The Distribution of Autophagosomes is Altered by the Phospholipase A2 Inhibitor ONO-RS-082

Honors Thesis

Presented to the College of Arts and Sciences,

Cornell University

in Partial Fulfillment of the Requirements for the

Biological Sciences Honors Program

by

Caroline Jovel

May 2020

Advisor: William J. Brown

Abstract

When cells are starved of essential nutrients, autophagy, a process of self-digestion, is induced for cell survival. Previous studies in mammalian cells have shown that a structurally intact Golgi complex negatively regulates the induction of autophagy. Other studies from the Brown lab have shown that the architectural integrity of the Golgi complex requires the activity of cytoplasmic Phospholipase A_2 (PLA_2) enzymes. For example, inhibitors of PLA_2 enzymes cause the intact Golgi complex to fragment into "mini-stacks." To further investigate the relationship between autophagy induction and Golgi architecture, HeLa cells were treated with the PLA_2 inhibitor ONO-RS-082 (ONO) to disrupt the Golgi, and its effect on autophagy was visualized by imaging the association of the autophagosome marker protein LC3. Normally, LC3-labeled nascent autophagosomes are evenly dispersed throughout the cytoplasm; however, I found that treatment with ONO caused autophagosomes to cluster in the center of the cell. This clustering occurred in both a time- and concentration-dependent manner. This result reveals a previously unknown connection between autophagy and the Golgi complex.

Introduction

Autophagy is the process by which a cell, when starved of essential nutrients, begins digesting parts of itself to provide minimal levels of nutrients for survival. Autophagy is conserved in all eukaryotic organisms, from single-celled yeast to mammals. Yoshinori Ohsumi and colleagues discovered the molecular mechanisms of autophagy with yeast as his model organism (Kirkin, 2019). They found that ubiquitin-like proteins, Atg5, Atg12, and Atg8, influence the formation of the autophagosome, a double membrane vesicle that digests the inside of the cell. By this process, parts of the cytoplasm are sequestered in the double-membrane vesicles. This

autophagic vesicle separates what is to be digested from the rest of the cytoplasm and transports the material to the vacuole of the yeast cell (or lysosomes in higher eukaryotes) to be degraded. Autophagy can be non-selective or selective: in the latter case, its digestive targets are selective, such as aggregated proteins or damaged organelles.

Autophagy has also been suggested to be involved in cancer (White, 2015) and neurodegenerative diseases (Netea-Maier et. al, 2016). Regarding cancer, selective autophagy is not understood thoroughly, but has been suggested to be vital in facilitating the tumor antigen presenting pathways and with "cancer cell-specific protein aggregates." The process can promote cancer by inactivating the tumor suppressor protein, p53, and regulating the metabolic pathways in mitochondria (White, 2015). Cancer cells are more autophagy-dependent than normal cells and this has been suggested to be due to lack of resources in the microenvironment and the increased metabolic demands from rapidly dividing cells (White, 2015). Neurodegenerative diseases have been linked with proposed autophagy defects such as in Parkinson's disease with mislocation of Rab1A-mediated Atg9, which is required for autophagosome formation, and amyotrophic lateral sclerosis (ALS) with mutations in the p62 gene, an autophagic receptor, that could potentially disrupt the clearing of protein aggregates commonly built up within these diseases (Nixon, 2013).

Because the molecular mechanisms of autophagy are not yet fully understood, connections between other cellular processes have been investigated. Membrane vesicle formation occurs from the Golgi complex, and autophagosomes are formed from a double membrane, therefore it has been suggested that the Golgi complex may play some role in autophagosome formation, although its exact role is unclear. A hallmark of autophagosome formation is the recruitment of proteins to membranes that initiate the formation of these newly formed organelles. One such

protein is LC3, which is recruited to nascent autophagosomes very early in the process of induction (Tanida, Isei et al., 2005.) These LC3-labeled autophagosomes are seen as small, punctate structures throughout the cytoplasm in mammalian cells.

Previous studies in mammalian cells have suggested that an intact Golgi complex ribbon negatively regulates autophagy (Gosavi et al., 2018). These authors showed that loss of intact Golgi ribbons resulted in compromised mechanistic target of rapamycin (mTOR) signaling leading to a dramatic increase in LC3-positive autophagosomes. mTor is a negative regulator of autophagy, and therefore any perturbation that results in loss of mTOR signalling will induce autophagy.

Previous studies from the Brown lab have shown that the dynamic maintenance of the steady-state architecture of the mammalian cell Golgi complex requires the activity of cytoplasmic Phospholipase A_2 (PLA_2) enzymes (de Figueiredo et. al, 1999). PLA_2 enzymes were also noted to be important for the formation of the Golgi complex (de Figueiredo et. al, 1998). For example, small molecule inhibitors of cytoplasmic PLA_2 enzymes were found to inhibit the assembly of an intact Golgi complex and to also induce the fragmentation of an intact Golgi complex into many smaller “mini-Golgis” (for reviews see Bechler et al., 2012; Ha et al., 2012). Indeed, a specific PLA_2 enzyme, platelet-activating factor acetylhydrolase IB (PAFAHIB) was discovered to be important for maintaining an intact Golgi complex (Bechler et al., 2010)

Currently, it is unknown how or why PLA_2 regulates the Golgi complex. Enzymatic activity of a PLA_2 is to hydrolyze the fatty acid of the SN_2 position of membrane phospholipids to generate a lysophospholipid and a free fatty acid. Previous work from the Brown lab has shown that activity of cytoplasmic PLA_2 enzymes is important for the formation of membrane tubules that appear to

link physical distinct Golgi stacks into one large, continuous ribbon. Thus, PLA_2 inhibitors would prevent the formation of these membrane tubules resulting in fragmentation of the golgi complex into separate mini-stacks.

Thus, the goal of this research is to confirm and extend previous studies by determining whether or not the formation of autophagosomes, as measured by LC3 recruitment, is dependent on an intact Golgi complex using PLA_2 inhibitors to fragment the Golgi complex.

Methods

HeLa Cell Culturing and Experimental Treatments

HeLa cells were cultured in a sterile, humid, 37℃ environment within a ventilated flask in Minimal Essential Media (MEM) supplemented with 10% NuSerum (MEM + NuSerum). When the cells were divided into separate flasks, they were washed with Puck's solution and treated with trypsin for about 30 seconds to dislodge the cells from the bottom of the ventilated flask. The cells were resuspended in MEM + NuSerum and placed into a new flask for standard growth conditions. For experimental treatments, cells were plated onto glass, microscope slide coverslips in 60 mm dishes. To induce autophagy, HeLa cells were incubated with MEM (no Nu-Serum) to starve the cells of nutrients or MEM and the PLA_2 inhibitor, ONO-RS-082 (ONO), in different concentrations to also fragment the Golgi complex.

Autophagosome and Golgi Complex Staining by Immunofluorescence

Autophagosome formation was detected by immunofluorescence using antibodies against LC3, and Golgi fragmentation was induced with a small molecular inhibitor of cytoplasmic PLA_2 enzymes ONO. Two types of experiments were conducted: concentration-dependence and time-course experiments. For the concentration-dependence experiments, cells were treated with 0, 5, 10, and 20 μM ONO. The four treatment groups each included two coverslips of HeLa cells. They were all treated for 30 min. Based on previous experiments conducted at the lab, at 30 min of autophagy induction, a significant enough difference could be seen to compare autophagosome dispersion. This experiment was repeated three times.

For the time-course experiments, HeLa cells were treated with 20 μM ONO at various time points for up to 15 min. This experiment was repeated a total of 3 times. A second time-course experiment included 7 groups of treated HeLa cells. There was a control group of cells that had not been treated at all, and cells incubated with MEM for up to 1 h. The experimental groups were cells treated with 20 μM of ONO for up to 1 h. This experiment was repeated 3 times. The experiments were stopped by placing cells in 3.7% formalin in Phosphate-buffered saline (PBS) fixative.

To stain both the autophagosomes and the Golgi complex, the coverslips containing the cells were submerged in fixative for 10 min using a fixing rack and washed three times with PBS for 5 min each. Subsequently, the coverslips were submerged in 0.01% saponin in PBS (PBSap) for 10 min to permeabilize cells before the primary antibody treatment. All subsequent antibody incubations/dilutions and washing steps were carried out using PBSap. The cells were incubated with a solution of 1:200, primary rabbit antibody, which labels autophagosomes, and 1:50, primary mouse antibody, anti-Giantin, which labels the Golgi complex, for 1 h. The cells were washed again three times with PBSap for 5 min each and then incubated with the secondary

antibodies in a solution of 1:500, anti-rabbit antibody-Daylight 488 labeled, and 1:500 anti-mouse antibody-Alexa Fluor 555-labeled, for 1 h. The cells were washed three times, each for 5 min, for the final time. The coverslips were then ultimately fixed to microscope slides under 5 μL of Prolong and allowed to air dry overnight.

Cell Imaging

Cells were imaged on a Axioscope II (Carl Zeiss, Inc.) with Plan-Apo 40x/NA1.4 air objective mounted to a Orca II camera (Hamamatsu Photonics), utilizing Openlab software (PerkinElmer). The contrast and brightness of the pictures were manipulated uniformly in each experiment with the FIJI computer software to better view the LC3 puncta.

Autophagosome Distribution Analysis

To quantify the distribution and clustering of the LC3 puncta within cells, the distance between the two furthest LC3 puncta (in pixels) was determined in 50 cells per treatment and control group in every experiment, which we call the Clustering Index. Once the data was extracted, the differences in the Clustering Index of LC3 puncta were averaged and compared between experiments and expressed as the percent change in Clustering Index from the control group to further normalize the data.

Results and Discussion

To determine if an intact Golgi complex affects the formation of autophagosomes, cells were treated with the PLA_2 inhibitor ONO, which is well documented to cause the fragmentation of the Golgi complex into separate mini-stacks (de Figueiredo, P et al., 1999). Then, newly formed autophagosomes were visualized by immunofluorescence of LC3, a protein that is recruited from

the cytoplasm to nascent autophagosomes. Consistent with the previous studies with ONO, I found that treatment of cells with 20 μM ONO for 30 min caused the Golgi to fragment into separate puncta as shown by immunofluorescence staining of the Golgi marker protein giantin (Fig. 1).

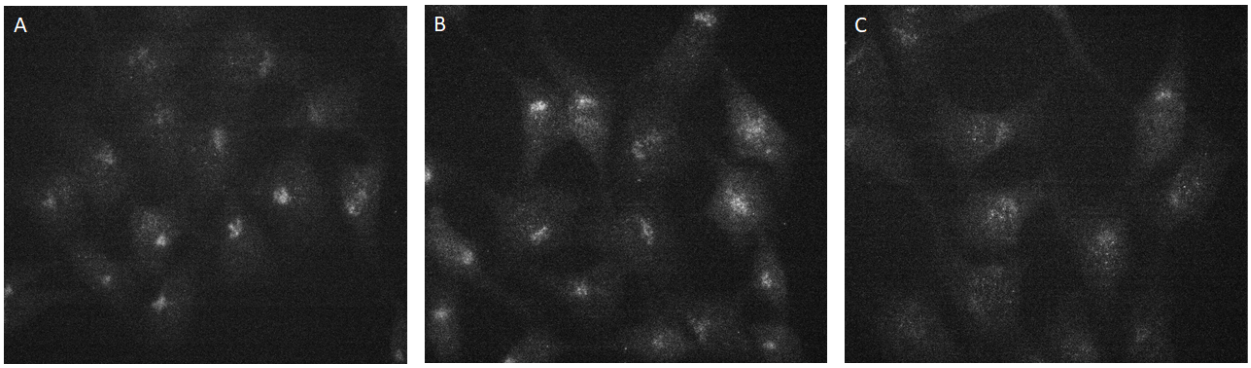

Figure 1. ONO Concentration Dependence and Giantin-labeled Golgi Complex Integrity. Cells that were treated with 0 (A), 5 (B), and 20 (C) μM of ONO for 30 min.

ONO Treatment Time Courses

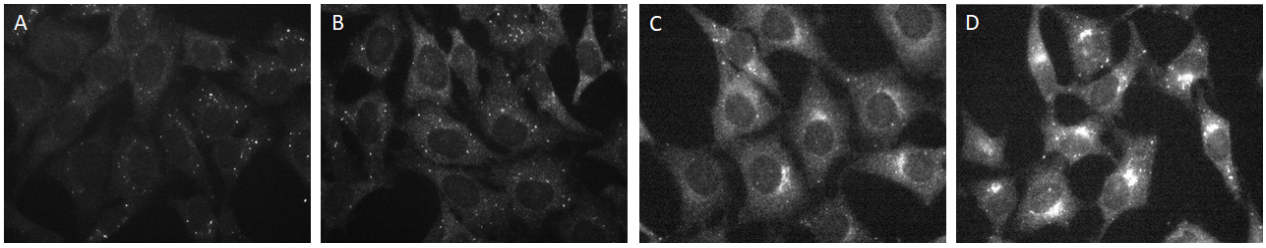

Figure 2. Cells in Regular Growth Media (MEM + Serum) for 15 min (A) and ONO Treated Cells in just MEM for 5 (B), 10 (C), and 15 min (D). Cells were labeled by immunofluorescence to show the distribution of LC3-labeled autophagosomes.

Based on these preliminary experiments, which determined that Golgi complexes were fragmented by 30 min in ONO, I conducted two sets of time-course experiments. In the first, I simply shifted cells to MEM containing ONO (20 μM) for various periods of time. Autophagosomes are induced when cells are starved for essential nutrients, but even in normal cell culture conditions (MEM plus serum supplement) HeLa cells exhibit a basal level of

autophagosomes as indicated by small LC3-positive puncta evenly distributed throughout the cytoplasm (Fig. 2A). After shifting cells to MEM plus ONO, a modest induction of autophagy was observed as autophagosomes became larger and brighter (Fig. 2B-D). This result is consistent with previous studies showing that fragmentation of the Golgi induces autophagy (Gosavi et al., 2018). Surprisingly, I also observed that ONO treatment dramatically altered the distribution of LC3-positive autophagosomes, which became increasingly clustered in the center of the cell (Fig. 2B-D). These results were quantified by measuring the distance between the furthest LC3-positive puncta in each cell, i.e., the clustering of autophagosomes. These analyses were consistent with the qualitative observations and revealed a statistically significant reduction in the distance between autophagosomes (Fig. 3).

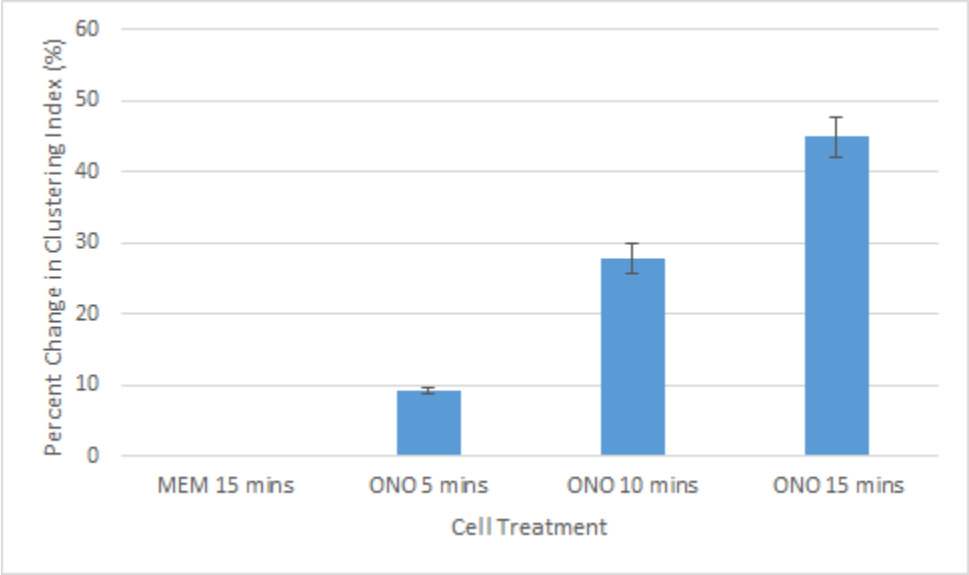

Figure 3. Distribution of LC3-labeled Autophagosomes Quantified at 5, 10, and 15 min in ONO. The control group was incubated in MEM alone for 15 min. Both the experimental and control group were standardized by experiment as a percent decrease from the control group. This graph also includes data from identical experiments run by Priscilla Kim (another undergraduate researcher in the lab). Each of us contributed an equal amount of data, which were combined into one analysis. N = 6 (T-test, $p = 0.008$ for 5 and 10 min, 0.002 for 5 and 15 min)

Having some concern that shifting cells to MEM (autophagy induction) and ONO (Golgi fragmentation) introduced two variables that might influence autophagosome distribution, I conducted a second set of experiments in which one set of cells were treated with ONO for various periods of time was matched by another that were treated with just MEM. In cells that were treated with MEM for up to 1 h, autophagosomes were fairly evenly distributed throughout the cytoplasm (Fig. 4A-D). However, in cells treated with ONO, autophagosomes became progressively redistributed and clustered in the middle of the cell (Fig. 4E-H). These results were again quantified by determining the mean distance between the furthest separated autophagosomes. Although incubation in MEM alone caused a modest increase in clustering, pairwise comparisons at each time point, between MEM alone and ONO treated, revealed a statistically significant increase in clustering at all time points (Fig. 5).

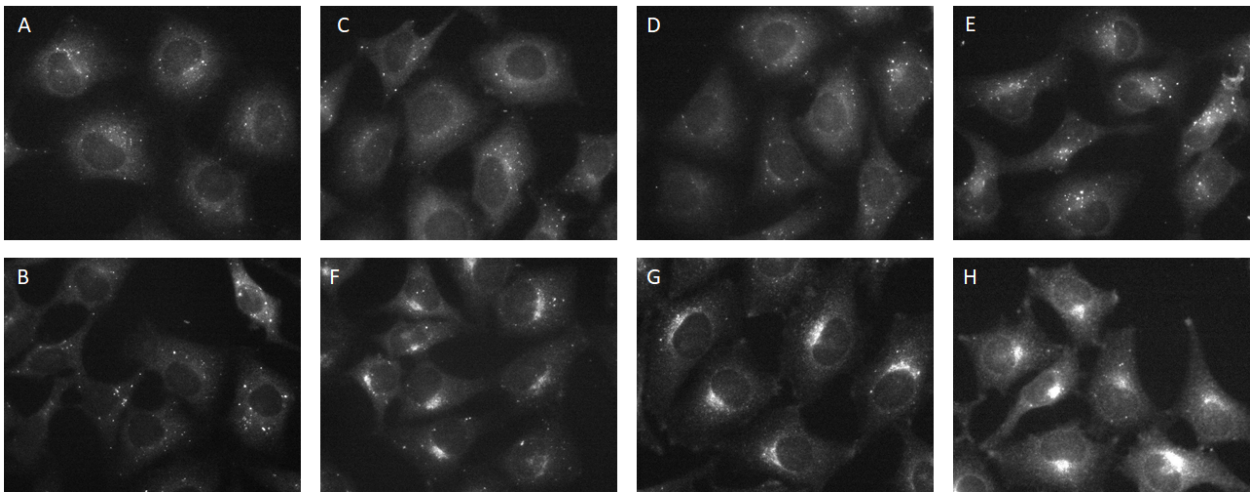

Figure 4. ONO and MEM Autophagy Induction Comparisons. (A-B) Control group where cells have had no ONO treatment. (C-E) Cells treated in media with MEM for 15 min, 30 min, and 1 h. (E) Time 0; (F-H) Cells treated in media with ONO for 15 min, 30 min, and 1 h. Cells were labeled by immunofluorescence to show the distribution of LC3-labeled autophagosomes.

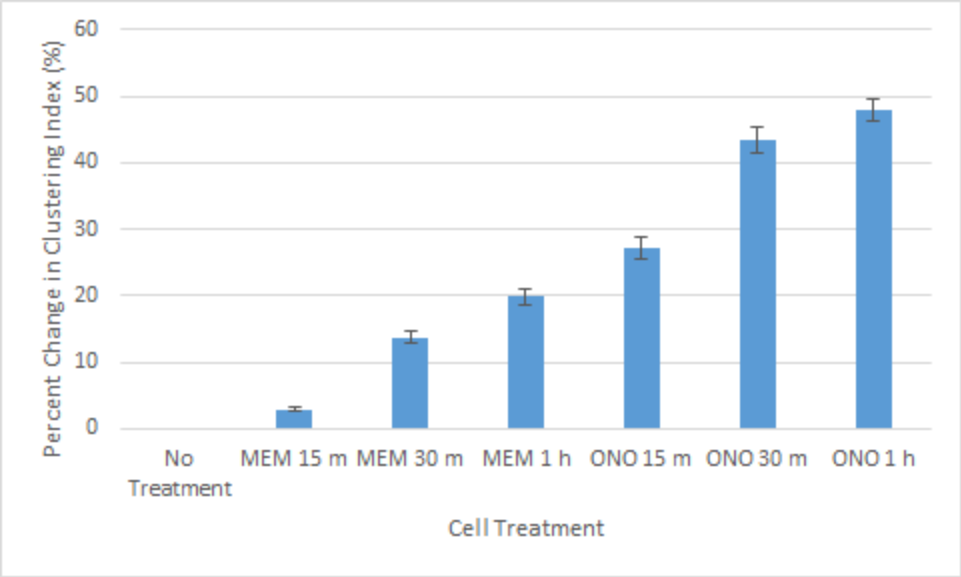

Figure 5. **Comparison of LC3-labeled Autophagosome Distribution in Cells Incubated in MEM or ONO.** N = 3 (T-test, p = 0.040 for 15 min, 0.013 for 30 min, 0.040 for 1 h)

ONO Concentration Dependence

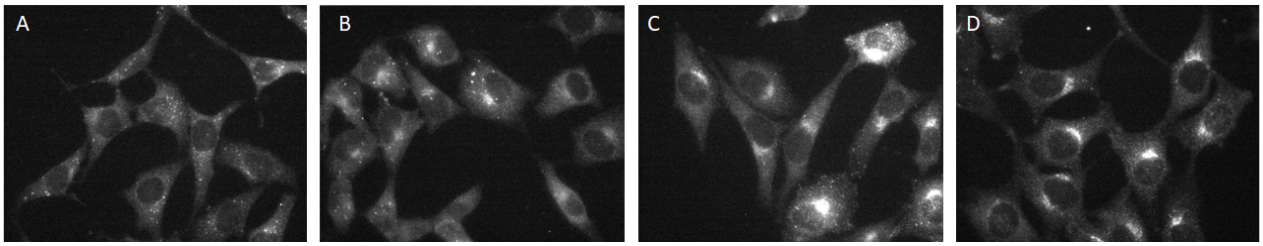

Figure 6. ONO Concentration Dependence and LC3-labeled Autophagosome Distribution. Representative micrographs of cells treated with 0 (A), 5 (B), 10 (C), and 20 (D) µM of ONO for 30 minutes.

To determine the minimal concentration of ONO needed to induce the clustering of autophagosomes, cells were treated with 0-20 µM ONO for 30 min. The fluorescent images revealed that even with 5 µM ONO, autophagosomes were found to be increasingly clustered in the center of the cell (Fig. 6B), which only became more pronounced with higher concentrations (Fig. 6C, D). Digital image analyses and quantification confirmed these results (Fig. 7).

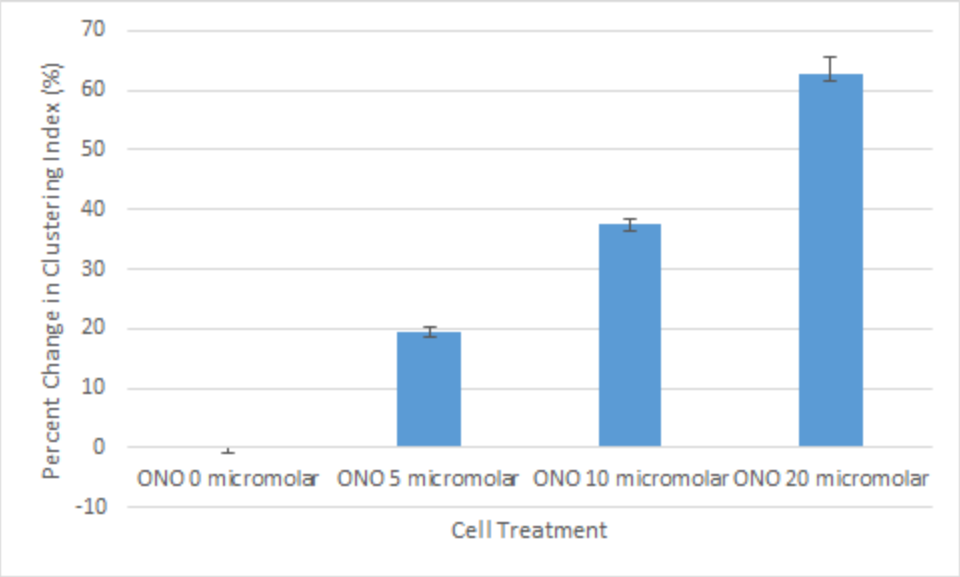

Figure 7. Distribution of LC3-labeled Autophagosomes Quantified at 5, 10, and 20 µM Concentrations. This graph also includes data from identical experiments run by Priscilla Kim (another undergraduate researcher in the lab). Each of us contributed an equal amount of data, which were combined into one analysis. N = 6 (T-test, p = 0.011 for 5 and 10 µM, 0.005 for 5 and 20 µM).

These results are consistent with previous work showing that an intact Golgi ribbon negatively regulates autophagy induction (Gosavi et al., 2018). Moreover, I found that ONO caused nascent autophagosomes to cluster in the center of the cell, which is a surprising and new observation. These results have prompted interesting possibilities for further research. The Golgi complex's integrity is suggested to have an impact on the formation of autophagosomes, although the process and reason why this occurs is yet to be discovered. One study suggests that membrane flow from the Golgi complex could be redirected to the phagophore (autophagosome) assembly site since the mutation of two post-Golgi Sec proteins, Sec2 and Sec4, vital for autophagy induction, produces an autophagy defect (Geng, Jiefei and Klionsky, Daniel, 2010.) Additionally, this study has contributed to the possibility that the flow of Golgi complex membrane flow switches from exocytosis (secretion) to the autophagy process when starved. Also notable, the LC3 puncta have clustered in a region that the Golgi normally resides and the reason why is

unknown. A possibility for this may involve microtubule motors such as kinesin and dynein and disruption of their regulation. Dynein is a (-)-end directed microtubule motor, which could bind to LC3 in different ways to tighten autophagosome association and facilitate movement to the center of the cell (Monastyrska, I., et al 2009).

At this point, we do not know if the clustering of autophagosomes to the center of the cell is a direct consequence of ONO-induced Golgi fragmentation. It is possible that ONO has an unexpected effect that is independent of Golgi fragmentation. Additionally, if autophagosome clustering is independent of Golgi fragmentation, PLA_2 could be directly changing lipid composition of the autophagosomes. Future studies should employ other PLA_2 inhibitors that also fragment the Golgi (de Figueiredo et al., 1998) and can help address whether or not the Golgi complex has an influence on autophagy. In addition, it will be interesting to see what effect, if any, knockdown or loss of PAFAHIB, which fragments the Golgi, would have on the distribution of LC3-positive autophagosomes. Finally, studies should examine the effect of ONO and other PLA_2 inhibitors on mTOR signaling. This research opens up an interesting new lead in autophagy research and will contribute to understanding its processes. By association, this research will potentially contribute to understanding cancer and neurodegenerative diseases.

Acknowledgements

The author would like to thank Dr. William Brown for his mentorship throughout the author's research exploration at Cornell University as well as for his wisdom and guidance. The author would also like to thank Priscilla Kim for the contribution of her additional trials.

References

Bechler, M. E., et al. "The phospholipase complex PAFAH Ib regulates the functional organization of the Golgi complex." *J. cell biology* vol. 190, 1, 45-53 (2010). doi/10.1083/jcb.200908105

Bechler, Marie E et al. "A PLA1-2 punch regulates the Golgi complex." *Trends in cell biology* vol. 22,2 (2012): 116-24. doi:10.1016/j.tcb.2011.10.003

de Figueiredo, P et al. "Membrane tubule-mediated reassembly and maintenance of the Golgi complex is disrupted by phospholipase A2 antagonists." *Molecular biology of the cell* vol. 10, 6 (1999): 1763-82. doi:10.1091/mbc.10.6.1763

de Figueiredo, P et al. "Evidence that phospholipase A2 activity is required for Golgi complex and trans Golgi network membrane tubulation." *Proceedings of the National Academy of Sciences* Jul 1998, 95 (15) 8642-8647. doi: 10.1073/pnas.95.15.8642

Geng, Jiefei and Klionsky, Daniel. "The Golgi as a potential membrane source for autophagy." *Autophagy*, 6:7, 950-951. (2010) doi: 10.4161/auto.6.7.13009

Gosavi, Prajakta et al. "The Golgi ribbon in mammalian cells negatively regulates autophagy by modulating mTOR activity." *Journal of cell science* vol. 131,3 jcs211987. 8 Feb. 2018, doi:10.1242/jcs.211987

Ha, Kevin D et al. "Regulation of the Golgi complex by phospholipid remodeling enzymes." *Biochimica et biophysica acta* vol. 1821,8 (2012): 1078-88. doi:10.1016/j.bbalip.2012.04.004

Kirkin, Vladimir. "History of the Selective Autophagy Research: How Did It Begin and Where Does It Stand Today?." *Journal of molecular biology* vol. 432, 1 (2020): 3-27. doi:10.1016/j.jmb.2019.05.010

Monastyrska, I., Rieter, E., Klionsky, D.J. and Reggiori, F. "Multiple roles of the cytoskeleton in autophagy." *Biological Reviews*, 84: 431-448. (2009) doi:10.1111/j.1469-185X.2009.00082.x

T. Netea-Maier, Romana et al. “Modulation of inflammation by autophagy: Consequences for human disease.” *Autophagy*, 12:2, 245-260 (2016). doi: 10.1080/15548627.2015.1071759

Nixon, R. “The role of autophagy in neurodegenerative disease.” *Nat Med* 19, 983–997 (2013). doi:10.1038/nm.3232

Tanida, Isei et al. “Lysosomal Turnover, but Not a Cellular Level, of Endogenous LC3 is a Marker for Autophagy.” *Autophagy* vol 1,2. 84-91 (2005) doi: 10.4161/auto.1.2.1697

White, Eileen. “The role for autophagy in cancer.” *The Journal of clinical investigation* vol. 125,1 (2015): 42-6. doi:10.1172/JCI73941