OPTIMIZATION OF AUTOPHAGIC CONTROLS IN HUMAN EMBRYONIC KIDNEY CELLS

An Undergraduate Thesis

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I. Abstract

Autophagy is a highly conserved and regulated process that plays an essential role in cell survival. It serves as a quality control system that degrades old or damaged material by the autolysosome and recycles it into new materials for the cell. This self-cannibalization process contributes to cellular homeostasis by maintaining a balance between synthesis, degradation, and recycling, thus preventing the accumulation of toxic substances. Rapamycin and chloroquine are biomaterials that induce or inhibit this response, respectively. Thus, these chemicals represent useful positive and negative controls for studying autophagy. However, studies that use these controls in human cells suggest that cellular responses to their effects are varied. As such, each lab must perform optimization to determine the appropriate dosing concentration and exposure time frame required to induce autophagy. Therefore, this study determined the time and doses required to induce or inhibit autophagy in the Human Embryonic Kidney (HEK) 293 cell line.

II. Introduction

Autophagy

Disturbing the homeostasis of a cell can trigger a wide variety of cellular stress responses, including cell repair mechanisms, temporary adaptation, autophagy, and cell death[1]. The cell's stress response depends on the severity and duration of the stressor. Before resorting to programmed cell death, a cell will try to break down old or damaged materials and recycle them for new uses. This is the process of autophagy, which quite literally translates to "self-eating" in Greek. When a cell activates autophagy, it will form autophagosomes around unneeded materials such as proteins or damaged organelles. An autophagosome then joins a lysosome to degrade this waste, referred to as an autophagolysosome. Getting rid of damaged material can increase efficiency of other cell pathways. Autophagy degrades the useful materials, which are then recycled and incorporated into new molecules[2]. This gives the cell more accessible and useful materials, and as a result, a better chance at survival. Typical scenarios that induce autophagy include oxidative, nutritional, or toxic stressors[3]. For example, during heat shock, the autophagic stress response is upregulated and the cell produces heat shock proteins and chaperones using the recycled material from the autophagy process[4].

In mammalian cells, three distinct types of autophagy co-exist: chaperone-mediated autophagy, microautophagy, and macroautophagy. Chaperone-mediated autophagy involves a specific degradation tag on the protein to allow the lysosome to recruit a chaperone protein and target it for degradation. Microautophagy is a non-selective process that directly engulfs and transports cytosolic materials into the lumen of the lytic organelle[5]. In macroautophagy, this cytosolic material is transported in intermediary autophagosomes and then fused with a

lysosome, as described earlier. This paper will focus on the cellular aspects of macroautophagy (henceforth referred to as 'autophagy') in HEK293 cells.

Autophagy regulates many cell types' survival, differentiation, development, and homeostasis and therefore needs to be highly conserved and regulated.
In turn, the dysregulation of autophagy associates with the pathogenesis of diseases, including neurodegenerative disease and cancer[6]. Different methods exist to study general autophagy in lab settings, including western blotting, gene expression, and tracking of fluorescently-tagged autophagy proteins. In each method, a protein specific to autophagy, Light Chain 3 (LC3), is often the study target (whether in protein form or mRNA indicating gene expression). Our lab uses a system to track fluorescently labeled LC3 and, therefore, autophagy. In this system, we transfected HEK293 cells with a dual reporter plasmid system containing the LC3 gene fused to two genes that code for fluorescent reporters, Green Fluorescent Protein (GFP) and mCherry, a red fluorescent protein (RFP). After transfection, cells will produce an abundance of LC3 tagged with the two reporters. Before inducing autophagy, cells will exhibit a diffuse cytosolic expression pattern of LC3 in both green and red. Upon inducing autophagy, the autophagosome membrane incorporates LC3 in high numbers, which can be visualized as small intense regions of fluorescence called "puncta." LC3 in the autophagosome membrane should fluoresce in both green and red, and then can be visualized with fluorescence microscopy and quantified[7].
Lysosomes will then fuse to the autophagosome- termed autophagolysosomes, to subsequently degrade material. In this later stage of autophagy, the GFP reporter protein, with a pKa of 6.0, is rendered nonfunctional and can no longer fluoresce. The mCherry RFP can be useful in this regard as it has a pKa of <4.5 and continues to display red fluorescence, even in acidic vesicles like autophagolysosomes[8]. Once fusion of the lysosomes has occurred, then late autophagy can

be identified in cells because puncta no longer fluoresce in both colors, only in red. Therefore, this transfection system is capable of tracking cells with little to no autophagy occurring (diffuse green and red fluorescence is observed throughout these cells), cells in early autophagy (green and red puncta are visible and can be overlaid in images to show colocalization), and cells in late autophagy (red only puncta are visible) (Figure 1).

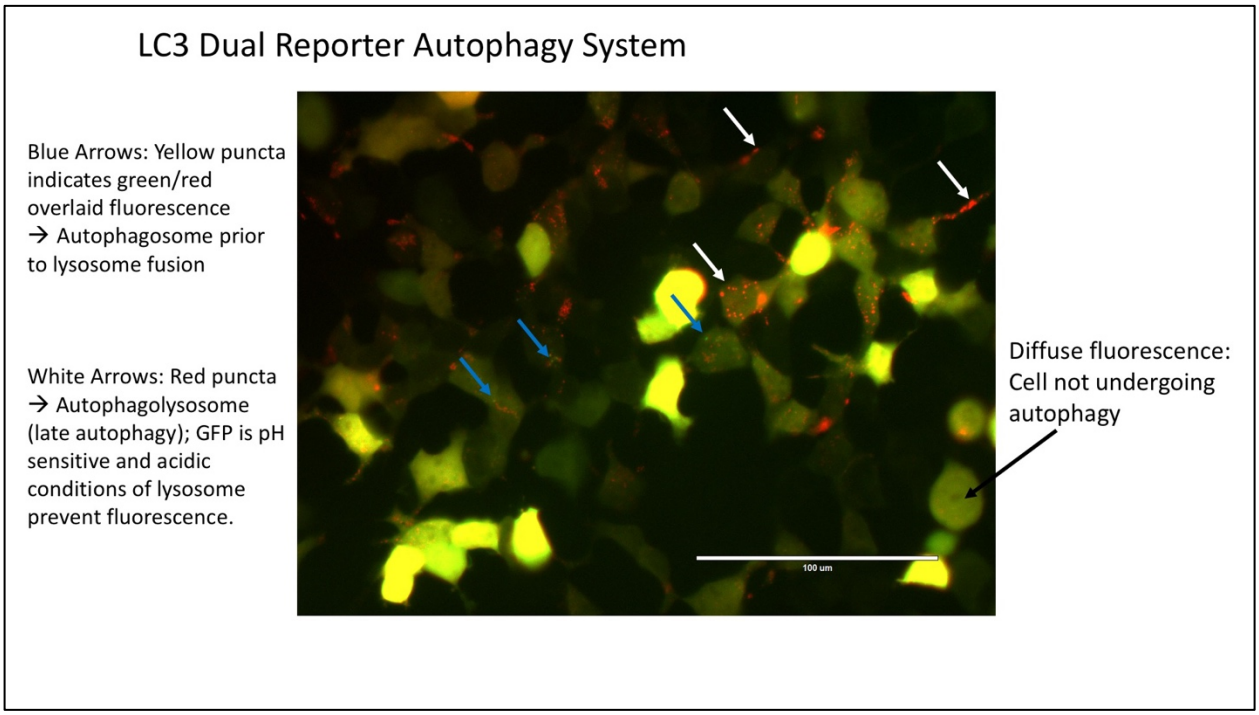


Figure 1. HEK cells transfected with the dual-reporter plasmid system. Arrows indicate cells at various stages in the autophagy process (blue arrow = early autophagy; white arrow= late autophagy; black arrow= pre-autophagy). Image provided by Dr. Heather Coan from previous studies.

Rapamycin and Chloroquine

As a highly conserved process, autophagy has several important signal transduction pathways involved in its regulation, including a nontranscriptional inhibition mechanism by mammalian target of rapamycin (mTOR). mTOR is an evolutionarily conserved serine/threonine kinase involved in catabolic processes that upregulate autophagy. Rapamycin is an mTOR

inhibitor (Figure 2), which induces autophagy by allosterically inhibiting mTORC1, one of two catalytic components of TOR[9]. Rapamycin has been the subject of clinical trials as a therapy for cancer because of its ability to inhibit tumor growth by blocking protein translation and inducing autophagic cell death[10]. However, rapamycin only seems to have inhibitory activity against TORC1 and not TORC2. Current research efforts are devoted to finding so-called 'rapalogs' that have inhibitory effects on both complexes[11]. It has been shown previously that rapamycin induces autophagy in a time and concentration dependent manner in a number of cell types, including well documented studies in HeLa cells[12] and neuroblastoma cells[13]. Labs often utilize HEK293 cells to study autophagy, and rapamycin is an excellent positive control for studies that investigate the process for various reasons. To this end, protocols can be found online for such studies, but they vary extensively in rapamycin dosage and time courses. Therefore, one main objective of my work was to develop a reproducible method for inducing autophagy with rapamycin in the HEK293 cells used in our laboratory. Optimization of rapamycin as a control will facilitate future research goals in the Coan lab aimed at better understanding the ability of certain biomaterials to induce autophagy.

Chloroquine (CQ) is an autophagy inhibitor that blocks the fusion of autophagosomes with lysosomes, thereby preventing autophagy from degrading material. Because of these mechanisms, CQ has also shown clinical promise in the treatment of cancer. CQ and its derivatives have displayed antitumor activity in melanoma as an mTOR and autophagy inhibitor[14] and can act as a potentiator to the effect of chemotherapy in glioblastoma multiformation[15] and colon cancer cells[16]. CQ can be used to study autophagy and is particularly useful as a negative control to better understand how inhibition of the pathway at a midpoint in the process (before lysosome fusion) would appear in autophagy experimental studies (Figure 2).

For example, in studies that track LC3 gene expression, CQ would not appear to alter autophagy because LC3 is upregulated upstream of CQ's effects. In western blotting studies that identify the presence of LC3 in an active vs "inactive form" LC3-II vs LC3-I, respectively, the presence of LC3-II indicates that the protein is active and has been incorporated into the autophagosome. Western blotting can identify LC3-II presence and is considered indicative of autophagy activation. However, this event also occurs upstream of chloroquine's activation, meaning that two of the three common methods used to assess autophagy (gene expression and western blotting) might show autophagy turned on despite being inhibited downstream. The plasmid transfection system used in our lab provides a clear-cut method of assessing autophagy after lysosome fusion. Therefore, the use of CQ as a negative control in studies of autophagy is critical to understanding how results might vary in the presence of a downstream inhibitor. Unfortunately, studies that utilize CQ in HEK293 cells vary in their methods, and so another aim of my work is to optimize protocols for using CQ as a control. Optimization of CQ is particularly critical to the Coan lab's goals because our previous studies hint at the upregulation of autophagy by specific biomaterial substrates – yet methods used to date do not track later autophagy, so the question remains as to whether these substrates activate or block the autophagy pathway. CQ comparisons will enable us to understand whether our biomaterials of interest are beneficial or detrimental to cell survival[17].

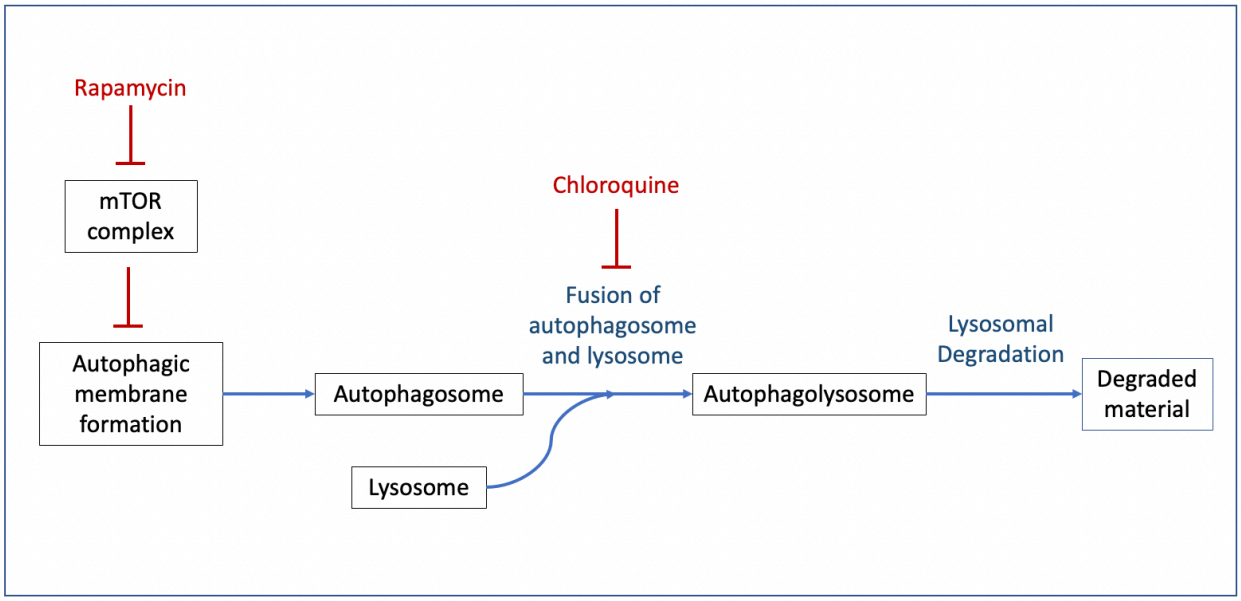


Figure 2. The progression through the autophagy pathway is shown with rapamycin and chloroquine controls indicating the location of their action on the pathway. Red lines indicate an inhibitory effect.

After the introduction of rapamycin to the HEK293 cells, we expect to be able to visualize the entire autophagy process using our transfection system. Before dosing, we should see diffuse fluorescence in the cells under the Green GFP light cube and diffuse fluorescence under the Texas Red light cube. After administering rapamycin, green and red colocalized puncta indicate early autophagy, whereas the presence of only red puncta identifies late autophagy. As rapamycin is an autophagy inducer, we should see all steps of the process represented over a long period of time. CQ is an autophagy inhibitor that blocks the fusion of the autophagosome with the lysosome. As such, we should not visualize red only puncta- an indication of late autophagy as blocking fusion of the lysosome halts the autophagy process at the autophagosome. So, in CQ-treated cells, we expect to see green and red puncta for a long period of time.

After optimizing rapamycin and CQ as autophagic controls in HEK293 cells, the next aim of this work is to determine whether cells can recover after initiating autophagy. This is important because an overarching goal of the Coan lab is to better understand induction of

autophagy by biomaterial substrates. However, despite some preliminary evidence by our lab suggesting that certain biomaterials can upregulate recycling pathways, we do yet know whether the process is beneficial to survival or whether it is a sign that the cells are responding to stress. To this end, we aimed to determine whether cells that initiate autophagy can also return to a level of homeostasis (baseline low levels of autophagy) after the induction. To accomplish this, we investigated whether supplementation with rapamycin and CQ can coax HEK293 cells to return to the normal diffuse fluorescence state, indicating that the pathway is no longer activated. If return to homeostasis is possible, then we can make comparisons to our biomaterials of interest to better understand whether their effects on cells are beneficial or detrimental.

III. Materials and Methods

Plasmids

Two plasmids were used in this study. pBABE-puro mCherry-EGFP-LC3B was a gift from Jayanta Debnath (Addgene plasmid # 22418 ; http://n2t.net/addgene:22418 ; RRID:Addgene_22418)[18] and was used for the experimental groups of cells while GFP-mApple, a gift from Dr. Robert Youker, was used in the control groups. These plasmids were received as bacterial (*E. coli*) agar stabs and were grown on kanamycin or ampicillin resistant agar plates. Isolation of the plasmids was conducted using the Invitrogen Plasmid Isolation kit. Verification of purity and concentration of plasmids was determined with a NanoDrop spectrophotometer.

Cell Cultures

The cells used for this study are HEK293 cells provided to Dr. Heather Coan's lab as a gift from Dr. Robert Youker. Cells are cryopreserved in 90% fetal bovine serum (FBS)/10% dimethyl sulfoxide (DMSO). Cells are thawed, cultured, and passaged based on standard protocols. Cell cultures are kept in a 5% CO_2 incubator at 37˚ C.

HEK293 Cell Culture and Maintenance

10% FBS/ Dulbecco's Modified Eagle Medium (DMEM) (Gibco Ref. # 11995-065) medium is made using 4 mL of FBS and 36 mL of DMEM. Cell plates containing HEK293 cells are retrieved from the incubator and checked for confluency under a microscope. Cells at 80-90% confluency are placed in a laminar flow hood in the cell culture room for passaging. Medium is removed and placed into a labeled waste container with bleach. 5mL of phosphate-buffered saline (PBS) is added to clear cell debris and remove dead cells. The solution is swirled

and then removed and placed into the waste container. Using 1.5 mL of TrypLe (Gibco Ref. # 12604-021), the cells are detached by coating the plate and placing the plate into the 37˚ C incubator. The plate is removed from the incubator and returned under the hood, where 3mL of cell growth medium is used to neutralize the TrypLe enzyme. The contents are pipetted into a labeled 15 mL tube and centrifuged at 1200 rpm for 5 minutes to pellet the cells. Spent medium is removed via pipetting after centrifugation and is placed into a labeled waste container. 3 mL of medium is then added to the pellet to reconstitute the cells. To determine cell concentration, 12 µL of Trypan Blue (Sigma Ref. # T8154) is used to dilute a 12 µL cell suspension sample by ½ and is mixed in a separate, labeled tube. Cells are counted using a hemocytometer, blue cells that take up the Trypan Blue dye are left uncounted, as they represent dead cells. Cells are seeded into 5 wells of a 6-well dish using the formula:

$$\text{amount of stock cells needed} = \frac{\left(150{,}000\,\frac{\text{cells}}{\text{mL}}\right) * 10\text{mL}}{(\#\text{ of counted cells} * 10{,}000) * 2}$$

After labelling a 6 well plate, 2mL of the new solution is added to each of the 5 wells used for the experiment. Any leftover cells are plated onto a new plate along with fresh medium for a total of 10mL in the dish and then are placed in the incubator for use in future experiments.

Plasmid Transfection into HEK293 Cells

For plasmid transfection, GFP-mCherry-LC3 and GFP-mApple were used. Three 2 mL tubes are set up in the laminar flow hood, labelled master mix (MM), LC3 and GFP. 500 µL of Opti-MEM (Ref. # 31985-062) is pipetted into the MM tube, 400 µL into the LC3 tube, and 100 µL into the GFP tube. This was calculated using:

Optimem in MM= #of total wells* 100µL

Optimem in LC3= #of LC3 wells* 100µL

Optimem in GFP= #of GFP wells * 100µL

Next, 12.5 µL of lipid Continuum (GEB Cat. # 400-700) is added to the MM tube calculated using:

Continuum in MM= # of total wells* 2.5 µL

The amount of LC3 and GFP plasmid needed was calculated using:

LC3 = 4000ng/ (conc. of LC3 plasmid on tube)

GFP = 500ng/ (conc. of GFP plasmid on tube)

The amount of LC3 calculated in µL was added to the LC3 tube and the amount of GFP calculated in µL was added to the GFP tube. The tubes were swirled gently and incubated at room temperature for 5 mins. After the 5-minute waiting period, 400 µL of the MM was pipetted into the LC3 tube and the remaining 100 µL was pipetted into the GFP tube. The two tubes were capped and swirled gently. They then remained untouched for 45 minutes to ensure the formation of lipid-plasmid complexes. Using a 6 well plate, 200 µL of the LC3 plasmid-containing tube were added to the first 4 wells of the plate while 200 µL of the GFP plasmid-containing tube were added to the 5th well. The well plate was labeled and placed into a 37˚ ˚C incubator for 24 hours to allow transfection to occur.

Cell Imaging

All images of cell fluorescence were taken using an Invitrogen EVOS FL Auto Cell Imaging System provided by the Western Carolina University Department of Biology. Light

cubes for both GFP and Texas Red were used to visualize fluorescence. Images were taken at 20X magnification with the pseudo color setting enabled.

Verification of Plasmid Transfection using Microscopy

After the wells are left in the incubator overnight, they are removed and taken to the EVOS microscope. Using the EVOS microscope, cells are visualized to see if transfection occurred as indicated by fluorescing cells. Time 0 images are taken of the cells in both light cube colors.

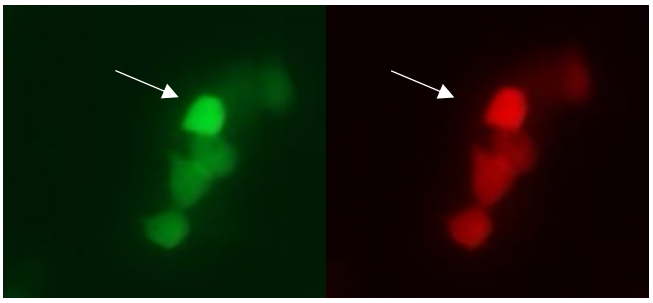
Image Analysis

All images taken of treatments and timepoints were subject to cell counts to determine: 1) the number of cells present in the field of view, 2) the number of cells present with green puncta, 3) the number of cells present with red puncta. This data was counted by hand and recorded in Excel separately. The percentage of cells containing puncta was determined by dividing the number of cells containing puncta over the total number of cells and multiplying this by 100. Three different locations per well were used to gather images per time point. The percentage of cells with puncta calculated was then averaged across the three locations and is displayed on the graphs in the results. Across all experiments, the number of total cells counted varied from a minimum of 10 cells to a maximum of 162 cells counted.

Cell counts were performed by altering the light and intensity of images to better visualize cells and puncta within. Figure 3 below is an example of how puncta from different stages of autophagy can be visualized when images are zoomed in, and intensity is altered. Note, images shown for each experiment in the results section are unaltered to show the entire field of

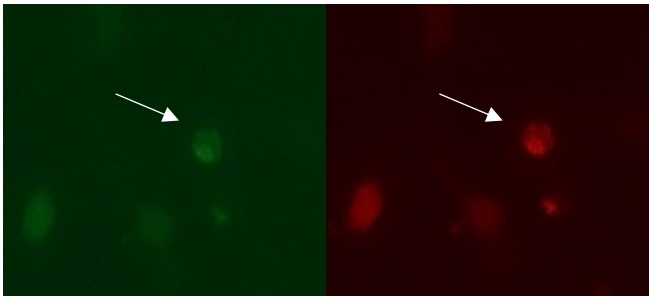
view that is then analyzed by zooming in and increasing light and intensity to obtain each cell count.

No Autophagy Example



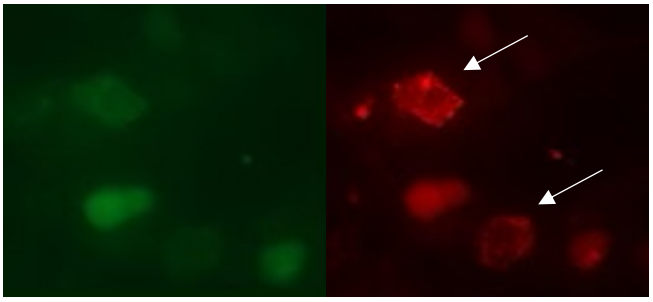
Arrows indicate cells with diffuse staining not undergoing autophagy. Image taken from well 4 in rapamycin recovery experiment at time 0 (Figure 8A).

Early Autophagy Example



Arrows indicate cells with both green and red puncta, before the lysosome fuses with the autophagosome, indicating early autophagy. Image taken from well 1 in the 0.3 μM rapamycin experiment at time 7.5 hr (Figure 4C).

Late Autophagy Example



Arrows indicate cells undergoing late autophagy as shown by only having red puncta. Image taken from well 1 in rapamycin recovery experiment at time 32 hr (Figure 8C).

Figure 3. Examples of cells during the stages of autophagy. Images were cropped and edited with high exposure and contrast to see a smaller proportion of cells clearly.

Statistical Analysis

Data was recorded in Microsoft Excel and analyzed via two-tailed unpaired T-Tests. P-values of less than or equal to 0.05 were deemed statistically significant.

Rapamycin Treatment

HEK293 cells were grown and successfully transfected with LC3 and GFP plasmids in a 6-well dish. Images of fluorescent cells were taken with the EVOS microscope 24 hours after transfection (time 0). Two of the four wells transfected with GFP-mCherry-LC3 were then exposed to either 0.3 μM or 0.5 μM of rapamycin, while the other two wells were left with no treatment. The last well was transfected with only the GFP-mApple and left alone as a control. To determine the amount of rapamycin needed per well the following calculation was used:

$$\text{amount of rapamycin needed in mL} = \frac{(0.3 \text{ or } 0.5\ \mu\text{M rapamycin}) * 2\text{mL}}{\text{concentration of stock rapamycin}}$$

Images of cells are taken with the EVOS microscope in both light cubes at specific time points (0, 4.5, 7.5, 20, 24, 27.5, and 32 hours). Between time points cell cultures are kept in 37˚C incubators. Cells with and without puncta are counted and data is recorded and graphed in Excel.

Rapamycin and Chloroquine Co-treatment

HEK293 are grown and successfully transfected with LC3 and GFP plasmids in a 6-well dish. Images of fluorescent cells were taken with the EVOS microscope 24 hours after transfection (time 0). The CQ experimental well was treated with 40 μM CQ. The amount used was calculated using:

$$\text{amount of chloroquine needed in mL} = \frac{(40\ \mu\text{M chloroquine}) * 2\text{mL}}{\text{concentration of stock chloroquine}}$$

The chloroquine and rapamycin combined experimental well was treated with 40 μM chloroquine and 0.3 μM rapamycin. The amount used was calculated in the same manner as previously described. The rapamycin-alone experimental well was treated with 0.3 μM rapamycin. The amount used was calculated as previously described. Images of cells were taken with the EVOS microscope in both light cubes at specific time points (0 and 18.5 hours). Between time points, cell cultures were kept in 37˚C incubators. Cells with and without puncta were counted and data was recorded and graphed in Excel.

Rapamycin Recovery Treatment

HEK293 cells were grown and successfully transfected with LC3 and GFP plasmids in a 6-well dish. Images of fluorescent cells were taken with the EVOS microscope 24 hours after transfection (time 0). Rapamycin experimental wells were dosed with 0.3 μM rapamycin and control wells were left alone. Images of cells were taken with the EVOS microscope using both light cubes at specific time points. Between time points, cell cultures were kept at 37˚C. After the 24-hour timepoint, spent media was removed and 10 mL of new media was added. This was to remove the rapamycin still left in the media. Cells with and without puncta were counted and data was recorded and graphed in Excel.

IV. Results

Rapamycin 0.3 μM

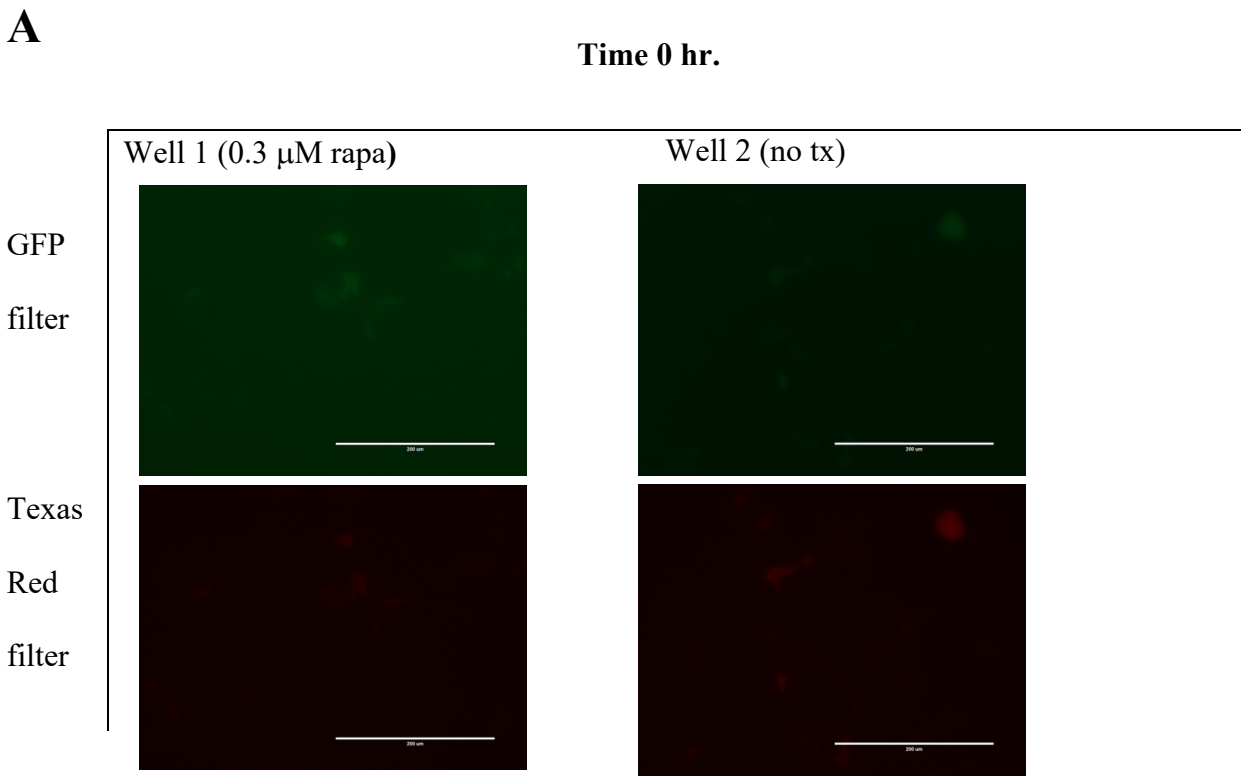
HEK293 cells transfected with the plasmid GFP-mCherry-LC3 and exposed to 0.3 μM rapamycin treatment showed a higher number of cells containing puncta under the Texas Red filter at around 7.5 hours as compared to the no treatment group. Before this timepoint, images at the 4.5-hour mark showed a relatively similar number of red puncta in both groups with an average of 4.9% (SD=3.6) cells with puncta/image in the experimental group and 4.2% (SD= 3.3) cells with puncta/image in the control group. After 7.5 hours, the distinction is clearer with 16.9% (SD= 8.4) of cells/image with red puncta as compared to 7.2% (SD=3.6) in the control. However, this difference is not statistically significant at $p = .078$.

When the cells were left for a full 24 hours with 0.3 μM rapamycin, there were an average of 50.9% (SD= 10.2) cells/image with puncta using the Texas Red filter. The cells that were left untreated showed puncta at this time point but had a significantly lower amount. The control group had an average of 23.7% (SD= 5.1) cells/image with puncta using the Texas Red filter, which was a statistically significant difference at $p = .003$. The two time-points after, 27.5 and 32 hours, also had an observably significant difference between the two groups. At 27.5 hours, comparisons of the treatment group and the control group was significant at $p = .001$. Further, at the 32 hour mark the distinction between the two groups was also clear at $p < .001$.

Figures 4A-F show cell images taken with the EVOS microscope in both light cubes. As the time points increase, in both treated and untreated cells, more red puncta appear. The treated cells, however, have a larger proportion of cells/image with puncta at timepoints past 24 hours. Figures 4G and 4H both show box and whisker plots of the data measured for each time point.

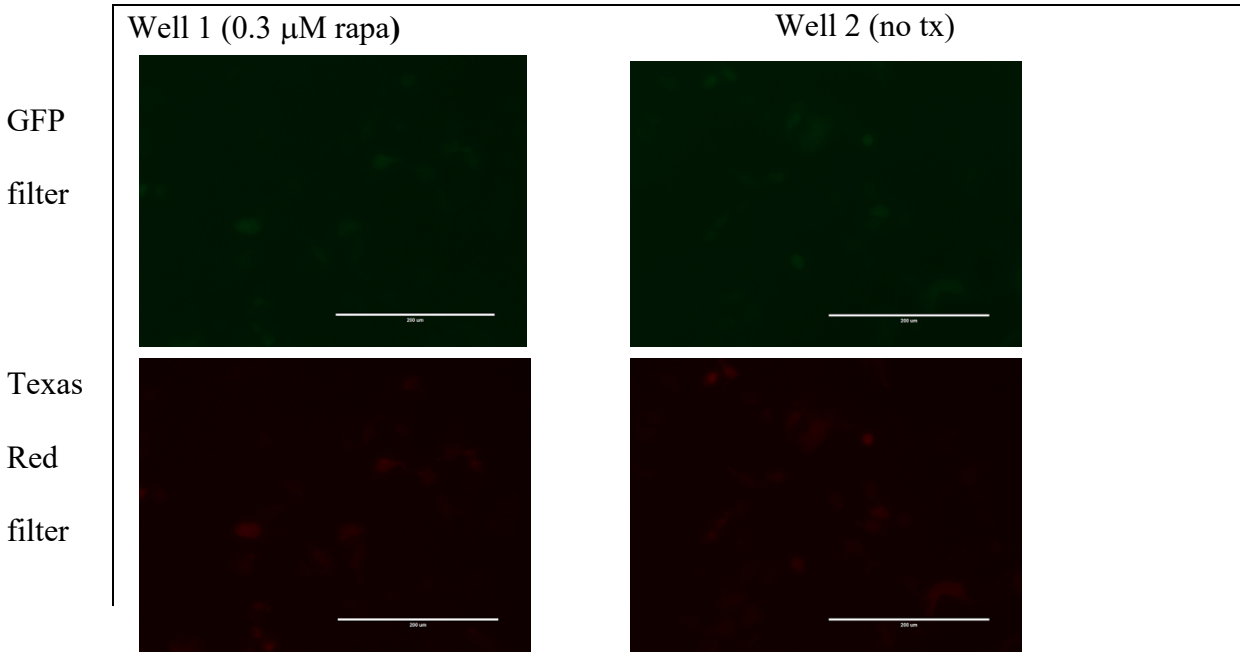
These figures show a numerical representation of the increasing number of red puncta over time. The rapamycin treated group has a sharper increase in puncta compared to the untreated group. At 24 hours, the differentiation between the two groups is observable as shown in the line graph of Figure 4I.

A



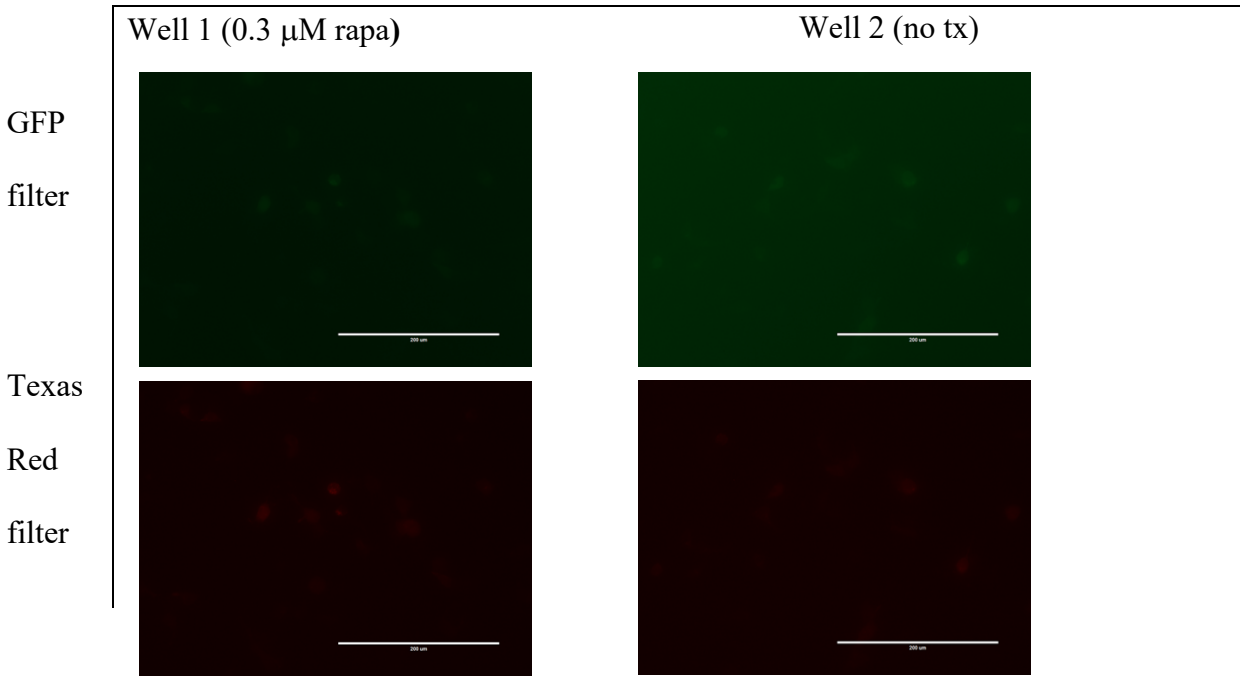
B

Time 4.5 hr.



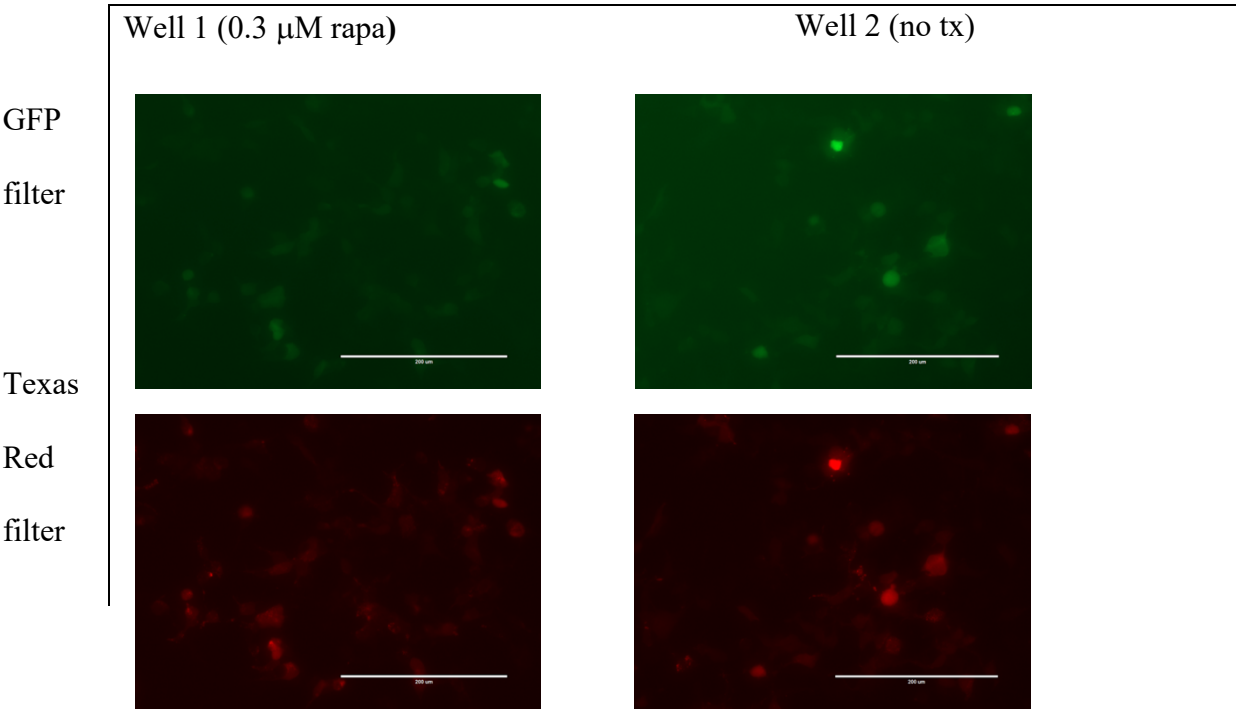
C

Time 7.5 hr.



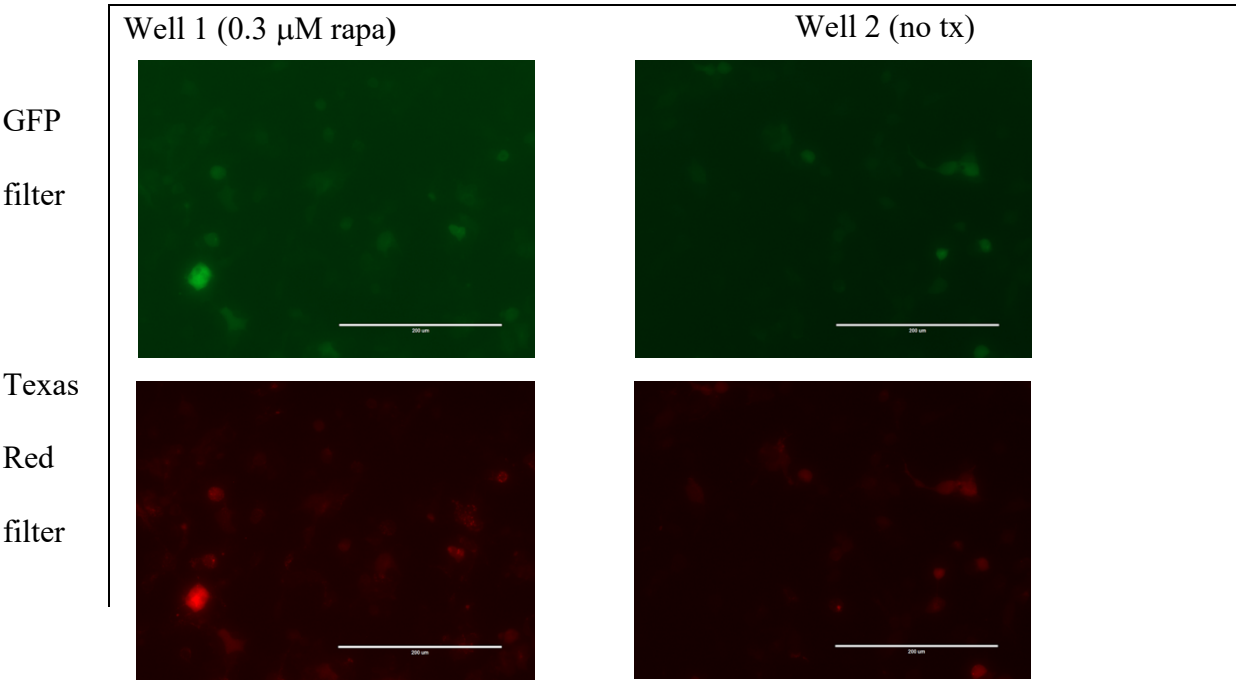
D

Time 24 hr.



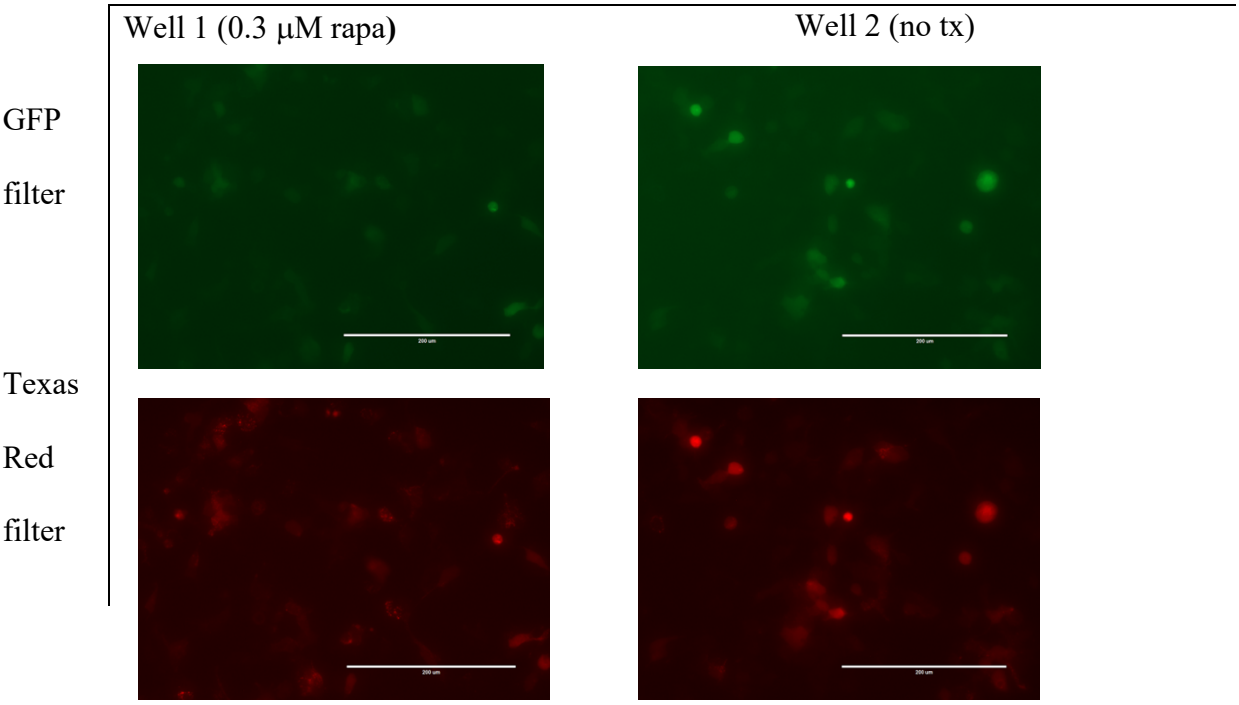
E

Time 27.5 hr.

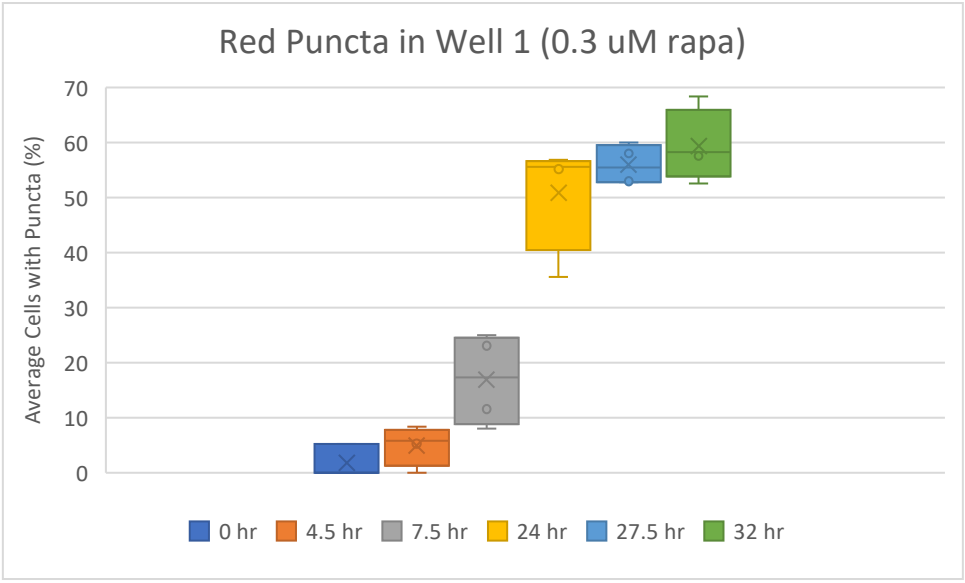


F

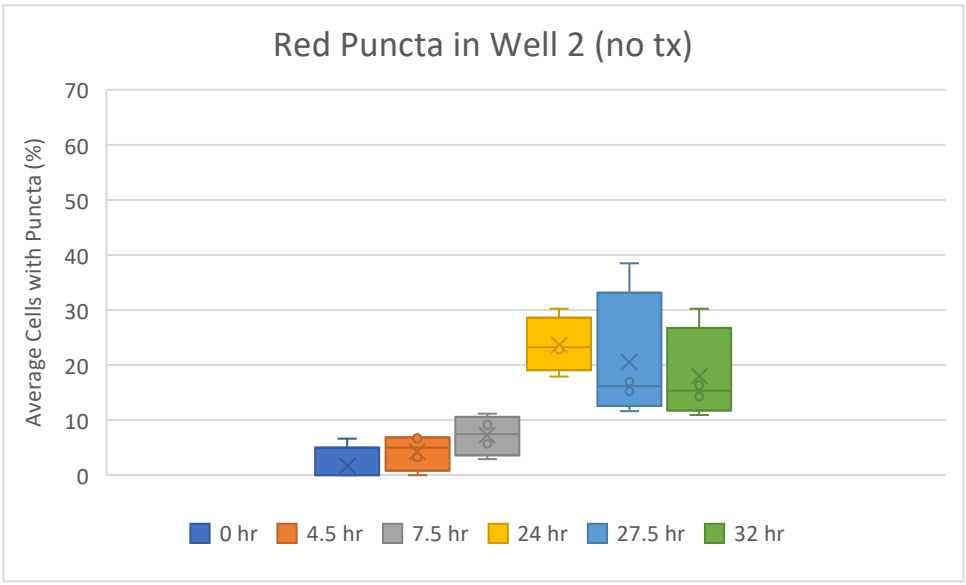
Time 32 hr.


Well 1 (0.3 µM rapa)
Well 2 (no tx)
GFP filter
Texas Red filter

G


Red Puncta in Well 1 (0.3 uM rapa)
Average Cells with Puncta (%)
70
60
50
40
30
20
10
0
0 hr
4.5 hr
7.5 hr
24 hr
27.5 hr
32 hr

H



I

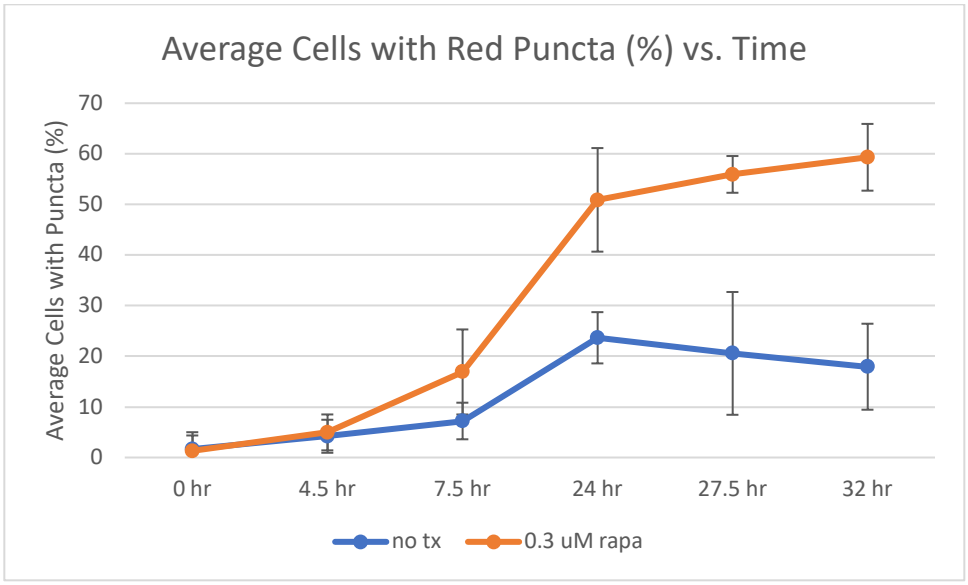


Figure 4. Rapamycin (0.3 μM) treated cells show an increase in red puncta over time in culture indicating flux through the autophagy pathway. A-F) Representative images are shown for HEK293 cells transfected with the LC3 plasmid. The cells fluoresce and form distinguishable green and red puncta at time points between 0-32 hours. Cells treated with 0.3 μM rapamycin are compared to untreated, transfected controls at the same time points. G) A box and whisker plot shows the average percentage of cells with red puncta at times 0, 4.5, 7.5, 24, 27.5, & 32 hrs in cells with treatment. Averages were calculated from each of the three images taken per well. H) A box and whisker plot shows the average percentage of cells with red puncta at times 0, 4.5, 7.5, 24, 27.5, & 32 hrs in cells with no treatment. Averages were calculated from each of the three images taken per well. I) The line graph shows averages from the treatment well and no

treatment well and demonstrates a clear difference between 0.3 μM rapamycin-treated cells and the no treatment controls starting at 24 hours.

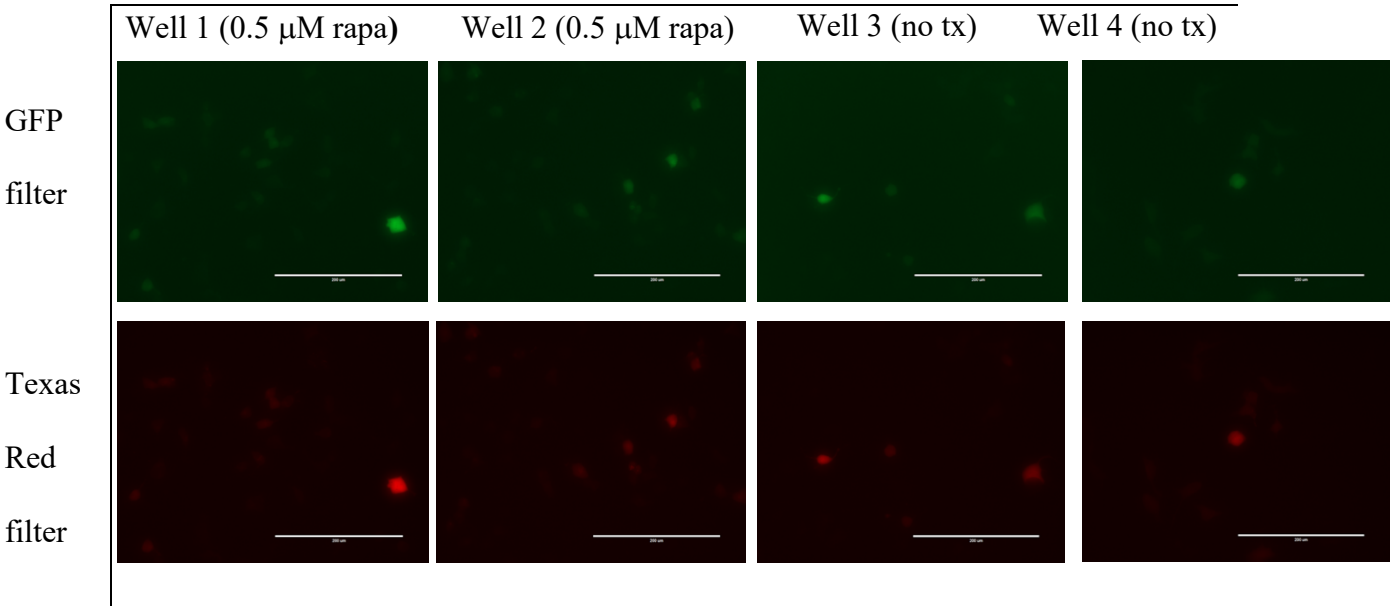
Rapamycin 0.5 μM

HEK293 cells transfected with the GFP-mCherry-LC3 plasmid and exposed to 0.5 μM rapamycin treatment showed a large number of cells containing puncta using the Texas Red filter as compared to cells with no treatment at the 20-hour timepoint. This timepoint was selected to preliminarily see if the cells could handle this concentration of rapamycin over a long period of time. Compared to the time 0 images in Figure 5A, Figure 5B shows more cells with puncta under the Texas Red filter. Well 1, dosed with 0.5 μM rapamycin, had an average of 67.7% (SD=13.5) cells/image with puncta at time 20 hours. Well 2, also dosed with 0.5 μM rapamycin, showed an average of 54.2% (SD=17) cells/image containing puncta using the Texas Red filter at time 20 hours. This is very different compared to 28% (SD=15.1) and 35.9% (SD=10.4) cells/image with puncta in untreated Wells 3 and 4, respectively. This is represented graphically in Figure 5C.

In Figure 5D, experimental Wells 1 and 2 were averaged and control treatment Wells 3 and 4 were averaged. This figure brings further attention to the stark difference in the proportion of cells/image with puncta between the two groups. The rapamycin-treated experimental group ($\bar{x}$= 37, SD=29.8) and the untreated control group ($\bar{x}$= 8.9, SD= 17.6) were significantly different with a p-value of .018.

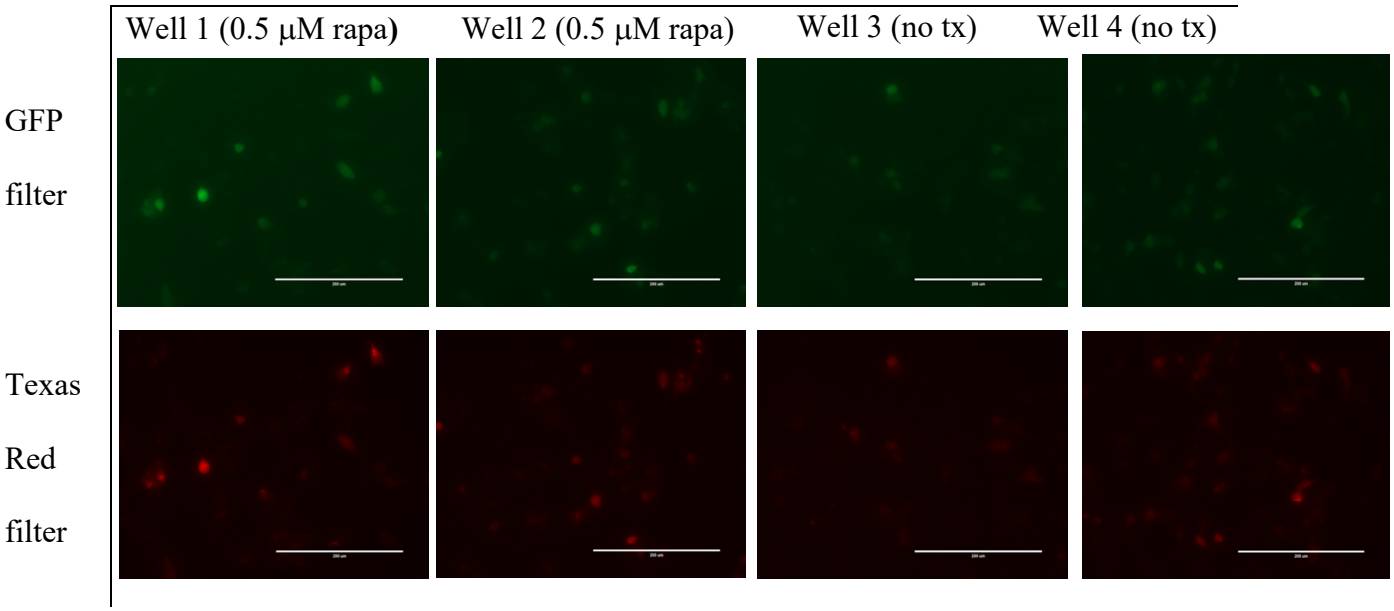
A

Time 0 hr.

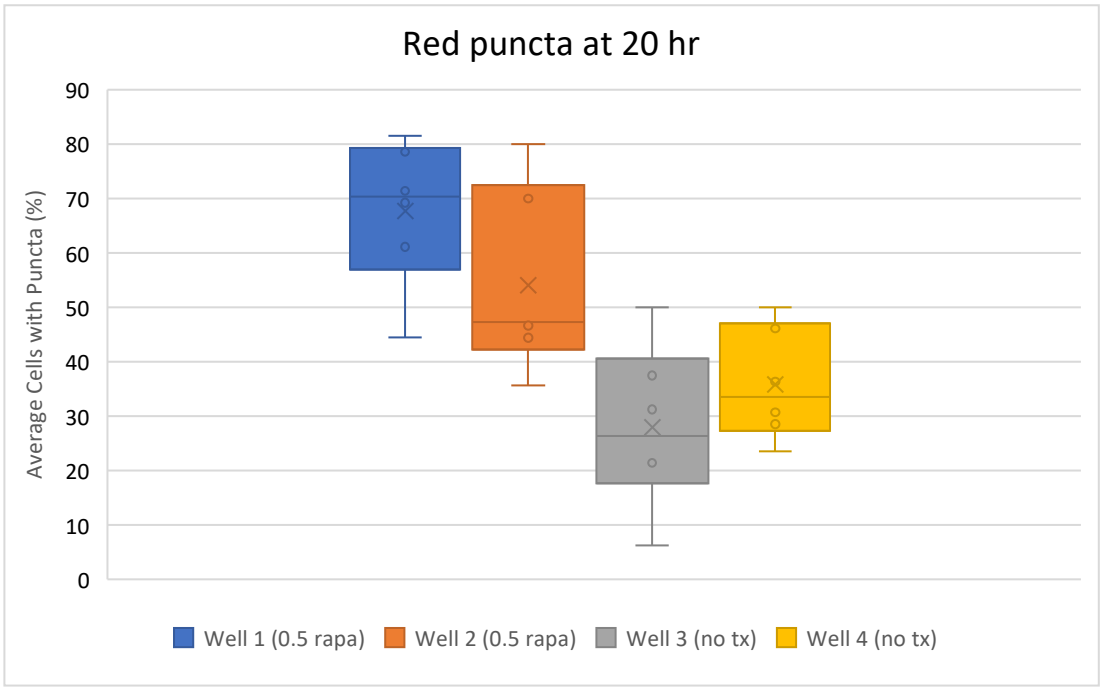


B

Time 20 hr.



C



D

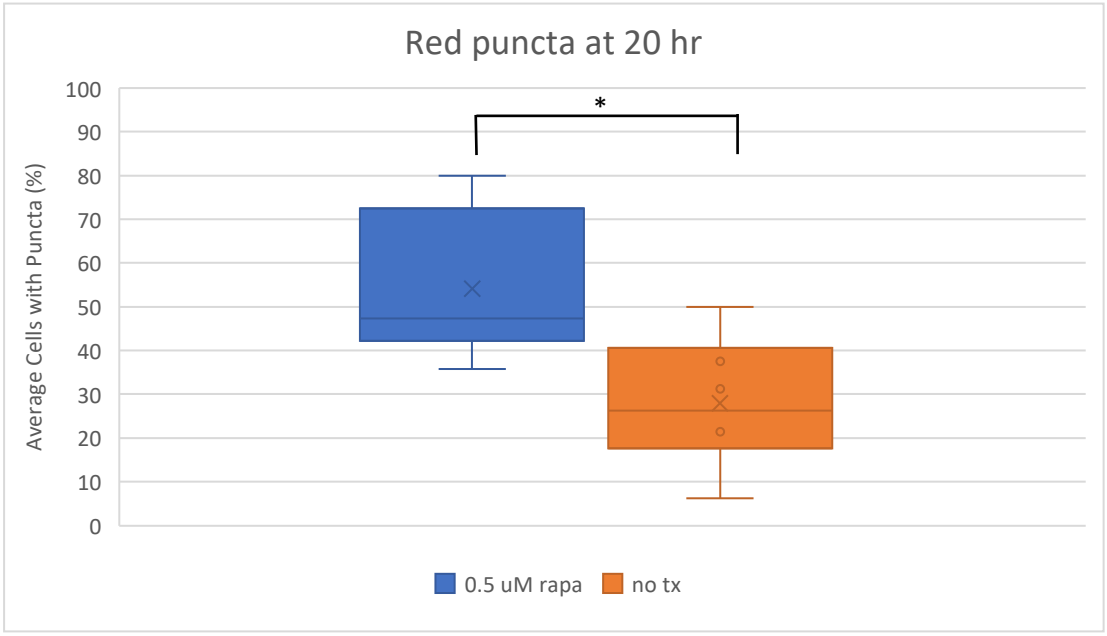


Figure 5. Rapamycin (0.5 μM) treated cells show a pronounced increase in red puncta over time in culture indicating flux through the autophagy pathway. A & B) Representative images are shown for HEK293 cells transfected with the LC3 plasmid. The cells fluoresce and form

distinguishable green and red puncta at time points between 0 and 20 hours. Cells treated with 0.5 μM rapamycin are compared to untreated, transfected controls at the same time point. C) A box and whisker plot shows the average percentage of cells with red puncta at time 20. Averages were calculated from each of the three images taken per well. Time 0 is not shown because cells showed no puncta at this time point. D) A box and whisker plot shows the percentage of cells with red puncta when same treatment wells are combined and averaged. Each box represents 2 wells with 3 images taken per well. *Represents a significant p-value of .018 vs the same time point control.

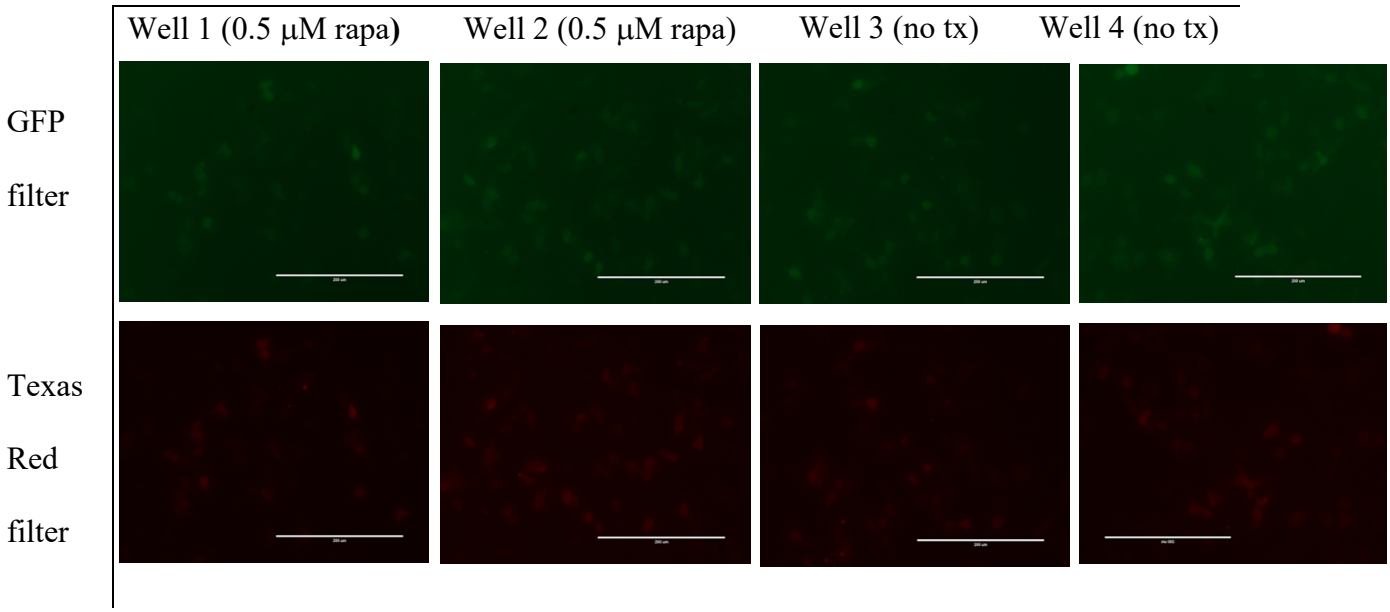
Establishing the timeline of rapamycin at a higher dosage was performed to better understand the progression from no autophagy to late autophagy at a variety of timepoints. In a second set of dosing experiments, cells were dosed with 0.5 μM rapamycin once more (Wells 1 & 2) while Wells 3 and 4 were left untreated. As expected, treated wells accumulated greater numbers of puncta faster than untreated wells. Images from each timepoint using both light cubes are shown in Figures 6A-E below. After dosing, the first time point imaged was at 15 hours and there is a visible difference in the two groups. At 15 hours, treated Wells 1 and 2 had 33% (SD=8.6) and 34.9% (SD=10.9), respectively, cells/image containing puncta using the Texas Red light cube (plots show this difference in Figure 6F). The control wells at this time point had an average of 5.3% (SD=1.7) and 8.4% (SD=2.1) cells/image with puncta. This was highly significant at a $p < .0001$. Box and whisker plots representing control wells are shown in Figure 6G.

All the timepoints after the 15-hour mark had extremely significant differences between the two groups with $p < .0001$ for each. The largest difference observed between the two groups occurred at 18 hours. A comparison between the four wells across all timepoints can be seen in Figure 6H below. At 24 hours, cells treated with 0.5 μM rapamycin had an average of 64.2% (SD= 11.1) cells/image with puncta as compared to no treatment wells with an average of 17.6% (SD=3.3) cells/image with puncta. In contrast, 0.3 μM rapamycin treatment resulted in an

average of 50.9% cells/image with puncta. That is a 20.7% increase in cells with puncta resulting from increased rapamycin dosing.

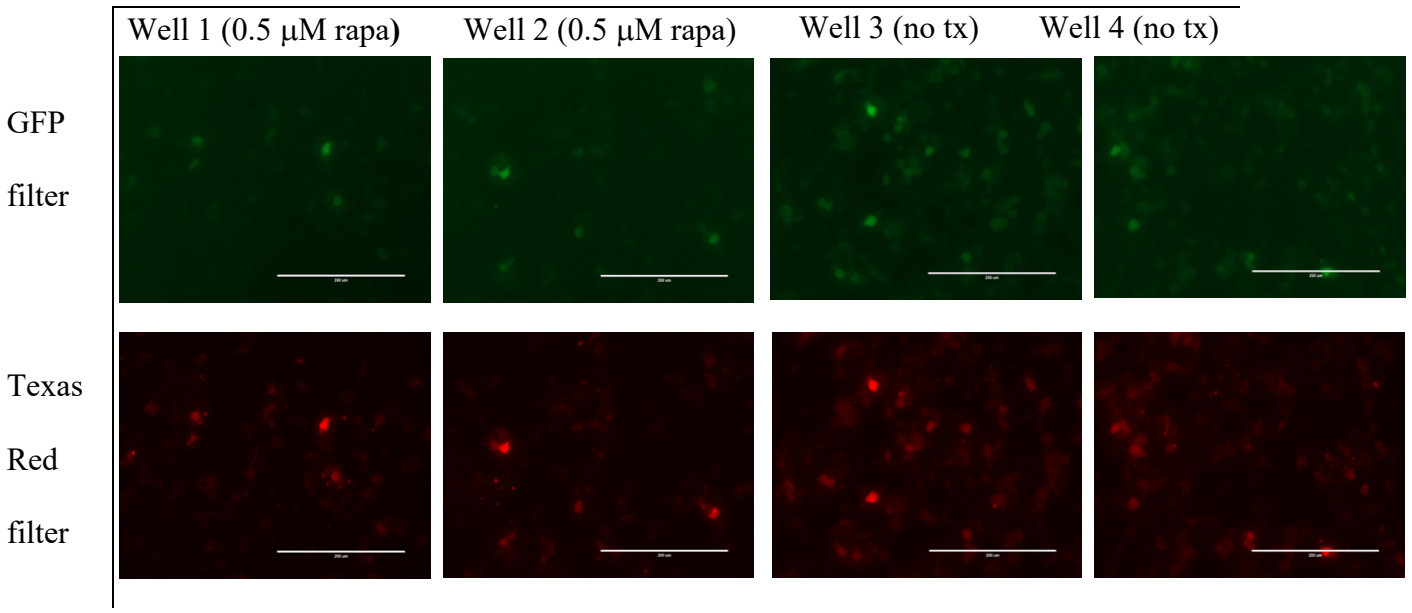
A

Time 0 hr.



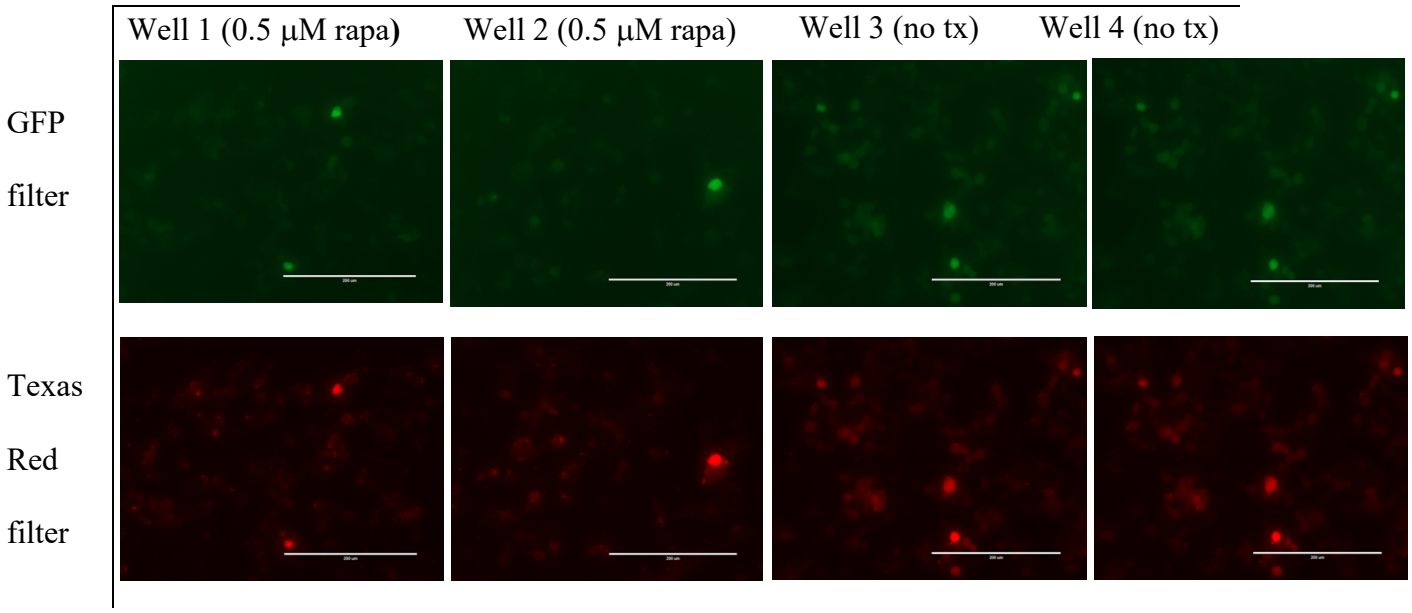
B

Time 15 hr.



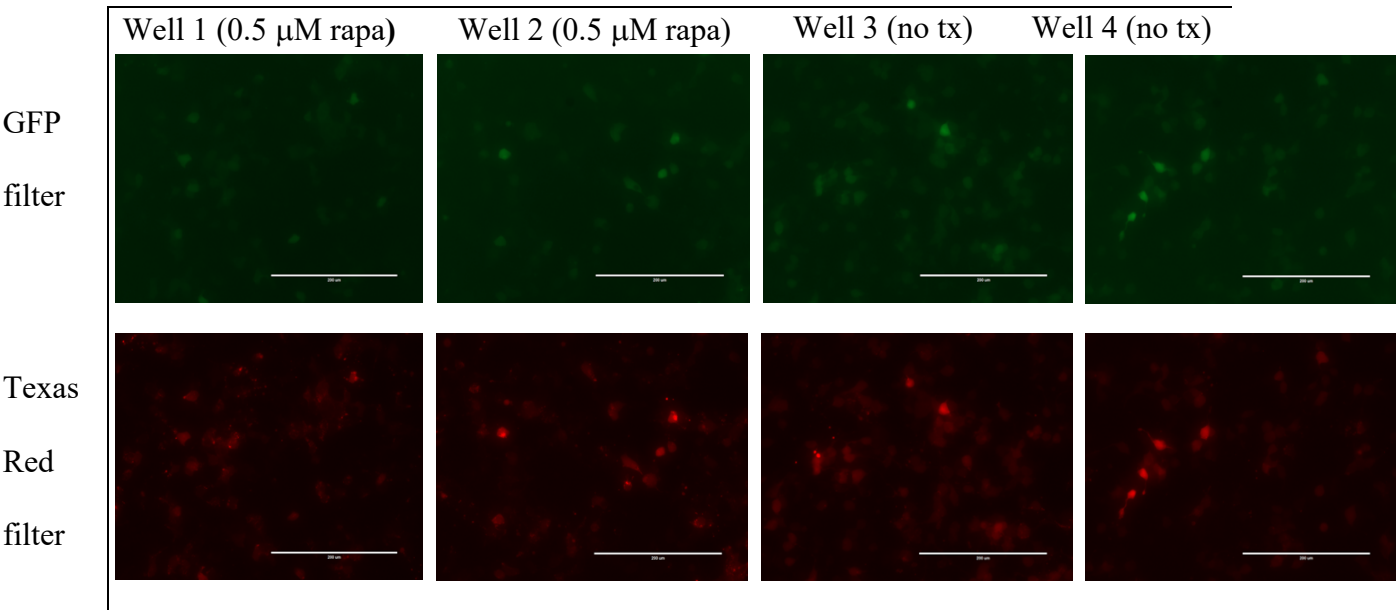
C

Time 18 hr.



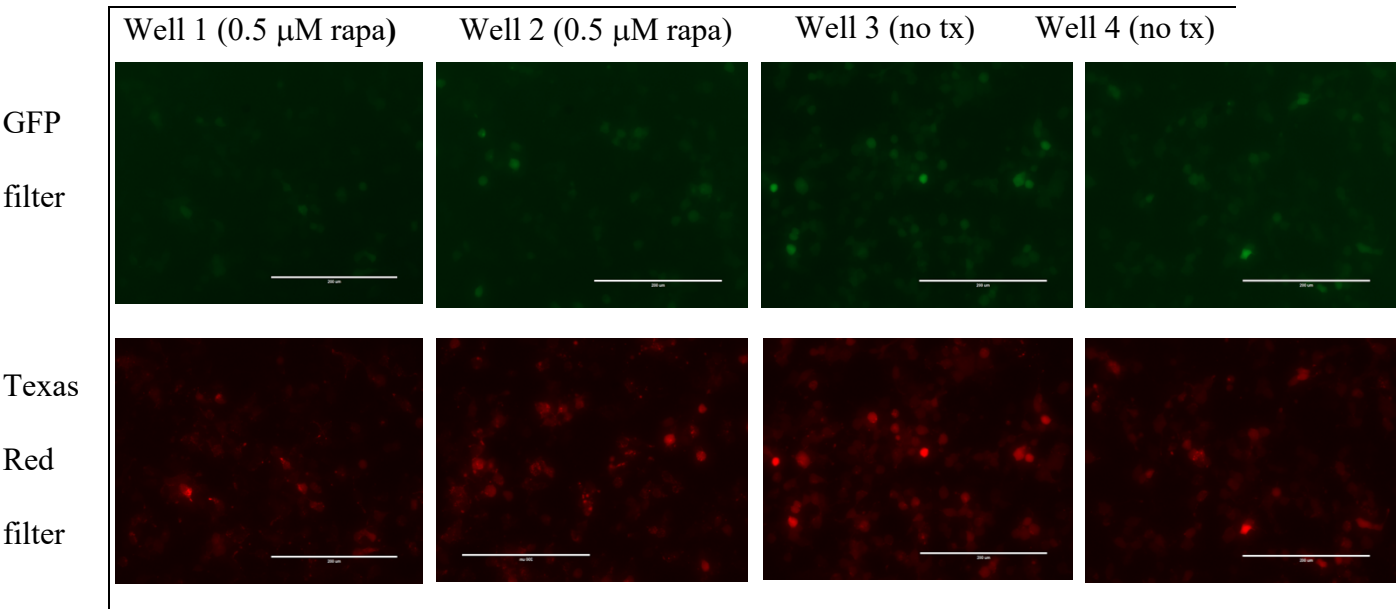
D

Time 21 hr.

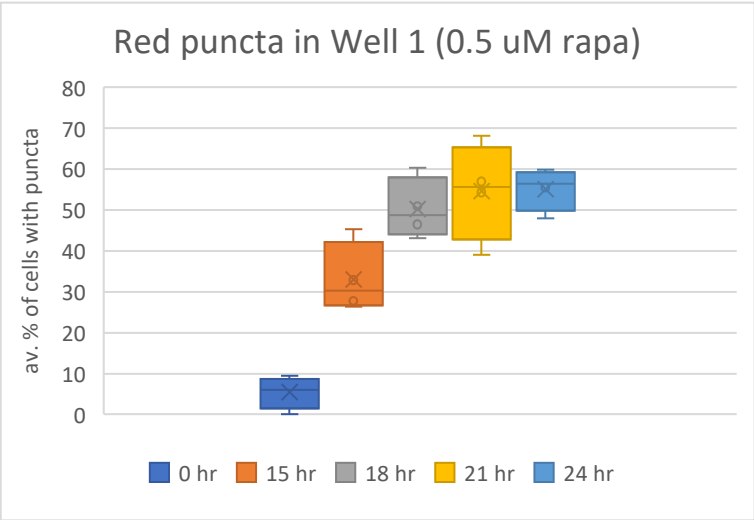


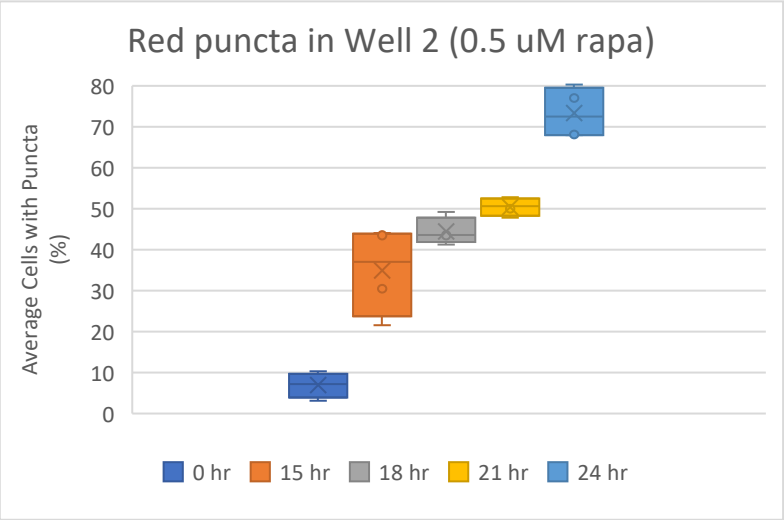
E

Time 24 hr.

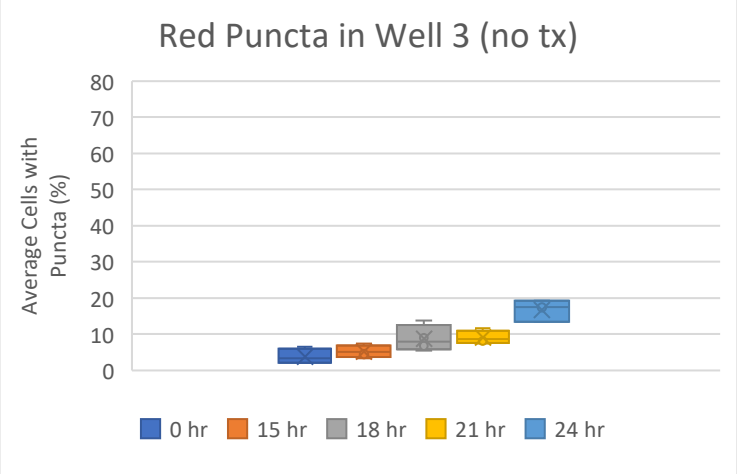


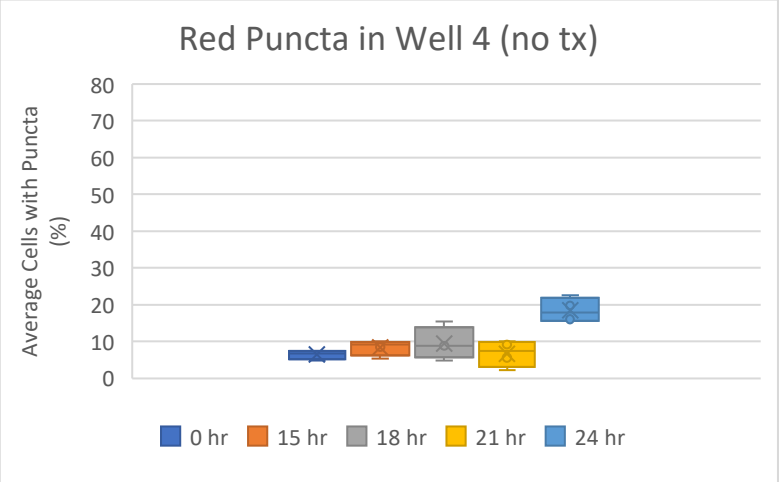
F


Red puncta in Well 1 (0.5 uM rapa)
av. % of cells with puncta
80
70
60
50
40
30
20
10
0
0 hr
15 hr
18 hr
21 hr
24 hr


Red puncta in Well 2 (0.5 uM rapa)
Average Cells with Puncta (%)
80
70
60
50
40
30
20
10
0
0 hr
15 hr
18 hr
21 hr
24 hr

G


Red Puncta in Well 3 (no tx)
Average Cells with Puncta (%)
80
70
60
50
40
30
20
10
0
0 hr
15 hr
18 hr
21 hr
24 hr


Red Puncta in Well 4 (no tx)
Average Cells with Puncta (%)
80
70
60
50
40
30
20
10
0
0 hr
15 hr
18 hr
21 hr
24 hr

H

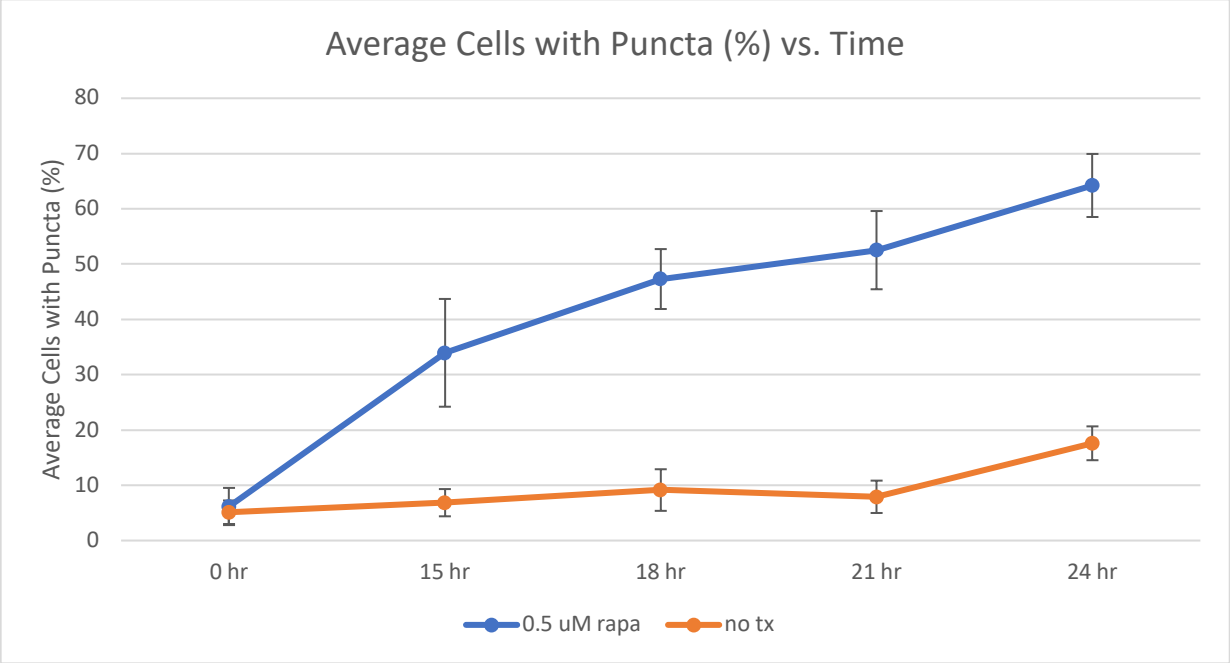


Figure 6. Rapamycin (0.5 μM) treated cells show a pronounced increase in red puncta over time in culture indicating flux through the autophagy pathway. A-E) Representative images are shown for HEK293 cells transfected with the LC3 plasmid. The cells fluoresce and form distinguishable green and red puncta at time points between 0 and 24 hours. Cells treated with 0.5 μM rapamycin are compared to untreated, transfected controls at the same time point. F) A box and whisker plot shows the average percentage of cells with red puncta at times 0, 15, 18, 21, & 24 hrs after treatment with 0.5 μM rapamycin. Averages were calculated from each of the three images taken per well. G) A box and whisker plot shows the average percentage of cells with red puncta at times 0, 15, 18, 21, & 24 hrs in cells with treatment. Averages were calculated from each of the three images taken per well. H) The line graph shows averages from all same-treatment wells and images for each of the time points and demonstrates a clear difference between 0.5 μM rapamycin-treated cells and the no treatment controls starting at 15 hours.

Rapamycin 0.3 μM and Chloroquine 40 μM Cotreatment

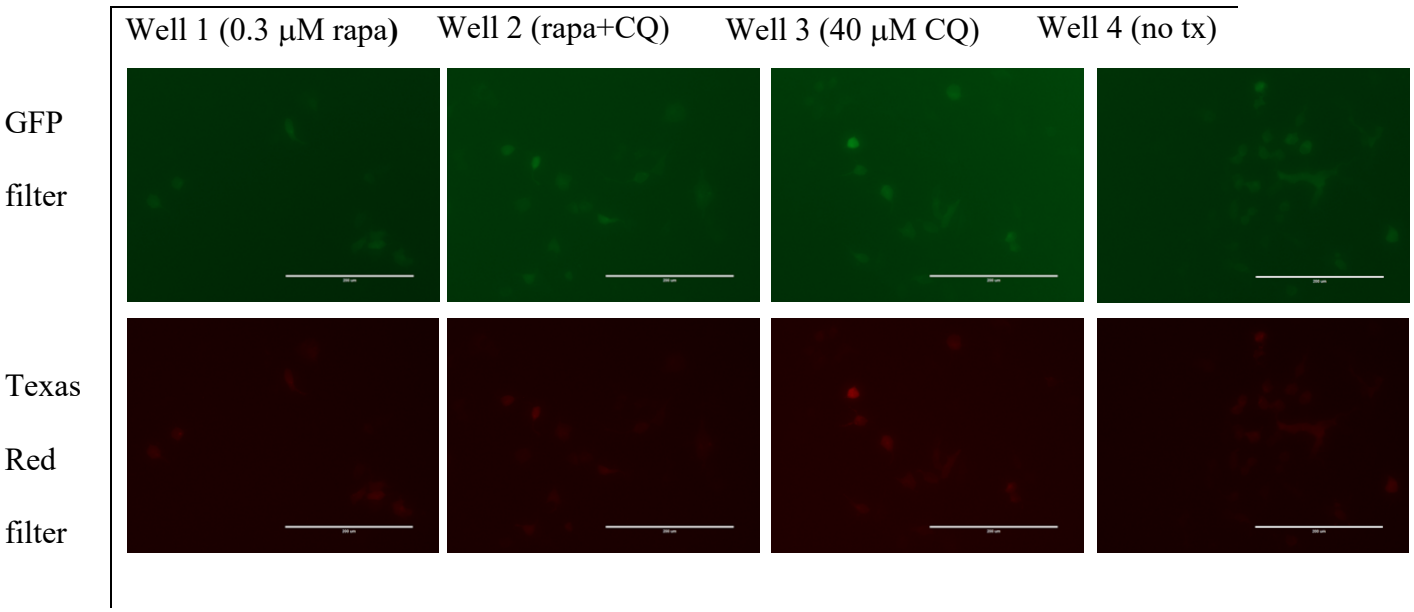
In this experiment, there were 3 experimental wells, 1 control well, and one for GFP-mApple. Well 1 was dosed only with 0.3 μM rapamycin and showed similar results at the 18.5 timepoint in terms of red puncta to the first experimental performed. Well 2 was dosed with 0.3

μM rapamycin along with 40 μM CQ. Cells in Well 3 were dosed only with 40 μM CQ and Well 4 was left untreated (no tx). Wells 1-3 showed a difference in the average amount of cells with red puncta after 18.5 hours, but this difference was not large enough to be statistically different. In the well treated with 0.3 μM rapamycin, 67% (SD=15.4) cells/image contained puncta using the Texas Red filter, as compared to 79.1% (SD= 10.6) in the combined rapamycin and CQ treatment and 73.8% (SD=13.7) in the CQ alone treatment. A t-test between Wells 1 and 2 showed that these differences are not significant at p= 0.246. Comparisons between Wells 1 & 3 and 2 & 3 showed similar results, p=0.532 and p= 0.568, respectively. However individual comparisons made to each of these wells with the no treatment Well 4 suggest a significant difference $p < .01$. Red puncta are visible in all treated groups in statistically indistinguishable numbers. All groups induce formation of red puncta compared to untreated cells (Figure 7C).

Visualizing the proportion of cells with puncta using the Green GFP filter shows statistically significant results between the experimental groups. Wells 1 (rapamycin-only treated cells) and 4 (no treatment) showed almost no green puncta. Well 2 containing the rapamycin and CQ combined treatment averaged 73.1% (SD=11.5) cells/image with green puncta at 18.5 hours. Chloroquine alone showed 53.8% (SD=9.2) of cells/image with green puncta (a t-test showed p= .0398 between Wells 2 & 3). T-tests between Wells 1 & 2 and 2 & 4 showed highly significant differences of $p < .0001$. Only the two treatments containing CQ had a significant number of green puncta at 18.5 hours, as shown in Figure 7B and C.

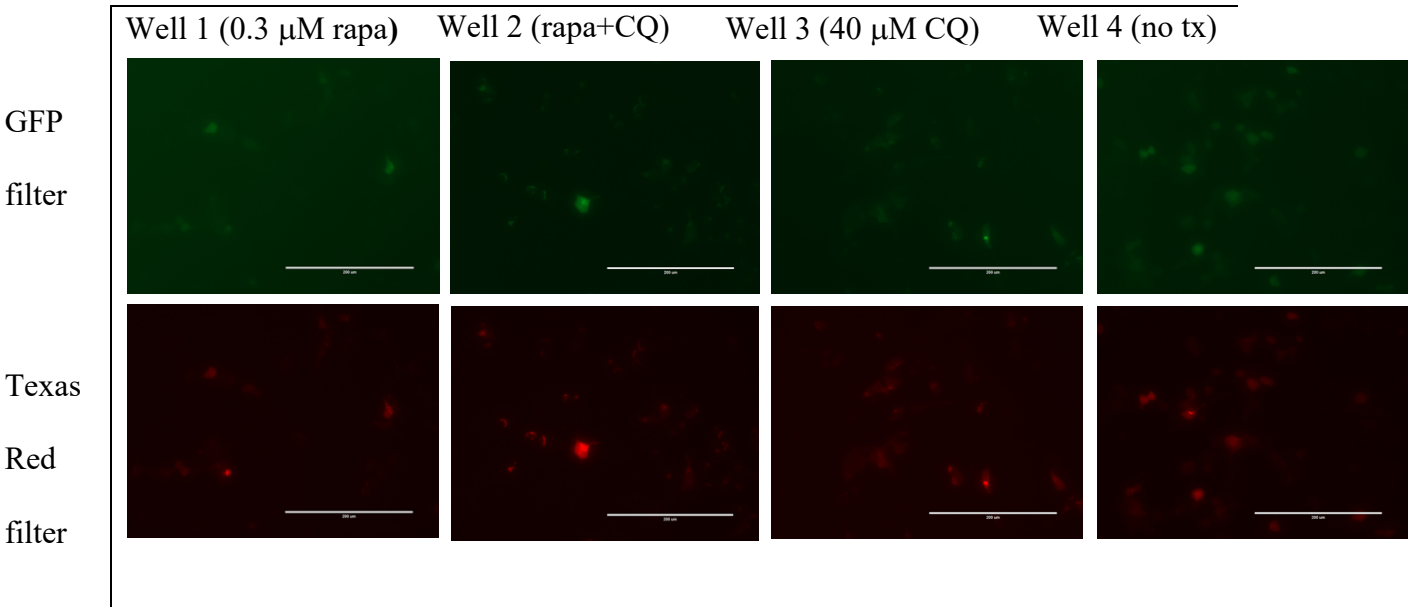
A

Time 0 hr.



B

Time 18.5 hr.



C

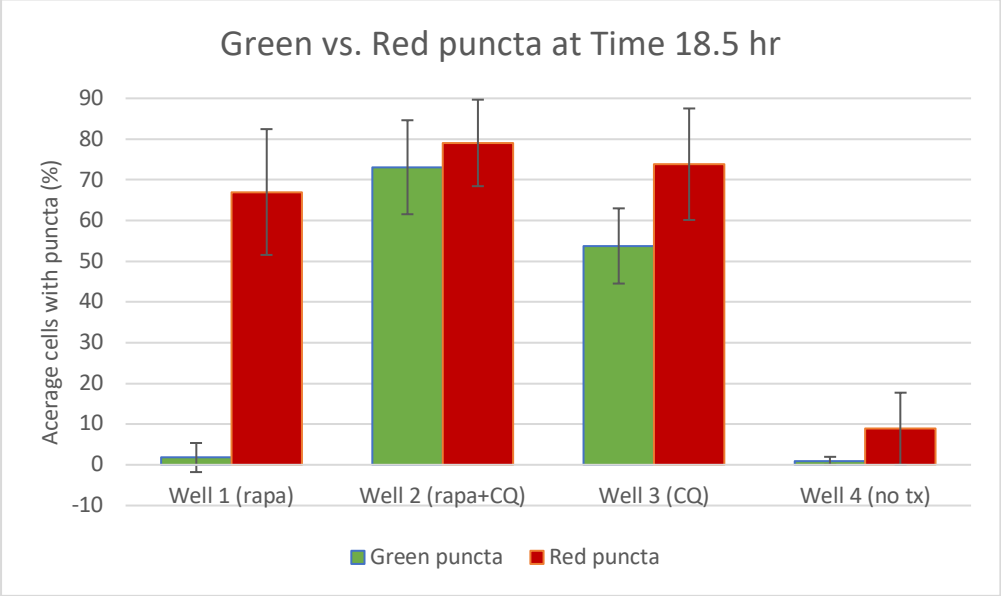


Figure 7. Rapamycin and Chloroquine treated cells demonstrate differences in progression from early to late autophagy. A&B) Representative images are shown for HEK293 cells transfected with the LC3 plasmid and treated with the following treatments: Well 1: Rapamycin (0.3 μM), Well 2: Rapamycin (0.3 μM) and Chloroquine (40 μM), Well 3: Chloroquine (40 μM), and Well 4: Untreated at time points 0 and 18.5hrs. *note the presence of green puncta in Chloroquine-treated cells indicating that autophagy has not progressed to lysosome-fusion. C) The bar graph shows the percentage of cells with green and red puncta for each treatment group. Of note, the rapamycin-treated cells show a high percentage of red puncta only indicating that late autophagy is predominantly occurring by 18.5hours. Both Chloroquine treatment groups show a high percentage of green and red puncta (as expected from a treatment that blocks lysosomal fusion, thereby preventing the GFP probe from being denatured).

Rapamycin Recovery

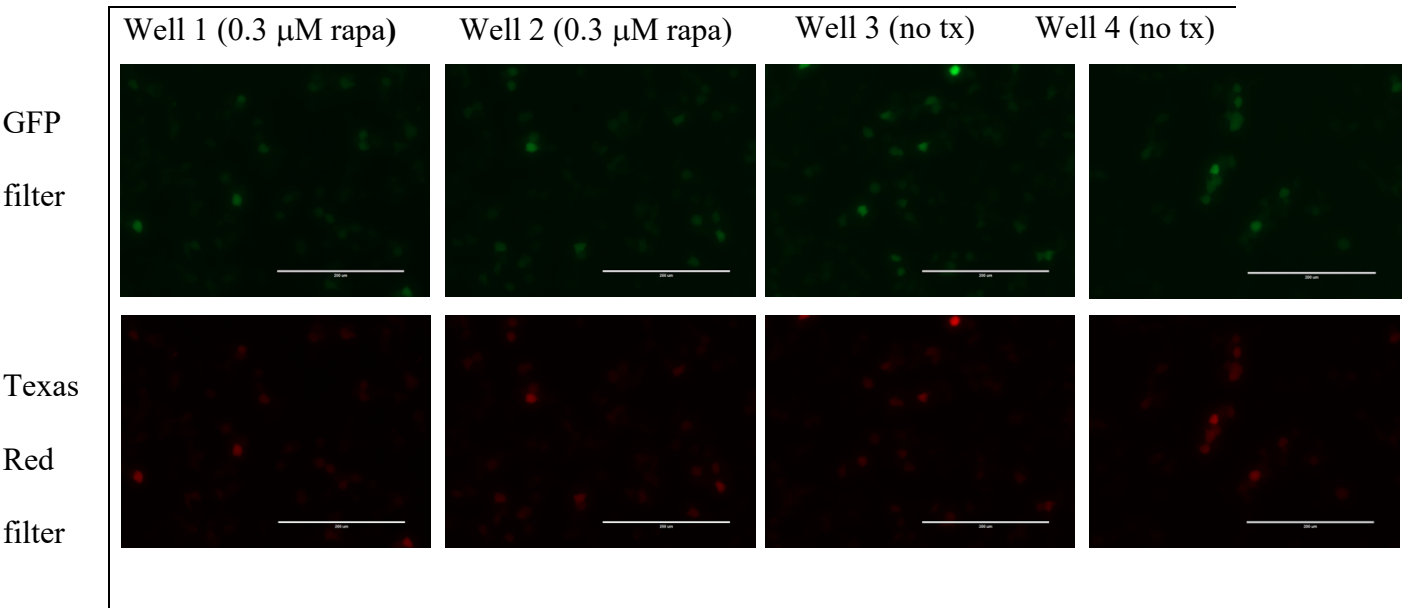
As a timeline has been established for the 0.3 μM rapamycin dose (shown in Figure 3 above) and because this dose is less concentrated than the higher 0.5 μM rapamycin dose, we assumed that it would be easier for cells to recover and return to baseline autophagy levels after exposure. In this experiment, we transfected cells with the LC3 plasmid and dosed one group with rapamycin (Wells 1 & 2) and left the other group untreated (Wells 3 & 4). Images were then taken at a variety of time points while changing medium each day in a multi-day time course to

coax cells to return to normal by continual supplementation of nutrients and removal of medium containing rapamycin in the treatment group (Figure 8A-D). At time zero, minimal green puncta and no red puncta was observed at all 4 wells. After dosing with 0.3 μM rapamycin and waiting for 24 hours, the expected difference between rapamycin-treatment and the no-treatment wells is visible. Wells 1 and 2 dosed with rapamycin showed a similar average of 23.5% (SD=11.3) and 22.6% (SD=9) cells/image displaying red puncta, respectively. In Wells 3 and 4 receiving no treatment, they showed 11.9% (SD=1.9) and 5.27% (SD= 0.3) cells/image with red puncta, respectively. When averaging the first two treatment wells ($\bar{x}$= 23.1, SD=9.13) and the last two no treatment wells ($\bar{x}$= 8.24, SD= 4.16), there is a statistically significant difference at p= .0049. Interestingly, there was a significant difference between Wells 3 and 4 both receiving no treatment (p= .0038), but this 24-hour timepoint is the only one where this difference was observed and might be explained by technical error- a stressed dish of cells.

At the 32-hour mark, averaged treatment groups ($\bar{x}$= 37.9, SD=6.96) and averaged non treatment groups ($\bar{x}$= 23.6, SD=16.2) when subject to a t-test, resulted in a significant difference with p= .0001. Even more significant at p <.0001 was the difference between the two groups at the 37-hour timepoint. The treatment wells ($\bar{x}$= 43.2, SD= 5.60) and non-treatment wells ($\bar{x}$= 29.7, SD= 17.1) were again averaged for this result. Figure 8E shows the red puncta over time in the two rapamycin-treated wells while Figure 8F shows the no treatment wells. Of note, rapamycin-treated cells showed a continual increase in red puncta over the time course of this experiment. The two rapamycin treated wells were averaged and the two no treatment wells were averaged to create Figure 8G for a better comparison between the two groups.

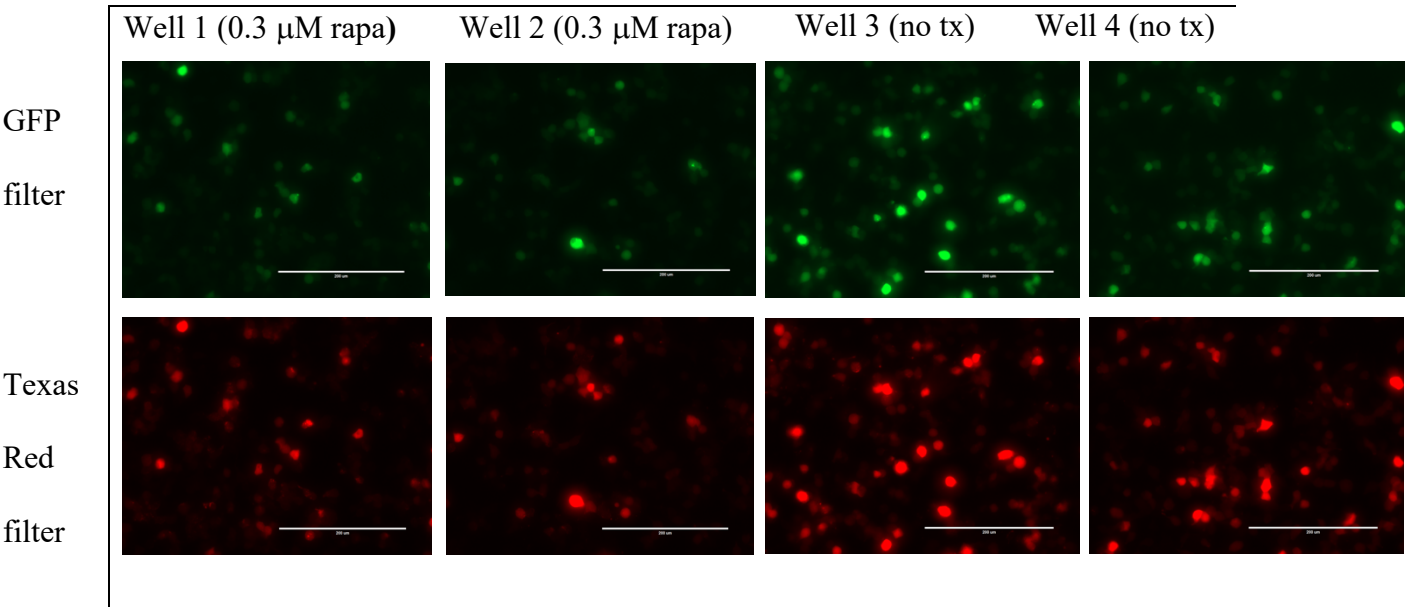
A

Time 0 hr.



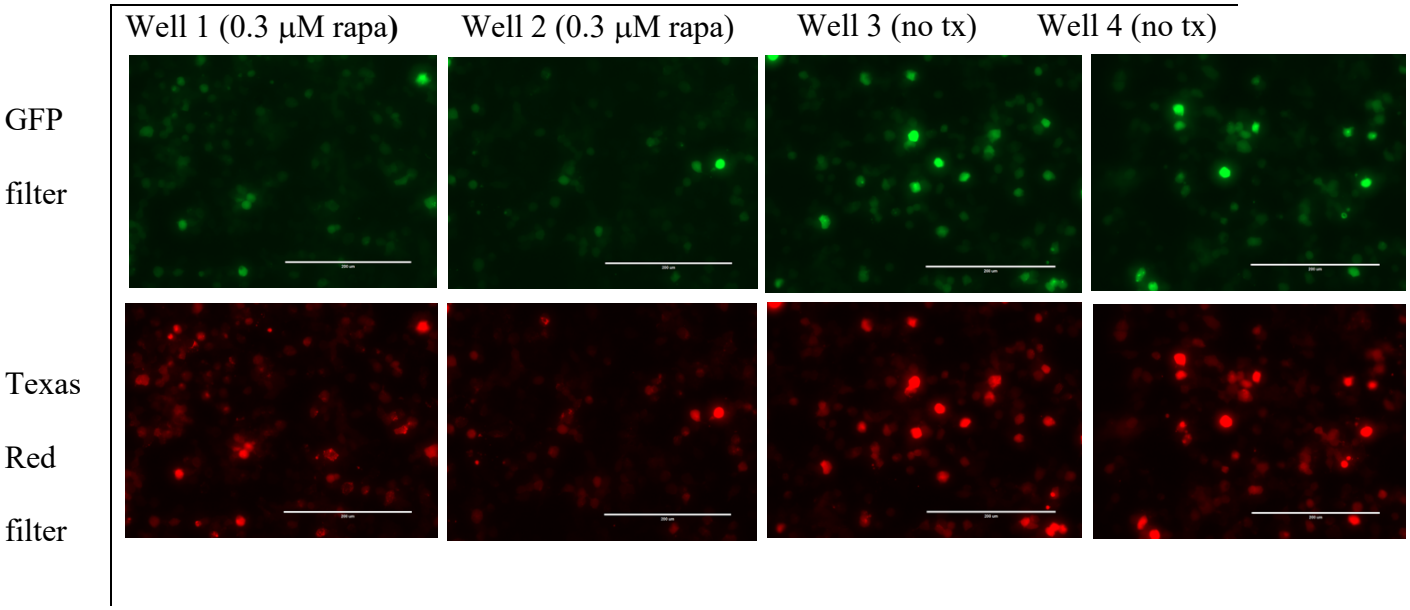
B

Time 24 hr.



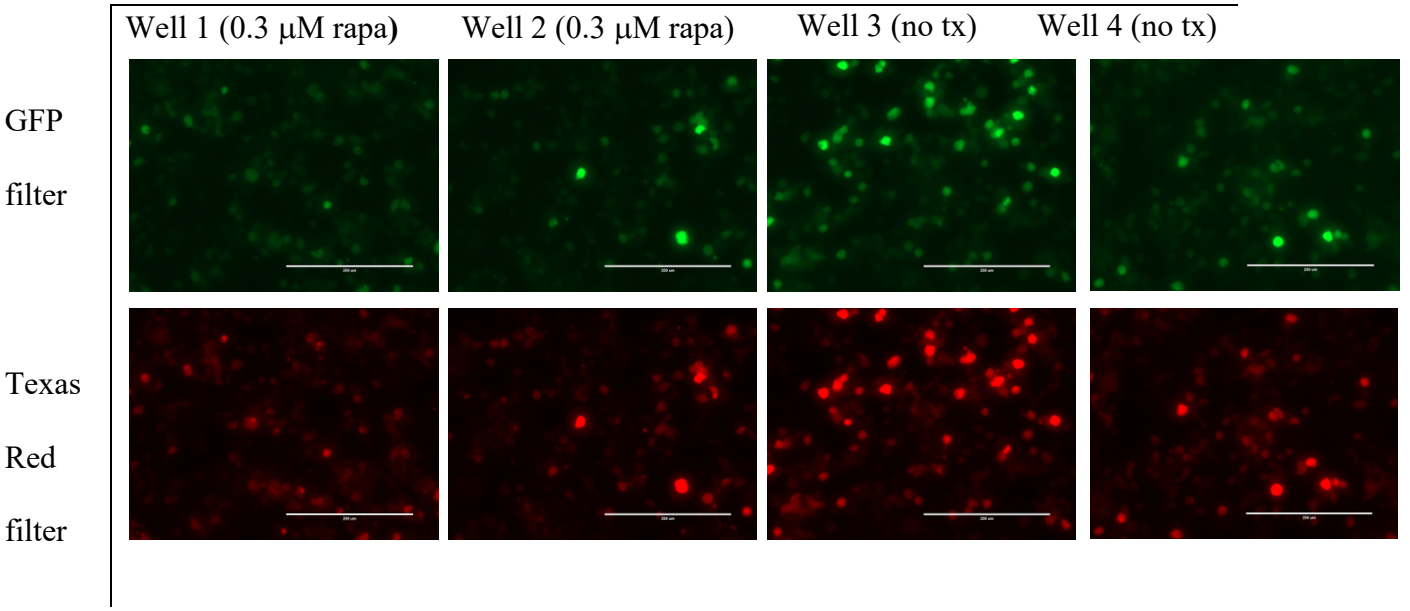
C

Time 32 hr.

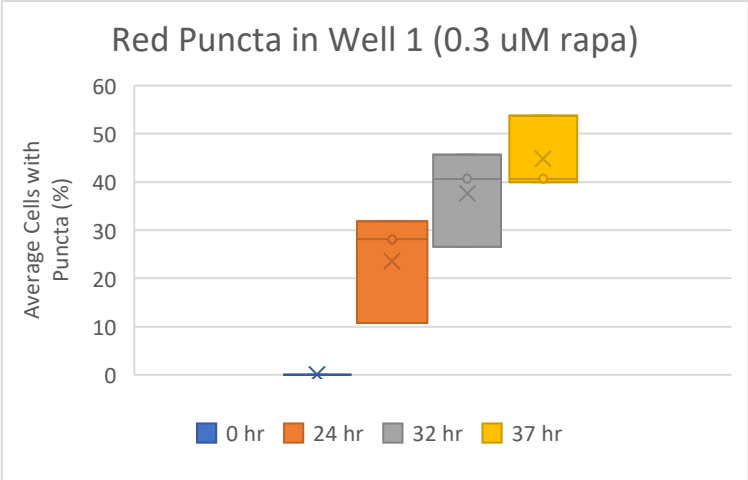


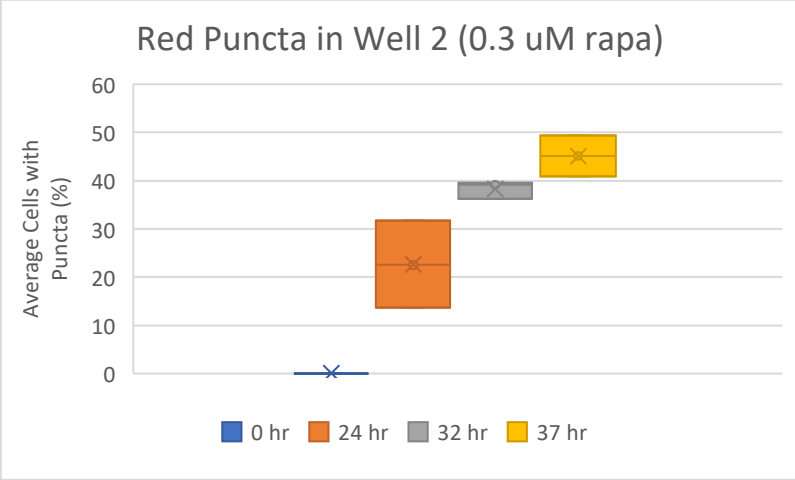
D

Time 37 hr.

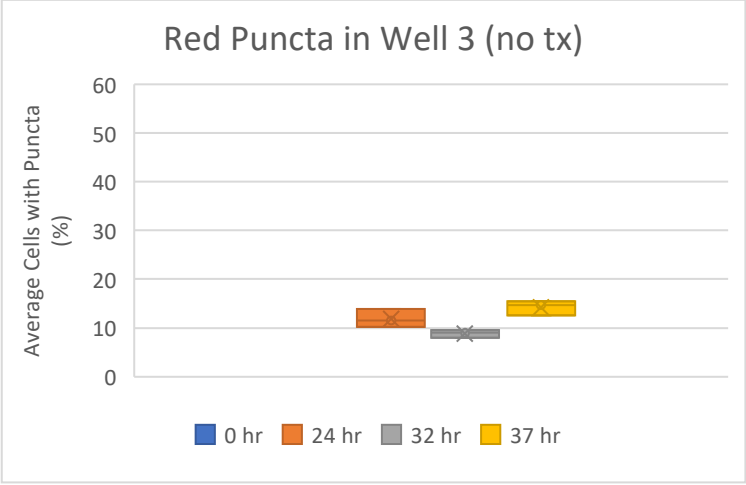


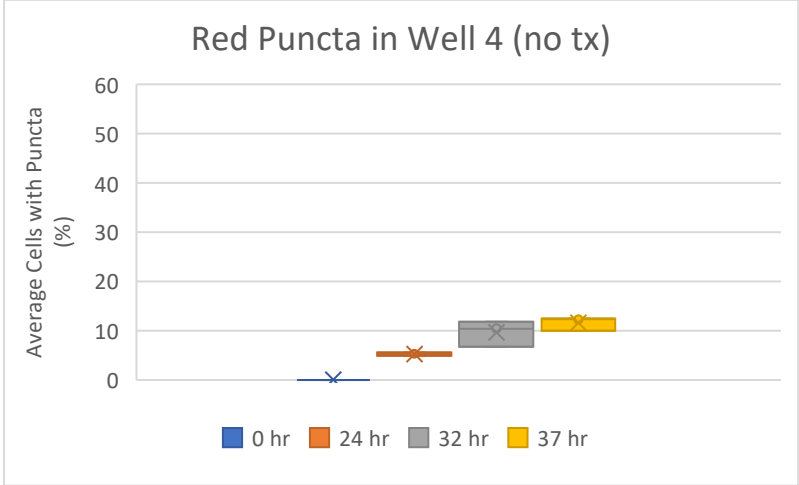
E


Red Puncta in Well 1 (0.3 uM rapa)
Average Cells with Puncta (%)
60
50
40
30
20
10
0
0 hr
24 hr
32 hr
37 hr


Red Puncta in Well 2 (0.3 uM rapa)
Average Cells with Puncta (%)
60
50
40
30
20
10
0
0 hr
24 hr
32 hr
37 hr

F


Red Puncta in Well 3 (no tx)
Average Cells with Puncta (%)
60
50
40
30
20
10
0
0 hr
24 hr
32 hr
37 hr


Red Puncta in Well 4 (no tx)
Average Cells with Puncta (%)
60
50
40
30
20
10
0
0 hr
24 hr
32 hr
37 hr

G

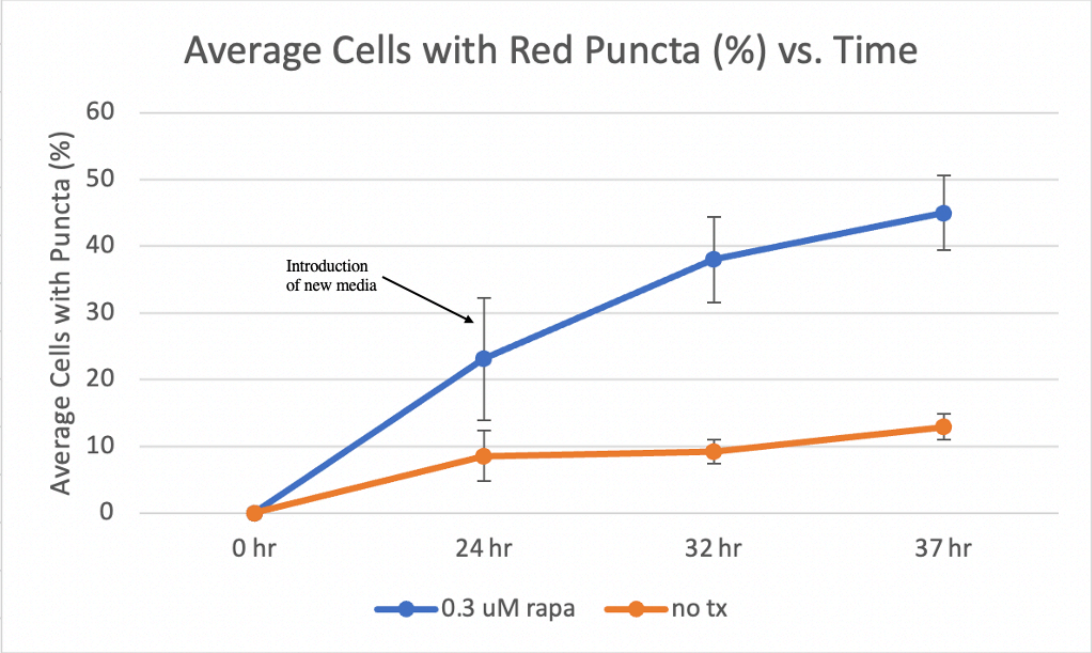


Figure 8. Rapamycin-treated cells show no indication of recovery or return to baseline over a 37-hour timeframe. Rapamycin-treated cells show increases in red puncta indicating increased numbers of cells undergoing late autophagy. A-D) Representative images are shown for HEK293 cells transfected with the LC3 plasmid and treated with rapamycin 0.3 μM (Wells 1 & 2) or no treatment (Wells 3 & 4). Cells were imaged over a 37-hour time course (0, 24, 32, & 37hrs) and allowed to recover from treatment after the 1st 24 hours through removal of culture medium containing rapamycin and supplementation with new medium. E) The box and whisker plots show the average percentage of cells/image containing red puncta per time point in rapamycin-treated wells. F) The box and whisker plots show the average percentage of cells/image containing red puncta per time point in untreated-treated wells. Of note, rapamycin-treated cells show no recovery within the time frames observed, yet untreated cells, when medium is changed, demonstrate low levels of autophagy- as would be expected in cells not induced to undergo the process. G) The line graph shows averages from all same-treatment wells and images for each of the time points and demonstrates a clear difference between 0.3 μM rapamycin-treated cells and the no treatment controls starting at 24 hours.

V. Discussion

In this study, we aimed to develop protocols for using rapamycin and chloroquine as autophagy controls in HEK293 cells. We also aimed to better understand whether cells can return to a baseline following exposure to these compounds. Our data will facilitate future Coan lab studies focusing on autophagy and biomaterial substrates that may alter the autophagy pathway.

Our rapamycin results suggest that 0.3 mM and 0.5 mM rapamycin induce autophagy, as expected, measured by a steady increase in the proportion of cells with red-only puncta, an indicator of late autophagy. Our data suggests that rapamycin's activity is time-dependent, becoming more pronounced and diverging from control cells with increased exposure time. However, there was an observable difference in these two experiments as the higher dosage of rapamycin produced a significantly higher proportion of cells with red puncta. This suggests that rapamycin's activity is likely dose-dependent. Of note, the higher dosage was more distinguishable from the untreated cells at an earlier time point. It may represent a better dose for experiments in the Coan lab to ensure reproducible effects that are easily distinguishable from the control cell response.

In all rapamycin experiments, we observed the entire autophagy process as expected, utilizing the fluorescent probe system selected for this project. Time 0 showed cells with mostly diffuse green and red fluorescence- an indication of transfection success. Cells at this time point were robustly expressing the LC3 transgene, of which the protein was dispersed throughout the cytoplasm (showing up as diffuse green and red fluorescence). However, autophagy was not actively running at this time point (as indicated by minimal to no puncta in the cells). Early time

points after induction of autophagy with the chemical inducer, rapamycin, showed the presence of cells containing green and red puncta. Puncta represent small, intensely fluorescing spots within the cell, indicating the aggregation of LC3 into autophagosomes. The presence of puncta with both green and red fluorescence indicates early autophagy before lysosome fusion. Later time points, after chemical induction of autophagy, generally showed cells with only red puncta. When green and red no longer show colocalization in this system, it indicates lysosome fusion to the autophagosome, which occurs late in the autophagy process. The lysosome's acidic contents denature the GFP protein preventing it from fluorescing at this stage in the process.

In rapamycin-treated cells, it was rare to observe both green and red puncta in high numbers of cells at any time point imaged. Yet, as expected, rapamycin led to late autophagy (higher percentages of cells with red-only puncta) at much higher rates than in non-treated cells. We believe that this suggests that the first phase of autophagy (autophagosome formation) may occur relatively early in the process and may be a very short-lived, transient phase. Therefore, our selected time points may have yet to catch high numbers of cells undergoing early autophagy. Timepoints that are closer together might better capture the early autophagy process but may not account for the short-lived, transient nature of early autophagy, and the benefit of knowing the exact timing of early autophagy might be outweighed by the tedious and time-consuming effort to identify such a short-lived process.

One result of note in the rapamycin-treatment experiments was that our untreated cells, over a long duration of time in culture, also started to show increased red puncta- indicating autophagy activation. This is likely due to our cells sitting in spent nutrient medium and overpopulating their dish, which are the very conditions that promote autophagy. It is problematic if we cannot distinguish between average levels of baseline autophagy and

autophagy induction via our chemical inducer. Therefore, in future experiments, it will be vital that we ensure the health of our control cells through continual media refreshes and by keeping the cells from becoming overpopulated on the dish by the end of the experiment. Long-duration experiments that last over 36 hours are not advisable, as they run the risk of non-treated cells exhibiting high levels of background autophagy.

In another set of experiments, we investigated the co-administration of the autophagy inducer, rapamycin, with the autophagy inhibitor, CQ. As expected, these experiments suggested that CQ blocks lysosomal fusion with autophagosomes, preventing the transition from early to late autophagy[17.] Cells treated with both rapamycin and CQ displayed green and red puncta at time points previously shown to contain red-only puncta (a sign of late autophagy). This lack of progression from green and red puncta to red-only puncta suggests that CQ is acting as expected and blocking the fusion of the lysosome. CQ, therefore, can act as an important negative control for future experiments in the Coan lab.

The CQ experiments observed an unexpected result: the average number of countable cells at 18.5 hours for the no-treatment group ($\bar{x}$=52.1) is statistically different from the CQ treatment group ($\bar{x}$=36.1) with a significance level of p=0.005, but not from the combined treatment of rapamycin and CQ ($p=0.018$). Repeated experiments are needed to see if autophagy inhibitors tend to reduce the number of cells fluorescing or if they are toxic to cells and induce cell death- thereby reducing cell numbers. Also of note is that CQ alone appeared to increase the presence of puncta in cells. Since CQ is not believed to induce autophagy, we hypothesize that blocking the pathway's flux could cause a buildup of high levels of autophagosomes in healthy cells. This buildup, typically maintained at a baseline homeostatic level, becomes detectable as puncta in our study. However, more work would be needed to understand this outcome better.

One final note regarding data obtained in rapamycin/chloroquine experiments is that the numbers of red and green puncta, in theory, should always be equal in cells treated with CQ because the probes are both attached to the same LC3 protein and should co-localize. However, reproducibly, each count yielded a smaller % of cells with green vs red puncta, possibly due to technical errors. We supplement our cell culture growth medium with a pH sensor called Phenol Red. This compound tends to obscure the wavelength the Green light cube uses but not the Texas Red light cube on the microscope. Therefore, Phenol Red in the medium may obscure lower fluorescing green puncta, potentially resulting in a lower count of green-only puncta.

In summary, monitoring cell changes induced by rapamycin and CQ is important for future work in the Coan lab. Honing these initial experiments and repeating them to show reproducible results should lead to reliable protocols for our particular HEK293 cell line. Additional studies that correlate western blotting results and potentially gene expression results with these controls will further strengthen our findings and provide a comprehensive understanding of how rapamycin and chloroquine affect autophagy.

Also important to our lab goals is a better understanding of whether cells can return to baseline after treatment with rapamycin or CQ. Rapamycin recovery experiments performed provide a first step toward developing our understanding of this process. To this end, we did not observe a change in cells allowed to recover for a minimal time period after exposure to rapamycin. Future work will increase the duration we observe cells while coaxing them to recover with supplemented nutrients to understand better whether cells can return to baseline following rapamycin or CQ treatment.

The experiments provided a sound basis for expanding our knowledge of autophagy controls and developing protocols for their use in HEK293 cells. However, changes to future

work will be needed to address the several limitations of these studies. One such limitation is the tedious and manual cell and puncta counting method. We attempted to automate cell counting using ImageJ, a free online software. However, the software appeared to miss a significant number of cells. Additionally, due to the lower efficiency and robustness of transfection with this system compared to certain plasmid systems, fluorescence was often low and posed challenges for automation in a software system for cell counts[19]. Therefore, we resorted to manual counting. Manual counts can introduce user error and individual bias, which can distort results when performed by multiple individuals. Such inconsistencies in data can arise as a result. Additionally, cells become difficult to distinguish from neighboring cells when riddled with puncta, leading to total cell count errors. The development of an automated counting system would significantly reduce user error in the future. Alternatively, having two or more individuals counting images and measuring intercoder reliability could be a good way to increase the accuracy of the cell count.

To gain a better understanding of cell recovery after autophagy induction, further trials are required involving different doses of rapamycin and varying time points for media changes. Once this is established, it would be interesting to investigate the effects of CQ, which inhibits the fusion of autophagosomes with lysosomes in autophagy. Currently, it remains unknown whether cells can restore their normal state after autophagy blockage. Since dysregulated autophagy holds clinical implications, it is crucial to determine whether autophagy induction and inhibition can be reversed.

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VII. References

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