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Characterization of a ULK1 Agonist

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CHARACTERIZATION OF A ULK1 AGONIST

by

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A thesis submitted in partial fulfillment of the requirements
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All requirements for graduation with Honors in the
Biomedical Engineering have been completed.

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CHARACTERIZATION OF A ULK1 AGONIST

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Evaluation of forskolin as an agonist of ULK1, as a proxy to autophagy modulation

Introduction and Background

Autophagy is a necessary cellular process in which old or malfunctioning components of the cell are degraded and recycled into their base components. This process is necessary, and the malfunction of autophagy can result in the build-up of poorly functioning organelles, the prevention of necessary cell death to improve overall tissue efficiency, and harm to the cell's overall metabolic state. It is the focus of this project to investigate the workings of autophagy in hepatic cells in both *in vitro* models, as well as *in vivo* as tested in a mouse model. Two depictions of autophagy are given below in figures 1 and 2.
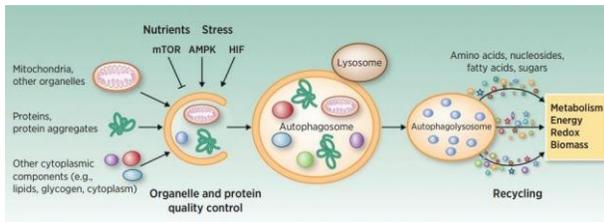
Figure 1: Base depiction of autophagy process. (SelfHacked, 2018)
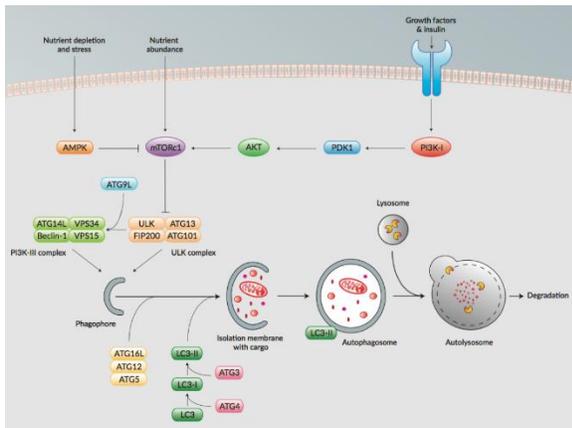
Figure 2: Protein interaction depiction of autophagy process. (InvivoGen, 2017)

ULK1 is one of the major proteins involved in the formation of the autophagosome, and the formation of the autophagosome is a critical step in the induction of autophagy. Thus, it was determined that identifying an agonist of ULK1 could be used as a proxy for potentially modulating autophagy expression. As such, two distinct questions were being asked with this project; whether an agonist for ULK1 could be identified, and if this agonist would then also act as a general agonist for autophagy.

For the purposes of this project, the identification of a ULK1 agonist was limited to hepatic cells. Thus, the results of this project are limited in scope to their effects in hepatic cells, as other cell types are likely to exhibit different metabolic conditions that may interact differently with any identified ULK1 agonist. As such, more investigation should be completed if the results of this project are to be extrapolated to other cell lines. In addition, due to the requirements to identify a ULK1 agonist and the number of experimental lines of investigation needed to confirm the function as a general autophagy agonist, this project is limited to the analysis of forskolin as both a ULK1 agonist and the characterization of its effects on autophagy. This factor had already been identified by Dr. Young Do Koo prior to the initiation of this project, due to background research into forskolin's purported agonistic activity to autophagy.

There is limited research into potential agonists of autophagy, and for which cell types these agonists are valid for. Basic research into the role of forskolin in autophagy suggests that it operates through the activation of protein kinase A (PKA), which in turn regulates cellular processes involved in stress response, metabolism, and survival processes, such as autophagy (Chiaradonna, Pirola, Ricciardiello, & Palorini, 2016). However, this research is limited to the fibroblast cell type, and as such does not control for accessory factors that forskolin may also activate. Furthermore, it has been shown that adenylyl cyclase has a critical role in the

induction of autophagy. (Chen, et al., 2013) Research into the metabolic pathway of autophagy has shown that forskolin acts as an activator of adenylyl cyclase, (Zhang, Zeng, Shen, Tian, & Yang, 2018) which may in turn result in the overall induction of autophagy. Given these correlations between forskolin and autophagy induction, it was determined to first investigate forskolin's role in autophagy prior to the screening of any additional drugs.

ULK1 levels were monitored as a proxy to autophagy modulation, and it was assumed that changes in the expression levels of ULK1 would cause downstream effects on autophagy due to the ease of formation of the autophagosome. Given that forskolin may have additional effects on accessory factors related to and modulated by the activation of autophagy, it was determined that these factors would also be monitored to ensure that accessory effects caused by the addition of forskolin were controlled for. In this manner, forskolin was screened as only a ULK1 agonist, while limiting any accessory effects. In addition, it was this concern over accessory effects that necessitated an extended screening of forskolin. Given the differences in metabolic processes in different cell types, it was unclear what exact effects forskolin would have in hepatic cell lines, and if these effects would even resemble those documented in previous research.

There are multiple metabolic controllers of autophagy, and while the entirety of the process is not fully understood, there is large support that ULK1 and ULK2, which are proteins found within the cell, modulate the formation of the autophagosome. The autophagosome is the structure that enables the autophagy process, as it forms to degrade and recycle the components present inside of it. As such, research performed in this project as well as in the overarching research scheme focused on understanding autophagy using ULK1 and ULK2 as factors to modulate the occurrence of autophagy. The importance of ULK1 can be visualized in figure 1, and the importance of ULK2 associated with ULK1 can be visualized in figure 2. Finally, an overview of autophagosome formation can be visualized on figure 3. To initiate autophagy induction and autophagosome formation, ULK1, ULK2, Atg13, FIP200 all complex together to initiate autophagosome formation, among other factors. It was this complex formation that initially led to the investigation of the roles of ULK1 and ULK2 in autophagy.
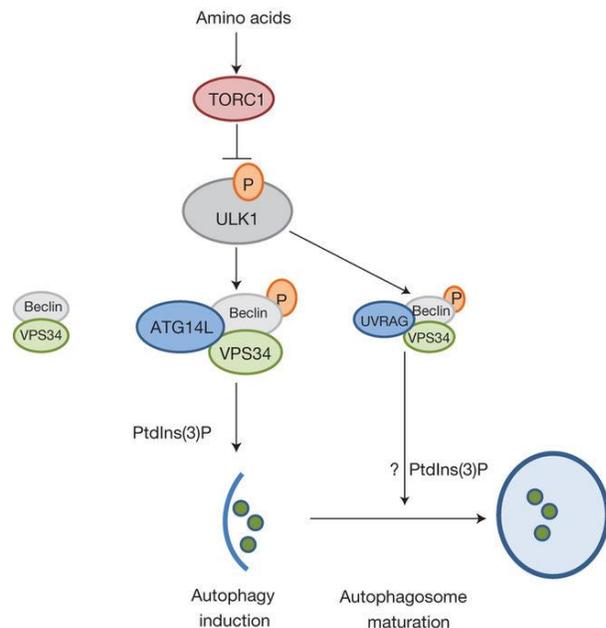
Figure 1: Depiction of the importance of ULK1 in triggering autophagy induction.
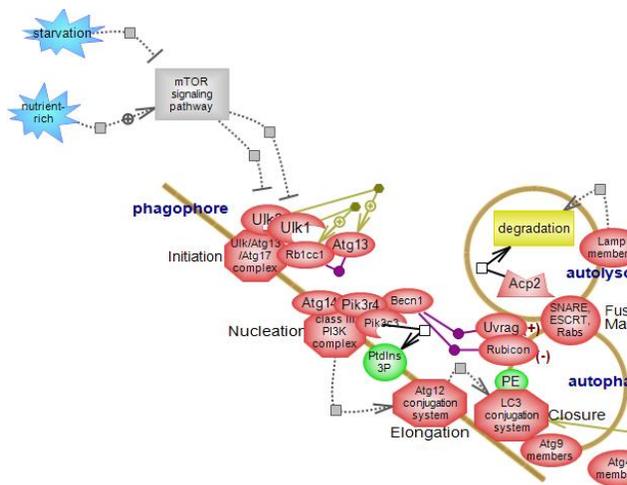
Figure 2: Depiction of the association between ULK1 and ULK2 in autophagy induction
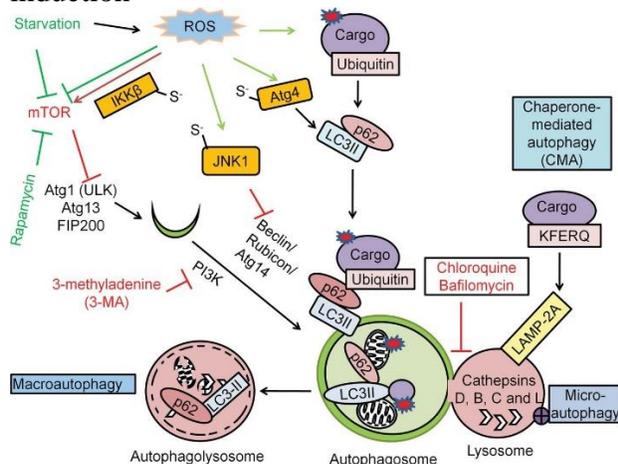
Figure 3: Depiction of the overall formation of the autophagosome, with contributing metabolic factors.

Given that the formation of the autophagosome utilizes a variety of ancillary metabolic factors and proteins, a selection of these factors was used to identify the effects of a ULK1 agonist towards autophagy. These selected factors included ATG13, GAPDH, FIP200, LC3B, p62, Bnip3, AKT, and Beclin1, along with the expression levels of ULK1. In this manner, the overall effect of a ULK1 agonist could be identified, and any accessory effects other than the increase of ULK1 transcription could be identified. These accessory effects

represented the largest concern for this project, as the agonist's primary effect should be to increase the activation of ULK1 to avoid changing the other experimental conditions of the overarching experiment. The levels of these metabolic factors were both identified using western blot analysis as well as through analysis of transcription levels through RNA analysis.

The main marker used in this experiment was the expression level of ULK1, as while it is known that ULK1 plays a major role in the formation of the autophagosome, the specific metabolic processes surrounding ULK1's involvement are being investigated. FIP200 was utilized as a marker of autophagy, as it has a known role in autophagy induction and complex formation with ULK1. (Liu, et al., 2013) In addition, ATG13 is also known to have a role in this complex and was utilized as a marker accordingly (Wei, Wang, Croce, & Guan, 2014). Next, p62 was identified as an important autophagy marker, as it has a defined role in the recognition of toxic waste needing removal through autophagy (Lippai & Low, 2014). As such, when a cell is autophagy-deficient, p62 begins to build up in the cell and can be measured as a marker of autophagy-deficiency. With the given goal for an autophagy activator, the expectation would be for p62 levels to decrease in autophagy-agonist treatment conditions, or at the very least remain constant. Beclin1 was identified as a marker of interest due to its known role in the induction of autophagy. Specifically, Beclin1 has defined roles in autophagosome nucleation and maturation (Kang, Zeh, Lotze, & Tang, 2011). LC3 (monitored as LC3B) was also noted as a reliable marker of autophagy, as it commonly reflects starvation-induced autophagy activity (Tanida, Ueno, & Kominami, 2008). GAPDH was utilized as a marker of autophagy induction due to starvation, as

literature indicates that GAPDH phosphorylation by AMPK—due to glucose starvation—increases Sirt1 activation and autophagy induction (Chang, et al., 2015). Bnip3 was also utilized as a key marker of autophagy, as Bnip3 acts as a potent inducer of autophagy in many cell types (Quinsay, Thomas, Lee, & Gustafsson, 2010).

In monitoring the effects of the proposed ULK 1 agonist, along with the effects of insulin addition, a new set of factors were utilized to characterize the effect on autophagy. Consistent with previous experimental conditions, ULK1 was maintained as the main marker of autophagy induction. Given that the desired effect was to increase the expression of ULK1, this factor needed to show increased levels in all experimental trials treated with forskolin. The next factor that was utilized was AKT, both in its non-phosphorylated and its phosphorylated forms. AKT has established roles in the inhibition of autophagy through the modulation of Beclin1, and as such its use as a marker indirectly analyzed the hypothesized agonist's effect on autophagy (Wang, et al., 2012).

In the overarching research scheme, both cell models and mouse models were developed into three breeds to display significantly reduced levels of ULK1, ULK2, or a double knock-down of ULK1 and ULK2. The purpose of these knock down treatments was to observe and record both the metabolic effects of reduced autophagy, such as an increase or decrease in related metabolic factors, as well as to observe phenotypic differences in the mouse model. These phenotypic differences were observed as an increased body weight, increased insulin resistance, and decreased glucose sensitivity in mice bred to display deficiencies in the autophagy process. However, these knock down models only allowed a one-way view into the overarching metabolic process. In order to fully understand the overarching process governing the observed results, the effects of increased autophagy also needed to be observed.

Prior to the completion of this project, no simple factor was known to cause a reliable increase in autophagy activity without also having significant auxiliary effects on unrelated metabolic factors. As such, the current landscape for autophagy activators was not useful nor present for the purpose of experimentally increasing autophagic activity while also keeping secondary metabolic factors constant. It was also determined that a drug treatment—as opposed to genetic manipulation—would prove most useful in the scope of the overarching research goal. This was determined as countering the knock-down effects of gene editing and selection with the effects of a ULK1 agonist drug could lead to interesting results. If the effects of genetic knock down could be countered with the identified agonist, for a removal of the metabolic effects caused by autophagy deficiency, significant support would be added to the research goal of supporting that autophagy has a significant impact in cellular metabolism and the diabetes disease state.

Methodology

After the cell samples were prepared, each set of samples were analyzed using a western blot analysis scheme. For all *in vitro* samples, the first step in this process was to extract all protein from the cell samples. This was conducted using RIPA buffer and a protease/phosphatase inhibitor lysis technique, in which the lysis buffer was added to each sample. The obtained solution was then vortexed and centrifuged, yielding a homogenized protein sample corresponding to each experimental condition. These protein samples were then

analyzed using a microBCA analysis step, which yielded the total concentration of all protein in each sample. Using this information, stock samples were prepared for western blot analysis in which protein was diluted using a 4x sample buffer and water to yield protein samples with identical total concentrations. Thus, while the concentrations of individual proteins of interest would vary between the samples, variance due to differing overall protein concentrations was eliminated.

The next step of the western blot analysis scheme was to then resolve the protein samples according to molecular weight and image the samples. First, the protein samples were resolved on a running step, on which proteins are resolved along an electrophoresis gel as a voltage gradient separated the various proteins through the gel based on their molecular weights. Higher molecular weight proteins travelled less distance in the gel than did lower molecular weight proteins. A ladder of known protein sizes was also resolved on the gel as a reference. Depictions of the running tank setup, and the samples and ladder resolved on the gel, are provided below in figures 4 and 5.
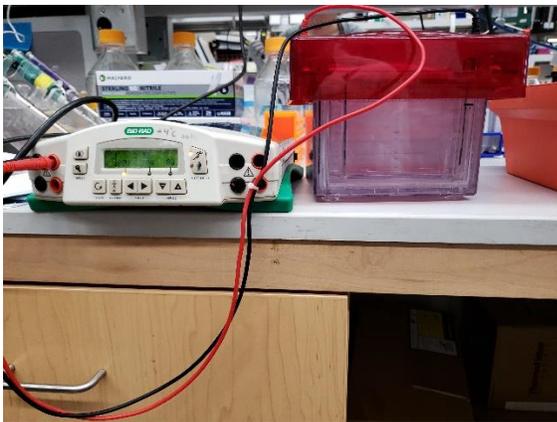
Figure 4: Depiction of running gel setup with connected power supply.
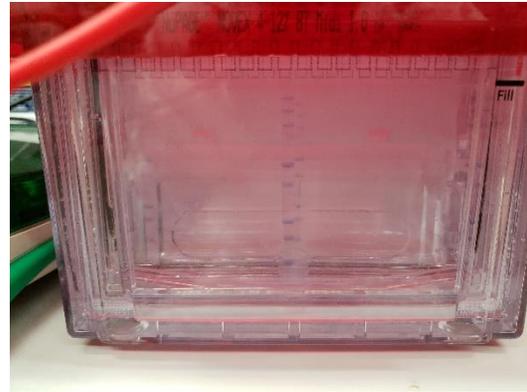
Figure 5: Depiction of protein ladder and samples resolved on running gel.

After the running gel had concluded, the proteins were then transferred to a nitrocellulose membrane in the transfer step. This was accomplished by stacking the running gel on top of a nitrocellulose membrane and applying a voltage gradient across this 'sandwich'. The voltage gradient caused the proteins contained within the gel to migrate and become bound into the nitrocellulose membrane. This transfer served to both affix the proteins into a stable position, as well as to expose portions of the proteins outside of the membrane for recognition using antibody selection. This transfer step was accomplished by either applying a 100 Volt gradient for only an hour, or by applying a 40 Volt gradient and allowing the transfer step to continue overnight. The transfer step was always conducted at 4°C to avoid degradation of data due to localized heating within nitrocellulose membrane. The results obtained using either technique are assumed to have provided identically reliable results, as no significant difference in effectiveness was observed while conducting the experimental protocol.

Following completion of the transfer step, the nitrocellulose membrane was divided into sections with only one protein of interest in each section. The proteins used as markers in this experiment were ULK1, FIP200, GAPDH, BNIP3, ATG13, P62,

LC3B, AKT, and pAKT. At this point, the empty space of the nitrocellulose membrane was blocked using skim milk to prevent nonspecific associations during the antibody detection step. A primary antibody for each protein of interest was incubated on its respective membrane piece overnight. Following this incubation, a secondary antibody—with an attached fluorescent tag—specific to the animal type of each respective primary antibody was added to each membrane section. This allowed detection of concentration of each protein of interest in each cell sample provided, as the complex of secondary antibody bonded to primary antibody bonded to protein allowed the relative quantification of protein concentration. These concentrations were always compared to a control set of cell samples for each experimental repetition.

The concentration of protein was quantified using the relative amount of fluorescence emitted, through the use of the fluorescent tag on the secondary antibody, for each protein of interest. The fluorescence detected on the spatial region on the membrane corresponding to the protein and conditions of interest was recorded using autoradiography methods. The proteins bound on nitrocellulose membrane were incubated with an enhanced chemiluminescent substrate, which activated the fluorescent tag. At this point, through the use of a dark room to avoid film spoilage, X-ray film was exposed to the membranes for varying time periods. The fluorescent tag would be detected and recorded by the X-ray film, with longer exposure times corresponding to a larger change on the X-ray film. After development of the film, distinct bands would present on the X-ray film that correspond to the relative concentration of each protein of interest. Through the comparison to controls, the relative concentrations of the proteins of

interest for each experimental condition could be quantified.

The initial experimental trials to screen forskolin as an autophagic inductor were conducted *in vitro*, varying the concentration of forskolin added as well as varying a starvation condition. Both the starvation and control conditions were treated with either no forskolin—as the control—and then with 1 micromolar or 10 micromolar forskolin experimental condition. The non-starvation cells were incubated with 10% FBS (fetal bovine serum), while the starvation condition cells were incubated without serum. This set of conditions was repeated twice to validate the results of the first trial.

The second set of experimental conditions evaluated using a western blot analysis scheme was to remove the starvation condition, and now include a condition where the expression of ULK1 was artificially knocked-down using siRNA. A control set was run along with this new experimental condition, incubating cells in either no forskolin, a 1 micromolar solution of forskolin, or a 10 micromolar solution of forskolin. The siRNA condition was repeated for both a control group—one in which the expression level of ULK1 was not knocked down—as well as for a group where siRNA was used to knock down the expression level of ULK1. For both the siRNA control and experimental group, cells were either treated with vehicle only—as a control—or were treated with a 10 micromolar solution of forskolin. This experimental protocol was repeated two times, to validate the results obtained in the first experimental trial.

The third set of experimental conditions evaluated using a western blot analysis scheme was to expose the *in vitro* samples to insulin as well as to forskolin. For this experimental trial, both an siRNA control group and an siULK1 group were

used. For each group, cells were either exposed to only vehicle (as a control group), to a solution containing 100nM forskolin for 15 minutes, to a 10 micromolar concentration of forskolin, or were exposed to both insulin and forskolin treatments. For this experimental condition, the proteins pAKT, AKT total form, GAPDH, and ULK1 were measured. This experimental condition was only conducted once. During all western blot experiments, if the possibility arose, replicates were created on the same membrane to test for reliability. These replicates were from the same cell sample and treatment day, and as such only prove to demonstrate that the results obtained for a given experiment were valid. They do not constitute an increase in overall sample size.

In addition to the maintenance of both cell culture colonies for *in vitro* testing and the use of western blot analysis, a series of mouse colonies were also maintained for the purposes of testing forskolin in an *in vivo* setting. Colonies of knock-down mice were bred and selected to either exhibit knock-down genotypes for ULK1, ULK2, or for ULK1/2. It was determined during the testing of the overarching hypothesis governing the involvement of ULK1 and ULK2 in diabetic symptom presentation that ULK2 did not have a profound impact, and as a result ULK2 mice were not prioritized during the breeding process. Starting in the fall of 2016, over the course of two years and over two hundred mice, the mice colony for ULK1 progressed from homozygous wild type mice, to heterozygous knock-down mice, to finally displaying a homozygous knock-down genotype during the spring of 2018. Over the same two year period, ULK2 mice also displayed a general trend towards containing purely homozygous knock-down mice, as a hundred and fifty mice were genotyped and differentiated based on ULK2 expression

level. It is expected that the ULK2 colony will contain purely homozygous knock-down mice by the end of 2018. Finally, the ULK1/2 double-knockdown mice colony was started in the spring of 2017, with approximately 150 mice bred and genotyped prior to the termination of the project.

The purpose of breeding the mouse colonies mentioned was to both test the overarching experimental conditions not pertaining to this project's specific hypothesis—e.g., the analysis of forskolin as a ULK1 agonist—as well as to develop an *in vivo* testing base for the forskolin treatments. While the effect of forskolin injection was not studied in the mouse model due to time constraints, the maintenance of the mouse colony is included within the methods as the mouse colony will likely be utilized in the future by other experimenters to characterize the *in vivo* response. Thus, while not directly used within this project, future experiments conducted to analyze the *in vivo* response to forskolin directly rely on the work conducted within this project.

Finally, the identification of the transcription levels of ULK1 and other factors of interest was identified using the creation of cDNA. This was accomplished through the extraction of RNA from cell culture, and subsequent creation of cDNA. RNA was extracted using a trypsin lysis buffer, with subsequent washes conducted using chloroform and ethanol. RNA was separated from other organic molecules using a centrifugation step, similar to how protein was extracted for western blot analysis. Following the extraction of RNA, concentration, purity, and contamination levels were identified using a NanoDrop machine, which utilizes Beer's Law to interpolate concentration of nucleic acids based on a solution's absorbance. Following confirmation that the samples had adequately low contamination and high

purity levels, cDNA stocks were created from each cell RNA sample using a standard thermocycler protocol. The created cDNA was then utilized for further polymerase chain reactions, to amplify a select RNA transcript. In this manner, the expression level of select RNA transcripts inside a cell could be identified by resolving amplified cDNA samples on an agarose gel. In this manner, relative amounts of RNA in each cell sample could be compared, utilizing the principal that different molecular weight DNA strands would resolve to different lengths on the agarose gel. The location in the gel where bands of interests resolved to could be compared to a standard ladder as well as to literature to confirm that the bands of interest were indeed the metabolic factors of interest. In this manner, controls from cell samples could be compared to experimental cell conditions to determine if the ULK1 agonist—or other added metabolic factors—altered the occurrence level of autophagy in the cell sample.

Results

The first western blot conducted on December 8th was used to evaluate the effects of forskolin on hepatic cells and yielded the following results. The loading scheme used is depicted in table 1.

Table 1: Loading scheme of Western Blot conducted on December 8th.
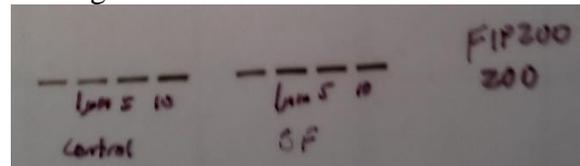
10% FBS				Serum Free (Starvation)			
Vehicle	1uM Forskolin	5uM Forskolin	10uM Forskolin	Vehicle	1uM Forskolin	5uM Forskolin	10uM Forskolin
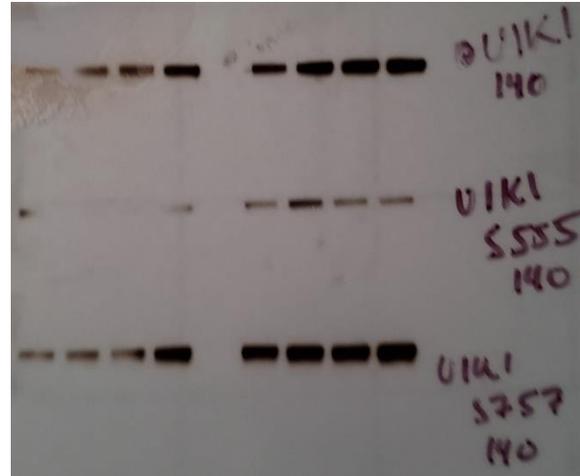
A: Expression levels of ULK1 for loading scheme detailed in table 1.
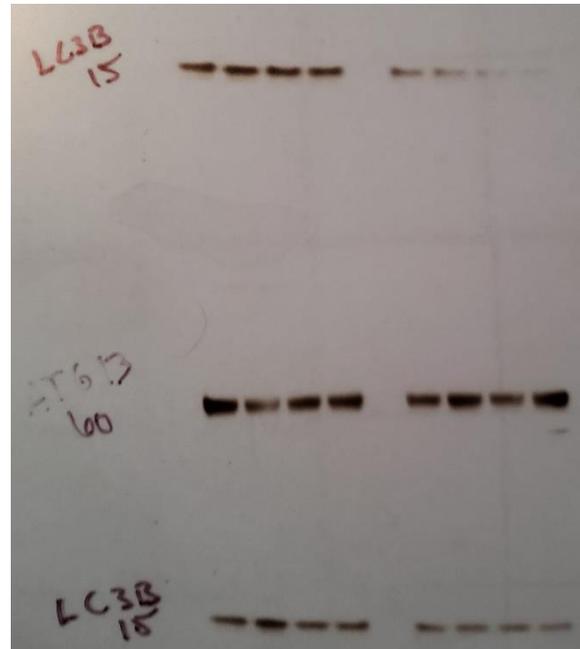
B: Expression levels of LC3B and ATG13 (with two replications of LC3B), for the loading scheme detailed in table 1.
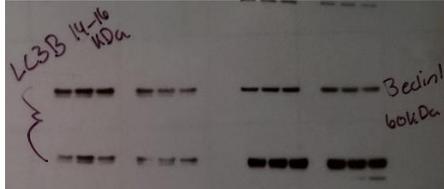
C: Expression levels of FIP200 for the loading scheme detailed in table 1.
Figure 6 (A-C): Expression levels of select factors for loading scheme detailed in table 1.

The next western blot was conducted on January 25th, 2018, and served to confirm the expression levels of the secondary factors of interest measured in the first western blot. The loading scheme used is diagramed in table 2, with the following figures depicting protein expression levels.

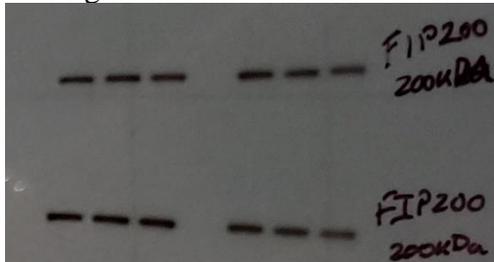**Table 2:** Loading scheme for western blot conducted on January 25th.

10% FBS			Serum Free (Starvation)		
Vehicle	1uM Forskolin	10uM Forskolin	Vehicle	1uM Forskolin	10uM Forskolin

A: Expression levels of LC3B and Beclin1, with two replications of LC3B, for the loading scheme detailed in table 2.
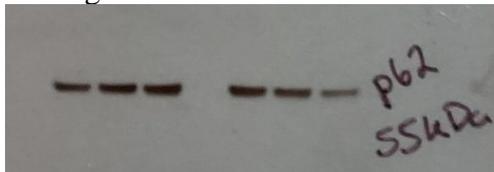
B: Expression levels of GAPDH for the loading scheme detailed in table 2.

C: Expression levels of FIP200, with two replicates, for the loading scheme detailed in table 2.
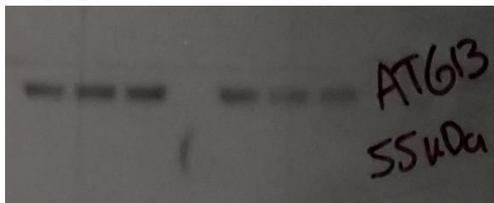
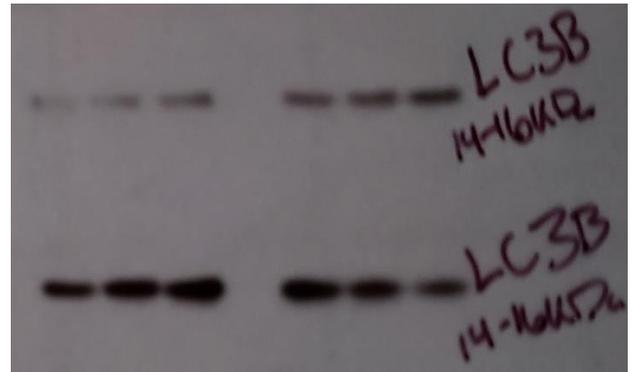**D:** Expression levels of ATG13 for the loading scheme detailed in table 2.

E: Expression levels of p62 for the loading scheme detailed in table 2.
Figure 7 (A-E): Expression levels of select factors for loading scheme detailed in table 2.

The next western blot was conducted on February 8th, and served as a final confirmation of the conditions used in the prior two western blots. A starvation condition was again used, with forskolin added in varying amounts as according to the loading scheme detailed in table 2. The following results were obtained.
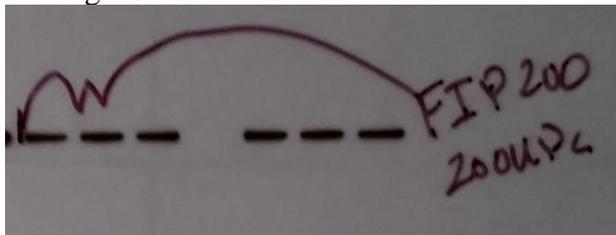
A: Expression levels of p62 for the loading scheme detailed in table 2.
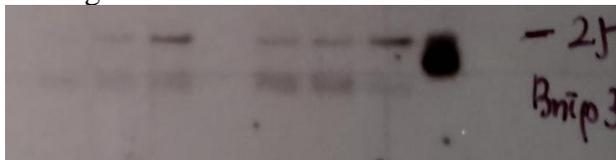
B: Expression levels of LC3B and FIP200, with two replications of LC3B, for the loading scheme detailed in table 2.
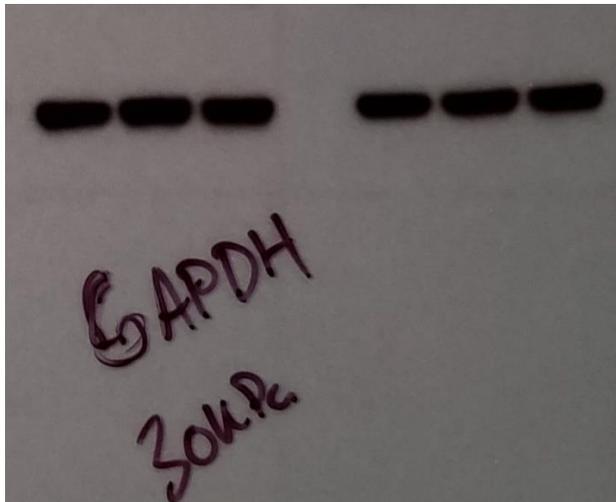
C: Expression levels of GAPDH for the loading scheme detailed in table 2.
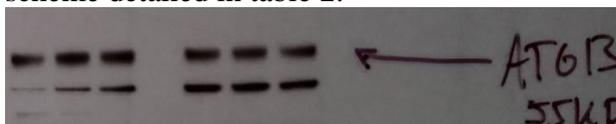

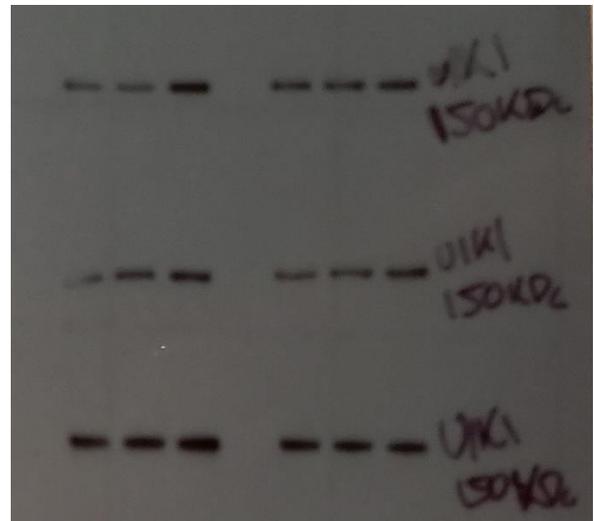**D:** Expression levels of FIP200 for the loading scheme detailed in table 2.

E: Expression levels Bnip3 for the loading scheme detailed in table 2.

F: Expression levels Beclin1 for the loading scheme detailed in table 2.

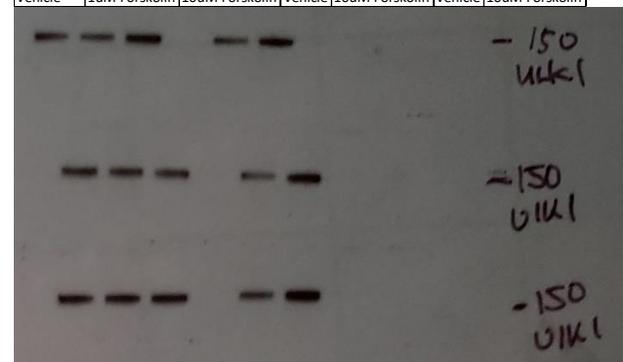**G:** Expression levels of ATG13 for the loading scheme detailed in table 2.

H: Expression levels of ULK1, with three replicates, for the loading scheme detailed in table 2.

Figure 8 (A-H): Replicate expression levels of select factors for loading scheme detailed in table 2.

The third western blot conducted in this project concluded on February 15th. The purpose of this experiment trial was to both confirm the results obtained in prior western blots—except for those obtained using starvation conditions—as well as to introduce the new experimental condition of siRNA use. The loading scheme used is depicted below in table 3.

Table 3: Loading scheme for western blot conducted on February 15th.

Replicate Trial			siCon		siULK1	
Vehicle	1uM Forskolin	10uM Forskolin	Vehicle	10uM Forskolin	Vehicle	10uM Forskolin

A: Expression levels of ULK1, with three replicates, for the loading scheme detailed in table 3.

B: Expression levels of p62 for the loading scheme detailed in table 3.
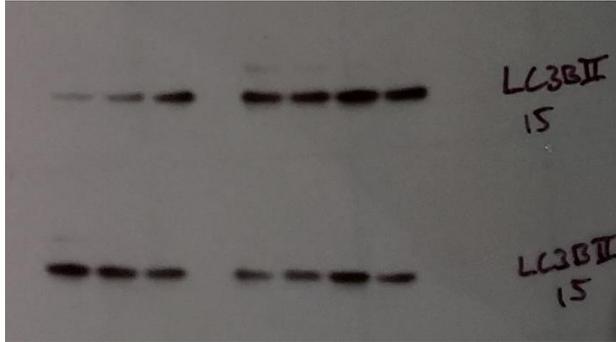
C: Expression levels of LC3B, with two replicates, for the loading scheme detailed in table 3.
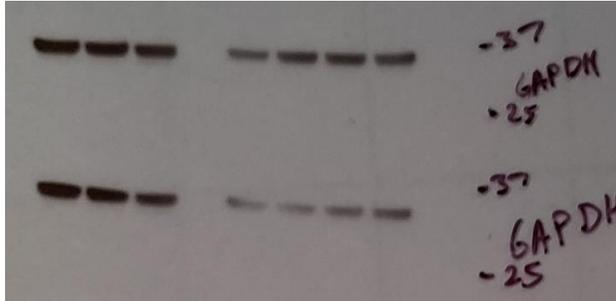
D: Expression levels of GAPDH, with two replicates, for the loading scheme detailed in table 3.

E: Expression levels of FIP200 for the loading scheme detailed in table 3.
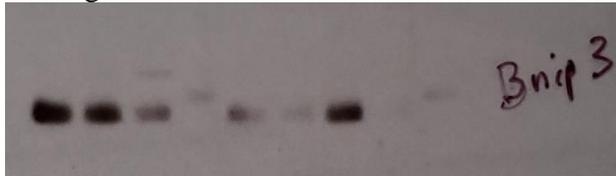
F: Expression levels of Bnip3 for the loading scheme detailed in table 3.
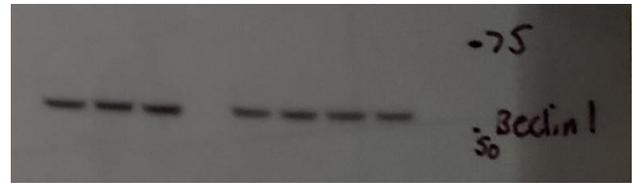
G: Expression levels of Beclin1 for the loading scheme detailed in table 3.

H: Expression levels of ATG13 for the loading scheme detailed in table 3.
Figure 9 (A-H): Expression levels of select factors for loading scheme detailed in table 3.

The fourth western blot conducted in this project concluded on March 1st. The purpose of this experiment trial was to confirm the results of the siRNA condition. Due to experimental error, only the expression levels for ULK were obtained. The loading scheme used is depicted below in table 4.

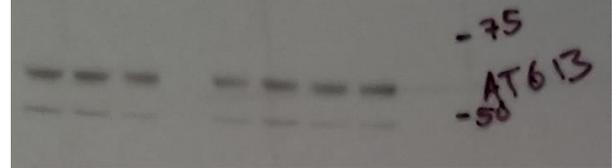**Table 4:** Loading scheme for western blot conducted on March 1st.

| siCon		siULK1		Replicate Trial		
Vehicle	10uM Forskolin	Vehicle	10uM Forskolin	Vehicle	1uM Forskolin	10uM Forskolin

Figure 10: Expression levels of ULK1 for the loading scheme detailed in table 4.

The fifth western blot conducted in this project concluded on March 29th. The purpose of this western blot was to investigate the effects insulin treatments would have, in addition to the effects of forskolin, when combined with the effects of siRNA. The loading scheme used is depicted below in table 5.

Table 5: Loading scheme for the western blot conducted on March 29th.

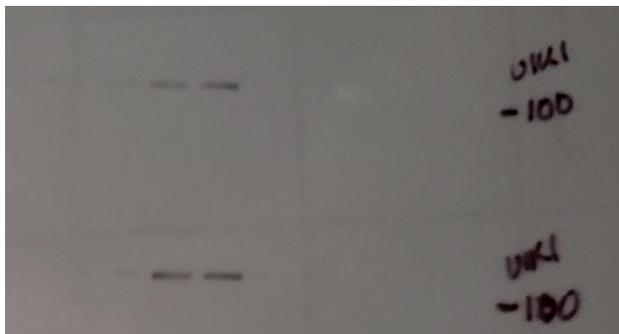| siCon				siRNA			
Vehicle	Insulin	Forskolin	Insulin + Forskolin	Vehicle	Insulin	Forskolin	Insulin + Forskolin
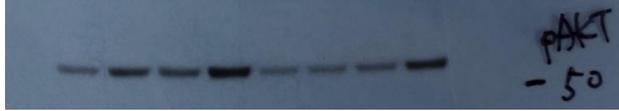
A: Expression levels of pAKT for the loading scheme detailed in table 5.

B: Expression levels of GAPDH for the loading scheme detailed in table 5.

C: Expression levels of AKT for the loading scheme detailed in table 5.
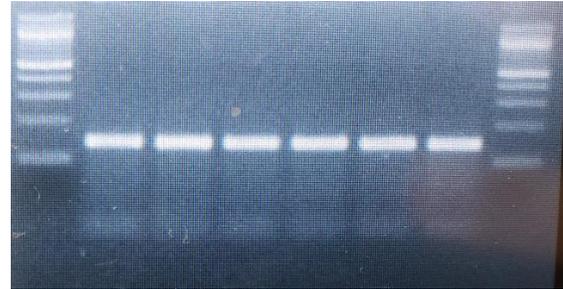

Figure D: Expression levels of ULK1, with two replicates, for the loading scheme detailed in table 5.

Figure 11 (A-D): Expression levels of select factors for loading scheme detailed in table 5.

In addition to the western blot experiments, an analysis using RNA transcription levels was conducted. The expression levels of ULK1 and GAPDH mRNA were evaluated and are depicted below in figure 12, for a loading scheme detailed in table 6.

Table 6: Loading scheme for mRNA analysis.

Vehicle	Vehicle	1uM Forskolin	1uM Forskolin	10uM Forskolin	10uM Forskolin

A: Expression levels of GAPDH.

B: Expression levels of ULK1.

Figure 12: Measured mRNA levels, using a cDNA amplification technique, using the loading scheme detailed in table 6.

Discussion

Analysis of the results from the initial western blots—those conducted to screen for ULK1 indication—indicate that forskolin is not a direct activator of autophagy. The results from the first western blot display a clear increase in ULK1 expression levels as a result of forskolin addition, in both non-starvation and starvation conditions. This result coincided with the increase in FIP200 levels with increasing concentrations of ULK1, as FIP200 complexes with ULK1. LC3B levels decreased markedly in starvation conditions, however there was no change in the non-starvation condition. This result was inconsistent with the expectation of autophagy induction. Finally, while ATG13

levels also remained consistent across experimental conditions, which was inconsistent with forskolin acting as an autophagy agonist. The mixed results of this experiment led to further replicates being conducted to confirm the results obtained.

The results obtained on January 25th yielded no significant aide to the hypothesis, as many of the results were inconsistent with forskolin acting as an autophagy inductor. FIP200 and ATG13 were observed to have no significant increase compared to control levels, a result inconsistent with autophagy induction. GAPDH was observed to decrease in starvation conditions, an effect opposite that of expectation. Also, GAPDH levels were observed to decrease, a result consistent if autophagy were inducted through a separate pathway than GAPDH, thus inhibiting GAPDH expression. LC3B expression was observed to have been invalidated due to the presence of a bubble present during the transfer step of the western blot, distorting the results such that they are unusable. The levels of p62 remained consistent across the non-starvation levels, indicating no build up due to autophagy deficiency. Finally, Beclin1 levels were observed to have no distinct increase, inconsistent with forskolin acting as an autophagy inductor. Due to the inconsistency of these results, it was determined that additional western blots were needed to continue to investigate whether forskolin acted as an autophagy inductor.

The following experiment served as an additional replication trial for the previous two western blot trials. The results of this experiment served to confirm that forskolin did not act as a direct autophagy agonist. While ULK1 levels were observed to greatly increase for increasing concentrations of forskolin, ATG13 levels were observed to remain relatively constant across experimental conditions. Finally,

FIP200 levels remained consistent across trials, indicating that while forskolin increased the overall expression of ULK1, it did not drastically increase the formation of the autophagosome. GAPDH levels remained consistent across forskolin levels, consistent with expectations as forskolin was not expected to modulate the starvation-induction process of autophagy. LC3B levels were inconsistent across replicates in the same western blot, and as such further replicates should be conducted to fully characterize the effect of forskolin on LC3B. Consistent with previous experiment trials, p62 levels remained consistent across experimental conditions. Finally, Bnip3 levels were observed to significantly increase for increasing levels of forskolin, suggesting that forskolin may still play some role in autophagy induction.

Further experimentation focused on the cellular response to forskolin in environments where ULK1 levels were artificially knocked-down due to the use of siRNA. This allowed for the identification of autophagy levels when the desired target of forskolin—ULK1—was removed. The results from the western blot conducted on February 15th continue to indicate that forskolin causes an increase in ULK1 levels, and the knock-down of ULK1 levels due to siRNA use was confirmed. The levels of p62 were observed to remain constant across all trials, consistent with a lack of autophagy deficiency. The levels of LC3B were observed to have no significant difference across trials. GAPDH levels were observed to remain constant across forskolin levels and siRNA treatments, consistent with the hypothesis that forskolin does not modulate starvation-induced autophagy. FIP200 levels were observed to decrease for siULK1 treatments, consistent with the expectation that knock-down of ULK1 would prevent the complex formation. Additionally, adding forskolin did not increase FIP200 in siULK1

conditions, supporting the hypothesis that forskolin only acts as an agonist to ULK1. ATG13 levels were observed to remain constant across trials, consistent with previous experiment trials. Beclin1 levels were observed to not significantly change across trials, inconsistent with the hypothesis that forskolin acts as a autophagy agonist but consistent with previous experimental results. Beclin1 levels were also observed to not change in siULK1 treatments, inconsistent with expectations and warranting further replications. Finally, Bnip3 levels were observed to decrease for increasing concentrations of forskolin, a result that is inconsistent with the hypothesis and with previous experimental results. This is likely due to Bnip3 being difficult to detect using the detection scheme used. Normal ECL solution was utilized for all other proteins of interest, however Bnip3 consistently required the use of a super-signal solution, which results in an increase in image distortion. As such, the results obtained are not reliable and should be replicated in future experimental trials prior to a definite conclusion being drawn on forskolin.

While the majority of the results for the western blot experiment conducted on March 1st were lost—due to a failure in the transfer step—the results for ULK1 levels remained intact. These results remained consistent with previous experiment trials, whereby ULK1 levels increased for increasing forskolin levels and were observed to significantly decrease for siULK1 conditions.

The final western blot conducted in the experimental plan served to explore the effects insulin treatment would have on autophagy induction. Insulin addition was expected to decrease the induction of autophagy, as the glucose level inside the cell would increase, causing the cell to be more metabolically active.

The experimental results show that ULK1 levels increased in forskolin addition conditions, with higher expression levels in insulin treatments. This coincides with the paradigm that ULK1 is involved in the insulin signaling pathway. GAPDH levels remained constant across trials, consistent with expectations and previous experimental results. Finally, pAKT levels were observed to increase in insulin treatment conditions and in forskolin treatment conditions, relative to the control. Further replications should be conducted to fully characterize the effect forskolin has on the insulin signaling pathway. However, the phosphorylation of AKT in forskolin treatment conditions, coinciding with an increase in ULK1 levels, is consistent with the proposal that forskolin increases ULK1 expression, which then has a role in the insulin signaling pathway.

Conclusion

The results obtained during this project are by no means complete nor satisfactory towards the definitive conclusion that forskolin acts as an autophagy inductor through the direct increase in ULK1 transcription. While the results of the experiments conducted suggest that forskolin is, indeed, a transcription agonist for ULK1, further research needs to be conducted to further support this hypothesis. In addition, the full characterization of autophagy related genes indicates that the increase in ULK1 was not matched by an equal increase in autophagy induction. Therefore, it is unlikely that forskolin acts in a direct manner to induce autophagy.

Due to time constraints, the experiments conducted in this experiment were limited to *in vitro* models only. However, further support could be obtained should a mouse model be utilized to characterize the autophagy response to forskolin, both in insulin-treatment

conditions and in standard conditions. In addition, further research should be conducted into both the side effects of forskolin addition—namely, the effects on other autophagy pathways—and into the RNA transcription levels of the proteins of interest.

Finally, the analysis of mRNA levels was mostly inconclusive. While the levels of GAPDH remained constant, consistent with prior western blot results, the levels of ULK1 were invalidated due to a faulty control. Given that the second vehicle sample tested as having an equal concentration of ULK1 as the 10uM forskolin treatment condition, no significant conclusion can be taken from the experiment. However, the observed trend still shows an overall increase in ULK1 expression for increasing concentrations of forskolin, and as such this experiment would benefit from replication to minimize the observed error in the control level of ULK1. Over the course of multiple experimental replicates, most of the inconsistencies between data were minimized and the cellular response to forskolin was characterized with reasonable confidence. Unfortunately, the cellular response did not indicate a direct induction of autophagy when ULK1 levels were increased. However, the role of forskolin and ULK1 in the insulin signaling pathway does hold promise as a line of investigation, and as such further experimentation should be done to fully characterize the autophagy response when both the insulin signaling pathway and forskolin levels are modulated.

This project serves as only a pilot study for future research endeavors. Due to the lack of statistical analyses, varying cell lines, and lack of *in vivo* models, the results can not be extrapolated beyond the scope of the current project. However, the results are promising and suggest that should further research be conducted to characterize the cellular response to forskolin, a modulator of autophagy that has connections to the insulin signaling pathway could be identified.

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