

Toolbox

Identification of novel autophagy regulators by a luciferase-based assay for the kinetics of autophagic flux

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Macroautophagy (hereafter referred to as autophagy) has recently emerged as an attractive target for the treatment of various degenerative diseases and cancer. The discovery of effective pharmaceutical regulators of autophagy has, however, been hindered by a lack of feasible assay systems for autophagic flux. Here, we present a luciferase-based reporter assay that measures autophagic flux in real time in living cells and demonstrate that this assay system is apt for the detection of dose- and stimulus-dependent differences in autophagy kinetics. Furthermore, by screening a small molecule kinase inhibitor library containing 80 compounds we identified 12 compounds as inducers of autophagic flux. Importantly, six inhibitors of the class I phosphoinositide 3-kinase—protein kinase B—mammalian target of rapamycin complex 1 axis, the central signaling pathway repressing autophagy, scored as autophagy inducers adequately validating the screen. We conclude that the assay system presented here allows easy and rapid monitoring of autophagy kinetics and is suitable for screening of small molecule libraries.

Introduction

Autophagy is the major intracellular catabolic pathway responsible for the turnover of cellular organelles and long-lived macromolecules.¹ It regulates diverse biological events including development, aging, cell death and microbial infections.²⁻⁴ 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. Finally, the degradation products are exported for reuse.

MAP1-LC3 (LC3), a mammalian homologue of yeast Atg8, is synthesized as a pro-protein that is rapidly cleaved at glycine 120 forming the cytosolic LC3-I. Upon induction of autophagy,

the C-terminal glycine of LC3-I is coupled to phosphatidylethanolamine by a ubiquitin-like conjugation reaction resulting in the formation of LC3-II,^{5,6} which associates with both membranes of the autophagosome. The fraction associated with the outer membrane is recycled whereas the fraction associated with the inner membrane is degraded in autolysosomes.⁷

The ability of LC3 to carry fluorescent fusion partners to lysosomes via the autophagic route has been amply documented.^{8,9} The degradation of such a fusion protein within the lysosomes might thus serve as a marker for autophagic flux, provided that appropriate controls for the autophagy-independent changes in the expression level of the protein are taken into account. In order to develop such an assay, we explored the use of *Renilla Reiniformis* luciferase (RLuc), which can be easily quantified within a broad dynamic range in cell lysates and living cells,^{10,11} as the LC3 fusion partner. After demonstrating the autophagy-dependent turnover of RLuc-LC3, we established an assay system based on the ratio of wild type and autophagy-deficient mutant of RLuc-LC3 for monitoring autophagic flux. Careful experimental and theoretical validation of this assay system allow us to conclude that it is suitable for the high throughput screening for modulators of the kinetics of autophagic flux.

Results and Discussion

An optimal reporter protein for autophagic flux must have a slow autophagy independent turnover. In order to decrease the autophagy-independent turnover of RLuc-LC3 fusion protein, we introduced a stabilizing C124A mutation into the RLuc protein.^{12,13} Then, we transfected murine embryonic fibroblasts (MEFs) transiently with RLuc-LC3 and firefly luciferase (FLuc, an internal control), and measured the luciferase activity in the cell extracts. To induce autophagy we treated the cells for 4 h with rapamycin, an inhibitor of mammalian target of rapamycin complex 1 (mTORC1), which resulted in a significant drop in the RLuc/FLuc ratio as compared to the untreated cells. This decrease depended on autophagy since MEFs deficient for autophagy genes (*Atg5* or *Atg7*) displayed a significantly lower response (Fig. 1A). The half-life of the enzymatic activity of FLuc has been reported to be 3 h,¹⁴ whereas the half-life of RLucC124A is 52 h.¹⁰ In order to overcome the possible source of inaccuracy associated

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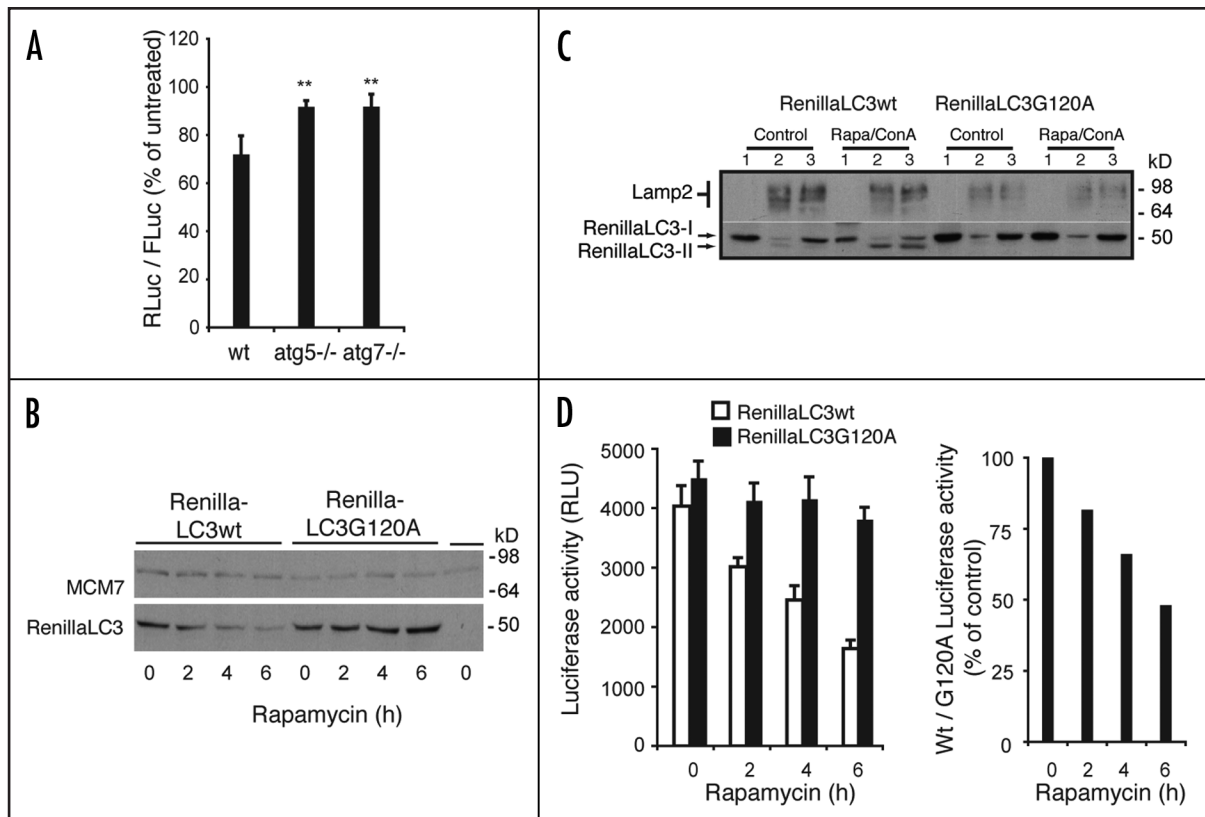


Figure 1. Rapamycin induces autophagy dependent decay of RLuc-LC3. (A) Wild-type, *Atg5*^{-/-} and *Atg7*^{-/-} MEFs were transiently transfected with plasmids expressing FLuc and RLuc-LC3wt. Cells were treated with 1 μ M rapamycin for 4 h. The ratio of RLuc/FLuc activity is expressed as percentage of that in untreated control cells. The values represent mean \pm SD from 3 independent experiments. **p value <0.01 as compared to wild-type cells. (B) Immunoblotting with antibodies against Renilla luciferase and MCM7 (internal control) in total extracts from MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} and treated with 250 nM rapamycin for 0, 2, 4 or 6 h. (C) Immunoblotting with antibodies against Renilla luciferase and Lamp2 of extracts from MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} either untreated (control) or treated with 250 nM rapamycin + 2 nM concanamycin A (ConA/Rapa) for 6 hours. Cytosolic extracts (1) or cytosol depleted total extracts (2) or total extracts (3). (D) Left part: RLuc activity in extracts of MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} and treated with 250 nM rapamycin for 0, 2, 4 or 6 h. The values represent mean \pm SD of a single experiment done in sextuplicate. Right part: The ratios of the luciferase measurements on the left were calculated at each time point and expressed as percentages of the ratio obtained from untreated cells. The average coefficient of variation for the RLuc-LC3wt and the RLuc-LC3^{G120A} expressing cells was 7.8% and 6.9%, respectively. Since the final data points represent a ratio between values obtained from the two cell lines, the resultant intra-assay coefficient of variation is 10.4%.

with different autophagy-independent stabilities of FLuc and RLuc^{C124A}, we tested the feasibility of establishing parallel populations of cells stably expressing RLuc^{C124A} fused to the wild-type LC3 (RLuc-LC3wt) and the LC3^{G120A} mutant (RLuc-LC3^{G120A}). RLuc-LC3^{G120A} is an ideal reference, since except for three base pairs in the DNA construct and a single amino acid in the protein, it is identical to the RLuc-LC3 construct. Yet, it is predicted not to undergo the cleavage- and lipidation-dependent changes required for autophagosomal localization.¹⁵ Therefore, a change in the relative levels of these two fusion proteins is likely to be mediated specifically by autophagy. Accordingly, immunoblot analyses of extracts from MCF-7 breast cancer cells stably expressing RLuc-LC3wt and RLuc-LC3^{G120A} and incubated with rapamycin for 6 h showed a gradual decrease in the level of RLuc-LC3wt but not in that of RLuc-LC3^{G120A} (Fig. 1B). The rapamycin-induced downregulation in the RLuc-LC3wt level was not accompanied by a detectable increase in the level of the faster migrating RLuc-LC3-II. To visualize RLuc-LC3-II and to further characterize the

fate of the fusion proteins after rapamycin treatment, we inhibited the lysosomal function by concanamycin A (ConA), an inhibitor of vacuolar H⁺-ATPase, and analyzed the digitonin extractable cytosolic fraction by immunoblotting. RLuc-LC3wt was readily extracted by digitonin indicating a cytosolic localization in control cells (Fig. 1C). After the treatment with rapamycin and ConA, the accumulation of RLuc-LC3-II was apparent, and akin to the membrane-bound reference protein, lysosome-associated membrane protein 2 (Lamp2), this faster migrating protein was not extractable by digitonin (Fig. 1C). Importantly, the RLuc-LC3^{G120A} was in the cytosolic fraction both in the control situation and after rapamycin/ConA treatment (Fig. 1C). These data are in good agreement with the expected membrane association of the lipidated form of RLuc-LC3wt and the inability of RLuc-LC3^{G120A} to be lipidated and associated with the membranes.

The luciferase reporter assay allowed an easy quantification of the observed difference in the levels of the two proteins and revealed more than a doubling in the turnover of RLuc-LC3wt as

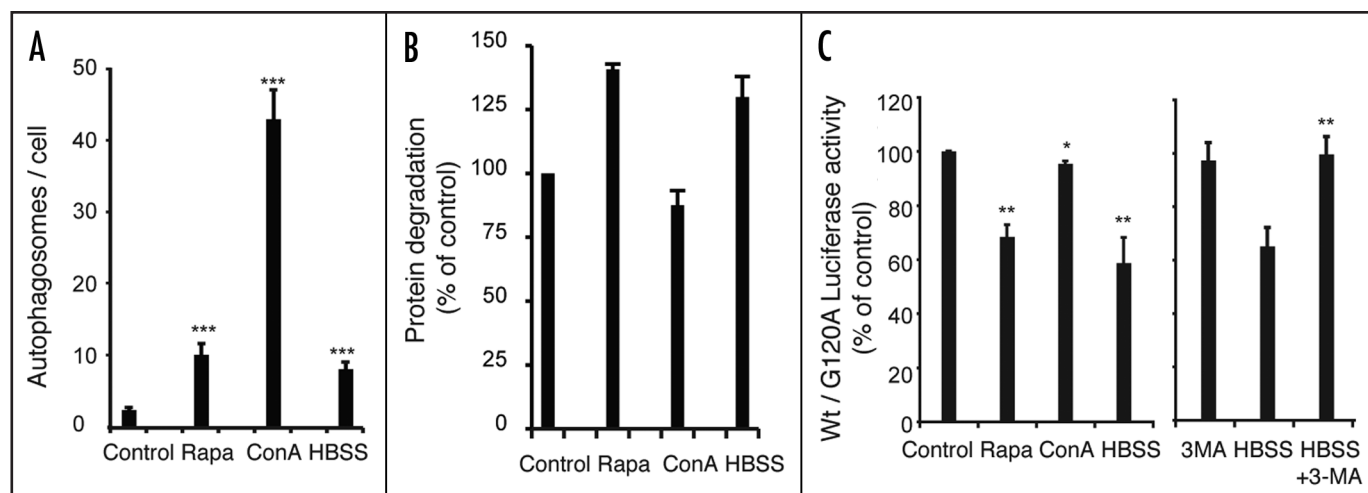


Figure 2. The RLuc-LC3wt/RLuc-LC3^{G120A} ratio reflects autophagic flux. (A) The average number of autophagosomes/cell \pm SEM counted from confocal microscopy images of MCF-7 cells expressing eGFP-LC3 left untreated (control) ($n = 70$) or treated with 250 nM rapamycin ($n = 36$), 2 nM ConA ($n = 49$) or Hanks balanced salt solution (HBSS) ($n = 48$) for 4 h. (B) MCF-7 cells were treated with 250 nM rapamycin, 2 nM ConA or HBSS for 4 h. The long-lived protein degradation is expressed as percentage of the value obtained from untreated cells and represent mean \pm SD for a representative experiment done in duplicate. (C) MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} were treated with 250 nM rapamycin, 2 nM ConA, HBSS, 10 mM 3-MA or HBSS + 10 mM 3-MA for 4 h. The values represent the mean ratio of luciferase activities from the two cell lines expressed as percentage of the ratio of untreated cells \pm SD ($n = 3$). * p value <0.05 , ** p value <0.01 and *** p value <0.001 as compared to untreated control cells (A and left in C), cells treated with HBSS (C, right).

compared to RLuc-LC3^{G120A} upon 6 h treatment with rapamycin (Fig. 1D). Thus, RLuc-LC3wt is targeted for autophagy-dependent degradation and this degradation can be readily detected by the reporter assay. It should be noted that RLuc-LC3^{G120A} as a cytosolic protein is also likely to be degraded by autophagy, albeit with a much lower rate than RLuc-LC3wt that is actively targeted to the autophagosomal membranes. Because the autophagy-mediated “unspecific” degradation rates of RLuc-LC3wt and RLuc-LC3^{G120A} are likely to be similar, we believe that autophagic bulk clearance of the reporter proteins has only a minor impact on the readout.

In order to further validate the assay, we exposed the cells to two well-documented inducers (rapamycin and starvation) and an inhibitor (ConA) of autophagic flux and compared the present assay with two established autophagy assays, i.e., the eGFP-LC3 intracellular localization assay that detects the accumulation of autophagosomes and the long-lived protein degradation assay that has been widely used to measure autophagic flux.⁸ As expected, all treatments lead to an increase in the number of eGFP-LC3 positive vesicles (Fig. 2A). The stronger increase in eGFP-LC3-positive autophagosome number in ConA-treated cells is likely to reflect the combined effect of stimulation of autophagosome formation via a pathway involving inhibition of mTORC1, perturbation of their further processing due to lysosomal dysfunction, and reactivation of eGFP fluorescence activity in autolysosomes upon neutralization of the pH.^{16,17} The long-lived protein degradation assay confirmed the expected stimulatory effects of rapamycin and starvation on autophagic flux, whereas ConA failed to stimulate the long-lived protein degradation (Fig. 2B). The luciferase reporter assay detected a similar lipidation-dependent decrease in RLuc-LC3 activity in starved and rapamycin-treated cells

(Fig. 2C). This is in agreement with the well-documented inhibitory effect of starvation on mTORC1.¹⁸ ConA had only a minimal impact on the readout demonstrating the distinction between the eGFP-LC3 localization assay showing a large increase in the number of eGFP-LC3-positive vesicles and the RLuc-LC3 assay as detection tools of autophagosome number and autophagic flux, respectively (Fig. 2A and C). The small stimulatory effect of ConA in the short (4 h) RLuc-LC3 assay may reflect the ability of ConA to inhibit mTORC1 combined with the fact that the disruption of the lysosomal pH gradient occurs gradually between 30–120 min post-treatment allowing an initial stimulation of the autophagic flux before the lysosomal function is severely compromised.¹⁶ The inability of the long-lived protein degradation assay to detect this small but significant stimulatory effect of ConA may, in turn, be due to the ConA-induced reduction not only in autolysosomal degradation but in all lysosomal proteolysis. We further tested if the autophagy inhibitor 3-methyl adenine (3-MA) could prevent the starvation-induced lipidation-dependent decrease in RLuc-LC3 reporter activity. Since this was the case (Fig. 1F), we conclude that the reporter assay data reflect starvation-induced autophagic flux.

To test if the luciferase assay can be used to detect autophagy induced by other less well-documented autophagy inducers than starvation and rapamycin, we tested the ability of etoposide (a topoisomerase II inhibitor), which has been reported to induce autophagy in apoptosis defective cells,¹⁹ to induce the lipidation-dependent degradation of RLuc-LC3 in MCF-7 cells. Indeed, MCF-7 cells treated for 12 h with 50 μ M etoposide showed a significant (49%) decrease in the RLuc-LC3wt/RLuc-LC3^{G120A} ratio, which was effectively inhibited by siRNA-mediated partial depletion of Beclin 1 (Fig. 3A). The eGFP-LC3 intracellular localization and the long-lived protein degradation assays confirmed the autophagy-promoting

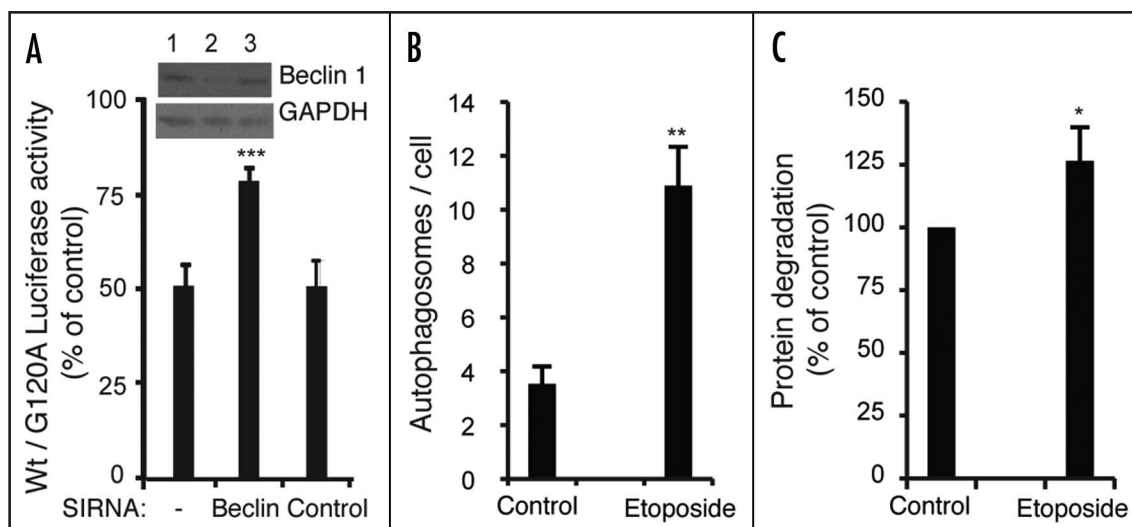


Figure 3. Etoposide treatment increase autophagic flux in MCF7 cells. (A) MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} were left untreated or treated with 50 μ M etoposide for 12 h after pretreatment with transfection vehicle alone or with siRNA directed against *BECN1* (Beclin 1) or with a control siRNA. The values represent the mean ratio \pm SD ($n = 4$) of luciferase activities from the two cell lines expressed as percentage of the ratio of untreated cells with corresponding siRNA treatments. The immunoblot on the top shows the levels of Beclin 1 and GAPDH (loading control) in total extracts of MCF-7 cells treated with transfection vehicle alone (1) or with Beclin 1 (2) or control (3) siRNA. (B) The average number of autophagosomes/cell \pm SEM counted from confocal microscopy images of MCF-7 cells expressing eGFP-LC3 left untreated (control) ($n = 45$) or treated with 50 μ M etoposide ($n = 44$) for 8 h. (C) MCF-7 cells were treated with 50 μ M etoposide for 12 h. The long-lived protein degradation is expressed as a percentage of the value obtained from untreated cells and represent mean \pm SD of three independent experiments. * p value <0.05 , ** p value <0.01 and *** p value <0.001 as compared to cells treated with control siRNA (A) or to untreated control cells (B and C).

effect of etoposide in MCF-7 cells (Fig. 3B and C). Importantly, these data demonstrate that etoposide also induces functional autophagy in cells capable of undergoing apoptosis and that the assay system is compatible with siRNA-mediated gene silencing.

Autophagy is a dynamic process that receives regulatory signals from several signaling pathways.²⁰ To appreciate this complexity it is crucial to follow autophagy in real-time. To confront this experimentally, we tested whether a luciferase substrate coelenterazine (EnduRenTM) optimized for use in living cells would allow kinetic analysis of autophagic flux in our assay system. Using this live cell kinetic assay, we analyzed the effect of rapamycin for 12 h with 1 h intervals between measurements and detected a lipidation-dependent gradual loss of RLuc-LC3 that was completely inhibited by 3-MA (Fig. 4A). Thus, EnduRenTM remarkably improved this assay system by allowing the kinetic analysis of the autophagic flux from the same well. This improvement made it possible to compare the kinetics and extent of autophagy induced by different treatments. To demonstrate this, we compared the kinetics of the RLuc-LC3wt/RLuc-LC3^{G120A} ratio in rapamycin- and etoposide-treated cells. The two drugs induced a lipidation-dependent decrease in reporter activity with strikingly different kinetics. The rapamycin response appeared less complex resembling exponential decay with 1 h lag-phase after drug addition indicating that the cells responded uniformly to the treatment (Fig. 4A, insert). The curve did, however, not move toward zero but instead it approached 27% suggesting the establishment of a new steady state of the RLuc-LC3wt/RLuc-LC3^{G120A} ratio. Interestingly, decreasing the concentrations of rapamycin to 0.5–1 nM increased the lag-phase after drug addition and resulted in curves approaching higher

steady state RLuc-LC3wt/RLuc-LC3^{G120A} ratios indicative of slower autophagy induction and smaller autophagic flux, respectively (Fig. 4B). The induction of autophagy by etoposide had a longer lag-phase than after rapamycin induction and the slope increased gradually reaching a maximum after approximately 7 h (Fig. 4A). In order to test whether the kinetics observed reflected the activity of mTORC1, we analyzed the phosphorylation of p70S6K in similarly treated cells. As expected, rapamycin induced a rapid and steady reduction of p70S6K phosphorylation to levels below the threshold for reliable densitometric quantification already 1 h after the addition of the drug, whereas etoposide induced a gradual reduction in mTORC1 activity correlating well with the kinetics of the lipidation-dependent decrease in the luciferase activity (Fig. 4A). To further examine the sensitivity of the assay, we made a comprehensive dose response curve for 6 h rapamycin treatment (8 pM–4 μ M). The maximal autophagic flux was achieved with 4–250 nM rapamycin, and concentrations above 250 nM were surprisingly less effective in inducing autophagic flux (Fig. 4C). These data suggest that high concentrations of rapamycin have an mTORC1-independent inhibitory effect on the autophagic flux.

The data presented above indicate that the RLuc-LC3wt/RLuc-LC3^{G120A} assay combined with EnduRenTM is suitable for a semiquantitative kinetic analysis of autophagic flux in living cells. In order to demonstrate its feasibility for screening, we applied it to screen a small molecule kinase inhibitor library containing 80 compounds (Table S1). We analyzed each compound in duplicate for 12 h with 2 h intervals. Based on the criteria presented in the legend, we classified 12 compounds as inducers of autophagic flux (Fig. 5). Importantly, six of the eight library compounds that

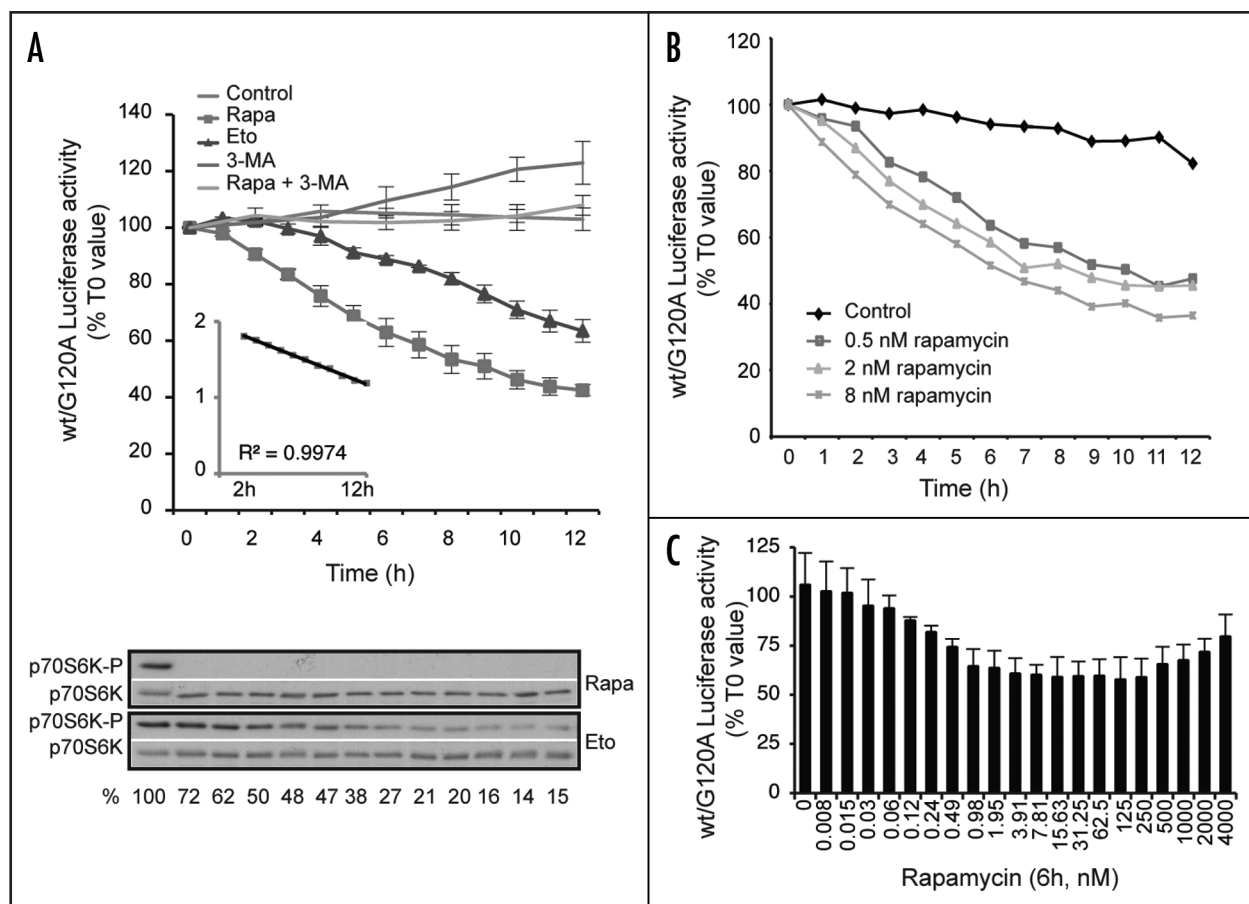


Figure 4. Analysis of the kinetics of autophagic flux in living cells. (A) MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} were plated in separate wells of a 96-well dish and incubated with 50 nM of the live cell luciferase substrate EnduRenTM for 2 h prior to addition of medium (Control; n = 3), 250 nM Rapamycin (Rapa; n = 3), 50 μ M Etoposide (Eto; n = 3), 10 mM 3-MA (n = 3) or Rapamycin (250 nM) + 3-MA (10 mM) (Rapa + 3-MA; n = 2). Luciferase activity was measured with 1–2 h intervals as indicated. The values represent the mean ratio \pm SEM of luciferase activities from the two cell lines expressed as percentages of the corresponding ratio in untreated cells at T0. The insert is a representation of the logarithm of the rapamycin values after subtraction of 27%, as a function of time (from 2 to 12 hours). The trendline and the corresponding R² were calculated with excel software. The value “27%” represents a hypothetical steady state of wt/G120A luciferase activity in the presence of rapamycin (established as the value giving a trendline with an R² closest to 1). Below: Immunoblot analysis of indicated proteins from MCF-7 cells treated with rapamycin or etoposide as above. The densitometry data from the etoposide experiment are shown under the blots. The values represent the p70S6K-P/p70S6K ratio as percentage of the value in untreated cells. (B) MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} were plated in separate wells of 96 wells dish and incubated with 50 nM of the live cell luciferase substrate EnduRenTM and the indicated amounts of rapamycin. Luciferase activity was measured with 1 h intervals for 12 h. (C) MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} were treated as in (B). The values represent the mean ratio \pm SD (n = 3) of luciferase activities after 6 h of rapamycin treatment from the two cell lines expressed as percentage of the corresponding ratio in untreated cells at T0.

inhibit the class I phosphoinositide 3-kinase (PI3-K)—protein kinase B (Akt)—mTORC1 axis, the central signaling pathway leading to the repression of autophagy, scored as inducers of autophagic flux adequately validating the screen. PI-103, a dual inhibitor of both class I PI3-K and mTORC1,²¹ emerged as an even more potent inducer than rapamycin suggesting that class I PI3-kinase may inhibit autophagy by mTORC1-dependent and independent pathways. Other hits included two inhibitors of protein kinase C (PKC) family. Together with a recent report showing that siRNA-mediated depletion of PKC delta and epsilon induces autophagy,²² these data suggest that PKC family members can act as negative regulators of autophagy.

Taken together, our data show that the assay measuring the kinetics of the RLuc-LC3wt/RLuc-LC3^{G120A} ratio is suitable

for the analysis of autophagic flux. Recently, other LC3-based reporter assays aimed at monitoring functional autophagy have been described. The Tandem Fluorescent-tagged LC3 allows visual distinction between autophagosomes before and after their fusion with lysosomes thus providing evidence for the presence of functional autophagy but limited information about the rate of the fusion events.¹⁷ The assay described by Shvets et al. measures the inhibition of GFP-LC3 fluorescence by flow cytometry after autophagy mediated transfer of GFP-LC3 into autolysosomes. This assay is restricted to living cells and only allows a single measurement on a cell population. In addition, it does not in the described format include quantifications from GFP-LC3^{G120A} in the assay readout, making it sensitive to autophagy independent changes in the stability of the reporter mRNA and protein. During the

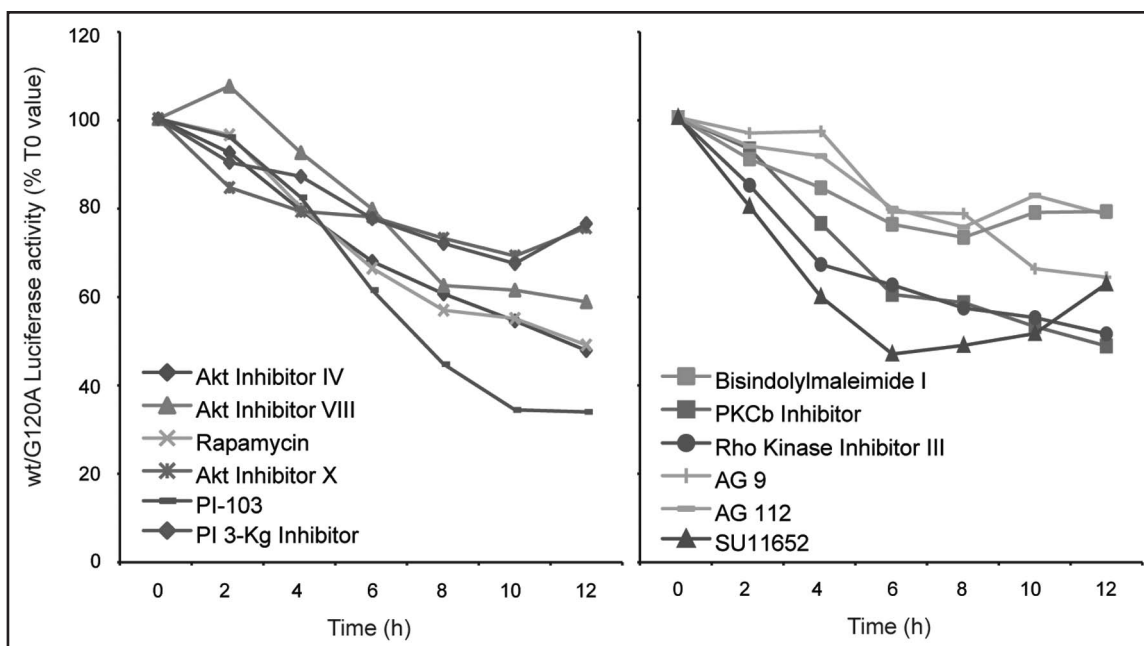


Figure 5. Screening of a small molecule kinase inhibitor library for autophagy inducers. MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3^{G120A} and pre-incubated with 50 nM EnduRenTM for 2 h were treated with 2.5 μ M of each of the 80 compounds (except for PI3-Kg inhibitor that was used at 1.25 μ M) in a Calbiochem small molecule kinase inhibitor library (Cat. 539744). Luciferase activity was measured with 2 h intervals. Based on theoretical considerations presented in Supplemental Figure S2, a compound was scored as an inducer of autophagic flux if the following criteria were fulfilled: The RLuc-LC3wt/RLuc-LC3^{G120A} ratio was reduced by 20% in at least one measurement during the time course, and the trend line through at least four consecutive measurements had an $R^2 > 0.8$ and a slope < 3 percentage points/h. The 12 compounds that scored as inducers are presented, and the values represent the mean ratio of luciferase activities from the two cell lines from a duplicate screen expressed as percentages of the corresponding ratio in untreated cells at T0 (a). Two compounds displayed toxicity (Lactate dehydrogenase release $> 20\%$ of total activity) and were excluded from the screen. For the entire data sets see Supplementary information (Table S1).

preparation of this manuscript an alternative luciferase assay aimed at measuring the autophagic flux of protein aggregates has been published.²³ This assay measures the selective autophagy mediated degradation of polyQ80-luciferase over polyQ19-luciferase. It remains to be studied whether this assay is useful for the study of other kinds of autophagy.

The assay system described herein is suitable for screening of small molecule or siRNA libraries for regulators of autophagic flux in real time in living cells. Thus, it offers new possibilities for the discovery of novel drug candidates and targets for the treatment of the multitude of diseases whose pathology involves deregulated autophagy.

Materials and Methods

Cell culture and treatments. *Atg5*^{-/-} mouse embryonic fibroblasts (MEFs) and appropriate wild-type control MEFs were kindly provided by Noboru Mizushima (Japan) and *Atg7*^{-/-} MEFs were kindly provided by Masaaki Komatsu (Japan).^{24,25} MEFs were grown in DMEM supplemented with 10% fetal calf serum, non-essential amino acids, penicillin and streptomycin. MCF-7 breast carcinoma cells were grown in RPMI-1640 (Gibco, 61870) supplemented with 6% fetal calf serum, penicillin and streptomycin. When plating for luminometry in living cells, RPMI-1640 without phenol red (Gibco, 11835) was used. MCF-7 cells that stably express eGFP-LC3 have been described previously.²⁶

Rapamycin, 3-MA, Concanamycin A and Etoposide were from Sigma-Aldrich. Aliquots of the kinase inhibitor library (Calbiochem, 539744) were kindly provided by Pekka Kallunki (Lundbeck A/S, Valby, Denmark).

Plasmid constructions. The expression vector pRLuc-LC3wt (rat LC3) was made by exchanging the open reading frame of eGFP in peGFP-LC3 (peGFP-C1 (Clontech) containing rat LC3) with that of *Renilla Reniformis* Luciferase using the restriction enzymes AgeI and BglII. The Renilla Luciferase open reading frame was obtained by PCR amplification of the plasmid pRL-null (promega) using primers with restriction sites (underlined) included as 5'-extensions. Upstream primer: TAG CGC TAC CGG TCG CCA CCA TGA CTT CGA AAG TTT ATG; downstream primer: ATC TAG ATC TGA GTC CGG ATT GTT CAT TTT TGA GAA CTC G.

Amino acid substitutions were made via site directed mutagenesis. The stabilizing mutation C124A in luciferase was introduced in pRLuc-LC3wt to create pRLucC124A-LC3wt using the primer CAT GAT TGG GGT GCT GCT TTG GCA TTT CAT TAT AG and the corresponding antiparallel primer.^{12,13} The G120A substitution was followed by a stop codon in the plasmid pRLucC124A-LC3G120A. The changes were introduced simultaneously in pRLucC124A-LC3wt with the primer: CTC CCA GGA GAC GTT CGC GTA AGC ACT GGC TGT TAC and the corresponding antiparallel primer.

Transfections. To create RLucC124A-LC3wt and RLucC124A-LC3G120A expressing MCF-7 cells, the cells were plated at a density of 12,000 cells/cm² in 58 cm² dishes and transfected 40 h later with 5 µg of appropriate plasmid DNA and 15 µl Fugene HD (Roche)/dish. Selection was performed in medium containing 400 µg/ml geneticin (Invitrogen, G-418).

Transient transfection of MEFs was performed by adding 80 ng pRLucC124A-LC3wt, 160 ng CMV-Luciferase (firefly), 860 ng empty vector and 4.5 µl of Fugene HD/well on cells plated at a density of 16,000 cells/cm² in 6-well plates 20 hours earlier. The cells were reseeded in 96-wells plates at a density of 15,000 cells/cm² 48 hours after the transfection and processed for the reporter assay the following day.

Transfection of siRNA was performed with Oligofectamine (Invitrogen) according to manufacturer's instructions using 33 nM of siRNA. The siRNA against *BECN1* (Beclin 1) and the control siRNA were described previously.²⁶

Extraction of cytosolic proteins. RLuc^{C124A}-LC3wt and RLuc^{C124A}-LC3^{G120A} expressing MCF-7 cells were plated in 5.8 cm dishes at 19,000 cells/cm², and treated the following day as indicated. Thereafter, the medium was removed and soluble cytosolic proteins were extracted by shaking the dishes for 15 min on ice in 1.2 ml of digitonin extraction buffer (100 µg/ml digitonin, 250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10 mM N-ethylmaleimide, 200 µM pefablock, 4 µM leupeptin, 3 µM pepstatin A, pH 7.5). The cytosolic extract was precipitated in 10% trichloroacetic acid and the pellet was dissolved in 350 µl SDS-lysis buffer. The cytosol-depleted cells were re-extracted in 350 µl SDS-lysis buffer. A parallel dish was extracted directly in 350 µl SDS-lysis buffer to obtain the total protein.

Reporter assays. Firefly- and Renilla-luciferase were detected using the reagents in the Dual-Luciferase[®] Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, cells were lysed in 50 µl/well of 1x lysis buffer and subjected to a single freeze/thaw cycle. Firefly-Luciferase was measured in black half area 96-wells plates (Costar, 3694) using 5 µl of lysate and 25 µl of Luciferase Assay Reagent II. Renilla-Luciferase was measured consecutively by further addition of 20 µl of Stop&Glo[®] Reagent to each well.

For the reporter assay in living cells, MCF7 cells stably expressing RLucLC3wt and RLucLC3G120A were plated in the uneven and even numbered columns of white 96 wells dishes (Nunc, 136101) respectively at 3.6 x 10⁴ cells/cm². The following day the cells were incubated for 2 h in 100 µl medium containing 50 nM EnduRenTM (Promega) and the luminescence was measured (Varioskan Flash plate-reader, Thermo Electron Corporation). The readout was obtained by calculating the ratio in luminescence between neighboring wells such that the ratio at T0 was defined to 100%. Thereafter, the drugs dissolved in EnduRenTM containing medium were added in the volume of 40 µl and luminescence measurements were performed at indicated intervals. It is important to notice that, when using EnduRenTM at a concentration of 60 µM as recommended by the manufacture, we observed inhibition of rapamycin induced autophagic flux in the reporter assay (an EnduRenTM dose response curve is shown in

Suppl. Fig. S1). To eliminate this effect we reduced the concentration of the substrate to 50 nM.

Immunoblotting. Cells were extracted in SDS-lysis buffer. Extracts were separated on SDS-PAGE and blotted onto nitrocellulose membranes. The primary antibodies used were: MCM7 DCS-141.1 (kindly provided by Jiri Bartek), Lamp-2 (Developmental Studies Hybridoma Bank, Clone H4B4), Beclin 1 (BD Transduction Laboratories, 612112), Renilla luciferase (Chemicon, Mab 4410), GPP (Santa Cruz, 9996), P70 S6K (cell signaling, 9202), P-P70S6K (Thr389) (cell signaling, 9206), GAPDH (biogenesis). The peroxidase coupled secondary antibodies were from Dako.

eGFP-LC3 translocation assay. The EGFP-LC3 translocation assay was performed as described previously.²⁶

Long-lived protein degradation assay. Long-lived protein degradation assay was performed with minor modifications to a previously reported protocol.²⁷ Briefly, MCF-7 cells were seeded at a concentration of 7.1 x 10³ cells/cm². After 18 h, 0.2 µCi/ml ¹⁴C-Valine (Amersham) was added for 24 h, the cells were washed three times in normal growth medium and incubated in normal growth medium supplemented with cold valine (5 mM) for 18 h. Thereafter, the cells were treated with drugs for 4 h in fresh growth medium or with Hanks Balanced Salt Solution (HBSS) as indicated. The growth medium was precipitated in 10% Trichloroacetic acid (4°C for 10 min), centrifuged for 5 min at 15,000 g, and the supernatant was collected. The cells were dissolved in 0.2 M NaOH (37°C for 10 min). The radioactivity in the supernatant and in the dissolved cells was measured in a scintillation counter (Wallac, Turku, Finland). Protein degradation was calculated by dividing the counts from the supernatants with the total counts.

Cell death assay. Cell death was monitored by lactate dehydrogenase release assay (Roche) essentially as described previously.²⁸

Statistical analysis. All experiments were performed at least three times unless otherwise indicated. A two-tailed unpaired t-test was used except when analyzing data expressed as percentage of control (Fig. 2C, left), where an one-sample t-test was used.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/FarkasAUTO5-7-Sup.pdf

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