(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 April 2014 (03.04.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/049580 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/IB2013/058996

(22) International Filing Date:
30 September 2013 (30.09.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
4080/CHE/2012 28 September 2012 (28.09.2012) IN

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/049580 A2

(54) Title: ASSAY TO MONITOR AUTOPHAGY, A METHOD AND KIT THEREOF

(57) Abstract: The present disclosure relates to a method of performing an assay to monitor the overall autophagic flux within a host cell particularly, macro autophagy, pexophagy and other autophagic pathways. The present disclosure also relates to a vector or expression construct and host cell suitable for being employed in the assay to monitor autophagy and a kit thereof.

"ASSAY TO MONITOR AUTOPHAGY, A METHOD AND KIT THEREOF"

TECHNICAL FIELD

The present disclosure relates to a method of performing an assay to monitor the overall autophagic flux within a host cell particularly, macroautophagy, pexophagy and other autophagic pathways. The present disclosure also relates to a vector or expression construct and host cell suitable for being employed in the assay to monitor autophagy and a kit thereof.

BACKGROUND OF THE DISCLOSURE

Autophagy is the major intracellular catabolic pathway responsible for the turnover of cellular organelles and long-lived macromolecules. It regulates diverse biological events including development, aging, cell death and microbial infections. Autophagy and related pathways involve degradation of either selective or non- specific cytoplasmic components via double membrane vesicles. The pathway is initiated when a newly formed double membrane, the phagophore, expands and confines a part of the cytoplasm resulting in the formation of an autophagosome. The subsequent fusion of the autophagosome with a lysosome results in the formation of an autolysosome and the degradation of the inner membrane and the cargo. This conserved pathway from yeast to humans plays critical role in cell survival during nutritional deprivation, clearance of damaged/superfluous organelles, protein aggregates and intracellular pathogens. Dysfunction of autophagy leads to cell death, cancer, neurodegenerative and other diseases.

Autophagy helps cell survival especially during nutritional deprivation or starvation by recycling redundant, excess and/or superfluous cytoplasmic constituents including proteins and organelles. Several intracellular pathogens including bacteria (Salmonella, Shigella, Mycobacterium, Group A Streptococcus, etc.) and viruses (herpes simplex virus, HIV, etc.) subvert autophagy to prevent their elimination by degradation in host lysosomal compartments.

Autophagy also has a neuroprotective role, as it clears large aggregates of mutant polyubiquitylated proteins resistant to proteasomal degradation. Furthermore, apart from neurodegenerative and infectious diseases, autophagy has been shown to be involved in heart diseases, atherosclerosis, certain myopathies, innate and adaptive immune responses, Crohn's disease and cancer. Thus, targeting autophagy is a potentially exciting avenue that is just

beginning to be exploited for disease and cancer cures. Recent studies have shown that modulating autophagy has positive outcomes in the diseases mentioned above. For e.g., modulating autophagic activity either genetically or biochemically, have resulted in increased killing of intracellular mycobacteria.

In view of the above points it can be deciphered that understanding autophagic flux becomes an important aspect in the field of cellular and molecular biology to address various disorders. There are various assay techniques to observe the authophagic pathways in the cell, but all these assays are not efficient and sensitive enough to understand the overall pathway involved in the autophagic flux which could be crucial to address the disorders. Most of the conventionally used assays to monitor autophagy are based on western blotting or microscopy and are qualitative in nature. Many high throughput assays have been developed in recent times to find out new drug candidates that affect autophagy. However, these assays do not measure the autophagic flux and cargo degradation in real time. (PMID: 17486044; PMID:18641457; PMID:21635740)

The instant invention aims at overcoming the above mentioned issues with a multiple assay which provides an effective alternative of a more robust, reproducible and sensitive kinetic assay to study pexophagy as well as general autophagy flux.

STATEMENT OF THE DISCLOSURE

Accordingly, the present disclosure relates to a method of performing an assay to monitor autophagy within a host cell, said method comprising acts of a) preparing at least one expression construct with a reporter gene b) transforming a host cell with the prepared expression construct c) culturing the transformed host cell in induction medium followed by starvation medium to induce autophagy within the host cell d) lysing the induced host cell of step (c) in a lysis buffer e) treating the lysed host cell with a substrate capable of reacting with the reporter to produce a light reaction, and f) analysing the reaction to monitor the autophagy; a vector or expression construct comprising a POT-1 promoter sequence and reporter gene optionally along with an SKL signal sequence; a host cell transformed with the vector or the expression construct as above; a method of identifying compound having or suspected of having an effect on autophagy, said method comprising act of a) performing steps a) to c) of the method as above to obtain the induced host cell; and b)introducing the compound in a high-throughtput assay plate, followed by contacting the induced host cell

with said compound and performing remaining steps d) to f) of the method as above to identify said effect on autophagy; and a kit to monitor autophagy, said kit comprising vector or expression construct as above or host cell as above, optionally along with components selected from a group comprising induction medium, starvation medium, lysis buffer, substrate and instruction manual or any combination thereof.

BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES

The features of the present disclosure will become more fully apparent from the following description taken in conjunction with the accompanying drawings. Understanding that the drawing depict only several embodiments in accordance with the disclosure and is therefore, not to be considered limiting of its scope, the disclosure will be described with additional specificity and detail through use of the accompanying drawing:

Figure 1 shows plasmid constructs for expression of firefly and *Renilla* luciferase in *S. cerevisiae* (MTCC 5858- wild type cells of *S.cerevisiae* having only *Renilla* luciferase MTCC 5859- wild type cells of *S.cerevisiae* having *Renilla* as well as firefly luciferase, MTCC 5860- *atg 1* mutant of *S.cerevisiae* having only firefly luciferase, MTCC 5861- *atg 1* mutant type having *Renilla* as well as firefly luciferase).

Figure 1 (a) shows the pPM5 vector comprising the following:

Vector Backbone – pRS305.Antibiotic Resistance – Ampicillin. Yeast Selection Marker – Leucine. Vector Description - Renilla luciferase gene downstream of Peroxisomal thiolase promoter of *Saccharomyces cerevisiae*(Sc POT1) to drive the cytoplasmic expression of *Renilla* luciferase gene (Rluc) during peroxisome biogenesis.

Figure 1 (b) shows the pPM10 vector comprising the following:

Vector Backbone – pRS306.Antibiotic Resistance – Ampicillin. Yeast Selection Marker – Uracil. Vector Description - Fluc gene with peroxisomal targetting sequence (SKL) cloned downstream of Sc POT1 promoter (Sc P_{POT1}) for peroxisomal expression of firefly luciferase gene. In this construct Sc P_{POT1}- Firefly is in the reverse orientation.

Figure 2 shows plasmid constructs for the expression of firefly and *Renilla* luciferase in *P.pastoris.*

Figure 2 (a) shows the pPM7 vector comprising the following:

Vector Backbone – pJCF-214.Antibiotic Resistance – Ampicillin.

Yeast Selection Marker – Arginine. Vector Description - *Renilla* luciferase gene downstream of Peroxisomal thiolase promoter of *Pichia pastoris* (Pp POT1) to drive the cytoplasmic expression of *Renilla* luciferase gene (Rluc) during peroxisome biogenesis.

Figure 2 (b) shows the pPM9 vector comprising the following:
Vector Backbone – pIB1.Antibiotic Resistance – Ampicillin. Yeast Selection Marker – Histidine. Vector Description - Fluc gene with peroxisomal targetting sequence (SKL) cloned downstream of Pp POT1 promoter for peroxisomal expression of firefly luciferase gene.

Figure 3 depicts a graph showing the activity of firefly and *Renilla* luciferase in wild type and mutant *S. cerevisiae* cells on induction of autophagy. Figure 3a shows the dual luciferase assay in the wild type *S. cerevisiae* cells. Figure 3b shows the dual luciferase assay in autophagy mutant. As can be seen upon induction of autophagy the activity of luciferase goes down with time in case of wild type cells, whereas there is no decrease in the autophagy mutants.

Figure 4 depicts a graph showing firefly luciferase assay in *P. pastoris*. The graph shows the decrease in firefly activity with time upon induction of autophagy. Luciferase assay is done in a 96 well plate with 3 independent assays done in triplicates and SD plotted as error bars.

Figure 5 shows the selective degradation of a cargo marker indicative of selective autophagy (pexophagy) which is followed over time in the yeast *Pichia pastoris*. The rates of degradation of the untreated cells of *Pichia Pastoris* are compared to the ones treated with 50 μM concentration of cyclic peptoids. The time taken for 50% decrease in cargo activity is taken as the criteria for comparing the control with the compounds (Figure 5a). The compounds that differ from the control by 3 SD (Standard Deviation) units are considered significant. The compound **4a** shows significant difference from the control. It increases the rate of degradation of the protein marker through autophagy. A dose response assay for **4a** shows a proportional increase in the rate of degradation with increasing concentration (Figure 5b). After the primary screening of the cyclic peptoids and the dose response is carried out in *Pichia pastoris*, the hit (4a) is validated by microscopic analysis in *Saccharomyces cerevisiae*. Microscopic validation studies in *S. cerevisiae,* for the degradation of peroxisomes through autophagy show a faster rate of peroxisome (GFP labelled) degradation in presence of 4a (50μM) as observed through appearance of diffused GFP in the vacuole

(arrowheads). In the presence of 4a (Figure 5d), GFP inside the vacuoles appear at earlier time points as compared to the untreated cells (Figure 5c).

Figure 6 shows the activity of firefly luciferase after addition of passive Lysis Buffer (Promega). Passive Lysis Buffer is added to cells in different eppendorfs at time t=0. Firefly luciferase substrate (D-luciferin from Promega) is added to every tube after 30 seconds interval and read in tube luminometer (Molecular Devices). The activity is taken for 5 minutes and is calculated in terms of % Relative Light Units (RLU). The graph shows that after the addition of lysis buffer the activity can be read within 90 seconds after which it starts decreasing.

Figure 7 shows a comparison between luciferase based assay and conventional Pot1-GFP processing assay. Figure 7a shows the decrease in the activity of wild type cells with time and the constant activity exhibited by the mutant without any decrease, with time. Figure 7b shows a Western for luciferase protein levels. Figure 7c shows a Western for Pot-1 GFP processing assay.

DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure relates to a method of performing an assay to monitor autophagy within a host cell, said method comprising acts of:

a) preparing at least one expression construct with a reporter gene;
b) transforming a host cell with the prepared expression construct;
c) culturing the transformed host cell in induction medium followed by starvation medium to induce autophagy within the host cell;
d) lysing the induced host cell of step (c) in a lysis buffer;
e) treating the lysed host cell with a substrate capable of reacting with the reporter to produce a lightreaction; and
f) analysing the reaction to monitor the autophagy.

In an embodiment of the present disclosure, method of identifying compound having or suspected of having an effect on autophagy comprises acts of:

a. performing steps a) to c) to obtain the induced host cell;

b. introducing the compound in a high-throughtput assay plate, followed by contacting the induced host cell with said compound and performing remaining steps d) to f) to identify said effect on autophagy.

In another embodiment of the present disclosure, the expression construct is prepared using vector backbone selected from a group comprising pRS305, pRS306, pIB1 and pJCF-214 or any combination thereof.

In yet another embodiment of the present disclosure, the reporter gene is selected from a group comprising firefly luciferase, *Renilla* luciferase and green fluorescent protein or any combination thereof.

In still another embodiment of the present disclosure, the expression construct comprises a ScPOT-1 promoter or PpPOT-1 promoter, optionally along with SKL signal sequence.

In still another embodiment of the present disclosure, the host cell is selected from a group comprising *Saccharomyces cerevisiae* and *Pichia pastoris*; and wherein the transforming of the host cell is carried out by lithium acetate method in *Saccharomyces cerevisiae* and by electroporation in *Pichia pastoris*.

In still another embodiment of the present disclosure, the induction medium is oleate medium comprising about 0.1% oleate, about 0.5% Tween-40, about 0.25% yeast extract, about 0.5% peptone and about 5mM phosphate buffer or wherein the induction medium is methanol medium comprising 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone and 0.5% methanol.

In still another embodiment of the present disclosure, the starvation medium is nitrogen starvation medium SD-N comprising, about 2% dextrose and about 0.17% yeast nitrogen base without amino acids and nitrogen source.

In still another embodiment of the present disclosure, the induction medium induces formation of peroxisomes in the host cell; and wherein the starvation medium induces autophagy, pexophagy, macro-autophagy or any combination thereof.

In still another embodiment of the present disclosure, the lysis buffer is passive lysis buffer having a pH ranging from about 6 to about 8, preferably 7.

In still another embodiment of the present disclosure, the substrate is luciferin which is added at a concentration ranging from about 8µl to about 12 µl, preferably 10 µl.
In still another embodiment of the present disclosure, the autophagy is monitored by measuring decay of the light reaction with respect to time.

In still another embodiment of the present disclosure, said method is employed for identifying modulators of autophagy, in detecting mutants which are partially blocked or completely blocked in autophagy and in detecting degraders of peroxisomes.

The present disclosure also relates to a vector or expression construct comprising a POT-1 promoter sequence and reporter gene optionally along with an SKL signal.

In an embodiment of the present disclosure, the vector or expression construct is selected from a group comprising sequence set forth as SEQ ID Nos. 1, 2, 3 and 4.

In another embodiment of the present disclosure, the reporter gene is selected from a group comprising firefly luciferase, *Renilla* luciferase and green fluorescent protein or any combination thereof.

In yet another embodiment of the present disclosure, the POT-1 promoter is *Sc*POT-1 promoter or *Pp*POT-1 promoter.

The present disclosure also relates to a host cell transformed with the vector or the expression construct as above.

In an embodiment of the present disclosure, the host cell is selected from a group comprising *Saccharomyces cerevisiae* and *Pichia pastoris.*

The present disclosure also relates to a method of identifying compound having or suspected of having an effect on autophagy, said method comprising act of:

a. performing steps a) to c) of the method as above to obtain the induced host cell; and
b. introducing the compound in a high-throughtput assay plate, followed by contacting the induced host cell with said compound and performing remaining steps d) to f) of the method as above to identify said effect on autophagy.

In an embodiment of the present disclosure, the compound is selected from a group comprising modulators of autophagy, mutants which are partially blocked or completely blocked in autophagy or degraders of peroxisomes.

The present disclosure also relates to a kit to monitor autophagy, said kit comprising vector or expression construct or host cell as above, optionally along with components selected from a group comprising induction medium, starvation medium, lysis buffer, substrate and instruction manual or any combination thereof.

In an embodiment of the present disclosure, the induction medium is oleate medium comprising about 0.1% oleate, about 0.5% Tween-40, about 0.25% yeast extract, about 0.5% peptone and about 5mM phosphate buffer or wherein the induction medium is methanol medium comprising 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 0.5% methanol.

In another embodiment of the present disclosure, the starvation medium is nitrogen starvation medium SD-N comprising, about 2% dextrose and about 0.17% yeast nitrogen base without amino acids and nitrogen source.

In yet another embodiment of the present disclosure, the lysis buffer is passive lysis buffer having a pH ranging from about 6 to about 8, preferably 7.

In still another embodiment of the present disclosure, the substrate is luciferin which is added at a concentration ranging from about 8µl to about 12 µl, preferably 10 µl.

In still another embodiment of the present disclosure, the autophagy comprises macro-autophagy, pexophagy, xenophagy and other related autophagic pathways or any combination thereof.

The present disclosure relates to a dual luciferase based assay in yeast to monitor macro-autophagy, pexophagy by a) creating plasmid constructs expressing firefly and Renilla luciferase b) generating yeast strains expressing these luciferase constructs, and c) developing autophagy assay using these strains.

In an embodiment of the present disclosure, the said assay is carried out using live yeast cells with two built-in reporters for two different autophagy pathways (general and selective). Since the assay consists of two reporters which can differentiate and effectively measure two different forms of autophagy, the assay has been referred as 'dual'.

In an embodiment of the present disclosure, pulse chase-like assay is employed, where the cargo, peroxisomal targeted firefly luciferase is built-up in peroxisomes and then its degradation is followed upon autophagy induction. The peroxisomal thiolase (POT1) promoter driven expression of firefly luciferase with C-terminus SKL sequence to target it to peroxisomes and *Renilla* luciferase, a cytosolic protein control/ macroautophagy indicator which serves as readouts for pexophagy and macroautophagy, respectively. The *Renilla* luciferase gene is without any targeting signal. Therefore, it goes to the cytoplasm (Proteins lacking any signaling sequence are by default targeted to the cytosol).

In another embodiment of the present disclosure, *Saccharomyces cerevisiae* and *Pichia pastoris* are employed to study macro-autophagy, pexophagy, xenophagy and other autophagic related pathways, in particular pexophagy and macro-autophagy.

In another embodiment of the present disclosure, peroxisomes in *Saccharomyces cerevisiae* are induced by growing the cells in oleate medium.

In another embodiment of the present disclosure, peroxisomes in *Pichia pastoris* are induced by growing the cells in methanol medium.

In another embodiment of the present disclosure, kinetic assay of pexophagy is used to detect mutants that are completely blocked or partially blocked in pexophagy. The degradation rates of all mutants tested are compared to the wild type. A mutant blocked completely in pexophagy would behave like the *Δatg1* mutant, showing no decrease in the luciferase activity with time. Other mutants such as the *Δatg5* and *Δatg8* mutants would also behave in

the same way as *Δatg1* mutant as all these are core autophagy genes. A partial blocker can be identified by comparing the rates of degradation of luciferase reporter of the mutant with the wild type cells.

In another embodiment of the present disclosure, the mutants, fast degraders that degrade peroxisomes much rapidly than the wild type are detected similarly, slow degraders are also detected.

In another embodiment of the present disclosure, the *Saccharomyces cerevisiae* shuttle vectors pRS306 (URA) and pRS305 (LEU) are used to clone the POT1 promoter and the firefly and *Renilla* luciferase genes, respectively.

In another embodiment of the present disclosure, the *Pichia pastoris* shuttle vectors pIB1 (HIS) and pJCF-214 (ARG) are used to clone the POT1 promoter and the firefly and *Renilla* luciferase genes, respectively.

In another embodiment of the present disclosure, the oleate responsive region of the POT1 promoter is amplified from yeast genomic DNA along with the firefly luciferase gene and *Renilla* luciferase gene.

In another embodiment of the present disclosure, peroxisomal thiolase fused to GFP (Pot1-GFP) is used to monitor the pexophagy in yeast.

In another embodiment of the present disclosure, Pot1-GFP assay is based on the immunoblot analysis.

In another embodiment of the present disclosure, autophagy assay provides the convenience of performing small molecule high through-put screening.

Summarising all the aspects disclosed above, in a nutshell, the present disclosure relates to an assay to monitor autophagy. Initially, a vector or an expression construct is prepared, having a reporter gene and a promoter, optionally along with a signal sequence, wherein the promoter is a peroxisome specific promoter. The expression of such reporter gene is driven by said promoter, which is activated by induction of peroxisomes.

This vector or expression construct is transformed into a host cell. In an embodiment, plurality of vectors or expression constructs are inserted into a single host cell, to monitor autophagy, by single or multiple reporter systems.

Upon transformation, the host cell comprising single or multiple vectors or expression constructs is cultured in induction medium to induce formation of peroxisomes. This host is thereafter transferred and harvested on nitrogen starvation medium to induce autophagy, including pexophagy and macro-autophagy within the host cell. The host cell is thereafter subjected to lysis by employing a lysis buffer.

Upon obtaining the cell lysate, which comprises the reporter, a suitable substrate capable of reacting with the reporter to produce a light reaction is added. This light reaction is measured and analysed to monitor the autophagy, specifically by measuring decay of the light reaction with respect to time.

The said assay is further employed to identify compounds having or suspected of having an effect on autophagy, by initially introducing the compounds in high throughput assay plate, followed by introducing the induced host cell into the assay plate. Thereafter similar steps of lysis and addition of substrate to observe the light reaction are performed. The change in decay of the emitted light, when compared to control conditions [without the test compound], will provide information on the effect of the test compounds on autophagy.

The invention is further illustrated by the following Examples. The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention. The Examples are also to be considered as part of the detailed description of the instant disclosure and not in isolation.

EXAMPLES

Example 1: Plasmid construction

Firefly luciferase construct in *Saccharomyces cerevisiae*

The Pot1promoter is PCR amplified from *S. cerevisiae* genomic DNA. The amplicon is subjected to agarose gel electrophoresis against a suitable marker. The amplified 251bp on the gel is eluted using Qiagen spin columns.

The lacZ cassette from the vectors pRS306 (URA) is removed by digesting with HindIII. Further, this digested vector is ligated with amplified POT1 promoter at the site of lacZ. Further, the firefly luciferase gene with the peroxisomal targeting signal (SKL) is amplified by polymerase chain reaction from a vector such as plasmid pRSVL. The amplified firefly luciferase gene is ligated downstream of the POT1 promoter of pRS306 to obtain firefly luciferase construct (Vector I).

Renilla luciferase construct in *Saccharomyces cerevisiae*

The Pot1promoter is PCR amplified from *Saccharomyces cerevisiae* genomic DNA. The amplicon is subjected to agarose gel electrophoresis against a suitable marker. The amplified 251bp on the gel is eluted using Qiagen spin columns.

The lacZ cassette from the vectors pRS305 (LEU) is removed by digesting with PvuII. Further, this digested vector is ligated with amplified POT1 promoter at the site of lacZ. Further, the Renilla luciferase gene is amplified by polymerase chain reaction from a vector such aspRL-CMV. The amplified renilla luciferase gene is ligated downstream of the POT1 promoter of pRS305 to obtain renilla luciferase construct (Vector II).

Firefly luciferase construct in *Pichia pastoris*

The Pot1 promoter is PCR amplified from *P.pastoris* genomic DNA. The amplicon is subjected to agarose gel electrophoresis against a suitable marker. The amplified 500bp on the gel is eluted using Qiagen spin columns.

The digested vector is ligated with amplified POT1 promoter. Further, the firefly luciferase gene with the peroxisomal targeting signal (SKL) is amplified by polymerase chain reaction from the vector such as plasmid pRSVL. The amplified firefly luciferase gene is ligated downstream of the POT1 promoter of pIB1 to obtain firefly luciferase construct (Vector III).

Renilla luciferase construct in *Pichia pastoris*

The Pot1promoter is PCR amplified from *P.pastoris* genomic DNA. The amplicon is subjected to agarose gel electrophoresis against a suitable marker. The amplified 600bp on the gel is eluted using Qiagen spin columns.

The digested vector is ligated with amplified POT1 promoter. Further, the renilla luciferase gene is amplified by polymerase chain reaction from a vector such as plasmid pRL-CMV, the amplified *Renilla* luciferase gene is ligated downstream of the POT1 promoter of pJCF-214 to obtain renilla luciferase construct (Vector IV).

The sequences of the vectors- pPM10 and pPM5 comprising firefly luciferase and *Renilla* luciferase genes respectively, which are expressed in *Saccharomyces cerevisiae* are as follows:

pPM10(6240 bp)

Vector backbone pRS306

Features:

EcoRI site: 2047

Firefly luciferase ORF: 3705-2053 (**in bold**)

XhoI site: 3706

Saccharomyces cerevisiae Pot1 promoter: 3961-3712(underlined)

KpnI site- 3962

SKL sequence (*in italics*)

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggatgccggga
gcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggcttaactatgcggcatcagagcagattgtactg
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pPM5(6669 bp)

Vector backbone- pRS305

Features:

PstI site: 3172

Saccharomyces cerevisiae Pot1 promoter: 3178-3427 (underlined)

HindIII site: 3428

Renilla luciferase ORF: 3434-4369 **(in bold)**

SalI site: 4370

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggatgccgggga
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cctttcgtc

pPM7

Vector backbone-pJCF-214

Features:

Eco RI: 395

Pichia pastoris Pot1 promoter: (underlined)

KpnI: 1003

Bam HI: 1944

Renilla luciferase ORF: **(in bold)**

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGA
CGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCG
CGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGC
AGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAA
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pPM9

Vector backbone-pIB1

Features:

SKL sequence: (*in italics*)

XmaI: 412

Pichia pastoris Pot1 promoter: (underlined)

SpeI/BcuI: 1019

PstI: 2678

Firefly luciferase ORF: **(in bold)**

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Example 2: Strain constructions

Strain construction in *Saccharomyces cerevisiae*

The vectors (Vector I and II) are transformed into *S. cerevisiae* haploid strains including wild-type (WT), and the *atg1* (systematic gene name, YGL180W), *atg5* (YPL149W) and *atg8* (YBL078C) deletion strains (Gietz and Woods 2002). The wild type and the deletion mutants are from the MATa collection, created by the *Saccharomyces* Genome Deletion Project. These strains are blocked in all autophagy related-pathways, including pexophagy. Transformation is carried out sequentially using the standard Lithium acetate transformation or electroporation procedure as below and the correct clones are selected for uracil (pFirefly- plasmid containing firefly luciferase gene) and leucine (pRenilla- plasmid containing renilla luciferase gene) auxotrophy, respectively.

Strain construction in *Pichia pastoris*

The vectors (Vector III and IV) are transformed into *P.pastoris* haploid strains including wild-type (WT, PPY12). Transformation is carried out using the electroporation procedure as mentioned below and the correct clones are selected for histidine (pFirefly) and arginine (pRenilla) auxotrophy, respectively.

Lithium acetate method of transformation in *Saccharomyces cerevisiae*:

Cells (~10^8 cells) in early logarithmic phase of growth are harvested, re-suspended in transformation mix (final concentrations: 33.3% PEG, 0.1M Lithium acetate, 270µg/ml salmon sperm DNA, 1-1.5 µg DNA) and subjected to heat shock at 42^{0}C for 40 minutes. Post heat shock cells of Saccharomyces cerevisae are harvested and plated onto the selection media plates SD-URA for pRS306-promoterPOT1-FLUC and SD-LEU for pRS305-promoterPOT1-RLUC.

Transformation in *Pichia pastoris* by Electroporation

Early logarithmic phase cells (50ml) are harvested and resuspended in 10ml YPD (1% yeast extract, 2% peptone, and 2% glucose) with 200µl 1M HEPES. Sterile 1M DTT (250µl) is added and incubated for 15 min at 30°C with tilting. Final volume is made to 50ml with cold sterile water. Cells are centrifuged at 3000 *g* x 3 min and washed 3 times with cold sterile water. Cells are washed with 5ml of 1M sorbitol (ice-cold) and final resuspension is done in 200µl 1M sorbitol. Forty five microliter of cells and 5µl of digested and purified plasmid DNA are added to 2mm electrocuvette and electroporation is done (2kV, 200 ohms, 25µF).

One milliliter of 1M ice cold sorbitol is used to resuspend the cells immediately and incubated at 30°C for an hour. Cells are pelleted down and plated on SD-HIS plates.

Example 3: Dual luciferase assay

WT, *atg1Δ*, *atg5Δ*, *atg8Δ* cells are grown to mid exponential phase in yeast nitrogen base (YNB without amino acids without ammonium sulphate- HIMEDIA, Catalogue number M151-100G)) with Ura and Leu dropout medium and transferred to oleate medium (0.1% oleate, 0.5% Tween-40, 0.25% yeast extract, 0.5% peptone and 5mM phosphate buffer)for the induction of peroxisomes.

Table 1: Ura Dropout media composition

Ingredients	**Percentage**
YNB	0.17%
Dextrose	2%
Ammonium sulphate	0.25%
Histidine	0.02%
Leucine	0.06%
Lysine	0.03%
Methionine	0.02%

Table 2: Leu dropout media composition

Ingredients	**Percentage**
YNB	0.17%
Dextrose	2%
Ammonium sulphate	0.25%
Histidine	0.02%
Uracil	0.01%
Na_2CO_3	1%
Lysine	0.03%
Methionine	0.02%

Table 3: Arg dropout media (SD-ARG Media) composition

Ingredients	**Percentage**

YNB	0.17%
Dextrose	2%
Ammonium sulphate	0.25%
Arginine	0.02%
Leucine	0.06%
Lysine	0.03%
Methionine	0.02%
Uracil	0.01%
Na_2Co_3	1%

Table 4: His dropout media (SD-HIS Media) composition

Ingredients	**Percentage**
YNB	0.17%
Dextrose	2%
Ammonium sulphate	0.25%
Histidine	0.02%
Leucine	0.06%
Lysine	0.03%
Methionine	0.02%
Uracil	0.01%
Na_2Co_3	1%

After 14-16 hour incubation, cells are washed with sterile water and re-suspended in nitrogen starvation medium (SD-N, 2% dextrose, yeast nitrogen base without amino acids and nitrogen source) to trigger pexophagy. Equivalent ODs of cells are collected at several time intervals from 0 to 24 hours post-induction of pexophagy. Each sample collected is used for two purposes: part of it is used for the dual luciferase assay and rest of it is used to prepare protein lysates for immunoblot analysis.

The cell suspension is subjected to lysis using passive lysis buffer (PLB) at a pH of 7 and dual luciferase assay is performed on the cell lysate with the dual luciferase reporter assay system (Promega Corp., Madison, WI) according to the manufacturer's instructions at different time interval to analyse the firefly luciferase stability in PLB. Further, 10 µl of luciferin i.e. the firefly luciferase substrate (from Promega Dual luciferase reporter assay system- Catalogue number E1980) is added immediately to the cell lysate and the luciferase

count is taken after every 30 second to analyse the decay of firefly luciferase with respect to time (Figure 6).

The data is used to calculate the ratio of peroxisomal firefly luciferase to cytosolic *Renilla* luciferase activity. As mentioned above, the rest of the lysate is also analysed by immunoblotting with anti-Pot1 antibodies.

Data analyses: Firefly luciferase activity decreases drastically within a few minutes after addition of substrate. The rate of decline in relative firefly luciferase activity is compared to that of Pot1 levels in course of starvation induction. Based on preliminary data and understanding of pexophagy, it is understood that the firefly luciferase activity will reflect pexophagy as indicated by Pot1 levels. When compared to firefly luciferase activity it was observed that the decrease in *Renilla* luciferase activity was much slower than the firefly luciferase activity.

Example 4: Pexophagy Assay

The processing of peroxisomal thiolase (POT1)-GFP, a peroxisomal matrix marker is used to assay pexophagy. During this process, the vacuolar processing of POT1-GFP occurs to yield stable GFP in the vacuole. This assay used two standards to measure pexophagy: 1) Level of thiolase degradation and 2) Level of GFP accumulation.

Wild type POT1-GFP strain is a laboratory strain with genomically tagged GFP to the C terminus of Pot1 (HIS selection marker) obtained from Dr. Rachubinsky. Wild type BY4741 and all knockout strains are obtained from European Saccharomyces Cerevisiae Archive for Functional Analysis (EUROSCARF).

Using genomic DNA of Pot1-GFP positive wild type strain (Rachubinsky lab) as template, the POT1-GFP-HIS cassette is amplified by using the following sets of primers:

Table 5: Primer sequences

Primer	Name	Sequence
Forward	oPGFP1	CATCCCTTGGGTTGTACTGG
Reverse	oPGFP2	CTATCGAATCTCCCCCATGA

Wild type and *atg1*Δ cells were transformed with POT1-GFP-HIS cassette by lithium acetate method. Transformants are screened by western blotting.

Pot1-GFP positive strains are allowed to grow till the A_{600} reaches 0.8-1 in YPD. Peroxisome biogenesis is induced by growing these cells in oleate medium for 12 hours. Cells are harvested, washed twice to remove traces of oleate and transferred to starvation medium without nitrogen, at inoculum density A_{600} 3, to induce pexophagy. Cells are collected at various time intervals after pexophagy induction and processed by TCA method as below.

TCA precipitation:

All samples are collected in 12.5% trichloroacetic acid final concentration and stored at -80°C for at least half an hour. Later, the samples are thawed on ice and centrifuged for 10 minutes at 16000 *g*, pellet is washed with 250 µl of ice cold 80% acetone twice and air dried. This pellet is re-suspended in 40 µl of 1% SDS- 0.1N NaOH solution. Sample buffer (5X, 10 µl) is added and boiled for 10 minutes before loading.

Immunoblotting

Total cell lysates are electrophoresed on 12%, SDS-PAGE for POT1-GFP processing pexophagy and transferred onto PVDF membrane at constant current of 2 Ampere for 30 minutes (Transblot turbo, BIORAD Inc, USA). Transfer is confirmed by Ponceau S staining of blot and the blots scanned and are used as loading controls. Blots are incubated overnight with 5% skim milk in primary anti-GFP mouse IgG antibody (Roche Diagnostics) at 1: 3000 dilution). Secondary antibody used at 1:10,000 is goat anti-mouse conjugated to HRP (Biorad). Blots are developed by using ECL substrate and images captured using auto capture program in Syngene G-Box, UK. Image J (NIH) is used for quantitation of band intensities.

Example 5: Correlation of luciferase assay with Pot-1 GFP processing assay

To validate the luciferase assay, it is compared to the conventional Pot1-GFP processing assay. In the luciferase assay (Figure 7a), the activity for the wild type cells goes down with time whereas the mutant shows no decrease in the activity which resembles the western for Pot1-GFP processing assay (Figure 7c), where the wild type cells show a decrease in Pot1-GFP levels and hence appearance of free GFP, whereas mutants show a complete block. Western for the luciferase protein levels also shows the same results (Figure 7b).This signifies that the gradual decrease in the luciferase enzyme activity of wild type cells correlates with a decrease in the POT1-GFP levels and the appearance of free GFP. Further,

as the degradation rates of mutants are compared to the wild type, it is evident that a mutant blocked completely in pexophagy would show no decrease in the luciferase activity with time. Similarly, a partial blocker can be identified by comparing the rates of degradation of luciferase reporter of the mutant with the wild type cells.

Example 6: Identification of small molecule modulators of autophagy

The dual luciferase assay is appropriate to identify small molecule modulators of the autophagy process. It is a kinetic assay which detects the decrease in the luciferase activity over time through autophagy. Therefore, through this assay the modulators of the autophagy pathway are detected in a high throughput format. For example, a set of 13 cyclic peptoids represented as compounds 4a to 4m are investigated for their ability to affect the autophagy process in *Pichia Pastoris*. Using the luciferase based assay, one cyclic peptoid represented as compound (4a) is detected that enhances the rate of autophagy which is further validated by microscopy. The said compound also shows a dose dependent increase in the rate of degradation of the luciferase reporter over time (Figure 5).

Methodology:

The luciferase based autophagy assay is done in the yeast *P. pastoris*, where degradation of cargo i.e. firefly luciferase is followed over time (upto 2 hours) upon induction of autophagy. Compounds are pre-added to the wells of 96 well plate. After transferring the transformed cells to starvation condition (SD-N media), the cells are then added to the plate already containing the compounds. Time taken for 50% decrease in cargo activity is plotted for untreated cells and the compounds at 50μM concentration for cyclic peptoids. Triplicate values for the control are plotted and a difference of 3 standard deviation units between the test and control is considered as significant.

After the primary screening of the cyclic peptoids in *Pichia pastoris*, microscopic validation studies are carried out in *Saccharomyces cerevisiae*.

Microscopic images are obtained using Zeiss confocal microscope. *Saccharomyces cerevisiae* cells are checked for selective degradation of peroxisomes through autophagy with or without the compound 4a.From this, it is validated that a cyclic peptoid represented as compound 4a is an enhancer of autophagy.

ADVANTAGES OF THE DUAL LUCIFERASE ASSAY:

1. As indicated in Figure 7, when the levels of firefly activity are compared with conventional assays like Pot1-GFP processing assay, the luciferase assay is found to be more sensitive. In the wild type situation, the firefly luciferase activity decreases to less than 50% within 2 hours (Figure 7a). Whereas the 50% decrease could be detected through Pot1-GFP levels only after 6 hours (Figure 7c). This indicates that smaller changes in the cargo flux can be detected better using the luciferase reporter than the conventional assays.
2. Since, the luciferase assay can be done in a plate format and for shorter time durations, it is highly amenable for high throughput studies for the detection of small molecule modulators of autophagy.
3. The dual luciferase assay can be employed to identify slow and fast degraders of peroxisomes; mutants which are partially blocked and completely blocked in pexophagy by comparing the rates of degradation of luciferase reporter of the mutant with the wild type cells.

SEQUENCE LISTING

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We Claim:

1. A method of performing an assay to monitor autophagy within a host cell, said method comprising acts of:
   a) preparing at least one expression construct with a reporter gene;
   b) transforming a host cell with the prepared expression construct;
   c) culturing the transformed host cell in induction medium followed by starvation medium to induce autophagy within the host cell;
   d) lysing the induced host cell of step (c) in a lysis buffer;
   e) treating the lysed host cell with a substrate capable of reacting with the reporter to produce a lightreaction; and
   f) analysing the reaction to monitor the autophagy.
2. The method as claimed in claim 1, wherein the expression construct is prepared using vector backbone selected from a group comprising pRS305,pRS306, pIB1 and pJCF-214 or any combination thereof.
3. The method as claimed in claim 1, wherein the reporter gene is selected from a group comprising firefly luciferase, renilla luciferase and green fluorescent protein or any combination thereof.
4. The method as claimed in claim 1, wherein the expression construct comprises a ScPOT-1 promoter or PpPOT-1 promoter, optionally along with SKL signal sequence.
5. The method as claimed in claim 1, wherein the host cell is selected from a group comprising *Saccharomyces cerevisiae* and *Pichia pastoris*; and wherein the transforming of the host cell is carried out by lithium acetate method in *Saccharomyces cerevisiae* and by electroporation in *Pichia pastoris.*
6. The method as claimed in claim 1, wherein the induction medium is oleate medium comprising about 0.1% oleate, about 0.5% Tween-40, about 0.25% yeast extract, about 0.5% peptone and about 5mM phosphate buffer or wherein the induction medium is methanol medium comprising 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone and 0.5% methanol.
7. The method as claimed in claim 1, wherein the starvation medium is nitrogen starvation medium SD-N comprising, about 2% dextrose and about 0.17% yeast nitrogen base without amino acids and nitrogen source.

8. The method as claimed in claim 1, wherein the induction medium induces formation of peroxisomes in the host cell; and wherein the starvation medium induces autophagy, pexophagy, macro-autophagy or any combination thereof.
9. The method as claimed in claim 1, wherein the lysis buffer is passive lysis buffer having a pH ranging from about 6 to about 8, preferably 7.
10. The method as claimed in claim 1, wherein the substrate is luciferin which is added at a concentration ranging from about 8µl to about 12 µl, preferably 10 µl.
11. The method as claimed in claim 1, wherein the autophagy is monitored by measuring decay of the lightreaction with respect to time
12. The method as claimed in claim 1, wherein said method is employed for identifying modulators of autophagy, in detecting mutants which are partially blocked or completely blocked in autophagy and in detecting degraders of peroxisomes.
13. A vector or expression construct comprising a POT-1 promoter sequence and reporter gene optionally along with an SKL signal sequence.
14. The vector or expression construct as claimed in claim 13, selected from a group comprising sequence set forth as SEQ ID Nos 1, 2, 3 and 4.
15. The vector or expression construct as claimed in claim 13, wherein the reporter gene is selected from a group comprising firefly luciferase, renilla luciferase and green fluorescent protein or any combination thereof.
16. The vector or expression construct as claimed in claim 13, wherein the POT-1 promoter is ScPOT-1 promoter or PpPOT-1 promoter.
17. A host cell transformed with the vector or the expression construct as claimed in claim 13.
18. The host cell as claimed in claim 17, wherein the host cell is selected from a group comprising *Saccharomyces cerevisiae* and *Pichia pastoris*.
19. A method of identifying compound having or suspected of having an effect on autophagy, said method comprising act of:
    a. performing steps a) to c) of claim 1 to obtain the induced host cell; and
    b. introducing the compound in a high-throughtput assay plate, followed by contacting the induced host cell with said compound and performing remaining steps d) to f) of claim 1 to identify said effect on autophagy.
20. The method as claimed in claim 19, wherein the compound is selected from a group comprising modulators of autophagy, mutants which are partially blocked or completely blocked in autophagy or degraders of peroxisomes

21. A kit to monitor autophagy, said kit comprising vector or expression construct as claimed in claim 13 or host cell as claimed in claim 17, optionally along with components selected from a group comprising induction medium, starvation medium, lysis buffer, substrate and instruction manual or any combination thereof.

22. The kit as claimed in claim 21, wherein the induction medium is oleate medium comprising about 0.1% oleate, about 0.5% Tween-40, about 0.25% yeast extract, about 0.5% peptone and about 5mM phosphate buffer or wherein the induction medium is methanol medium comprising 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 0.5% methanol.

23. The kit as claimed in claim 21, wherein the starvation medium is nitrogen starvation medium SD-N comprising, about 2% dextrose and about 0.17% yeast nitrogen base without amino acids and nitrogen source.

24. The kit as claimed in claim 21, wherein the lysis buffer is passive lysis buffer having a pH ranging from about 6 to about 8, preferably 7.

25. The kit as claimed in claim 21, wherein the substrate is luciferin which is added at a concentration ranging from about 8µl to about 12 µl, preferably 10 µl.

26. The methods as claimed in claims 1 and 19 and the kit as claimed in claim 21 wherein the autophagy comprises macro-autophagy, pexophagy, xenophagy and other related autophagic pathways or any combination thereof.

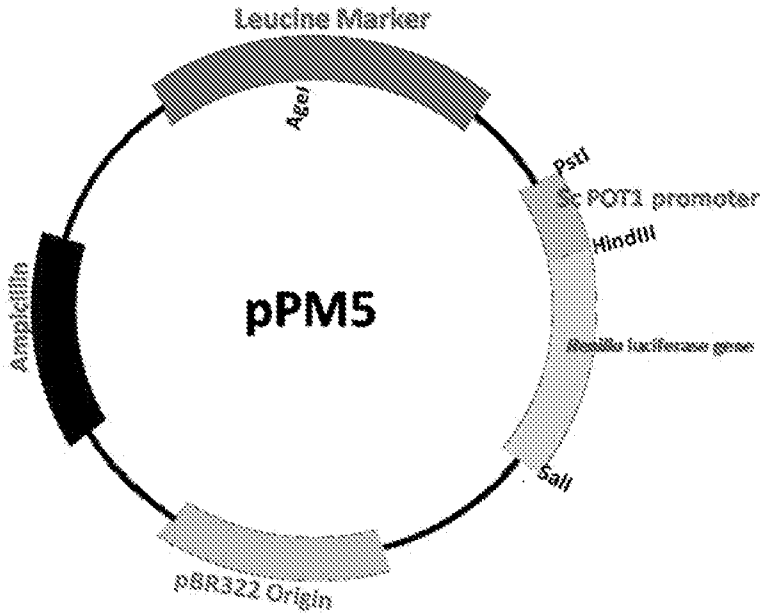


Figure 1 (a)

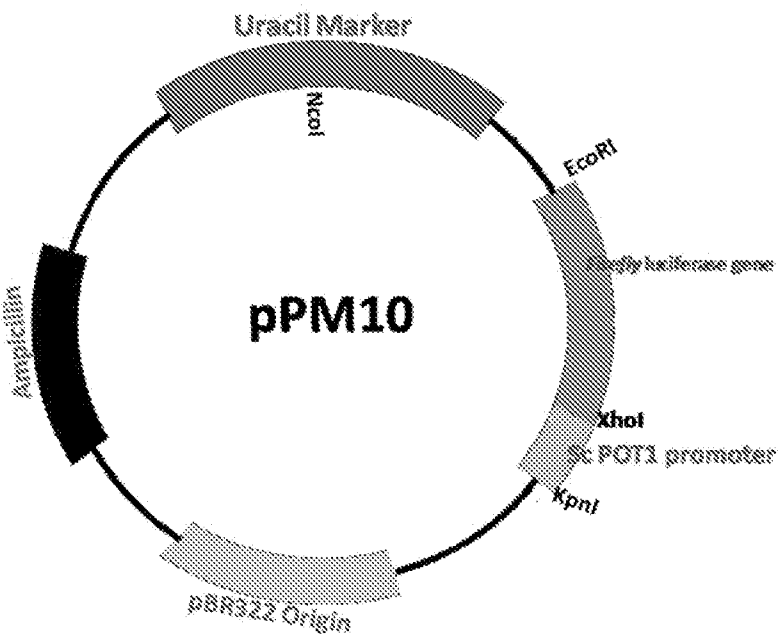


Figure 1 (b)

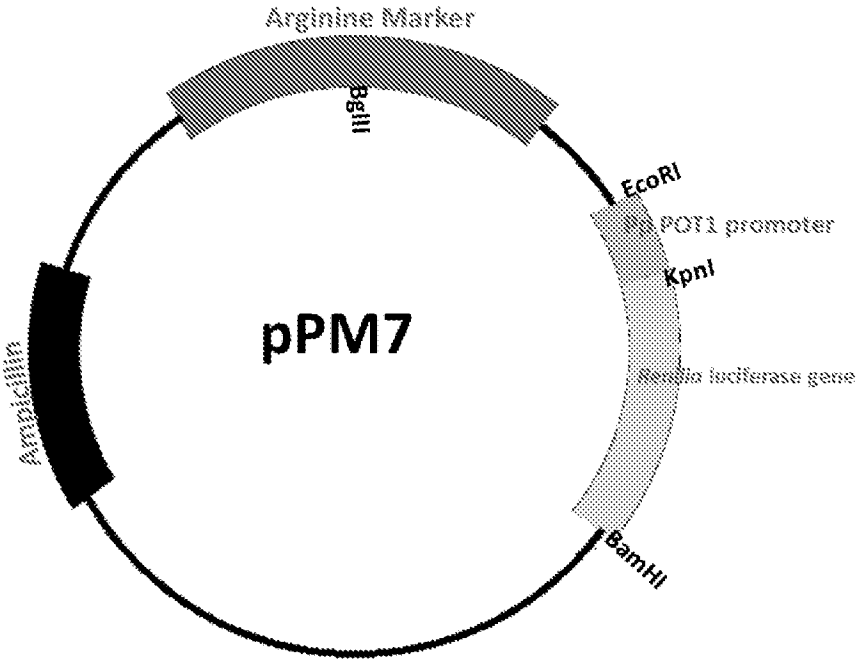


Figure 2(a)

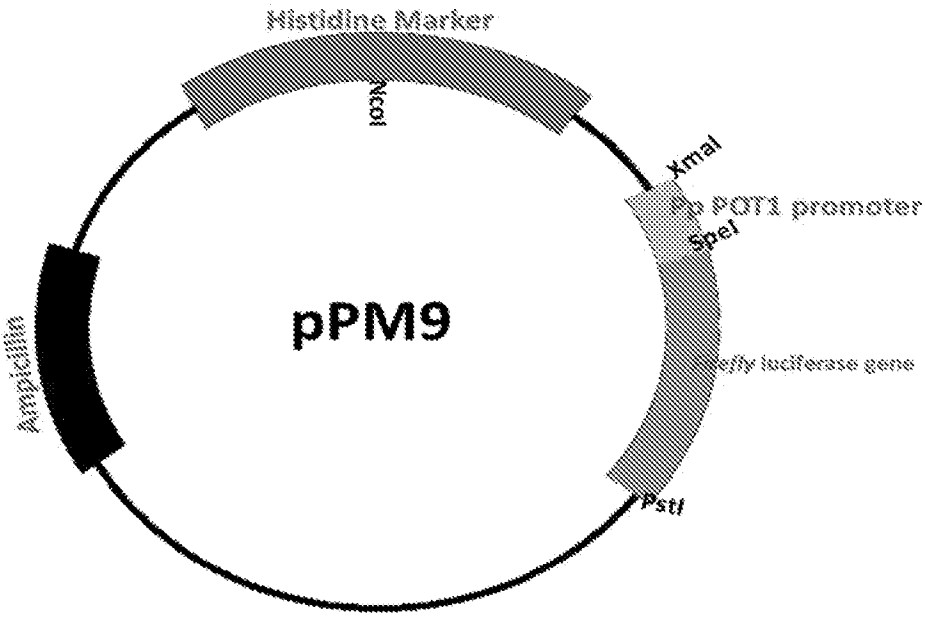


Figure 2 (b)

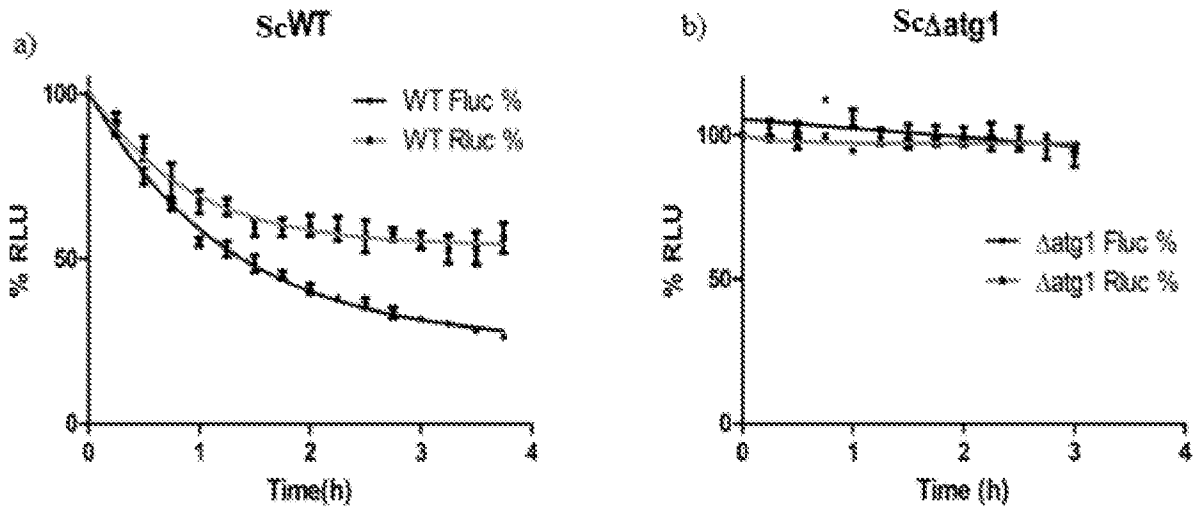


Figure 3

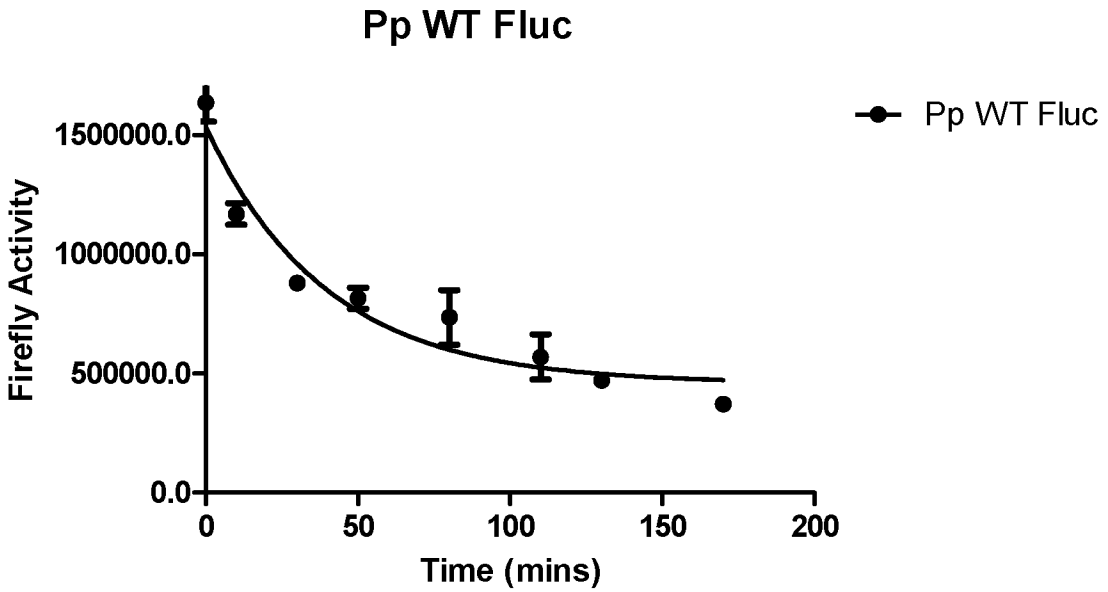
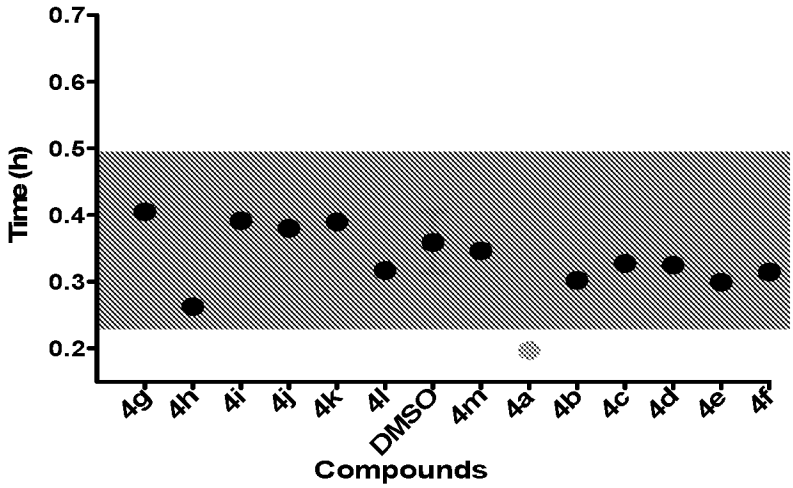


Figure 4

a)


0.7
0.6
0.5
0.4
0.3
0.2
Time (h)
4g
4h
4i
4j
4k
4l
DMSO
4m
4a
4b
4c
4d
4e
4f
Compounds

b)

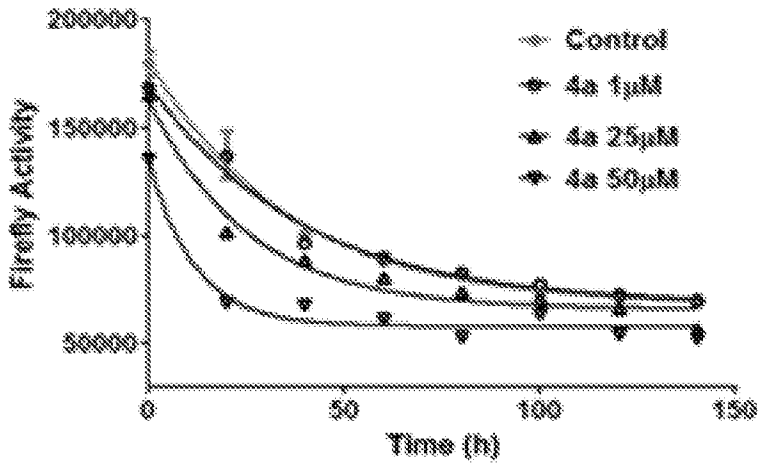

200000
150000
100000
50000
Firefly Activity
0
50
100
150
Time (h)
Control
4a 1μM
4a 25μM
4a 50μM

Figure 5

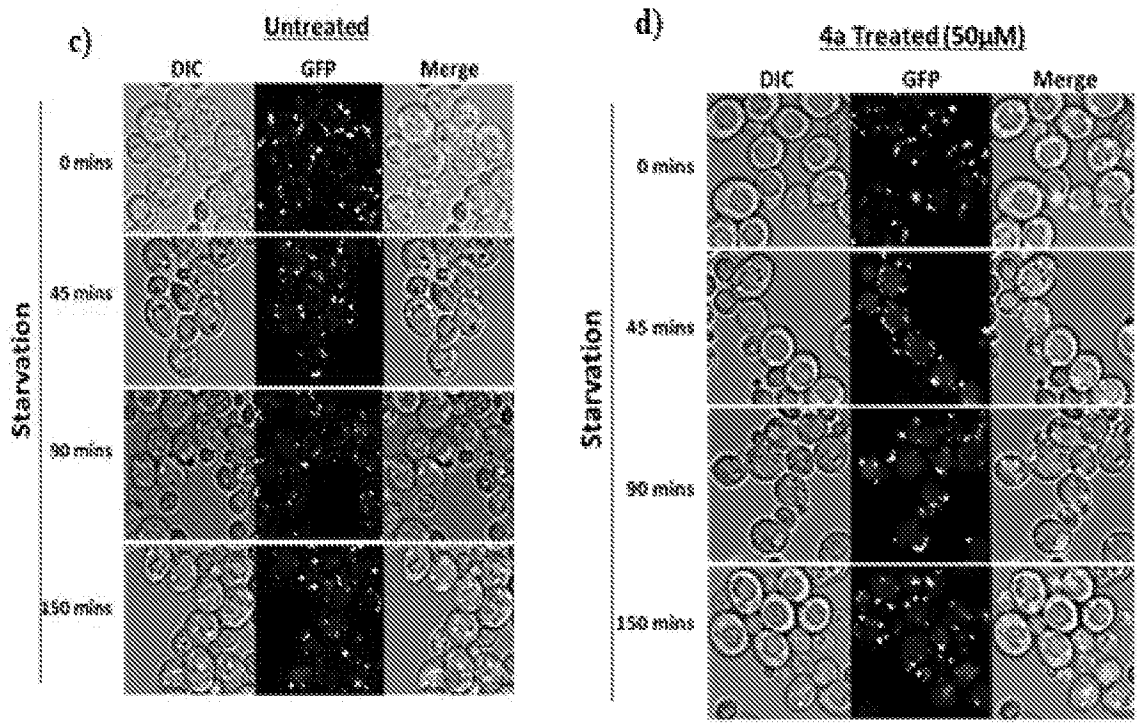


Figure 5(continued)

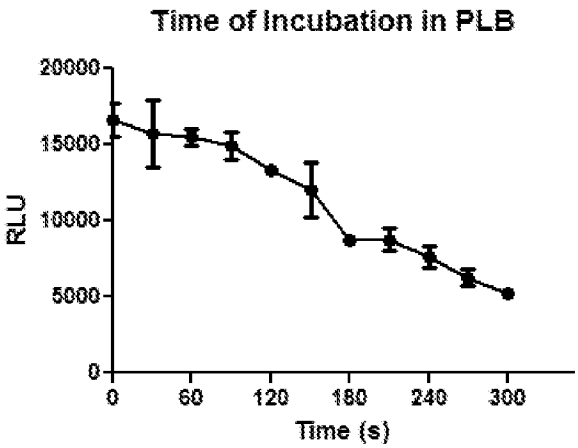


Figure 6

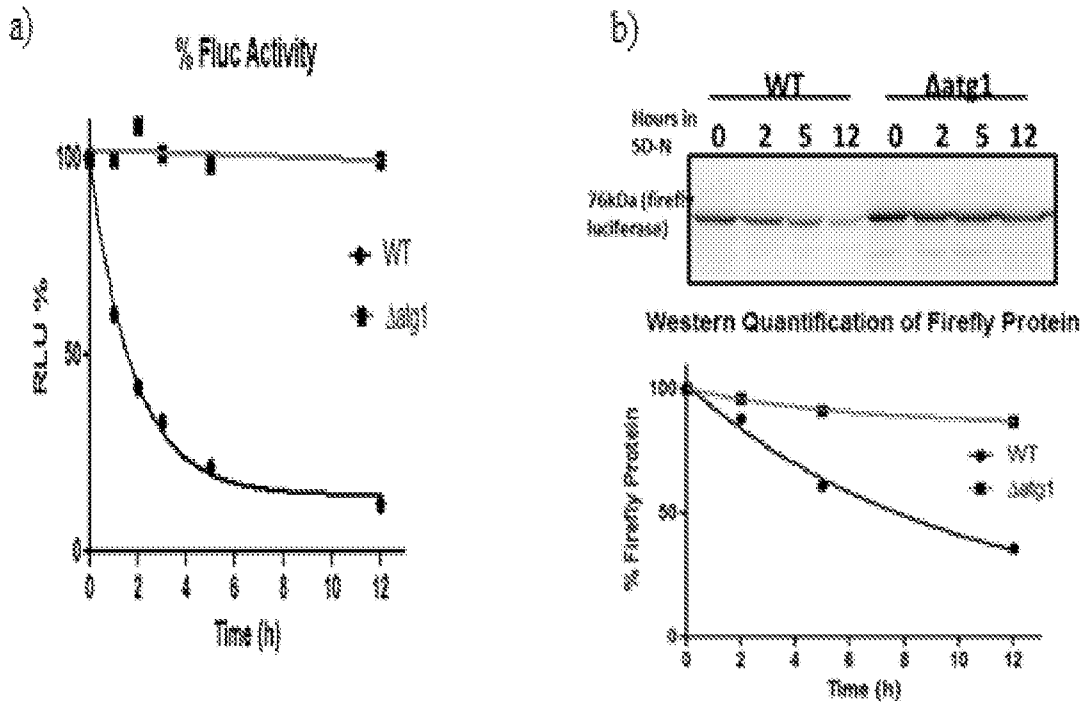

a)
% Fluc Activity
100
50
0
0 2 4 6 8 10 12
Time (h)
WT
Δatg1
b)
WT
Δatg1
Hours in SD-N
0 2 5 12 0 2 5 12
Western Quantification of Firefly Protein
100
50
0
0 2 4 6 8 10 12
Time (h)
WT
Δatg1

Figure 7

c)

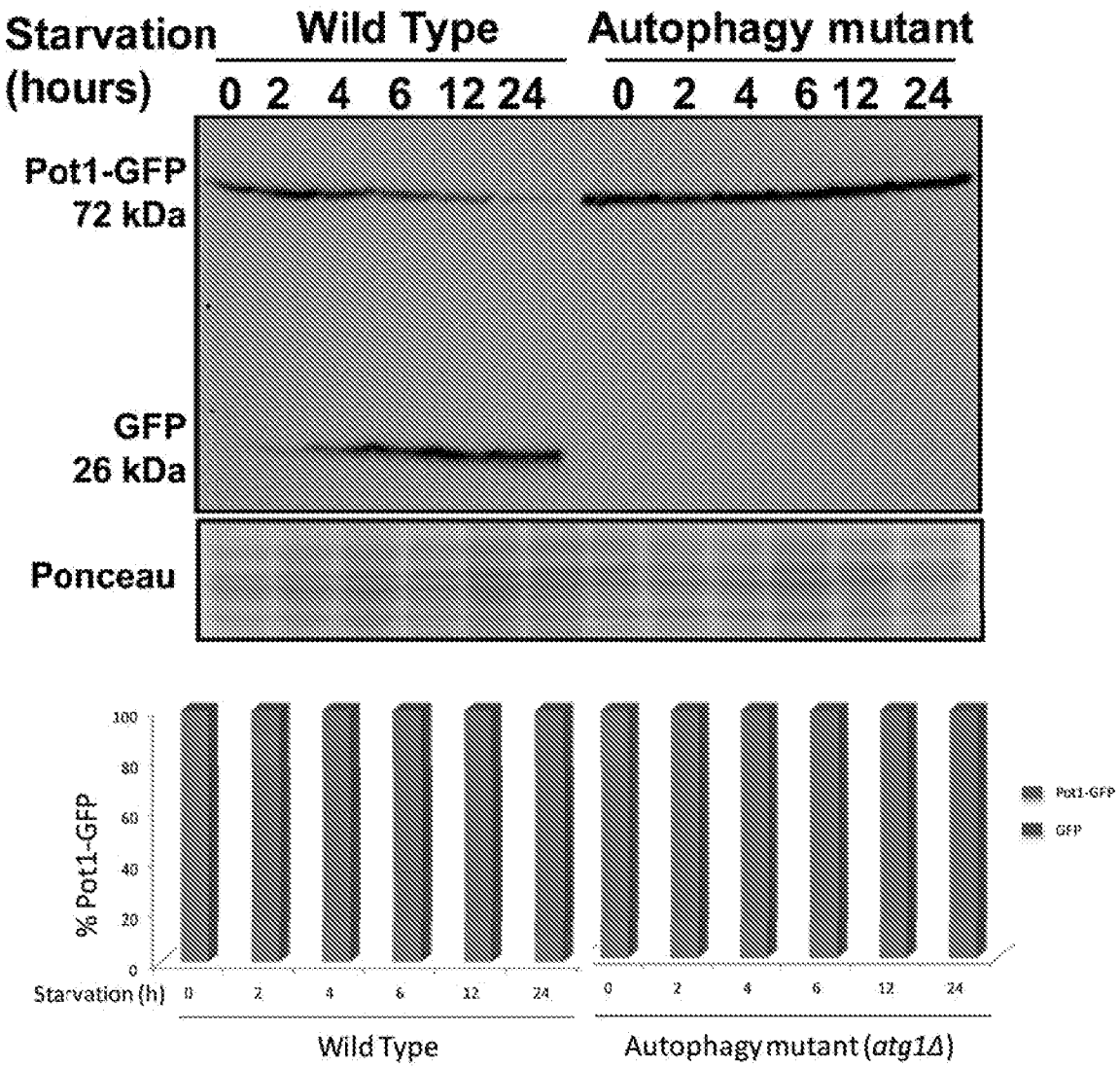


Figure 7 (Continued)