-B), implicating the complex in late-stage aggrephagy. ATG13 and ATG101 are also necessary components in the ULK1 initiatory complex, suggesting that the ATG13-ATG101 may have a role in the initiation of aggrephagy. Further experimentation is required to fully elucidate the mechanism of aggrephagy regulation by the ATG13-ATG101 complex.

Our data show that ATG9A colocalizes with p62 condensates independent of ATG13 (Figure 1, Figure 2). In fact, ATG9A colocalization with p62 is dramatically increased in the absence of ATG13. Additionally, we show that ATG9A colocalizes with p62 independent of FIP200 (Figure 1A,C). This suggests the possibility of a mechanism whereby ATG9A is recruited to p62-ubiquitin

condensates independent of the canonical ULK1 complex. Already, Agudo-Canlejo et al. show that the biophysical properties of p62 condensates support the remodeling of lipid membranes, suggesting that p62 condensates themselves may regulate aggrephagy induction and recruitment of ATG9A vesicles.²⁰ The characterization of this mechanism presents an additional avenue of future study.

Here we have characterized a function of ATG13 that is independent of the ULK1 complex. Previous data from Kananngara and Poole et al. along with the data presented here suggest that the ATG13-ATG101 complex performs functions independent of the canonical ULK1 complex, nearly all of which are presently uncharacterized.¹⁷ Using the split TurboID system, we plan to use mass spectrometry proteomics to study the protein interactome of the ATG13-ATG101 complex within and independent of the canonical ULK1 complex. This study will direct future studies of the functions of the ATG13-ATG101 complex independent of ULK1.

In conclusion, this study uncovers a mechanism in which the ATG13-ATG101 complex regulates ATG9A-mediated aggrephagy independent of ULK1. This complex acts to promote the degradation of p62-ubiquitin condensates. Additionally, this study presents the discovery of an ATG13-ATG101 complex, which interacts with ATG9A independent of both ULK1 and FIP200. Lastly, this study describes the development of a protein fragment complementation assay using the biotin ligase TurboID to further study the mechanism of aggrephagy regulation by the ATG13-ATG101 complex. Taken together, the data presented here provide a better understanding of the mechanism of aggrephagy regulation

and aid in the identification of therapeutic targets for diseases in which protein aggregation is a primary etiology.

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