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# Lysosomal pH and the Control of Genes Involved in Inflammation and Degradation

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# Lysosomal pH and the Control of Genes Involved in Inflammation and Degradation

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The endosomal/lysosomal system is essential for cell survival. The regulation of lysosomal pH is critical for lysosomal function and has been known to play a pivotal role in aging and disease. The pH within the lysosomes is essentially increased in certain diseases and our preliminary data suggests that it may also increase with age. It is known that the methods used for lysosomal pH measurement are extremely difficult and technique sensitive. In this study, we searched for key genetic markers to help identify the presence of chronic elevation of lysosomal pH. This will allow us to utilize the speed, specificity and sensitivity of laboratory confirmation with quantitative polymerase chain reaction (qPCR) as an alternative method to direct measurement of lysosomal pH. In our study, we demonstrated a trend towards an increased expression of TcfEB and vATPase genes in the presence of long-term lysosomal pH elevation. Therefore, these two genes could potentially be used as markers to recognize the presence of chronic lysosomal pH elevation in diseased cells. In contrast, a short-term lysosomal pH elevation showed a decreased expression of IL-1b, IL-18 and TcfEB highlighting the time-dependent nature of genetic expression. Both genes, TcfEB and vATPase, might be used as important tools for the rapid detection of disease or infection in clinical specimens and are also particularly suitable in optimizing the therapeutic management of diseased cells.

## **Degree Type**

Thesis

## **Degree Name**

MSOB (Master of Science in Oral Biology)

## **Primary Advisor**

Kathleen Boesze-Battaglia, PhD

## **Subject Categories**

Dentistry | Oral Biology and Oral Pathology

# **Lysosomal pH and the Control of Genes Involved in Inflammation and Degradation**

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## **Abstract**

The endosomal/lysosomal system is essential for cell survival. The regulation of lysosomal pH is critical for lysosomal function and has been known to play a pivotal role in aging and disease. The pH within the lysosomes is essentially increased in certain diseases and our preliminary data suggests that it may also increase with age. It is known that the methods used for lysosomal pH measurement are extremely difficult and technique sensitive. In this study, we searched for key genetic markers to help identify the presence of chronic elevation of lysosomal pH. This will allow us to utilize the speed, specificity and sensitivity of laboratory confirmation with quantitative polymerase chain reaction (qPCR) as an alternative method to direct measurement of lysosomal pH. In our study, we demonstrated a trend towards an increased expression of TcfEB and vATPase genes in the presence of long-term lysosomal pH elevation. Therefore, these two genes could potentially be used as markers to recognize the presence of chronic lysosomal pH elevation in diseased cells. In contrast, a short-term lysosomal pH elevation showed a decreased expression of IL-1 $\beta$ , IL-18 and TcfEB highlighting the time-dependant nature of genetic expression. Both genes, TcfEB and vATPase, might be used as important tools for the rapid detection of disease or infection in clinical specimens and are also particularly suitable in optimizing the therapeutic management of diseased cells.

## **Introduction**

Lysosomes are specialized organelles within cells involved in degrading cellular macromolecules and making their components available to the cell as nutrients (Mindell 2012). The enzymes present in the lysosome (e.g. proteases, glycosidases and lipases) are responsible for the breakdown of material delivered to the lysosome either by phagocytosis from outside the cell, such as with bacteria, or from inside the cell, such as organelles and proteins through autophagy. When defects occur within the lysosome, incomplete degradation of materials can result, leading to a number of possible pathological conditions known collectively as lysosomal storage diseases. These pathologic disorders are generally caused by specific mutations in any of the enzymes responsible for degradation of a particular material, leading to an accumulation of undigested material that prevents proper cellular functions (Lieberman et al., 2012). One example of this would be Tay-Sachs disease, where defects in the beta-N-acetylhexosaminidase A enzyme (Schultz et al., 2011) required for degradation of glycolipids cause a buildup of lipids in the brain, resulting in progressive deterioration of neural tissue.

However, lysosomal storage diseases may occur due to defects in the lysosome in general, rather than due to specific defects in any one enzyme. Because the degradative enzymes present in the lysosome operate optimally within a small range of acidic pH of 4.5 to 5.0, lysosomes maintain a characteristic internal pH that is essential for facilitating its function (Mindell 2012). It is well established that the pH gradient within the lysosome is generated by the action of a V-type ATPase, a proton-pumping membrane protein that uses the free energy of ATP hydrolysis to drive protons against their electrochemical gradient into the lysosomal lumen (Grabe et al., 2000). If the pH is not maintained in this small range, the activity of the enzymes is reduced and material accumulates in the lysosome. Therefore, anything that might change lysosomal pH, such as age or drugs, can cause

a buildup of material, resulting in a lysosomal storage disease phenotype (Liu et al., 2008). Thus, the regulation of lysosomal pH is central for the basic housekeeping of all cells (Pillay et al., 2002).

Little is known about how exactly the accumulation of undigested materials results in pathology, however many cellular processes have been shown to be altered in lysosomal storage diseases. One such change is an increase in the presence of pro-inflammatory cytokines, such as IL-1 $\beta$  or IL-18 (Masters 2012). While pro-inflammatory cytokines may initially help clear the excess undigested material through recruitment of macrophages, long term activation of the inflammatory pathway can be detrimental and cause a pathology of its own. Enzymes termed “the inflammatory caspases” are activated by cellular sensors of danger signals, the inflammasomes, and subsequently convert pro-inflammatory cytokines into their mature, active forms to activate the inflammatory response (Vladimer et al., 2013). In addition, the inflammasomes regulate non-conventional protein secretion of alarmins and cytokines, glycolysis and lipid biogenesis, and the execution of an inflammatory form of cell death (Lamkanfi et al., 2012). By acting as key regulators of inflammation, energy metabolism and cell death, the inflammasome can exert profound influences on innate immunity, infectious and non-infectious inflammatory diseases (McIntire et al., 2009).

In the current study, our first step was to understand how a cell responds to increases in lysosomal pH. In order to do so, we treated two types of cells routinely used in our lab (an adult retinal pigmented epithelial cell line named ARPE-19 cells and a AG07623 human fibroblast cell line) with drugs known to alkalinize lysosomal pH, Bafilomycin A1 (Baf A1) and Chloroquine (CHQ). Baf A1 is known to selectively inhibit vacuolar type ATPase (vATPase) on the lysosomal membrane and therefore increase the lysosomal pH (Pivtoraiko et al., 2010). CHQ is an uncharged molecule at neutral pH, which allows it to cross the lysosomal membrane where it is deprotonated in

the acidic environment (Gonzales-Noriega et al., 1980). It is thought that this buildup of CHQ in its cationic form disrupts the balance of charges needed to maintain proton transport into the lysosome.

In order to examine how a cell responds to an elevation in lysosomal pH, including the possible production of inflammatory cytokines, we examined how expression of a number of genes related to lysosomal pH or inflammation changed after treatment with CHQ or Baf A1. Of particular interest were TcfEB, the transcription factor controlling lysosomal function and autophagy (Settembre et al., 2011), and vATPase, which is responsible for lysosomal pH maintenance (Graves et al., 2008). Also, TcfEB has been known to control vATPase expression (Llopis et al., 2011). Analyzing inflammatory genes, IL-1 $\beta$  and IL-18 was helpful in gaining some insight into the feedback mechanisms related to inflammatory cascade. We also analyzed the gene expression of the so-called “inflammasome”: NLRP3 and caspase-1, which are involved in the production and processing of pro-inflammatory cytokines (Lamkanfi et al., 2012). Our project consisted of confirming these changes in gene expression and understanding whether changing lysosomal pH is itself sufficient to alter gene expression.

In this study, the quantitative polymerase chain reaction (qPCR) method was used to analyze gene expression and is known to be an extremely rapid, specific and sensitive technique (Maurin et al., 2012). In contrast to qPCR, the direct measurement of lysosomal pH is extremely challenging and difficult (Liu et al., 2008). Therefore, by searching for a gene, which is up regulated with lysosomal alkalization, we could help identify a potential genetic marker to recognize the presence of chronic elevation of lysosomal pH in aging, disease or drug treatment. This has widespread implications on the diagnosis and therapeutic management of diseased cells that are known to have a long-term increase in lysosomal pH.

## **Materials and Methods**

All experiments took place in the Levy Building at the University of Pennsylvania School of Dental Medicine.

### *Cell Culture of ARPE-19 Cells*

The human ARPE-19 cell line was obtained from the American Type Culture Collection (Manassus, VA) and maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture (DMEM)/ F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Lonza, Allendale, NJ, USA) at 37°C in a humidified 5% CO<sub>2</sub> incubator. Prior to experimentation, the ARPE-19 cells were sub-cultured onto 6-well cell culture plates (BD Falcon) and allowed to reach approximately 80% confluence before they were utilized.

### *Cell Culture of Fibroblast Cells*

The AG07623 human fibroblast line utilized in our experiments was obtained from the Coriell Institute for Medical Research in Camden, NJ, a part of the NIA Aging Cell Culture Repository. Fibroblast cells were maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 5% CO<sub>2</sub> incubator. Prior to experimentation, the fibroblasts were sub-cultured onto 6-well cell culture plates and allowed to reach approximately 80% confluence before they were utilized.

### *Treatment of Cells to Increase Lysosomal pH*

After allowing ARPE19 cells to grow to approximately 80% confluence, the medium was removed and replaced with either 200nM Baf A1 or 30 $\mu$ M CHQ dissolved in medium. Baf A1 is known to selectively inhibit vATPase on the lysosomal membrane and therefore increase the lysosomal pH (Pivtoraiko et al., 2010). CHQ is an uncharged molecule at neutral pH, which allows it to cross the lysosomal membrane where it is deprotonated in the acidic environment (Gonzales-Noriega et al., 1980). The experiment was performed in triplicates for statistical purposes. For Baf A1 treatment, the cells were incubated for 3 hours prior to RNA extraction, for CHQ treatment the cells were incubated for either 4 or 24 hours. Fibroblasts were treated with 10 $\mu$ M CHQ in the same manner for 6 hours prior to RNA extraction. All drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO). The ARPE19 and fibroblast cells in the control group were maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

### *RNA Extraction and Quantification*

Following incubation with either CHQ or Baf A1, the media in each well was removed and replaced with 1ml of TRIzol reagent (Invitrogen). Total RNA was then extracted according to manufacturer's guidelines. The purified RNA in solution was quantified using a spectrophotometer. The minimum purity required for the qPCR experiment exhibited an A260/A280 ratio of 1.8.

### *Reverse Transcription and Quantitative PCR*

Following RNA quantification, reverse transcription was performed with 1  $\mu$ g of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) to convert mRNA to cDNA. The cDNA was then amplified using the SYBR Green PCR Master Mix and a 7300 Real-Time PCR system. To



amplify our gene of interest, small oligonucleotide primers were designed specifically using Primer3 software online (<http://frodo.wi.mit.edu/>). The exact sequences of the primers used in our study are listed in Table 1. The experimental protocol was 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Changes in gene expression were determined using the  $\Delta\Delta C_T$  method, which compares differences in  $C_T$  values between control and experimental samples, using a housekeeping gene (for these experiments,  $\beta$ -actin) as a calibrator.

Gene	Forward (5' → 3')	Reverse (5' → 3')
$\beta$ -actin	AGAAAATCTGGCACCACACC	GGGGTGTGGAAGGTCTCAA
IL-1 $\beta$	TCCCCAGCCCTTTTGTGTA	TTAGAACCAAATGTGGCCGTG
IL-18	GGAATTGTCTCCCAGTGCAT	ACTGGTTCAGCAGCCATCTT
NLRP3	CTTCTCTGATGAGGCCAAG	GCAGCAAAGTGGAAAGGAAG
Caspase-1	ACCTCTGACAGCACGTTCT	CCTTCGGTTTGTCTTCAA
TcfEB	GTCCGAGACCTATGGGAACA	CGTCCAGACGCATAATGTTG
vATPase	GAAGAAGTCCAAGGCTGTGC	TTCAGGAAGAGGCAGACGTT

**Table 1 - PCR Primer List**

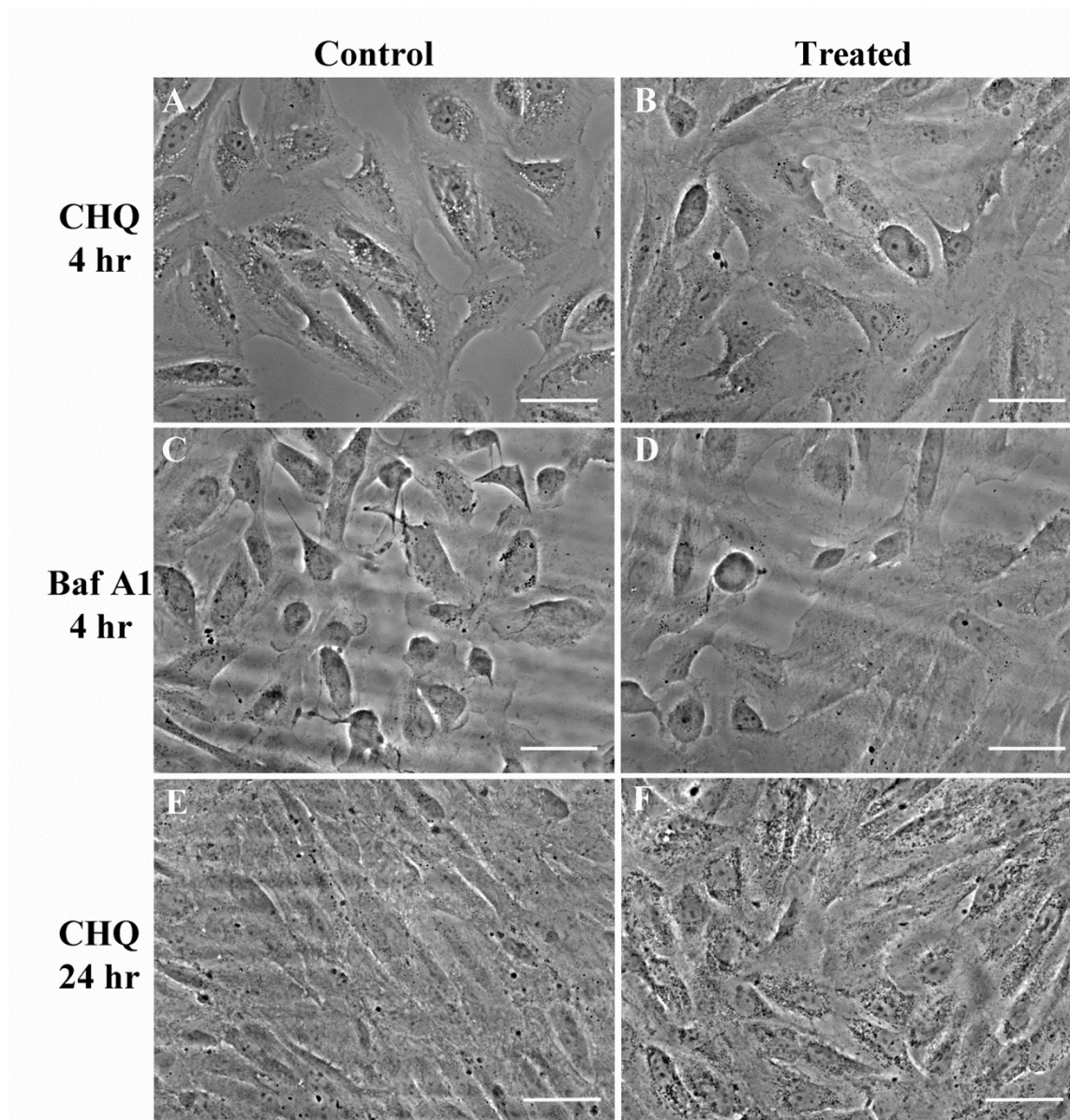
### *Statistical Analysis*

A paired student's t-test was used to assess if the gene expression of the control and experimental group were statistically different from each other. The results were considered statistically significant if  $p < 0.05$ .

### **Results**

In order to determine if gene expression is affected by altered lysosomal pH, we incubated our cells with either CHQ or Baf A1, which increases lysosomal pH and thereby reproduce the cellular conditions present in aging or disease (Gonzales-Noriega et al., 1980, Pivtoraiko et al., 2010). To ensure that our treatments did not cause cell death, which would skew our results, ARPE19 cells were treated with either CHQ or Baf A1 in the concentrations and durations used in our experiments and observed under a microscope for gross changes in cell morphology. As shown

in Figure 1, treatment with either 30 $\mu$ M of CHQ (for 4 or 24 hours) or 200 nM Baf A1 (for 3 hours) did not result in a noticeable change in either growth rate or gross morphology of the ARPE-19 cells. Cells treated with CHQ for 24 hours showed increased phase-dense particles, consistent with increased undigested material in these cells (Figure 1).



**Figure 1 - Microscopic analysis of APRE19 cells treated with drugs to raise lysosomal pH.**

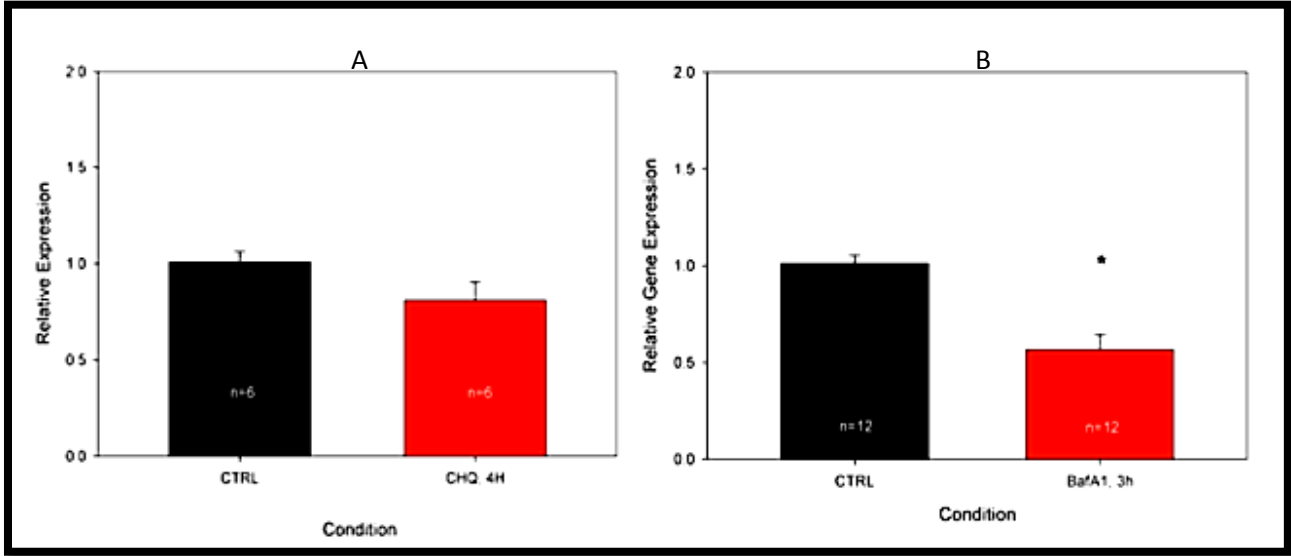
Phase-contrast photomicrographs of ARPE19 cells treated with (A, B) 30 $\mu$ M CHQ for 4 hours, (C, D) 200nM Baf A1 for 3 hours or (E, F) 30 $\mu$ M CHQ for 24 hours. The left-panel photomicrographs (A, C, E) are cells treated with control media, while the right-panel micrographs (B, D, F) are drug-treated. Photos were taken with a 40X Phase 2 objective, the calibration bar in each represents 50 $\mu$ m.

To examine pH-mediated changes in gene expression, we stimulated both ARPE19 cells and human skin fibroblasts with CHQ and/or Baf A1 and performed qPCR analyses on the RNA extracted from these cells. The genes examined included TcfEB, vATPase, IL-1 $\beta$ , IL-18, NLRP3 and Caspase-1.

#### *Transcription factor EB (TcfEB)*

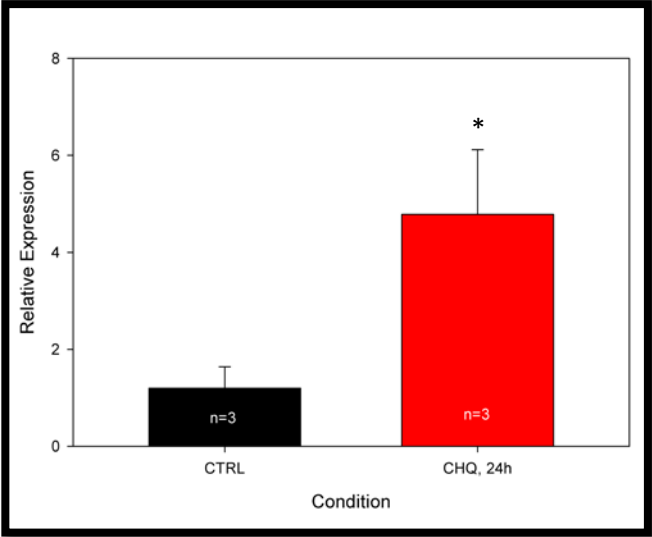
The TcfEB, a transcription factor responsible for activating lysosomal biogenesis, coordinates the autophagy pathway by driving expression of autophagy and lysosomal genes (Settembre et al., 2011).

Short-term treatment of ARPE19 cells with Baf A1 (200nM, 3hrs) resulted in a decrease in TcfEB expression of approximately 50% ( $p < 0.05$ ,  $n = 12$ ) (Figure 2A). A similar short-term treatment with CHQ (30 $\mu$ M, 4 hours) exhibited a small, but statistically insignificant decrease in TcfEB (Figure 2B). Conversely, long-term treatment of ARPE19 cells with CHQ (30 $\mu$ M, 24hrs) resulted in an increase in TcfEB gene expression (Figure 3) by approximately 4.5-fold ( $n = 3$ ). Human skin fibroblasts treated for 6 hours with CHQ (10 $\mu$ M) suggested no effect ( $n = 2$ ) on the expression of the TcfEB transcription factor (Figure 4).



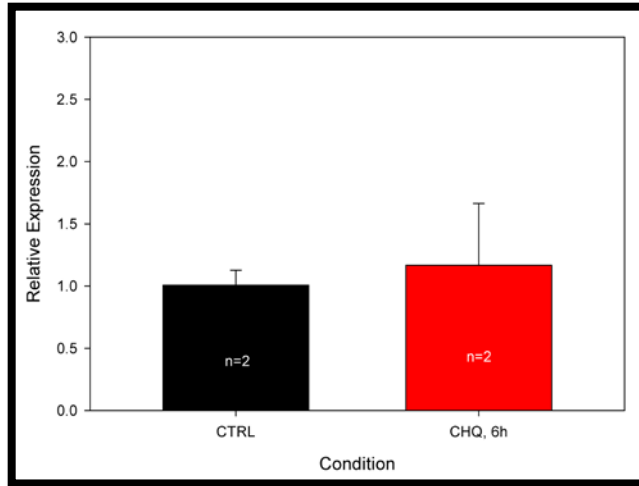
**Figure 2 - TcfEB expression in ARPE19 cells following short-term increases in lysosomal pH.**

Changes in TcfEB gene expression in ARPE19 cells following treatment with (A) 30µM CHQ for 4 hours or (B) 200nM Baf A1 for 3 hours. \*p< 0.05.



**Figure 3 - TcfEB expression in ARPE-19 cells following long-term elevation in lysosomal pH.**

Changes in TcfEB gene expression following 24-hour treatment of ARPE19 cells with 30µM CHQ. The control cells showed a similar gene expression at 24 hours compared to the earlier time points.

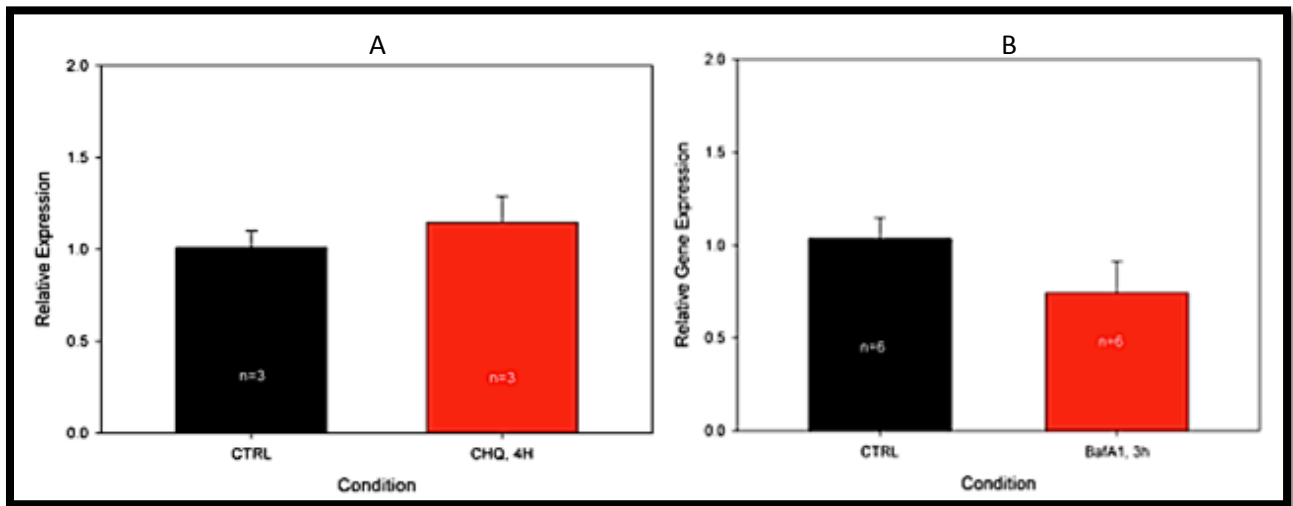


**Figure 4 - TcfEB expression in human skin fibroblasts following lysosomal pH elevation.**

Changes in TcfEB gene expression following 6-hour treatment of fibroblasts with 10 $\mu$ M CHQ.

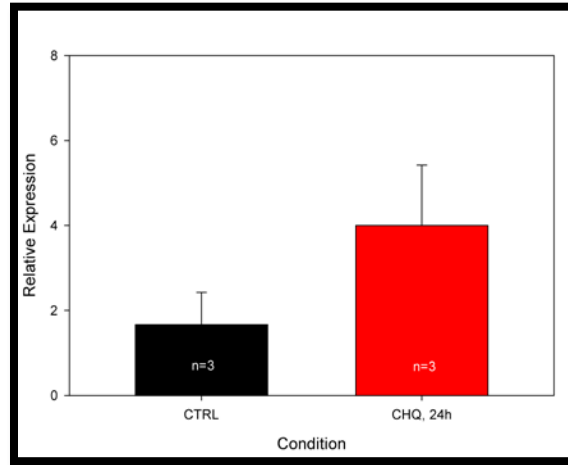
*vATPase*

Lysosomal acidification is absolutely essential for lysosomal function and is mediated by a V-type proton ATPase (Graves et al., 2008). Short-term elevation of lysosomal pH with either CHQ (n=3) or Baf A1 (n=6) did not significantly alter vATPase gene expression (Figure 5). However, long-term treatment of ARPE19 cells with CHQ is suggestive of a possible increase in the expression of the vATPase gene (Figure 6), albeit not significantly  $p>0.05$  (n=3). Preliminary results also suggest that alkalinizing lysosomal pH in human skin fibroblasts may increase vATPase gene expression (n=2) (Figure 7).



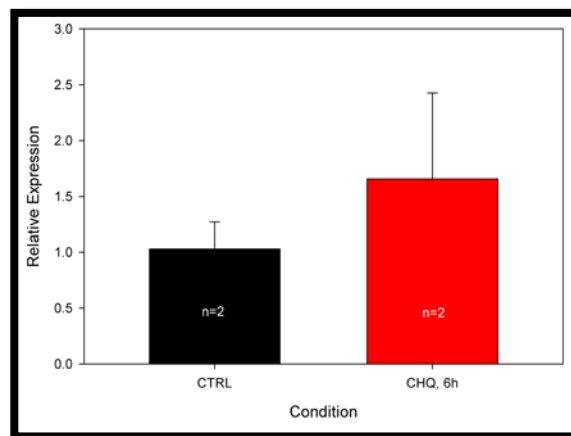
**Figure 5 - vATPase expression in ARPE19 cells with short-term elevation of lysosomal pH.**

Changes in vATPase gene expression in ARPE19 cells following treatment with (A) 30 $\mu$ M CHQ for 4 hours or (B) 200nM Baf A1 for 3 hours.



**Figure 6 - vATPase expression in ARPE19 cells following long-term elevation of lysosomal pH.**

Changes in vATPase gene expression following 24-hour treatment of ARPE19 cells with 30 $\mu$ M CHQ.



**Figure 7 - vATPase expression in fibroblasts following alkalization of lysosomal pH.**

Changes in vATPase gene expression following 6-hour treatment of human skin fibroblasts with 10 $\mu$ M CHQ.

### *IL-1 $\beta$ and IL-18*

IL-1 $\beta$  and IL-18 are pro-inflammatory cytokines released following the activation of the inflammasome (Masters 2012). The analysis of IL-1 $\beta$  and IL-18 gene expression in the presence of increased lysosomal pH gives us some insight into possible mechanisms that could exist between the initial message and ultimate end product of inflammasome activation.

ARPE-19 cells exhibited a significant decrease in gene expression for both IL-1 $\beta$  and IL-18 following short-term elevation of lysosomal pH with 200nM Baf A1 (decreases of 36%, n=12 and 22%, n=9, respectively) (Figure 8). In contrast, long-term stimulation of ARPE19 cells with 30 $\mu$ M CHQ did not result in any change in inflammatory gene expression (Figure 9).

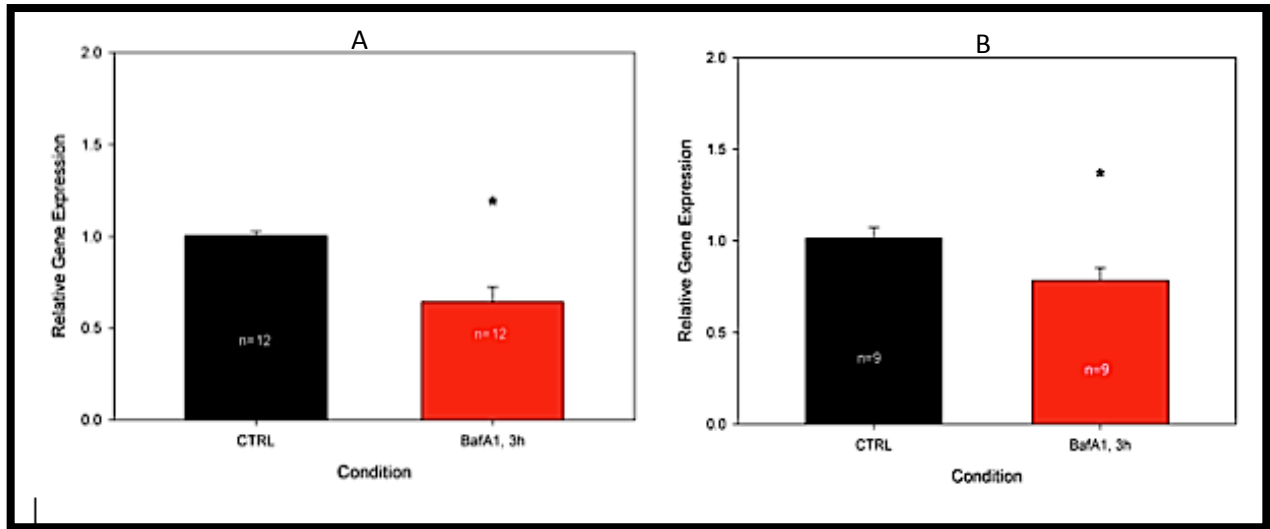


Figure 8 - Effects of short-term elevation of lysosomal pH on inflammatory cytokine gene expression.

Changes in gene expression for (A) IL-1 $\beta$  or (B) IL-18 following 3 hr treatment with 200nM Baf A1. \*p<0.05.

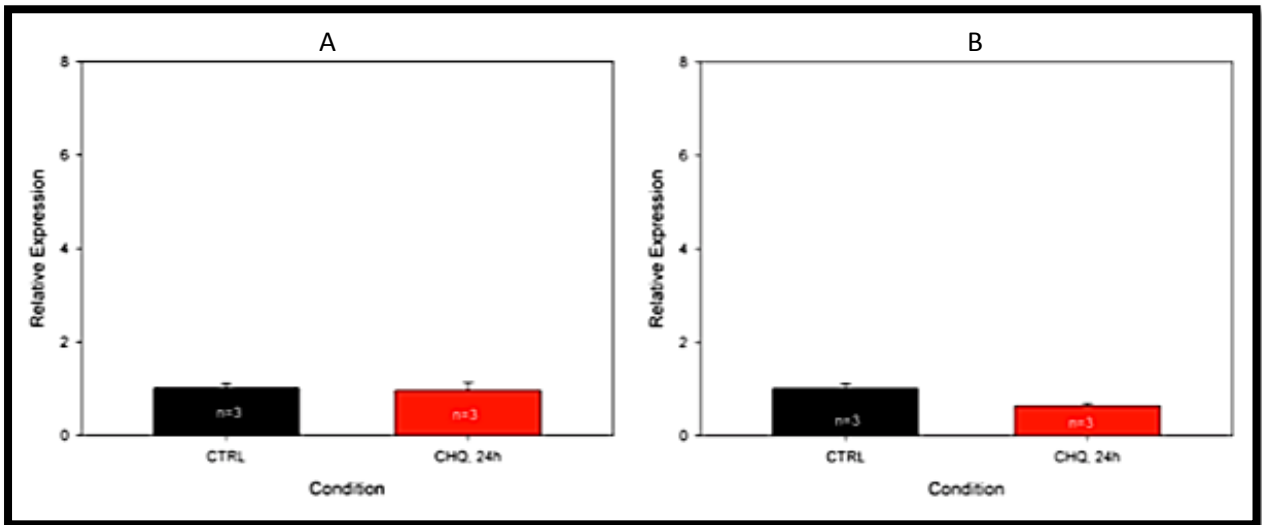


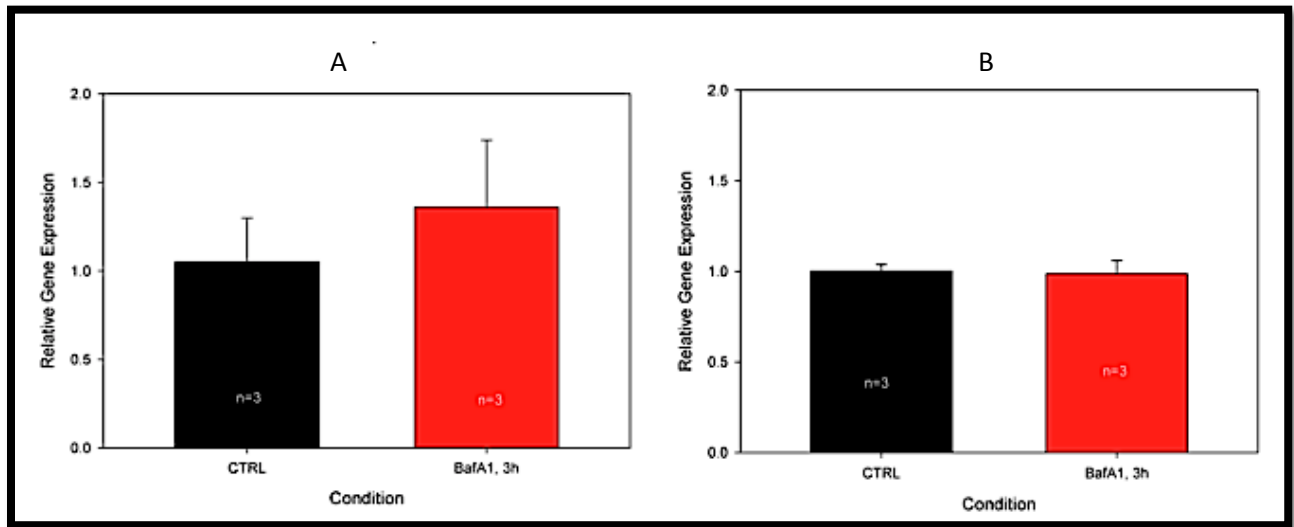
Figure 9 - Effects of long-term elevation of lysosomal pH on inflammatory cytokine gene expression.

Changes in gene expression for (A) IL-1 $\beta$  or (B) IL-18 following 24 hr treatment with 30 $\mu$ M CHQ. \*p<0.05.



## *NLRP3 and Caspase-1*

NLRP3 and Caspase-1, components of the inflammasome, are involved in the production and processing of pro-inflammatory cytokines such as IL-1 $\beta$  or IL-18 to their active forms (Vladimer et al., 2013). The gene expression of both inflammasome genes with short-term Baf A1 treatment (200nM, 3 hr) did not show any significant variation (Figure 10) (n=3 each).



**Figure 10 - Changes in inflammasome gene expression following short-term elevation of lysosomal pH.**

Changes in gene expression for (A) NLRP3 and (B) Caspase-1 following a 3-hour stimulation with 200nM Baf A1.

## **Discussion**

The relationship between the lysosomal pH and the genes involved in autophagy and inflammation is complex. It has been established that the regulation of lysosomal pH is essential for cell survival (Baltazar et al., 2012). Lysosomes are the stomachs of the cell-terminal organelles on the endocytic pathway where internalized macromolecules are degraded (Graves, et. al. 2008). They contain a wide range of hydrolytic enzymes and depend on maintaining acidic luminal pH values for efficient function. Because pathology can result when the acidic environment of the lysosome is not maintained, elevation of lysosomal pH with drugs, such as Chloroquine and Bafilomycin, helps give

us some insight into the inflammatory signals and feedback mechanisms that contribute to disease or aging (Nujić et al., 2012).

TcfEB is known to activate the transcription of a number of genes responsible for the formation of lysosomes as well as genes involved in different steps of autophagy, thereby increasing the number of lysosomes and autophagosomes (Spampanato et al., 2013). Activation of TcfEB promotes faster fusion of autophagosomes to lysosomes and enhances autophagic degradation. In our experiments, long-term lysosomal pH elevation resulted in a considerable increase in the TcfEB gene expression, suggesting a compensatory feedback mechanism in response to prolonged alkalization (Figure 11). This increase would probably result in an increase in the formation of new lysosomes in an attempt to compensate for decreased lysosomal function due to lysosomal pH elevation. Also, since TcfEB is a transcription factor that controls vATPase expression (Llopis et al., 2011), its increased expression could be a mechanism to help re-acidify the lysosomes. In contrast, the short-term lysosomal pH elevation resulted in a decrease in TcfEB gene expression, suggesting that cells respond to a short-term increase in lysosomal pH in a different manner than in the long-term. This short-term decline in expression may be suggestive of a mechanism to slow down the autophagy pathway in an attempt to let lysosomes “catch up”.

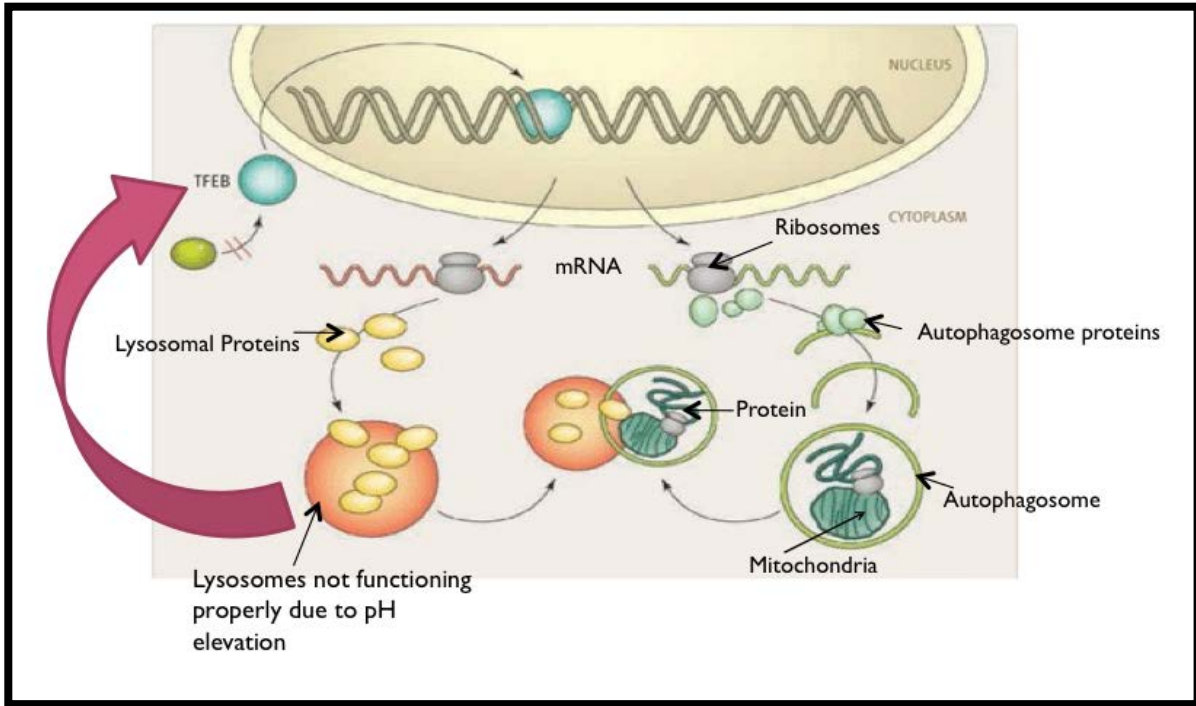
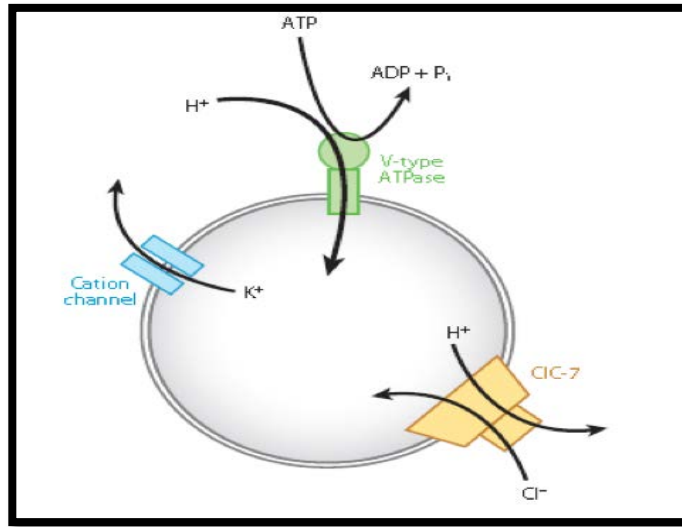


Figure 11. Phosphorylation of TcfEB (upper left) by ERK2 retains it in the cytosolic compartment. Upon starvation, reduced ERK2-dependent phosphorylation of TcfEB mobilizes it to the nucleus, where it activates a transcription program that controls the formation of both lysosomes (lower left) and genes involved in different steps in the autophagic process (lower right). The TcfEB-mediated increase in number of lysosomes and autophagosomes and their faster fusion enhances autophagic degradation. The pink arrow suggests that there is a compensatory feedback mechanism whereby decreased lysosomal function can lead to an increase in the production of TcfEB. This figure is taken from Science 17 June 2011: vol. 332 no. 6036 1392-1393.

Maintenance of an acidic pH is critical to facilitate function of degradative lysosomal enzymes. The pH gradient is generated by the action of Vacuolar-type  $H^+$ -ATPase (V-ATPase), a membrane protein that uses ATP hydrolysis to drive protons against their electrochemical gradient into the lysosomal lumen (Graves et al., 2008)(Figure 12). Because an acidic pH is so critical to lysosomal function, we wanted to know more about how it is regulated and therefore we examined how an elevation of lysosomal pH influences vATPase mRNA expression. Short-term lysosomal pH elevation elicited no change in gene expression, however a longer incubation time significantly increased vATPase gene expression (Figure 5,6). This implies that long-term pH elevation causes an

up-regulation of the vATPase gene in order to assist in forming more vATPase membrane proteins that will in turn facilitate lysosomal pH acidification and regulation.



**Figure 12. Lysosomal transporters involved in pH homeostasis. The V-type ATPase (green) uses the metabolic energy of ATP hydrolysis to drive protons into the lumen. This process builds a net positive charge inside the lumen of the lysosome. This figure is taken from Mindell et al., 2012.**

Inflammatory cytokines such as IL-1 $\beta$  and IL-18 are an ultimate product of inflammasome activation and have been associated with various diseases (Fettelschoss et al., 2011). Lysosomal storage diseases are known to be a group of pathologies that normally activate the inflammasome and inflammatory cascade (Schultz et al., 2011). In our experiments, the gene expression of the inflammatory cytokines IL-1 $\beta$  and IL-18 in the short-term actually decreased. This is possibly because our experiments did not use a long enough time period to generate a strong impact on inflammatory gene expression caused by lysosomal alkalinization. Lysosomal Storage Diseases are chronic disorders and may only exhibit increased cytokine production after a long period of lysosomal function. Additionally, our experiments only examined cytokine gene expression and not actual cytokine protein production. Therefore, cytokine production may still be increased following lysosomal alkalinization.

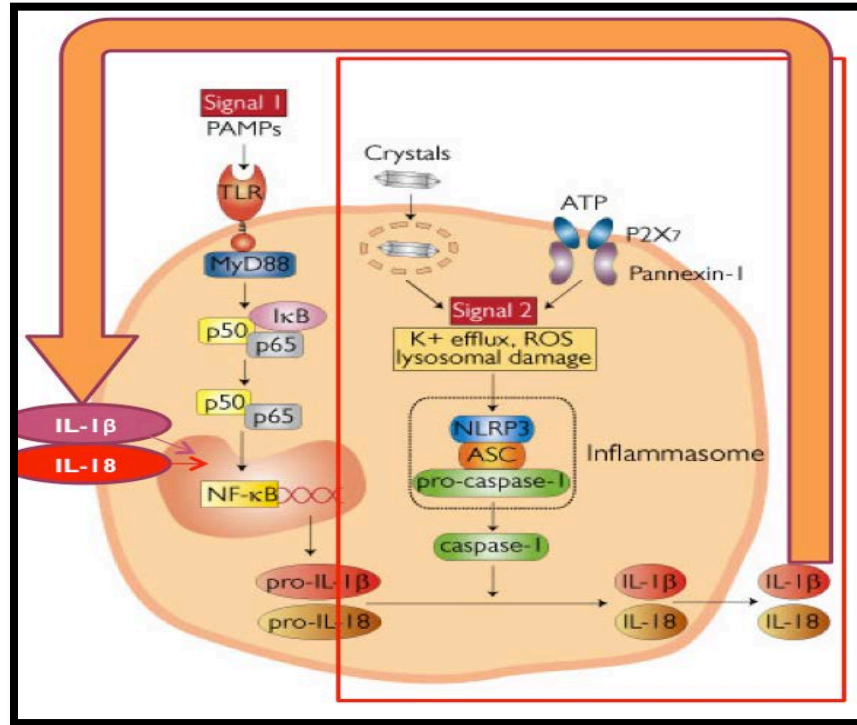


Figure 13. The figure shows the maturation and release of IL-1 $\beta$  and IL-18 requiring two distinct signals: the first signal leads to synthesis of pro-IL-1 $\beta$  and pro-IL-18 and other components of the inflammasome, such as NLRP3 itself; the second signal results in the assembly of the NLRP3 inflammasome, caspase-1 activation and IL-1 $\beta$  secretion. The orange arrow suggests a possible negative feedback system whereby IL-1 $\beta$  and IL-18 cytokine release down regulates IL-1 $\beta$  and IL-18 message and vice versa. This figure is taken from Invivogen November 2009.

The so-called “inflammasome”, NLRP3 and Caspase-1, is involved in the production and processing of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-18 (Figure 13). Their gene expression showed no significant change in the short-term elevation of lysosomal pH. Again, this may imply that our treatment was not long enough to see an increase in the inflammasome gene expression as is seen in pathologies such as Lysosomal Storage Diseases.

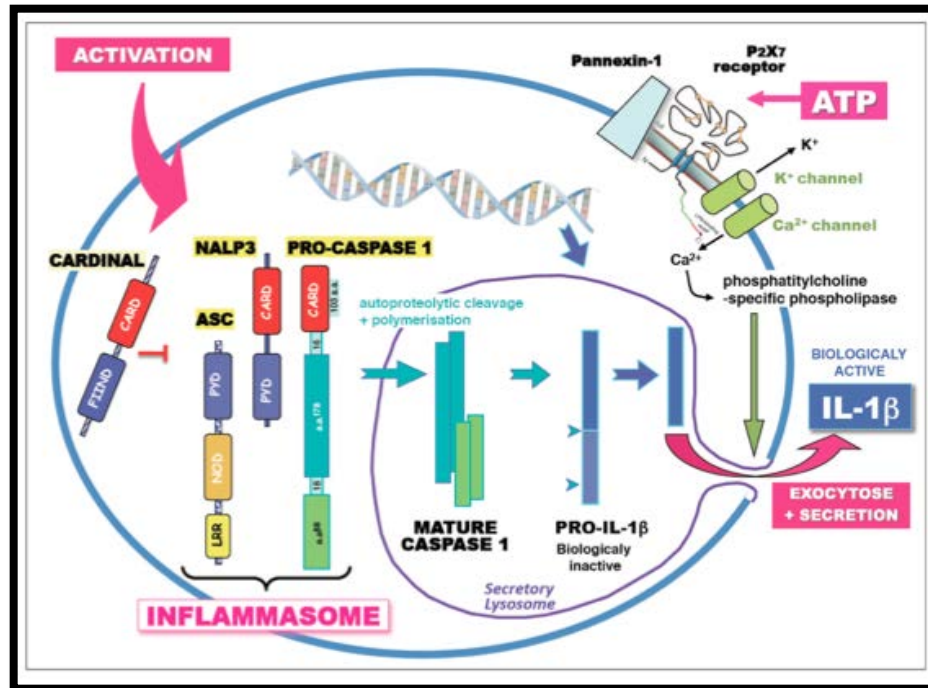


Figure 14. The activation of the inflammasome. Interleukin-1beta (IL-1beta) release requires the activation of different molecules gather under the name of "inflammasome" by Jürg Tschopp. The activation of inflammasome leads to the active form of caspase-1, the enzyme required for the maturation of IL-1beta. This figure is adapted from Bull. Assoc. Anciens Elèves Inst. Pasteur 2007, 49: 58.

Recently, quantitative real-time PCR tests have been extensively developed in clinical microbiology laboratories for routine diagnosis of infectious diseases. It allows early, sensitive, accurate and specific laboratory confirmation of cellular genetic response (Maurin et al., 2012). It requires a relatively small amount of material and can accurately distinguish very small changes in total gene number (Shah et. al 2013). Conversely, direct measurement of lysosomal pH in cells is problematic. The procedure requires the culture of the cells to be studied in large numbers, and the dye used for the actual measurement has been shown to alkalinize lysosomes itself, skewing the results. Therefore, instead of measuring the lysosomal pH directly in diseased cells, a genetic marker for lysosomal pH elevation would provide more accuracy and predictability. As we have shown in the current study, long-term elevation of lysosomal pH in ARPE-19 cells caused an increase in TcfEB and vATPase, which may allow the use of TcfEB and vATPase mRNA as a potential genetic

marker for diseases in which there is increased lysosomal pH. This can be used for future research purposes where the genetic markers would help analyze cellular responses to different drugs or conditions.

## **Conclusion**

This study demonstrates that increases in lysosomal pH can lead to an up regulation of genes specific to the regulation of lysosomal function. We also introduce the idea of using these genetic markers to indicate when lysosomal pH is elevated, offering a possible alternative to the conventional direct lysosomal pH measurement. This has widespread implications on the diagnosis and therapeutic management of diseased cells that are known to have a chronic elevation of lysosomal pH. Further studies, *in vitro* and *in vivo*, would be helpful in gaining more insight into the expression other key inflammatory genes such as Nuclear factor kappa B (NFkB).

## **References**

Baltazar GC, Guha S, Lu W, Lim J, Boesze-Battaglia K, Laties AM, Tyagi P, Kompella UB, Mitchell CH. Acidic nanoparticles are trafficked to lysosomes and restore an acidic lysosomal pH and degradative function to compromised ARPE-19 cells. *PLoS One*. 2012;7(12):e49635.

DiCiccio JE, Steinberg BE. Lysosomal pH and analysis of the counter ion pathways that support acidification. *J Gen Physiol*. 2011 Apr;137(4):385-90.

Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer HD, Johansen P, Senti G, Contassot E, Bahmann MF, French LE, Oxenius A, Kündig TM. Inflammasome activation and IL-1 $\beta$  target IL-1 $\alpha$  for secretion as opposed to surface expression. *Proc Natl Acad Sci U S A*. 2011 Nov 1;108(44):18055-60.

Gonzalez-Noriega A, Grubb JH, Talkad V, Sly WS. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J Cell Biol*. 1980 Jun;85(3):839-52.

Graves AR, Curran PK, Smith CL, Mindell JA. The Cl<sup>-</sup>/H<sup>+</sup> antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature*. 2008 Jun 5;453(7196):788-92.

Grabe M, Wang H, Oster G. The mechanochemistry of V-ATPase proton pumps. *Biophys J*. 2000 Jun;78(6):2798-813.

Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol*. 2012;28:137-61.

Lieberman AP, Puertollano R, Raben N, Slaugenhaupt S, Walkley SU, Ballabio A. Autophagy in lysosomal storage disorders. *Autophagy*. 2012 May 1;8(5):719-30.



Liu J, Lu W, Reigada D, Nguyen J, Laties AM, Mitchell CH. Restoration of lysosomal pH in RPE cells from cultured human and ABCA4 (-/-) mice: pharmacologic approaches and functional recovery. *Invest Ophthalmol Vis Sci.* 2008 Feb;49(2):772-80.

Llopis- Peña S, Vega-Rubin-de-Celis S, Schwartz JC, Wolff NC, Tran TA, Zou L, Xie XJ, Corey DR, Brugarolas J. Regulation of TFEB and V-ATPases by mTORC1. *EMBO J.* 2011 Jul 29;30(16):3242-58.

Masters SL. Specific inflammasomes in complex diseases. *Clin Immunol.* 2012 Dec 21. S1521-6616(12)00306-3.

Maurin M. Real-time PCR as a diagnostic tool for bacterial diseases. *Expert Rev Mol Diagn.* 2012 Sep;12(7):731-54.

McIntire CR, Yeretssian G, Saleh M. Inflammasomes in infection and inflammation. *Apoptosis.* 2009 Apr;14(4):522-35.

Mindell JA. Lysosomal acidification mechanisms. *Annu Rev Physiol.* 2012;74:69-86.

Nujić K, Banjanac M, Munić V, Polančec D, Eraković Haber V. Impairment of lysosomal functions by azithromycin and chloroquine contributes to anti-inflammatory phenotype. *Cell Immunol.* 2012 Sep;279(1):78-86.

Pillay CS, Elliott E, Dennison C. Endolysosomal proteolysis and its regulation. *Biochem J.* 2002 May 1;363(Pt 3):417-29.

Pivtoraiko VN, Harrington AJ, Mader BJ, Luker AM, Caldwell GA, Caldwell KA, Roth KA, Shacka JJ. Low-dose bafilomycin attenuates neuronal cell death associated with autophagy-lysosome pathway dysfunction. *J Neurochem.* 2010 Aug;114(4):1193-204.

Schultz ML, Tecedor L, Chang M, Davidson BL. Clarifying lysosomal storage diseases. *Trends Neurosci.* 2011 Aug;34(8):401-10.

Schwake M, Schröder B, Saftig P. Lysosomal membrane proteins and their central role in physiology. *Traffic.* 2013 Jul;14(7):739-48.

Settembre C, Ballabio A. TFEB regulates autophagy: an integrated coordination of cellular degradation and recycling processes. *Autophagy.* 2011 Nov;7(11):1379-81.

Shah T, Zabaneh D, Gaunt T, Swerdlow DI, Shah S, Talmud PJ, Day IN, Whittaker J, Holmes MV, Sofat R, Humphries SE, Kivimaki M, Kumari M, Hingorani AD, Casas JP. Gene-centric analysis identifies variants associated with interleukin-6 levels and shared pathways with other inflammation markers. *Circ Cardiovasc Genet.* 2013 Apr;6(2):163-70.

Spampanato C, Feeney E, Li L, Cardone M, Lim JA, Annunziata F, Zare H, Polishchuk R, Puertollano R, Parenti G, Ballabio A, Raben N. Transcription factor EB (TFEB) is a new therapeutic target for Pompe disease. *EMBO Mol Med*. 2013 May;5(5):691-706.

Turk B, Turk V. Lysosomes as "suicide bags" in cell death: myth or reality? *J Biol Chem*. 2009 Aug 14;284(33):21783-7.

Vladimer GI, Marty-Roix R, Ghosh S, Weng D, Lien E. Inflammasomes and host defenses against bacterial infections. *Curr Opin Microbiol*. 2013 Feb;16(1):23-31.

## Literature Review

# Lysosomal pH and the Control of Genes Involved in Inflammation and Degradation

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In 1968, Coffee and De Duve showed that enzymes present in the lysosome (e.g. proteases, glycosidases and lipases) are responsible for the breakdown of protein delivered to the lysosome. They were also one of the first to note that the hydrolytic enzymes contained in the lysosome required an acidic pH for optimal function and the maintenance of this characteristic internal pH within the lysosome is essential for facilitating its function. The hydrolysis of acid-denatured human or bovine globin at 37° by extracts of highly purified rat liver lysosomes was most extensive between pH 4.4 and 5.6. After exhaustive digestion under these conditions, the ninhydrin-positive material released by enzymatic hydrolysis amounted to 70% of that released by acid hydrolysis. The main products of hydrolysis were free amino acids (42% of the total amino acid residues of the protein) and small peptides, mostly dipeptides. The peptides that were resistant to the lysosomal enzymes were found to be partly degraded when incubated at pH 8.0 with a high speed supernatant from rat liver. Acid-denatured bovine serum albumin was also attacked by the lysosomal enzymes, although less efficiently than globin. Similar tests performed with undenatured globin, serum albumin, peroxidase, invertase, and ferritin indicated that the susceptibility of these proteins to lysosomal digestion depended on their sensitivity to denaturation under the incubation conditions. Furthermore, the relative stability of the proteins in the system in vitro paralleled their reported ability to persist intact within liver lysosomes in vivo. The information provided by these

experiments may be directly relevant to the physiological process of protein degradation within lysosomes.

In 1985, Chung investigated whether Chloroquine could inhibit the lysosomal enzymes when  $\alpha$ -N-benzoyl-DL-arginine-2-naphthylamide is used as a substrate. His observations showed that Cathepsin B was inhibited in a pH dependant manner. Chloroquine at 1mM inhibited the naphthylamidase activity of the enzyme by about 15, 30 and 50% at pH of 5, 6 and 7, respectively, and therefore its inhibitory activity seemed to be increasing with increased buffer pH. The degree of inhibition of Cathepsin B by Chloroquine was strictly dependant on the buffer pH, with more basic pH resulting in greater inhibition. This pH-dependant inhibition by Chloroquine appeared to be due to the increased affinity of the compound to Cathepsin B at higher pH, as shown by the pre-incubation and dialysis experiments and by kinetic analysis. Therefore, this study illustrated how Chloroquine is likely to affect the intracellular protein breakdown by directly inhibiting one of the major lysosomal proteases, Cathepsin B. It also showed that Chloroquine was responsible for the inactivity of the acidic lysosomal proteases as a result of the elevation of the lysosomal pH.

Ohkuma et al in 1982 established the fact that the lysosomal pH gradient is generated by the action of a V-type ATPase, a proton-pumping membrane protein that uses the free energy of ATP hydrolysis to drive protons against their electrochemical gradient into the lysosomal lumen. Fluorescein isothiocyanate-conjugated dextran was introduced preferentially into hepatic lysosomes by intraperitoneal injection into rats. The pH in isolated lysosomes, measured by fluorescein fluorescence, was approximately 5 and gradually increased in KCl (to 7.0) at 25 degrees C. In the presence of  $Mg^{2+}$ , ATP caused acidification of lysosomes that was reversed by the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone.  $Mn^{2+}$ ,  $Co^{2+}$ , and  $Fe^{2+}$  could replace  $Mg^{2+}$  but  $Ca^{2+}$  could not.  $Cu^{2+}$ ,  $Zn^{2+}$ , and  $Cd^{2+}$  were inhibitory. A membrane-permeant anion, in practice

chloride, was required for this acidification. ATP-driven acidification was sensitive to N-ethylmaleimide and quercetin but insensitive to oligomycin, ouabain, and vanadate. There were some differences between "normal" lysosomes and tritosomes (a lysosome that is loaded with Triton); the acidification was resistant to azide and N, N'-dicyclohexylcarbodiimide in normal lysosomes but sensitive to these reagents in tritosomes. Therefore, these results provide evidence for the presence of an electrogenic proton pump, a V-type ATPase driven by MgATP (H<sup>+</sup>-ATPase) on the lysosomal membrane.

Lysosomal Storage Diseases (LSDs) were first defined as lysosomal enzyme deficiency states in 1965 by H.G. Hers based on his discovery that the glycogen storage disorder known as Pompe disease exhibits an absence of acidic  $\alpha$ -glucosidase activity. Hers' conceptual breakthrough provided the foundation for understanding literally dozens of additional so-called "storage" disorders, including the gangliosidoses and other sphingolipidoses, the mucopolysaccharidoses, the glycoproteinoses, and so forth. What became readily apparent in time, however, was that non-lysosomal enzymes, as well as soluble and transmembrane proteins of late endosomes and lysosomes, when defective, could also cause lysosomal storage defects essentially identical to conditions lacking a specific lysosomal hydrolase. Hence, Platt et al in 2004 showed an understanding of the latter type of LSDs, which include I-cell disease, multiple sulfatase deficiency, Niemann-Pick type C disease, mucopolipidosis IV, Danon disease, juvenile neuronal ceroid lipofuscinosis and others. Today, LSDs are recognized as a cohort of nearly 60 different inherited disorders, with each sharing a genetic defect that renders the lysosomal system dysfunctional and unable to degrade specific materials normally processed within the cell. As a consequence, many tissues and organ systems are affected, including brain, viscera, bone and cartilage, with early onset central nervous system (CNS) dysfunction predominating. Whereas clinical features of these

disorders vary widely, most are fatal within the first two decades of life following many years of worsening disease. The progressive nature of phenotype development is one of the hallmarks of LSDs. Schultz et al in 2012 showed a number of examples of LSDs including Tay-Sachs disease, where defects in the beta-N-acetylhexosaminidase A enzyme for degradation of glycolipids cause a buildup of lipids in the brain, resulting in progressive deterioration of neural tissue.

Spampanato et al in 2013 proposed that the therapeutic approach for lysosomal storage disorders (LSDs) relies upon the ability of transcription factor EB (TFEB) to stimulate autophagy and induce lysosomal exocytosis leading to cellular clearance. He showed that TFEB is a viable therapeutic target in Pompe disease, a paradigm of LSDs, characterized by both lysosomal abnormality and dysfunctional autophagy. There was an over expression of TFEB in a new muscle cell culture system and in mouse models of the disease that reduced glycogen load and lysosomal size, improved autophagosome processing, and alleviated excessive accumulation of autophagic vacuoles. Therefore, the results suggested that TFEB is an important transcription factor specific for the lysosomal function and autophagy.

In 2011, Llopis et al set out to examine whether TcfEB may be involved in mTORC1-dependent regulation of V-ATPases. An antibody recognizing TcfEB was generated in collaboration with Bethyl, and they evaluated the effects of TcfEB knockdown on V-ATPase expression. Among eight TcfEB shRNAs tested, two were found that substantially lowered TFEB levels. TcfEB depletion down regulated V-ATPase expression, particularly in Tsc2-deficient cells, in which baseline levels were up regulated. These data show that TcfEB is required for mTORC1-induced V-ATPase expression suggesting that there is a relationship between TcfEB and V-ATPase mRNA expression.

In 2008, Liu et al showed how the regulation of lysosomal pH is critical for lysosomal function and has been known to play a pivotal role in aging and disease. They hypothesized that anything that might change lysosomal pH, such as age or drugs, can cause a buildup of material because of slow enzyme activity, resulting in accumulation of partially digested material and a lysosomal storage disease phenotype. Consequently, they investigated if treatment to decrease lysosomal pH levels may enhance degradative activity. They found that pharmacologic elevation of cAMP could restore an acid pH and improve degradative function. In this study, the pharmacologic identification of intracellular cAMP as a key mediator of lysosomal acidification offers a variety of potential options to correct a compromised pH level. The extension of these approaches to RPE cells from ABCA4<sup>-/-</sup> (Stargardt's disease) mice demonstrated for the first time that elevated lysosomal pH is a specific defect in these animals, with the magnitude of the lysosomal alkalinization in adult mice predicted to substantially lower lysosomal enzyme activity in situ.

Tseng et al., in 2013 evaluated the effect of lysosomal alkalinization and destabilization on NLRP3 inflammasome activation in Adult Retinal Pigment Epithelial (ARPE) cells and investigated the mechanisms by which inflammasome activation may contribute to the pathogenesis of age-related macular degeneration (AMD). Expression of the IL-1 $\beta$  precursor, pro-IL-1 $\beta$ , was induced in ARPE-19 cells by IL-1 $\alpha$  treatment. Immunoblotting was performed to assess expression of NLRP3 inflammasome components (NLRP3, ASC, and procaspase-1) and pro-IL-1 $\beta$  in ARPE-19 cells. Lysosomes were destabilized using the lysosomotropic agent L-leucyl-L-leucine methyl ester (Leu-Leu-OMe). Active caspase-1 was detected using FAM-YVAD-FMK, a fluorescent-labeled inhibitor of caspases (FLICA) specific for caspase-1. Immunoblotting and ELISA detected IL-1 $\beta$ , and cytotoxicity was evaluated by LDH quantification. The results showed that NLRP3 up regulation occurs in the RPE during the pathogenesis of advanced AMD, in both geographic atrophy and



neovascular AMD. Therefore, the destabilization of RPE lysosomes induced NLRP3 inflammasome activation, which may contribute to AMD pathology through the release of the pro-inflammatory cytokine IL-1 $\beta$  and through caspase-1-mediated cell death, known as "pyroptosis." This study provides a link between lysosomal pH elevation and inflammation via inflammasome activation leading to pathology.

The drug, Bafilomycin A1 is a potent and highly specific inhibitor of the v-ATPase, typically inhibiting at nanomolar concentrations. In 2002, Bowman et al has shown that subunit c of the integral V (0) domain participates in Bafilomycin A1 binding, and that this site resembles the Oligomycin binding site of the F-ATPase. Wang et al in 2005 investigated whether the a subunit of the V-ATPase might participate in binding Bafilomycin A1. Twenty-eight subunit a mutations were constructed just N-terminal to the critical Arg (735) residue in transmembrane 7 required for proton transport, a region similar to that shown to participate in Oligomycin binding by the F-ATPase. The mutants appeared to assemble normally and all but two showed normal growth at pH 7.5, whereas all but three had at least 25% of wild-type levels of proton transport and ATPase activity. Of the functional mutants, three displayed K (i) values for Bafilomycin A1 significantly different from wild-type (0.22 +/- 0.03 nm). These included E721K (K (i) 0.38 +/- 0.03 nm), L724A (0.40 +/- 0.02 nm), and N725F (0.54 +/- 0.06 nm). Only the N725F mutation displayed a K (i) for Concanamycin (0.84 +/- 0.04 nm) that was slightly higher than wild-type (0.60 +/- 0.07 nm). These results suggest that subunit a of V-ATPase participates along with subunit c in binding Bafilomycin A1. Therefore, Bafilomycin A1 is known to selectively inhibit V-ATPase on the lysosomal membrane and therefore increase the lysosomal pH.

In 1980, Gonzales-Noriega et al showed different effects of the drug Chloroquine on normal and I-cell fibroblasts (mucopolipidosis II, a lysosomal enzyme storage diseases). Chloroquine treatment

of normal fibroblasts had three effects: (a) greatly enhanced secretion of newly synthesized acid hydrolases bearing the recognition marker for uptake, (b) depletion of enzyme-binding sites from the cell surface, and (c) inhibition of pinocytosis of exogenous enzyme. Only the third effect was seen in I-cell fibroblasts. These studies are consistent with a model for enzyme transport that proposes two pathways for delivery of enzyme to lysosomes, an intracellular pathway from the endoplasmic reticulum to lysosomes, and a quantitatively less important pathway involving enzyme pinocytosis by cell surface receptors. They suggest that both pathways depend on the phosphomannosyl enzyme receptor, and that Chloroquine disrupts both pathways by impairing receptor recycling. In this way, Chloroquine elevates the lysosomal pH and disturbs lysosomal function.

Overall, the literature shows that the regulation of lysosomal pH is critical for lysosomal function and plays a pivotal role in aging and disease. The transcription factor, TcfEB has been described as a master regulator of lysosomal function and is also known to regulate vATPase activity. When defects occur within the lysosome, incomplete degradation of materials can result, leading to a number of possible pathological conditions known collectively as lysosomal storage diseases. These pathologic disorders are generally caused by specific mutations in any of the enzymes responsible for degradation of a particular material, leading to an accumulation of undigested material that prevents proper cellular functions. Little is known about how exactly the accumulation of undigested materials results in pathology, however many cellular processes have been shown to be altered in lysosomal storage diseases. One such change is an increase in the presence of pro-inflammatory cytokines, such as IL-1 $\beta$  or IL-18. While pro-inflammatory cytokines may initially help clear the excess undigested material through recruitment of macrophages, long term activation of the inflammatory pathway can be detrimental and cause a pathology of its own. Also, by acting as key regulators of inflammation, energy metabolism and cell death, the

inflammasome can exert profound influences on innate immunity, infectious and non-infectious inflammatory diseases.

## References

Bowman BJ, Bowman EJ. Mutations in subunit C of the vacuolar ATPase confer resistance to bafilomycin and identify a conserved antibiotic binding site. *J Biol Chem.* 2002 Feb 8;277(6):3965-72.

Coffey JW, De Duve C. Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. *J Biol Chem.* 1968 Jun 25;243(12):3255-63.

Chung Chin Ha. pH-Dependant inhibition of Cathepsin B by Chloroquine. *Korean Biochem. J.* 1986. Volume 19. No. 1. pp 93-98.

Gonzalez-Noriega A, Grubb JH, Talkad V, Sly WS. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J Cell Biol.* 1980 Jun;85(3):839-52.

Hers HG. Alpha-Glucosidase deficiency in generalized glycogenstorage disease (Pompe's disease). *Biochem J.* 1963 Jan;86:11-6.

Liu J, Lu W, Reigada D, Nguyen J, Laties AM, Mitchell CH. Restoration of lysosomal pH in RPE cells from cultured human and ABCA4 (-/-) mice: pharmacologic approaches and functional recovery. *Invest Ophthalmol Vis Sci.* 2008 Feb;49(2):772-80.

Llopis- Peña S, Vega-Rubin-de-Celis S, Schwartz JC, Wolff NC, Tran TA, Zou L, Xie XJ, Corey Dr, Brugarolas J. Regulation of TFEB and V-ATPases by mTORC1. *EMBO J.* 2011 Jul 29;30(16):3242-58.

Ohkuma S, Moriyama Y, Takano T. Identification and characterization of a proton pump on lysosomes by fluorescein-isothiocyanate-dextran fluorescence. *Proc Natl Acad Sci U S A.* 1982 May;79(9):2758-62.

Platt FM, Walkley SU. *Lysosomal Disorders of the Brain.* Oxford University Press. 2004.

Schultz ML, Tecedor L, Chang M, Davidson BL. Clarifying lysosomal storage diseases. *Trends Neurosci.* 2011 Aug;34(8):401-10.

Spampanato C, Feeney E, Li L, Cardone M, Lim JA, Annunziata F, Zare H, Polishchuk R, Puertollano R, Parenti G, Ballabio A, Raben N. Transcription factor EB (TFEB) is a new therapeutic target for Pompe disease. *EMBO Mol Med.* 2013 May;5(5):691-706.

Tseng WA, Thein T, Kinnunen K, Lashkari K, Gregory MS, D'Amore PA, Ksander BR. NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization:

implications for age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2013 Jan 7;54(1):110-20.

Wang Y, Inoue T, Forgac M. Subunit a of the yeast V-ATPase participates in binding of bafilomycin. *J Biol Chem.* 2005 Dec 9;280(49):40481-8.