The Effects of NAADP binding proteins, JPT2 and LMS12 on the levels of autophagy

Soinbhe McAllister

soinbhe.mcallister.22@ucl.ac.uk

Supervised by Dr Alexander Agrotis

Cell & Developmental Biology

Funded by the Laidlaw Foundation 2023

Introduction

Autophagy is a process in which a cell digests its own contents in a waste and recycling pathway (Yu, et al., 2018). A cell is constantly digesting its contents to build new organelles by breaking down old proteins into amino acids to build new proteins, which is the basal level of autophagy. The basal level can be upregulated when the cell needs new material, for example during nutrient starvation. This process helps repair cell damage and increases the cell's chance of survival. Therefore, researching how to regulate autophagy has significant therapeutic potential for conditions caused by cell stress, like Parkinson's (Chang, et al., 2022). Calcium ion signalling plays a huge role in cellular function, and there are calcium ion channels called two pore channels, TPCs, on lysosomes, which are organelles involved in autophagy (Marchant, et al., 2022). Two proteins called JPT2 and LMS12 are involved in activating the TPCs and may play an indirect role in autophagy. My aim was to gain a deeper understanding of the link between calcium signalling and autophagy by researching the effect of the presence/absence of JPT2 and LMS12 on the level of autophagy.

Background

During cell starvation, there are signals emitted that rely on the information of the cell's metabolic state (Yu, et al., 2018). A kinase complex called ULK1 is inhibited under nutrient-rich conditions but is activated when the cell is deprived of nutrients. When there is not enough food for the cell, remodeling occurs when the ULK1 complex is activated, which initiates the formation of the phagophore assembly site (PAS), a specialized structure where autophagosome formation begins. The phagophore nucleates and extends to form a cup-shaped double membrane, making

the autophagosome. There are two bilayers present in an autophagosome. It has been postulated that organelles like the endoplasmic reticulum and Golgi apparatus are the origin of the autophagosomal membrane (Tong, et al., 2010). Upon fusion with the lysosome to form the autolysosome, the inner membrane and contents/cargo are degraded via lysosomal enzymes. The small molecules resulting from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and the maintenance of cellular functions under starvation conditions.

For this project, we used cells from the U2 OS cell line, which are epithelial cells cultivated from the bone tissue of an osteosarcoma patient (Niforou, et al., 2008).

To measure autophagy, we can measure the levels of two autophagy markers, LC3 and p62. They are both proteins involved in the autophagy pathway. During autophagosome formation, LC3-I is converted to LC3-II through a process of lipidation, which involves the conjugation of LC3-I with phosphatidylethanolamine, and LC3-II is then recruited to the autophagosome membrane (Tanida, et al., 2008). LC3-II remains covalently bonded to the membrane until autophagosome formation is completed, making it a good marker for autophagy. The amount of LC3-II is clearly correlated with the number of autophagosomes. p62 is a multifunctional protein that serves as a cargo receptor in autophagy (Jiu, et al., 2016). Since p62 acts as a cargo receptor, it becomes a target for autophagic degradation. When autophagy is in operation, both the cargo attached to p62 and p62 itself are taken up by autophagosomes, ultimately transporting them to lysosomes for breakdown.

Consequently, the presence of active autophagy leads to a reduction in p62 levels. Reduced levels of p62 are therefore associated with an activated autophagy pathway. By quantifying the levels of proteins, we can measure the levels of autophagy.

Nicotinic acid adenine dinucleotide phosphate, NAADP, is a second messenger that releases Ca2+ by activating the TPCs on lysosomes (Marchant, et al., 2022). NAADP activates TPCs indirectly by binding to a NAADP binding protein, like JPT2 and LMS12, associated with the TPC complex. Since JPT2 and LMS12 have been identified as NAADP binding proteins, their absence should disable the activation of TPCs and possibly have an effect on autophagy. To test this, two samples are used. The first are mock cells, or ‘wild-type cells’, where no gene editing has been done. The second are double knock-out cells (DKO), where the genes for JPT2 and LMS12 were removed using gene editing.

To accurately study autophagy, it's useful to look at basal autophagy compared to autophagy under nutrient-starved conditions. We can manipulate this by simulating cell starvation with drug treatments such as Torin-1. Torin-1 is an inducer of autophagy as it's a selective ATP-competitive inhibitor of mTOR (mammalian target of Rapamycin) kinase (Andersson, et al., 2016). Under normal conditions with sufficient nutrients and growth factors, mTORC1 is active and phosphorylates proteins in autophagy, inhibiting the initiation of autophagy. Torin-1 inhibition of mTORC1 dephosphorylates autophagy-related proteins, enabling the initiation of autophagy. The dephosphorylation promotes the formation of the phagophore, an initial structure of the autophagosome.

To measure autophagic flux by looking at the amount of autophagosome formation, the fusion of autophagosomes with lysosomes must be prevented, and the process of autophagy cannot finish. This can be done by another drug called Bafilomycin A1, an inhibitor of autophagy. Bafilomycin A1 inhibits the acidification of the lysosome through its interaction with V-ATPase, an enzyme that is a proton pump (Mauvezin & Neufeld, 2015). It inhibits lysosomal acidification by preventing the passage of protons into the lysosomal lumen from V-ATPase. If the lysosome is not acidified, the activity of its proteases is prevented so that the engulfed cargo cannot be degraded, and autophagy does not occur.

Torin-1 stimulates the formation of autophagosomes, and Bafilomycin-1 prevents the formation of the autolysosome. Therefore, by applying both treatments to a sample, there will be a large increase in autophagosomes, indicated by a high expression of both LC3-II and p62. Basal autophagy is observed by comparing a sample with no treatments to a sample with Bafilomycin. Torin-induced autophagy is observed by comparing a sample with Torin-1 to a sample with Torin-1 and Bafilomycin.

Methodology

Western blotting is a laboratory technique used for separating and identifying proteins by utilizing gel electrophoresis to distinguish proteins within a mixture based on their molecular weight and thus type. The same Western blot protocol was followed for each repeat of the experiment.

1. Sample preparation: 8 samples were used per experiment. 4 samples were the mock cells, the other 4 were the DKO. The cells were suspended in a nutrient

rich medium and underwent cell splitting so that the number of cells in each sample were the same. Each sample was 120 µL in volume, with one sample per well in a well plate. The treatments were prepared by adjusting their concentrations. Torin-1 was to be at a concentration of 250nM in the samples, and Bafilomycin A1 at a concentration of 100nM. These were adjusted to their concentrations by adding a solvent, DMSO. DMSO was also used for the control sample. For each of the 2 sets: one sample was left as a control by adding 4 µl of DMSO, one sample was treated with 2 µl of Bafilomycin A1 and 2 µl of DMSO, one sample was treated with 2 µl of Torin-1 and 2 µl of DMSO, and the last sample was treated with 2 µl of Bafilomycin A1 and 2 µl of Torin-1. The samples were then incubated at 37°C for 3 hours.

2. Lysing cells: The cells were lysed so the cell membranes broke down and the non-soluble parts were separated from the proteins. A lysis buffer was made with 1% protease inhibitor, to prevent denaturing of the proteins. The samples were aspirated over ice to remove the medium, and then washed with phosphate-buffered saline. 120 µl of the lysis buffer was added to each well, then pipetted up and down over the wells. When doing this, the pipette was scraped across the surface to detach the cells and suspend them in the lysate. The samples were then transferred into chilled tubes and centrifuged at 4°C, so that the non-soluble parts can collect at the bottom to form the pellet, and the proteins remain in the liquid to make the supernatant. Tubes containing 30 µl of 4x LSB and 12 µl 1M DTT were prepared. 78 µl of each sample was transferred into the tubes containing LSB and DTT, so the volume in each tube was 120 µl again. The samples were then incubated at 95°C for 5 minutes. This was done because denatured proteins have a more consistent charge-to-mass ratio as the charges on the amino acid residues are evenly

distributed along the length of the linearized protein. A uniform charge distribution allows proteins to migrate through the gel primarily based on their size molecular weight, allowing more accurate separation of proteins of different sizes.

3. Gel electrophoresis: Protein ladders were used to aid in estimating the size of proteins separated during gel electrophoresis. They were used as points of reference because they contain a mix of proteins of known molecular weight. A gel tank was filled with MES running buffer. With gel loading tips on a pipette, the samples and protein ladder were loaded into the gel. The tank was attached to the electrodes and the gel was run for 35 minutes at 200V.

4. Transfer: The gel was removed from the tank and a transfer membrane was placed on top of the gel. The gel and transfer membrane were sandwiched between filter papers and sponges soaked in transfer buffer. The membrane is placed as such so that the negatively charged proteins can migrate from the negative cathode to the positive anode. This sandwich was placed into a tray in the correct orientation so that the transfer membrane is closer to the anode. The tray was put into the tank and filled with transfer buffer, and the remainder of the tank was filled with distilled water. The tank was attached to the electrodes and the transfer was run for 1 hour at 30V.

5. Blocking: The membrane was blocked for 1 hour in a blocking solution of TBST with 5% milk powder, to prevent antibodies from binding to the membrane non-specifically.

6. Antibody incubation: The primary antibody was added to a tube of blocking solution at the appropriate dilution. The membrane was placed inside the tube and

left in a cold room at 4 °C overnight. The primary antibody recognizes and binds specifically to the target proteins, p62 and LC3, through antigen-antibody interactions. The membrane was then removed and placed into another tube with just TBST and washed 3 times to remove the primary antibodies. The secondary antibody was added to a tube of blocking solution at the appropriate dilution. The membrane was placed inside this tube and incubated at room temperature for 1 hour. Secondary antibodies recognise and bind to the primary antibodies that are already bound to the target proteins. The secondary antibodies are conjugated with an enzyme, catalyse a chemical reaction when that produces chemiluminescence for imaging. The membrane was then removed and placed into another tube with just TBST and washed 3 times to remove the secondary antibodies.

7. Imaging: The membrane was coated with an ECL substrate, which the enzyme acts upon, then placed into a polyethene sheet for imaging. The sheet with the membrane was then placed in an imaging machine. A colorimetric photo was taken to capture an image of the protein ladder blot, and a chemiluminescent photo was taken to capture an image of the target protein blots.

8. Actin control: The membranes were incubated with primary and secondary antibodies specific to actin, and then imaged for actin using the same previous steps. Actin is used as a loading control for a western blot to normalize the levels of protein detected by confirming that protein loading is the same across the gel.

Results

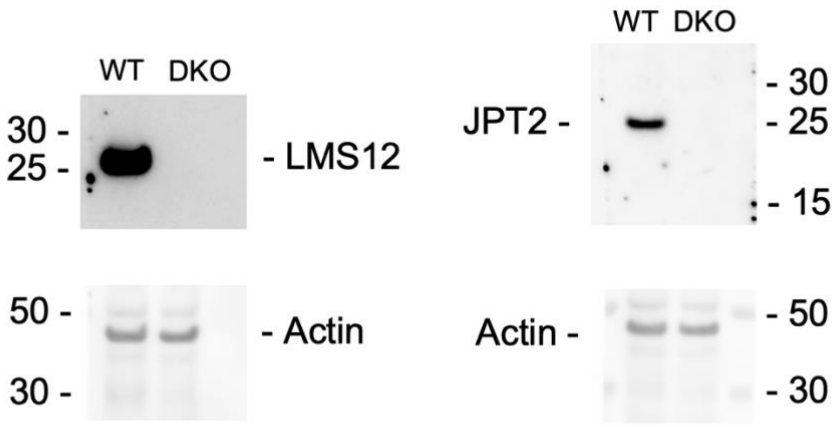

Figure 1 – A western-blot analysis of wild type cells and DKO cells expressing JPT2 and LMS12.

Firstly, a western blot was done for LMS12 and JPT2 for both wild-type and double knockout cells to confirm that the double knockout cells were indeed missing the proteins LMS12 and JPT2.

The chemiluminescent imaged blots were pasted into PowerPoint, where they were then annotated and scaled using the protein ladder colorimetric image. The numbers on the Y-axis indicate kDa, kilodaltons, a unit of measurement of the molecular masses of proteins.

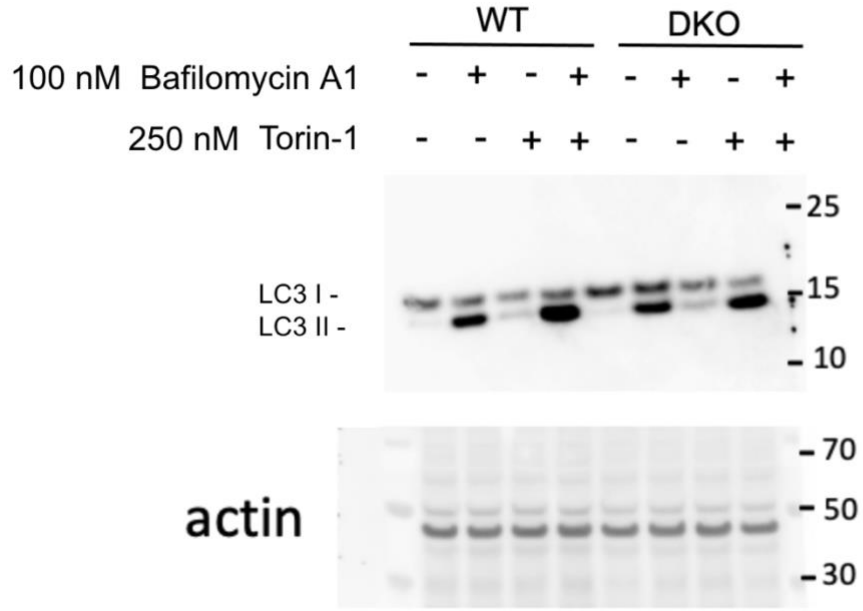

Figure 2 – A western-blot analysis of wild type cells and DKO cells expressing LC3 after 3-hour treatments of Baf A1 and Torin-1.

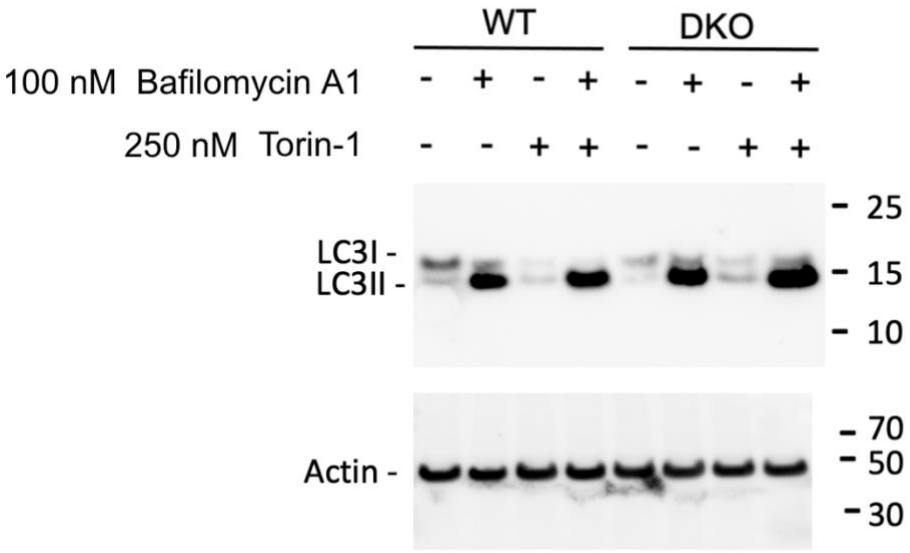

Figure 3 – A western-blot analysis of wild type cells and DKO cells expressing LC3 after 3-hour treatments of Baf A1 and Torin-1.

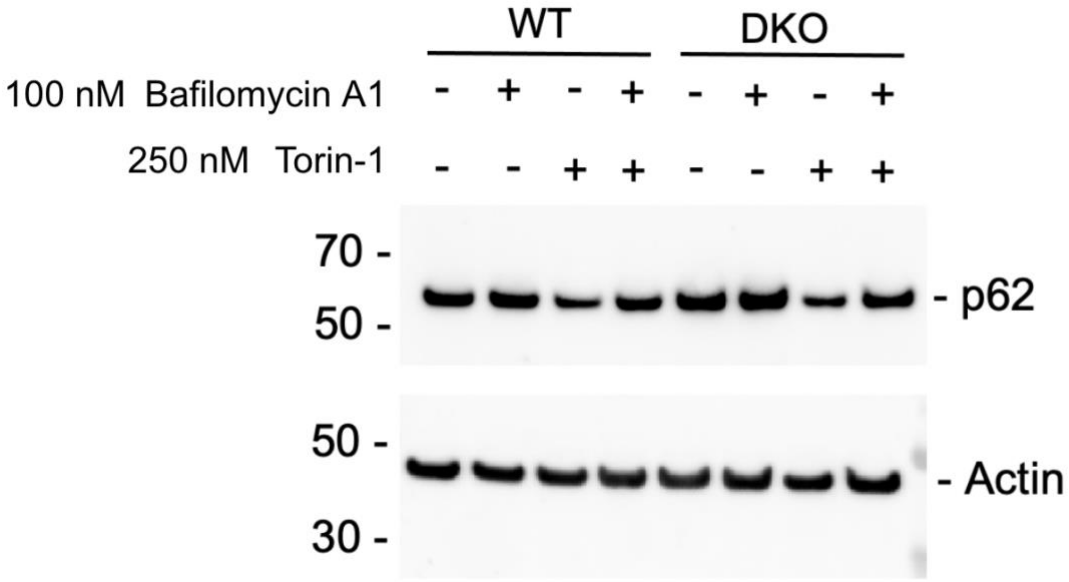

Figure 4 – A western-blot analysis of wild type cells and DKO cells expressing p62 after 3-hour treatments of Baf A1 and Torin-1.

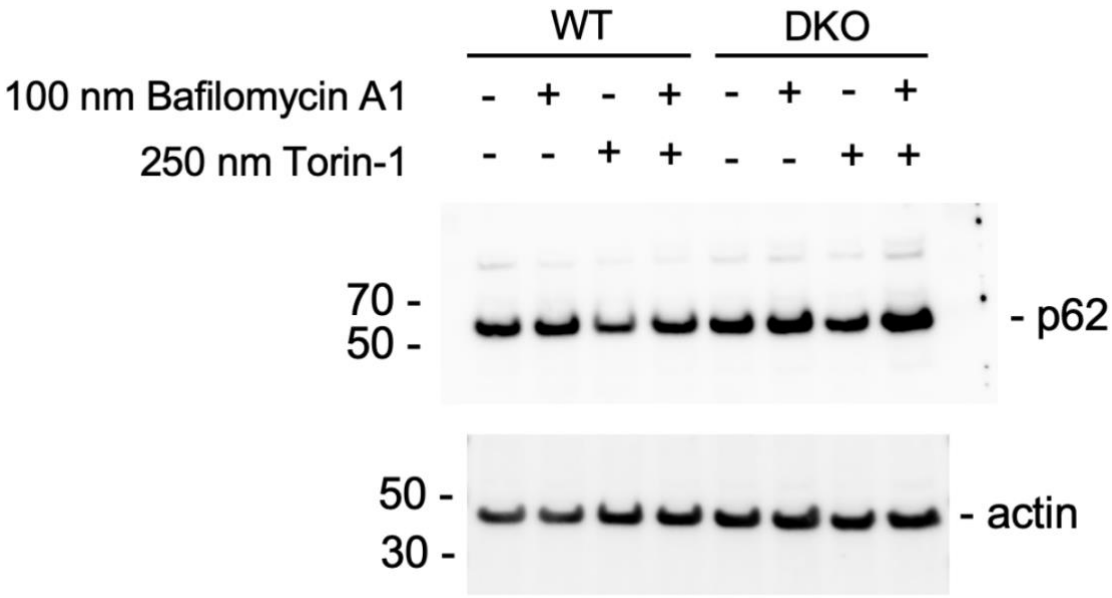

Figure 5 – A western-blot analysis of wild type cells and DKO cells expressing p62 after 3-hour treatments of Baf A1 and Torin-1.

Using a software called Fiji, the imaged blots were quantified by giving us the relative amounts of p62 and LC3 in each sample. This was repeated to obtain the relative amounts of actin for the same membranes. The relative amounts of both p62 and LC3 were divided by the relative amounts of actin. Then, these amounts were all normalised to the wild-type control sample, which was the reference sample. In Western blot analysis, normalization adjusts protein band signals for loading and transfer differences. A reference sample is set to 1, and all other samples are expressed relative to it.

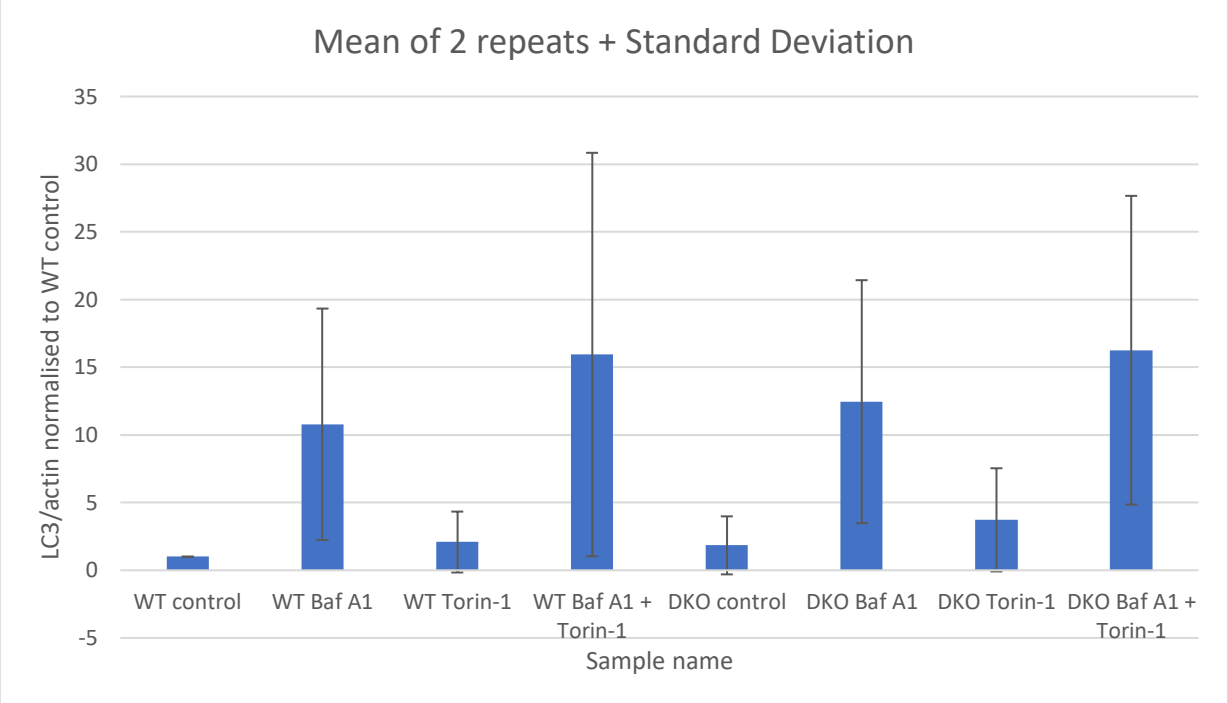

Figure 6 – A chart showing the relative amounts of LC3 protein in each sample divided by the relative amounts of actin normalised to the wild-type control sample.

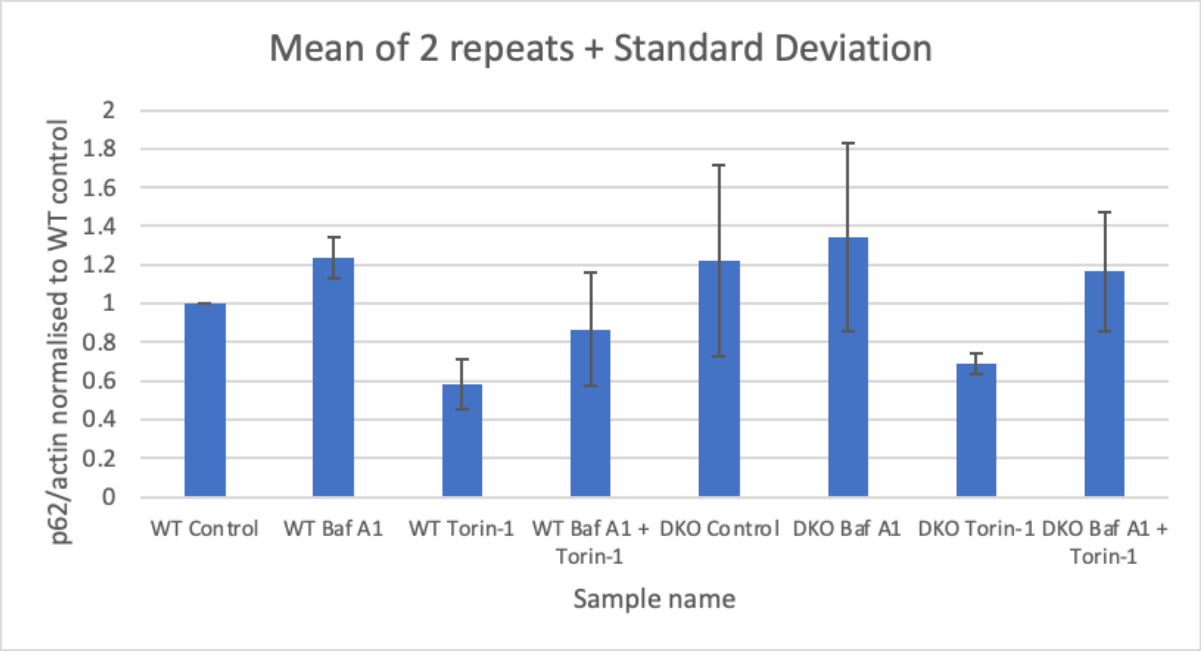

Figure 7 – A chart showing the relative amounts of p62 protein in each sample divided by the relative amounts of actin normalised to the wild-type control sample.

Interpretation: After analysing the results by studying the charts and standard deviation, it was decided that there was no significant difference observed in basal autophagy or Torin-1 induced autophagy for both p62 and LC3-II.

Conclusion

There is no significant difference in autophagy levels between the wild-type and DKO cells.

Reflections and Discussion

This project presented many challenges. While tricky and some unsolvable, they were extremely rewarding. After reflecting on the methodology, I have identified future steps that I or another student could take to repeat this experiment and procure more conclusive results, and possibly expand the findings.

Firstly, doing a Western Blot analysis is only a semi-quantitative analysis, making the interpretation of the results difficult. Since it is semi-quantitative, it relies on signal intensity comparisons using chemiluminescent antibodies. However, the correlation between signal strength and protein quantity is not consistently linear. Therefore, a twofold increase in signal may not equate to a twofold increase in protein expression, which can pose challenges for precise protein level quantification. Western blotting involves relative, not absolute, quantification, limiting precision and the ability to detect subtle protein expression changes. This was further complicated by the fact that there was some unclear blotting, making it difficult to quantify the levels of one type of protein in a blot. Future steps would include using electron microscopy, which offers direct visualization of autophagic structures, resulting in the absolute quantification of autophagosomes and autolysosomes to assess autophagic activity.

The Western Blot process was very complex with many steps, and at first, it was daunting for someone like me with limited lab experience. Through repeating it many times, I began to understand the process and could perform it independently. However, this did not stop some errors from occurring, as one misstep in the many

stages of the process could severely affect the blot. In the future, I would like to continue research to refine my abilities in laboratory techniques, including Western Blotting. I believe that by doing more repeats of the Western Blotting process, I could reduce the chance of human error and produce more precise results. This could also mean I could do more repeats of the experiment, by reducing the time it takes to do one repeat. More repeats would increase the reliability of the results and reduce the standard deviation.

Bafilomycin A1 may have some unwanted effects since it interacts with the V-ATPase proton pump on the lysosome, which is also the location of the TPCs and where JPT2 and LMS12 would bind to NAADP. Therefore, Bafilomycin A1 may interfere with the NAADP activation of the TPCs. Future steps would involve finding alternative autophagy inhibitors and testing them with this project. My supervisor suggested to me to use combination inhibitors that affect proteases rather than the channels in the autophagy pathway. Protease inhibitors that specifically target enzymes involved in autophagy, such as cathepsins, can provide a more direct and specific way to inhibit autophagy. This specificity reduces the risk of off-target effects that can occur when using lysosomal inhibitors like bafilomycin A1, which may affect multiple cellular processes beyond autophagy.

The autophagy markers we used also have their own limitations that have been reported in the literature on autophagy. Firstly, p62 is a multifunctional protein involved in various cellular processes, including autophagy. It can act as a selective autophagy receptor for ubiquitinated cargo, but it also participates in other pathways like proteasomal protein turnover. Therefore, changes in p62 levels may not solely

indicate autophagic activity and can be influenced by unrelated factors (Puissant et al., 2012). Although elevated levels of LC3-II are commonly associated with the initiation of autophagy, they can also occur due to the inhibition of autophagosome-lysosome fusion or the impairment of lysosomal degradation. In such situations, LC3-II could accumulate without corresponding autophagic progression, which could potentially lead to misinterpretation of the readings (Abeliovich et al., 2016). To mitigate the limitations of LC3 and p62, consider incorporating additional autophagy markers. Beclin-1, ATG5, and ATG7 are examples of proteins involved in autophagy that, when used in addition to p62 and LC3, can provide a more precise assessment of autophagy.

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