Università degli Studi di Padova

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Corso di Laurea in Biotecnologie

Elaborato di Laurea

Transfection optimization of the Hereditary Spastic Paraplegia associated gene SPG11 and identification of the optimal conditions for autophagy induction

Tutor: Ch.ma Prof.ssa Elisa Greggio

Dipartimento di Biologia "A. Vallisneri"

Laureanda: Ilaria Talli

Matricola: 1118722

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Abstract

Hereditary Spastic Paraplegias (HSPs) are a heterogeneous group of neurological disorders, characterized by the degeneration of distal axons of the corticospinal tracts, and affected patients manifest spasticity and weakness of the lower limbs. There are 72 genetic loci with 55 spastic paraplegia genes (SPGs) which have been identified. The great majority of Hereditary Spastic Paraplegias are transmitted through Mendelian inheritance modes, and they can be autosomal dominant or recessive forms, but also recessive X-linked forms. Among Autosomal Recessive forms (ARHSP), mutations in SPG11 encoding spatacsin cause the most common form of the disease. Although its functions are still unclear, it is thought that spatacsin plays a pivotal role in the regeneration of lysosomes from autolysosomes, a process that would be deregulated if mutations in SPG11 are present. Ectopic expression of SPG11 in the neuronal cell line SH-SY5Y may help to evaluate the role of spatacsin in the processes of autophagy and autophagic lysosome reformation (ALR). Autophagy describes the segregation and delivery of cytoplasmic components, including proteins and organelles, for the degradation by the hydrolytic enzymes of the lysosomal machinery. It is a critical process for the healthy functioning of the cells, since failure in autophagy is a major reason for the accumulation of cell damage and aging.

Le Paraplegie Spastiche Ereditarie (HSPs) sono un gruppo eterogeneo di malattie neurologiche, caratterizzate dalla degenerazione della parte distale degli assoni del tratto corticospinale, e i pazienti affetti manifestano spasticità e debolezza degli arti inferiori. Sono stati identificati 72 loci genetici e 55 geni associati (SPGs). La maggior parte delle Paraplegie Spastiche Ereditarie sono trasmesse secondo ereditarietà mendeliana, e possono essere distinte in autosomiche dominanti e recessive e recessive legate al cromosoma X. Tra le forme autosomiche recessive (ARHSP), le mutazioni nel gene SPG11 che codifica per la proteina spatacsina causano la forma più comune della patologia. Nonostante le sue funzioni siano ancora poco chiare, si pensa che la spatacsina abbia un ruolo fondamentale nella rigenerazione dei lisosomi a partire dagli autolisosomi, processo che sarebbe deregolato se fossero presenti mutazioni nel gene SPG11. L'espressione esogena del gene SPG11 in linee di cellule SH-SY5Y può contribuire a comprendere il ruolo della proteina spatacsina nei processi di autofagia e rigenerazione di lisosomi autofagici (ALR). L'autofagia descrive la segregazione e la distribuzione di componenti del citoplasma, incluse proteine e organelli, per la degradazione da parte di enzimi idrolitici dei lisosomi. È un processo fondamentale per il corretto funzionamento cellulare, dal momento che la mancanza del processo autofagico è motivo di accumulo di danni ed invecchiamento cellulari.

Introduction

Hereditary Spastic Paraplegias

Hereditary Spastic Paraplegias are clinically and genetically heterogeneous inherited neurodegenerative disorders characterized by progressive weakness and spasticity in the lower limbs. The pathology of the disease involves the degeneration of the axonal terminal portion in the corticospinal tracts with additional neurological signs and cognitive impairment first noticed during childhood. To date, 72 genetically distinct forms are currently recognized, and 55 spastic paraplegia genes have been identified. Several HSP genes have been associated with cellular functions related to organelle and membrane trafficking, axonal transport and autophagy. HSP can be inherited via Mendelian modes, and so autosomal dominant and recessive forms and recessive X-linked forms can be distinguished. However, the Autosomal Recessive forms are the most common, and mutations in SPG11 encoding spatacsin are the major cause. Moreover, ARHSP linked to SPG11 mutations can come with other specific phenotypical signs, that are white matter lesions, cerebellar signs and the thinning of the corpus callosum (TCC), which is a flat bundle of neuronal fibres that connects the left and the right cerebral hemispheres. Nevertheless, mutations in SPG15 encoding spastizin are responsible for ARHSP with thin corpus callosum.

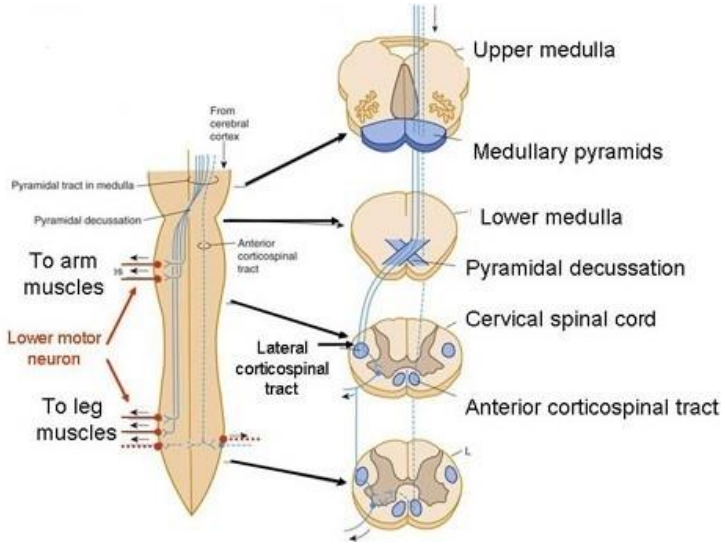


Figure 1 | Schematic illustration of the course of corticospinal tract fibres in the spinal cord, together with cross section at representative levels. From Waxman SG, *Clinical Neuroanatomy*, 26th ed.

Most mutations of SPG11 are small deletions or insertions, and they lead to the production of a truncated protein, which is consequently affected by a loss of function. Hence, the main cause for the TCC is the presence of these mutations, which can come with mental retardation and cognitive impairment. As a matter of fact, the main clinical symptoms are dysarthria, dysphagia and upper limb spasticity, associated with bladder dysfunction and axonal, motor or sensorimotor peripheral neuropathy[1].

[1] Stevanin *et al.* (2007), *Mutations in SPG11, encoding spatacsin, are a major cause of spastic paraplegia with thin corpus callosum*, Nature Genetics 39: 366-372

Macroautophagy

Macroautophagy is a cellular process through which the cells are able to degrade their constituents and membranes in stressful conditions; this way, the cellular components are processed and re-introduced into the metabolic pathways in different forms. The basal levels of macroautophagy are low, but they may rise in stressful or pathological conditions. Macroautophagy is implicated in different physiological cellular processes, such as starvation, embryonic development, quality control of endogenous proteins and organelles, degradation of pathogens and immune response regulation. In some human pathological conditions, the macroautophagic process may be impaired and this situation may lead to the accumulation of non-degraded vesicles; examples can be found in Parkinson's Disease and Alzheimer's Disease.

The macroautophagic process involves different steps, which may be summarized as follows:

- Vesicle formation, development and elongation
- Autophagosome formation
- Fusion between autophagosome and cellular lysosomes (autolysosome)

Macroautophagy regulation is under the control of several different proteins; however, one of the most relevant proteins involved is LC3. It is found as an endogenous non-processed form, pro-LC3, and is activated after autophagy induction to the form LC3-I. If LC3-I is conjugated with phosphatidylethanolamine, it is converted to the form LC3-II; the latter is sufficiently lipophilic to insert into the autophagosome membranes. Experimentally, measuring the levels of LC3-II through the Western Blot (14 KDa) permits the evaluation of the number of autophagosomes and autolysosomes. LC3-II levels are found to be higher in starvation samples indicating an augmented number of autophagosomes.

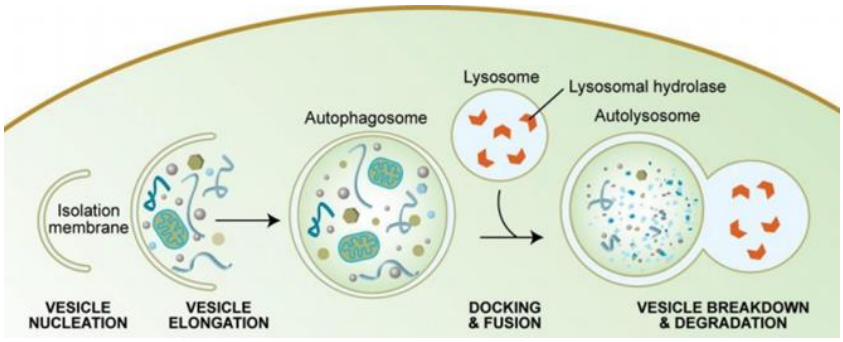


Figure 2 | Schematic illustration of the macroautophagic process. From Kirkegaard, Taylor and Jackson (2014), *Nature Rev Microbiol*.

SPG11

SPG11 is a protein of 2443 aminoacids (278 KDa); its cellular functions are still unclear even though it was discovered back in 2007. Thus, understanding the pathophysiological role of SPG11 is appealing for a number of reasons. It has been

proposed that it acts as a scaffold protein during vesicle formation, interacting with SPG15 (spastizin) and the AP-5 complex, which is thought to play a role in endosomal sorting[2]. Secondly, the protein is supposed to regulate autophagy and the lysosomal pathway, since its loss is the main cause of autophagy deregulation in many neurodegenerative disorders, together with the loss of SPG15. In fact, it is believed that SPG11 and SPG15 also play a fundamental role in the autophagic lysosome reformation (ALR), to generate free lysosomes. Impaired ALR is expected to lead to exhaustion of lysosomes available for fusion with autophagosomes and therefore accumulation of autolysosomes[3]. The process is evident in cells which are particularly sensitive to autophagy defects, such as cortical neurons and Purkinje cells; a reduction of lysosomes available for fusion causes a defect in autolysosomal degradation followed by accumulation of undegraded material and neuronal death[3].

SPG11 has a predicted secondary structure containing α-solenoids that are similar to those found in clathrin heavy chains subunits. It also possesses an N-terminal, β-propeller-like domain, which is thought to interact with AP-5. The interaction between SPG11, SPG15 and AP-5 results in the formation of early endosomal compartments, in which SPG11 and SPG15 function as a scaffold and docking site, respectively. Through knockdown techniques, Hirst *et al.* demonstrated that the loss of either SPG11, SPG15 or AP-5 complex results in perturbed trafficking of late endosomes and lysosomes (positive for LAMP1)[2].

SPG11 is expressed in various areas of the human nervous system: layer V of the motor cortex, spinal cord, hippocampus, cerebellum, dentate nucleus and pons. In neurons, it is prevalently found in neuronal cell bodies, proximal neuritis and axons of the motor neurons in the spinal cord. The fact that it is ubiquitously expressed and highly conserved suggests that it has an essential biological function. However, its subcellular distribution is still unclear. It is shown to have a diffuse cytoplasmic distribution and partial co-localization with several cellular markers. It co-localizes with microtubules, endoplasmic reticulum, vesicles involved in protein trafficking and rarely with mitochondria. Instead, it never co-localizes with markers for endosomes, lysosomes and Golgi apparatus. Moreover, similar distribution is found for spastizin encoded by SPG15, suggesting that both SPG11 and SPG15 function in common cellular pathways to maintain axonal integrity. Since they are found in vesicles involved in protein trafficking, it is tempting to assume that spatacsin might play a substantial role in axonal transport. In fact, loss of spatacsin function may affect transport in axons, leading to the degeneration of the corticospinal tracts[4].

[2] Hirst *et al.* (2013), *Interaction between AP-5 and the hereditary spastic paraplegia proteins SPG11 and SPG15*, Molecular Biology of the Cell: 2558-2569

[3] Varga *et al.* (2015), *In Vivo Evidence for Lysosome Depletion and Impaired Autophagic Clearance in Hereditary Spastic Paraplegia Type SPG11*, PLoS Genet 11

[4] Murmu *et al.* (2011), *Cellular distribution and subcellular localization of spatacsin and spastizin, two proteins involved in Hereditary Spastic Paraplegia*, Molecular and Cellular Neuroscience 47: 191-202

Hirst *et al.* reported co-localization of spatacsin and late endosomal marker LAMP1.

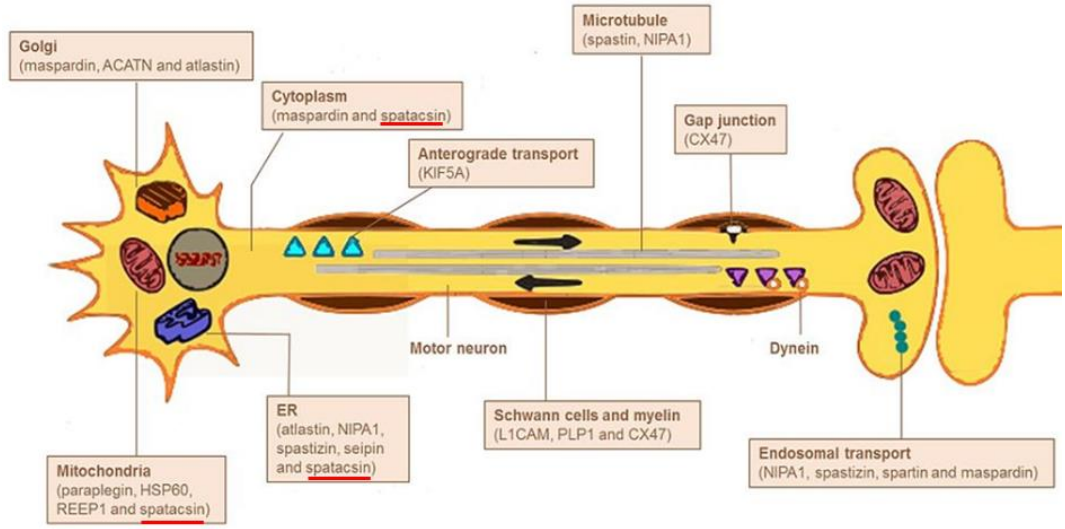


Figure 3 | Overview of HSP pathogenesis on cellular level. Identified affected genes in each pathway are depicted.

Mutations inside SPG11 are distributed throughout the gene, and they are mostly truncating mutations. The presence of HSP-TCC is the best indicator to test SPG11 in patients, but other phenotypic signs are mental retardation, cognitive deterioration, lower motor neuron involvement and white matter lesions in the periventricular regions; gait instability is the first sign at onset of Hereditary Spastic Paraplegia in all patients. Most of the mutations have been identified in families from the Mediterranean basin, but others have been found in families from Scandinavia, Japan and South America[5].

Spatacsin and macroautophagy

Spatacsin function in the macroautophagic process is mainly due to its role in the autophagic lysosomes reformation (ALR). If there is impaired ALR, there is evidence of exhaustion of lysosomes available for fusion with autophagosomes and consequent accumulation of autolysosomes. The autolysosomes are full of undegraded material, which results in pathologic accumulation of substance characteristic of neurodegenerative disorders. Impairment in the ALR process affects cells which are particularly sensitive to autophagy defects, such as cortical neurons and Purkinje cells; they eventually undergo a reduction of lysosomes available for fusion and so defects in autolysosomal degradation followed by accumulation of undegraded material and neuronal death.

[5] Stevanin *et al.* (2008), *Mutations in SPG11 are frequent in autosomal recessive spastic paraplegia with thin corpus callosum, cognitive decline and lower motor neuron degeneration*, Brain 131: 772-784

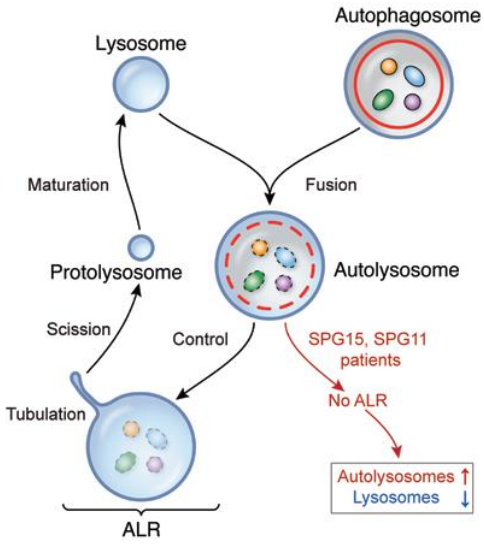


Figure 4 | Autophagosomes fuse with lysosomes to form autolysosomes. After degrading materials, the lysosomal tubule emanates from the autolysosome and becomes the protolysosome, which is destined to become a lysosome after maturation. This process is called ALR.

In cells from patients with SPG15 or SPG11, fusion of the lysosome and the autophagosome occurs normally, but ALR is blocked by impaired initiation of lysosomal tubulation from the autolysosome, which eventually results in accumulation of autolysosomes and exhaustion of free lysosomes.

From Chang *et al.* (2014), *Spastic paraplegia proteins spastizin and spatacsin mediate autophagic lysosome reformation*, J Clin Invest.

Tracking autophagy with LC3-II and p62

Macroautophagy starts with the formation of vesicles, continues with the formation of autophagosomes and ends with the fusion between autophagosomes and cellular lysosomes. Several mechanisms to promote and inhibit autophagy have been recognized. Torin treatment inhibits mTOR, which is an inhibitor of autophagy, therefore leading to an increment of the macroautophagic flux. Treatment with Chloroquine inhibits the acidification of lysosomes, therefore blocking the fusion between autophagosomes and cellular lysosomes. Starvation acts in the same way as Torin treatment, by the inhibition of mTOR.

Reporting LC3-II and p62 localization makes autophagy studies possible. p62 can be used as a marker for the induction of autophagy, the clearance of protein aggregates and the inhibition of autophagy, while LC3-II is a useful label to track the binding of p62 and the subsequent recruitment of autophagosomes, since it inserts inside the autophagosome membranes.

Aim of the thesis

Given the difficulties in detecting, monitoring and purifying endogenous SPG11 protein, in this thesis exogenous SPG11 was transfected in the form of a plasmid. To gain insights into the physiological function of SPG11, one approach is to identify the interactor partners. Since the future aim of this project is to investigate SPG11 interactome to gain insights into its biological functions, the protein will be overexpressed in fusion with a Flag-tag in the neuronal cell line SH-SY5Y. Inserting a Flag will help detecting the protein itself as well as purifying it, since antibodies directed to the Tag are more specific than the ones directed against spatacsin.

More specifically, one of the aims of my project was to evaluate the transfection efficiency of Flag-SPG11 in the neuroblastoma cell line SH-SY5Y comparing two different transfection reagents: Polyethylenimine (PEI) and Lipofectamine 2000. A second aim was to optimize conditions for autophagy induction. Starvation, pharmacological inhibition of mTOR (with Torin1) and inhibition of lysosomes acidification (with Chloroquine) were performed and autophagy induction was evaluated, by Western Blot with antibodies against LC3-II and p62.

Materials and Methods

SH-SY5Y cell cultures

SH-SY5Y is a cell line derived from human cells and used in scientific research. This cell line is derived from the SK-N-SH cell line, which was isolated from a bone marrow biopsy of a patient affected with neuroblastoma. SH-SY5Y cells are often used *in vitro* as models to test neuronal function and differentiation. The cells grow in two distinct ways: some grow into clumps floating in the media, while others form clusters which stick to the dish. Indeed, the ability to form aggregates is a reminiscence of their cancerous nature. They have a drop-like shape and some neurite-like processes.

The growing medium is a mixture of DMEM and Ham's F12 in 1:1 proportions, with 10% Fetal Bovine Serum (FBS). The DMEM contains 1.5 g/L sodium bicarbonate, 2 mM L-Glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. The cells should be grown at 37 degrees Celsius with 95% air and 5% carbon dioxide, into flasks for cell culture adhesion. Ham's F12 is a nutrient mixture. FBS comes from the blood drawn from a bovine fetus; it contains several growth factors, allowing for versatility in many different cell culture applications. The growing medium also contains antibiotics. The cells are grown until they are dense and considered to be confluent, so that they can be split and used in different types of laboratory experiments.

SH-SY5Y cell line can spontaneously differentiate into two different phenotypes in vitro, the neuroblast-like phenotype and the epithelial-like one. However, the cells mainly differentiate along the neuronal line, thanks to exogenous differentiating agents such as retinoic acid, phorbol esters, and specific neurotrophins (Brain-Derived Neurotrophic Factor). SH-SY5Y cell line has a dopamine-β-hydroxylase activity to convert dopamine to norepinephrine, which gives the cells the double nature of being adrenergic and dopaminergic.

Transfection

Transfection is the process of introducing nucleic acids into eukaryotic cells. It relies either on physical treatment or on chemical or biological carriers. One chemical method is to use cationic polymers, such as polyethylenimine (PEI): the negative charges on the DNA bind to the polycation and the complex is taken into the cell via endocytosis. Another method is the lipofection, or liposome transfection. It is a technique used to inject genetic material via liposomes, which are vesicles with a phospholipid bilayer that can merge with cell membrane. It is usually carried through the use of positively charged lipids to form aggregates with the negative charges on the DNA, and one example is Lipofectamine 2000.

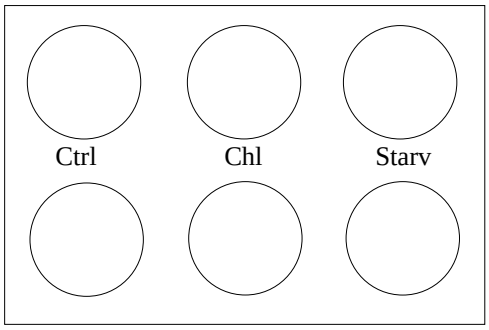
Transfection is used to introduce a particular plasmid, which contains SPG11 fused at the N-terminal with a Flag-tag, a highly immunogenic, 8 aminoacid peptide sequence (DYKDDDDK) recognized by commercially available antibodies.

Endogenous SPG11 is only slightly expressed and so antibodies directed against it are not appropriate. After transfection, the antibodies used are directed against the Flag linked to the protein, so that the process is more efficient.

Starvation

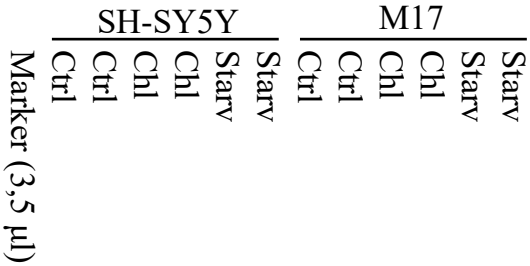
Starvation is a metabolic condition in which cells are deprived of the basal nutrients. The cells are forced to grow without the essential nutrients, leading to cell suffering. Extending the period in which the cells are kept in non-optimal conditions can be useful to drive experiments concerning macroautophagy. In fact, a consequence of the lack of nutrients is the rise of the macroautophagic process. After the starvation experiment, a Western Blot is carried out to visualize the levels of LC3, rising when macroautophagy occurs, and p62, a protein which is degraded when the macroautophagic process takes place. In particular, the starvation medium for this experiment was DMEM without FBS and Hank's Balanced Salt Solution (HBSS), which can guarantee the correct pH and osmotic balance.

The SH-SY5Y cells (2 ml) are grown into a 6-wells plate in a medium containing DMEM and FBS. After 24 hours, in two wells the growing medium is changed with one that does not contain FBS for 22 hours. In this way, cells cannot take advantage from the growth factors of the FBS. After that, the growing medium is changed with HBSS for 2 hours (Starv). Two other wells are then treated with Chloroquine for additional 24 hours (Chl), and the last two wells are control samples (Ctrl). This way, it is possible to test different starvation conditions, evaluating the levels of LC3 and p62.



A parallel experiment was carried out with M17 cells, to compare LC3 levels in two different cell lines.

After the starvation experiment, cell lysis is performed in Sample Buffer (80 µl Laemmli 1X). The steps of the process are described as follows: the growing medium is removed and cells are washed in PBS. After that, the lysis is carried out in Sample Buffer; the cellular lysis solution is picked and boiled for 10 minutes; after 20 minutes in the spin-dryer, the supernatant is separated from the pellet and collected to perform a Western Blot in Running Gel 15% (2,482 ml H_2O, 2,625 ml Acrylamide 40%, 1,75 ml Tris 1.5M, 70 µl SDS 10%, 70 µl Aps 10%, 3 µl Temed) to identify the lanes of LC3 and p62.



Western Blot

The Western Blot, also known as Immuno Blot, is an analytical technique used in molecular biology to detect specific proteins purified or from a cellular extract. The sample material undergoes protein denaturation and gel electrophoresis; subsequently, it is possible to identify different lanes corresponding to the different proteins, which separate due to their dimensions. To make proteins accessible for antibody detection, they are transferred onto a membrane made of nitrocellulose. The membrane is placed into a solution of non-fat, dry milk, which contains several proteins that can saturate the membrane, therefore preventing the antibody from making non-specific bonds to proteins other than its target. This process is called blocking. Afterwards, the membrane is washed in Tween Tris Buffer Saline (TTBS) in three cycles of ten minutes each. Subsequently, the membrane is placed in a solution with the primary antibody for one hour: the primary antibody is able to bind only to its specific target protein. After the incubation with the primary antibody, another three washings are carried out with TTBS. Lastly, the membrane is placed in a solution containing the secondary antibody, which binds to the primary one. The peculiarity of the secondary antibody is the presence of a reporter enzyme, which enhances the signal after a specific reaction therefore allowing the detection of the target protein. Three more final washings are carried out with TTBS.

The primary antibodies are

- αp62 abcam 109012 [R] for p62, in 1:5000 dilution in TTBS
- αLC3 Novus Bio [R] for LC3, in 1:5000 dilution in TTBS
- anti β-actin A1978 Sigma [M] for actin, in 1:20000 dilution in TTBS

The secondary antibodies are

- anti-Rabbit for p62 and LC3, in 1:15000 dilution in TTBS
- anti-Mouse for actin, in 1:80000 dilution in TTBS

The detection is performed in the darkroom. A photographic film is exposed to the results of the Western Blot: the time of exposure to the Western Blot membrane varies for different proteins; afterwards, the photographic film is placed into different solutions for the detection: developing solution, water and fixing solution. Lastly, the film is washed with water again. The film is transparent and allows visualization of the lanes where the protein of interest is positioned. The Western

Blot also allows to infer the molecular weight of the protein, since the corresponding lane can be compared with the molecular weights standards, and to detect the signal intensity, which is a quantitative parameter for the protein level.

A Western Blot was performed to identify the presence of LC3 and p62 in SH-SY5Y cells, which have been previously treated with Torin1 and Chloroquine, to understand their effect in the macroautophagic process. The Running Gel was 15% (2,482 ml H2O, 2,625 ml Acrylamide 40%, 1,75 ml Tris 1.5M, 70 µl SDS 10%, 70 µl Aps 10%, 3 µl Temed) and the Stacking Gel was standard (0,877 ml H2O, 0,25 ml Acrylamide 40%, 0,832 ml Tris 0.3M, 20 µl SDS 10%, 20 µl Aps 10%, 2 µl Temed). The TRIS-Glycine Buffer 1X (3,02 g TRIS, 18,8 g Glycine, 1 g SDS) is used and the voltage is 100 V. 10 µl of the samples are arranged as follows:

	SH-SY5Y								M17			
Marker (3,5 µl)	Ctrl	Ctrl	DMSO	DMSO	Torin	Torin	Chl	Chl	Torin	Torin	Chl	Chl

At the same time, a Western Blot with the same samples was performed in a Pre-Casted gel 4-20%, with the same distribution of the samples in the wells; the only exception is that the marker is loaded together with the first sample (SH-SY5Y Ctrl) since there are only twelve wells. The TRIS-MOPS-SDS (6,06 g TRIS, 10,46 g MOPS, 1 g SDS) is used and the voltage is 100 V. The reason why two different gels were used is to understand which one is the most adequate for this kind of experiments.

The second Western Blot is performed as an experiment to test transfection of exogenous Flag-tagged SPG11 in SH-SY5Y cells with the use of two different transfection reagents, PEI and Lipofectamine 2000. The antibody used for the Western Blot is directed against the Flag-tag allowing the detection of spatacsin. The peculiarity of the antibody is that it is directly associated with Horseradish Peroxidase (HRP), which provides the signal for the detection without the use of the secondary antibody. A Pre-Casted Gel 4-20% is used in this experiment, otherwise it would not be possible to detect SPG11; the TRIS-MOPS-SDS (6,06 g TRIS, 10,46 g MOPS, 1 g SDS) is used and the voltage is 100 V. 10 µl of the samples are arranged as follows:

SH-SY5Y

Marker (3,5 µl)
Ctrl
Ctrl
Ctrl Lipo 1:2
Lipo 1:1
Lipo 1:1
Lipo 1:2
Lipo 1:2
PEI 1:3
PEI 1:3 [HEK]

The primary antibodies are

- anti β-actin A1978 Sigma [M] for actin, in 1:20000 dilution in TTBS
- anti α-Flag-HRP Sigma for SPG11, in 1:10000 dilution in TTBS

The secondary antibody is used for actin: it is an anti-Mouse in 1:80000 dilution in TTBS. SPG11 does not require a secondary antibody: the first antibody is kept for 1.5 hours and then the membrane is washed three times with TTBS.

Results

Induction of autophagy after Torin1 and Chloroquine treatments

Western Blots of LC3-II and p62 were performed to evaluate the effectiveness of Torin1 and Chloroquine treatment in inducing autophagy. Torin1 activates the macroautophagic process (increased autophagy flux), while Chloroquine inhibits the acidification of lysosomes therefore preventing the fusion and degradation of the autophagosomes (accumulation of undegraded material). Analyzing the gels, it is possible to notice that LC3-II levels are increased in Torin1 treated samples, confirming that autophagy is induced (Figure 5). When cells were treated with Chloroquine, LC3-II not only increased with respect to LC3-I (non lipidated form) but also accumulated, indicating that degradation is impaired, as expected. LC3-II is inserted into the autophagosome membrane, the number of which is higher after treatment with Torin1, which stimulates macroautophagy, and Chloroquine, which prevents the degradation of autophagosomes. The control samples did not display any detectable LC3-II, indicating that SH-SY5Y cells do not perform basal autophagy. Looking at the lanes where there are M17 cells, the results are similar, indicating that both SH-SY5Y and M17 cells are adequate models for these types of analyses. Moreover, comparing the two different gels, it is possible to notice that the two lanes of LC3-I and LC3-II respectively are more evident in the gel with 15% of acrylamide. Importantly, the 15% gel allows a better separation between LC3-I and LC3-II.

Concerning p62, its levels appear higher when cells are treated with Chloroquine. In fact, Chloroquine inhibits autolysosome formation, therefore leading to accumulation of p62 as undegraded material inside the autophagosomes. On the other hand, after Torin1 treatment, which stimulates macroautophagy, there is a lower presence of autophagosomes and of undegraded material, which matches with the observed lower levels of p62 compared to controls.

Actin quantities are similar in all the conditions since actin is a ubiquitously-expressed protein inside the cells. This control is important to rule out the possibility that different levels of p62 or LC3-II observed are not due to different protein content.

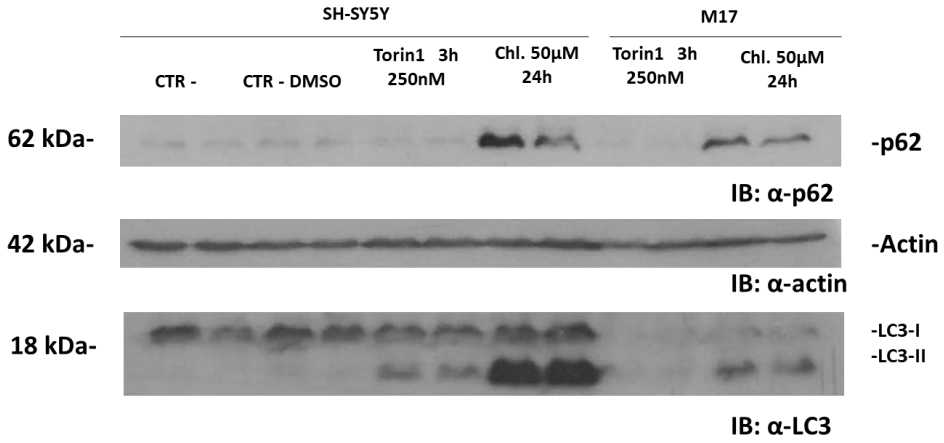


Figure 5 | SH-SY5Y cells treated with Torin1 and Chloroquine. Samples were loaded in 15% acrylamide SDS-PAGE.

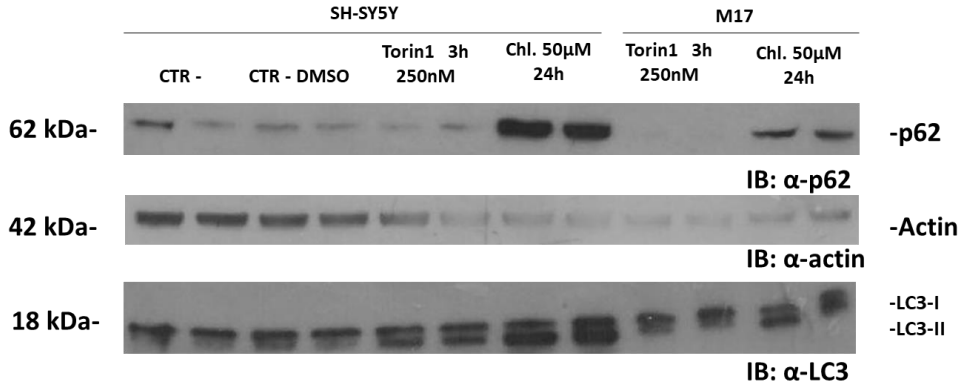
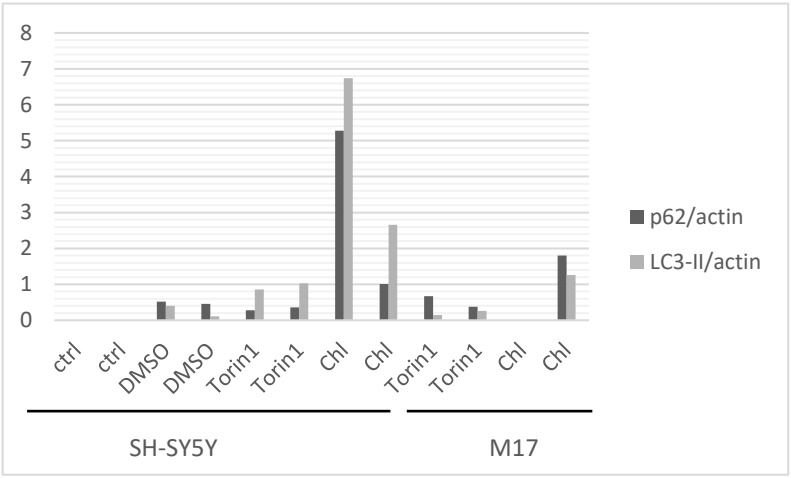
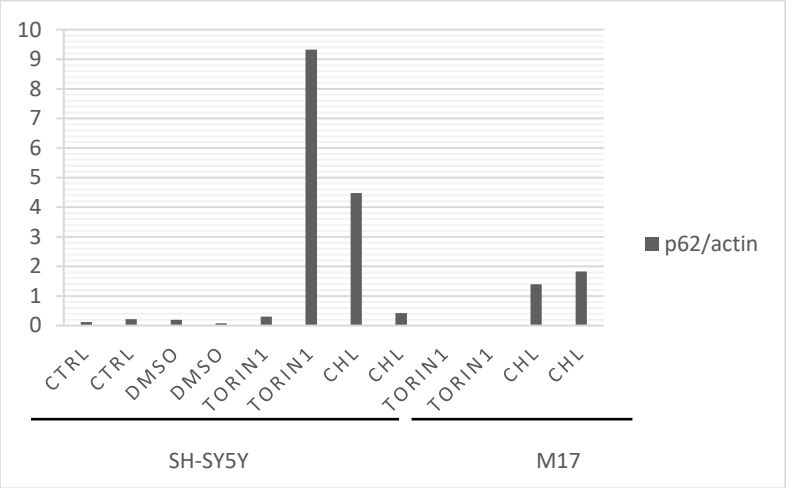


Figure 6 | SH-SY5Y cells treated with Torin1 and Chloroquine. Samples were loaded in 4-20% acrylamide SDS-PAGE.

The levels of p62 and LC3 are normalized over the levels of actin control. Note that quantification was not possible for all samples due to lack of signal on Western Blot. The quantification of LC3-II and p62 derives from one single experiment; to carry out a statistical analysis, future experiments have to be performed (at least three experiments).



Graph 1 | SH-SY5Y cells treated with Torin1 and Chloroquine (15% acrylamide gel).



Graph 2 | SH-SY5Y cells treated with Torin1 and Chloroquine in (4-20% acrylamide gel).

Starvation experiment

Starvation stimulates macroautophagy because cells suffer from a lack of nutrients. When this occurs, cells start degrading the endogenous components to gain metabolic energy through the formation of autophagosomes, which contain the lipidated form of LC3. The Western Blot performed reveals high levels of LC3-II and p62 in SH-SY5Y cells treated with Chloroquine, as expected (positive controls). In fact, Chloroquine causes the arrest of the macroautophagic process before the fusion between autophagosomes and cellular lysosomes, therefore leading to an increase of autophagosomes and LC3-II. Moreover, treatment with Chloroquine shows high levels of p62 because the macroautophagic process stops and autophagosomes accumulate inside the cells. Regarding the samples subjected to starvation, the quantity of LC3-II is almost absent. A similar situation occurs when analyzing p62: starvation samples present slightly visible lanes corresponding to p62. This could be explained due to a technical problem. When proteins were extracted, the samples were highly viscous probably due to an excess of DNA. Thus, before loading into the SDS-PAGE, we triturated the samples with a syringe to break the DNA. As shown in Figure 7, actin is present in the controls, but only traces of LC3 could be detected. Since this experiment is difficult to interpret, I would recommend repeating the experiment in the future to probe the ability of starvation in stimulating macroautophagy in SH-SY5Y cells compared with Torin1.

The amount of LC3-II and p62 were normalized against actin. As expected form previous experiment, actin levels are lower in M17 cells compared to SH-SY5Y cells. The quantification was not possible for all the samples due to weak signals.

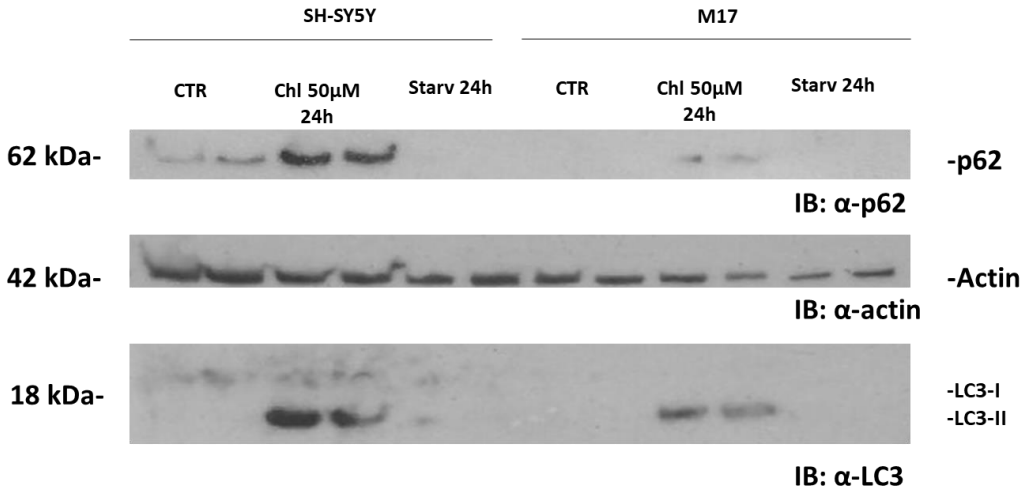
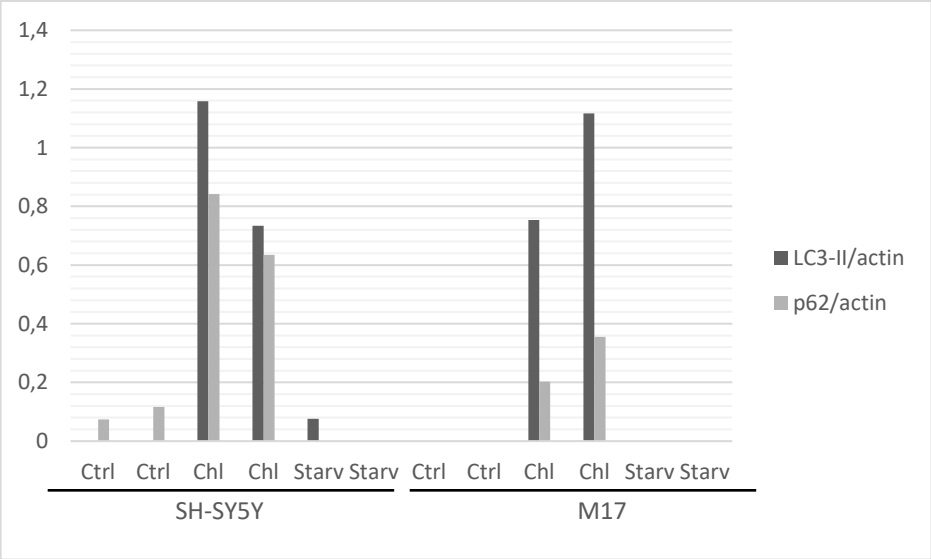


Figure 7 | Starvation experiment in 15% acrylamide gel.



Graph 3 | Starvation experiment in 15% acrylamide gel.

Optimization of Flag-tagged SPG11 transfection

The efficiency of Flag-tagged SPG11 transfection was tested by Western Blot. This allowed us to evaluate the levels of exogenous SPG11 under different transfection conditions. In particular, two transfection agents were tested, PEI and Lipofectamine 2000, in different ratios to evaluate optimal transfection conditions. Based on the Western Blot in Figure 8, transfections with Lipofectamine 2000 in 1:2 ratio resulted the best condition: the protein expressed appear of the expected size (278 KDa), the yield is good and there are no apparent degradation bands. In contrast, transfection with PEI, while it yielded more protein, was associated with presence of a degradation band around 140 KDa (Figure 8). This is not cell type specific since this degradation band was present also in HEK cells. Why PEI causes protein degradation is unknown and future investigations are needed to address this

issue. As expected, the control samples showed no bands corresponding to SPG11, therefore allowing us to confirm that the signal at 278 KDa is specific. Finally, actin loading is almost the same in every sample, confirming that similar amount of total proteins was loaded.

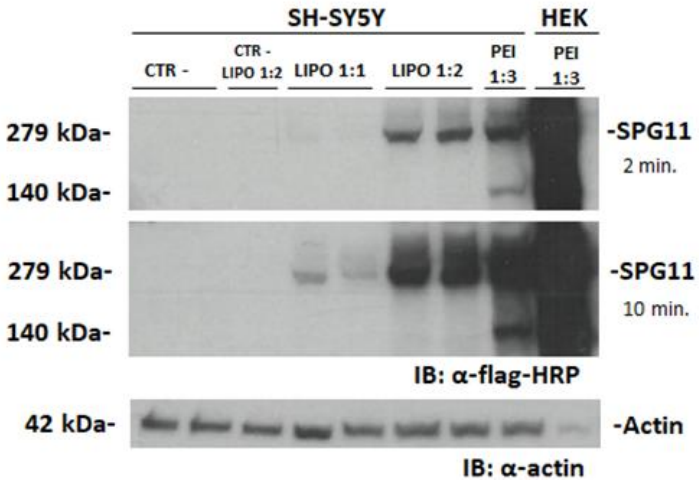


Figure 8 | SH-SY5Y cells transfected with exogenous Flag-SPG11 in 4-20% acrylamide gel.

Discussion

SPG11 mutations represent the major cause of ARHSPs; however, there is little information about the structure, the cellular localization and the function of spatacsin. The lack of robust antibodies against SPG11 prevents us from conducting studies on the endogenous protein. Therefore, the transfection of exogenous Flag-SPG11 by using a specific plasmid will be necessary to evaluate the localization, the function, the interactome and the levels of spatacsin, since antibodies used are directed against the Flag. In this project, we set out to optimize the best transfection conditions which will represent the starting point for future experiments. Our results suggest that treatment with Lipofectamine 2000 in 1:2 ratio is the most suitable condition to transfect Flag-SPG11 in the neuroblastoma SH-SY5Y cell line. In fact, the results show a single band corresponding to the expected molecular weight MW of spatacsin. In contrast, the treatment with PEI appears more aggressive for cells: while band at the expected MW of full-length spatacsin is present, there is also an additional band corresponding to a MW of 140 KDa, probably reflecting a degradation product. In addition, these putative degradation fragment is present also in an other cell line, namely HEK 293T, indicating that it is the transfection reagent rather than the cell line to cause the artefactual product.

With respect to the optimization of autophagy induction, SH-SY5Y cells were treated with Torin1 and Chloroquine and also underwent starvation. The results were analyzed by Western Blot with antibodies against LC3 and p62. It is well known that the levels of LC3 are the results of the balance between its nuclear synthesis and macroautophagic degradation; in particular, its quantity increases in situations where macroautophagy is stimulated, with the lipidated form (LC3-II) increased and inserted into the autophagosome membranes. Treatment with Torin1 induces the macroautophagic process by acting as an inhibitor of mTOR, which is an inhibitor of autophagy. Therefore, an augmented number of autophagosomes results in an increased level of LC3-II. Instead, Chloroquine acts differently: it is a compound that inhibits the degradation of autophagosomes by blocking their fusion with lysosomes. The consequence is that there is an accumulation of autophagosomes inside the cells, which corresponds to an increased level of LC3-II.

Based on the results collected along this internship, we can conclude that treatments with Torin1 and Chloroquine were effective in modulating autophagy, leading, as predicted, to different outcomes, because they intervene at different stages of the macroautophagic process *(red arrows, Figure 9).* Moreover, it is worth noting that starvation also takes part in the process, mimicking the situation where mTOR is inhibited *(green arrow, Figure 9)*, therefore connecting the two experiments described in this project.

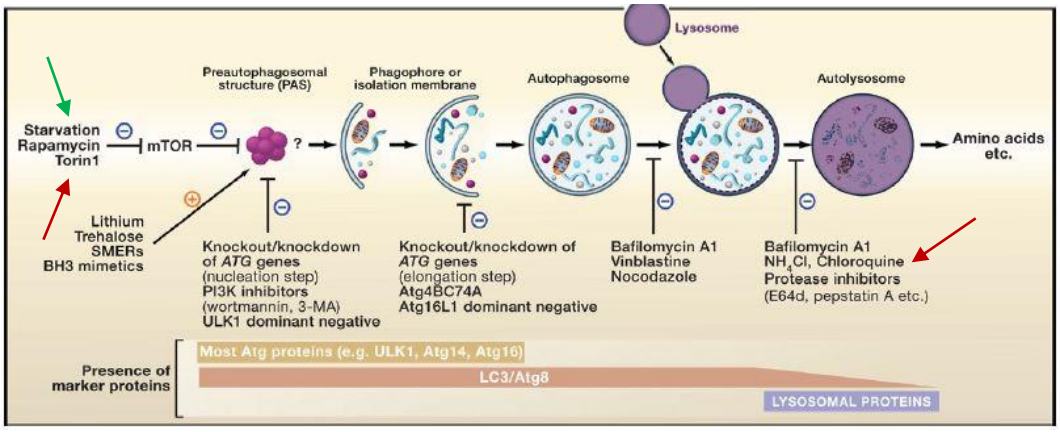


Figure 9 | A partial list of treatments and reagents that modulate autophagy are indicated. From Mizushima *et al.* (2010), *Methods in Mammalian Autophagy Research*, Cell 140.

Regarding the starvation experiment, it stands out that the treatment with Chloroquine induces an augmented quantity of LC3 due to the accumulation of autophagosomes which are not degraded through the fusion with cellular lysosomes. The cells which underwent starvation show very low quantities of LC3-II; the lipidated form of LC3 accumulates inside the autophagosome membrane and can be considered as a reference when analyzing macroautophagy. These are degraded when the cells activate macroautophagy upon a proper stimulus. p62 can be considered as an indicator, too. Its levels are high in samples treated with Chloroquine, because it accumulates inside the non-degraded autophagosomes; it is almost absent in starvation samples, but its role is clear: p62 is degraded when the cells activate macroautophagy, since it accumulates inside the autophagosomes which are degraded when the macroautophagic flux works.

It is therefore tempting to assume that Torin1 treatment is an adequate condition for autophagy induction in cell line SH-SY5Y, since the results obtained mirror the expectances: the corresponding levels of LC3-II are higher than in the other conditions, such as treatment with Chloroquine, which however shows a slight increment if compared to the basal levels in control samples. p62 quantity rises, too, demonstrating that there is more autophagic flux. Starvation shows a very low quantity of the proteins LC3-II and p62 compared to control samples. Starvation induces the macroautophagic process, trying to refill the cells with new metabolic energy which cannot be obtained fully from metabolism because of the lack of nutrients. The low levels of LC3-II and p62 in starvation cells are an indication of the fact that starvation leads to the activation of macroautophagy. Starvation should be considered a valid treatment to study macroautophagy even though more experiments should be performed to verify and fully understand this type of method for autophagy induction.

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