UNIVERSITAT DE BARCELONA

Final Degree Project

Study of the role of the Gq protein in mitophagy

Main field: Biochemistry and Molecular Biology
Secondary fields: Cellular Biology and Genetic Engineering

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Abbreviations

Abbreviations

- **6XDNA:** 6X loading buffer
- **Atg9A**: Autophagy-related protein 9A
- **Bp**: Base pairs
- **DAG**: Diacyl glycerol
- **ddH_2O**: Double distilled water
- **DMEM**: Dulbecco's Modified Eagle's medium
- **FIS1**: Mitochondrial fission 1 protein
- **FBS**: Foetal Bovine Serum
- **GAP**: GTPase Activating Protein
- **GEF**: Guanosine Exchange Factor
- **GPCR**: G-protein coupled Receptor
- **HEK 293 cells**: Human Embryonic Kidney 293 cells
- **IP3**: Inositol trisphosphate
- **LB**: Lysogeny broth
- **LC3**: Microtubule-associated protein 1A/1B-light chain 3
- **MEF cells**: Mouse Embryonic Fibroblasts cells
- **MTOC**: Microtubule Organizing Centre
- **OMM**: Outer Membrane of Mitochondria
- **PCR**: Polymerase Chain Reaction
- **Phox and Bem1 domain**: PB1 domain
- **PIP2**: Phosphatidylinositol 4,5-bisphosphate
- **PLC**: Phospholipase C
- **PINK1**: PTEN-induced kinase 1
- **Rab GAP**: Rab GTPase activating protein
- **Rab7**: Ras-related protein
- **RILP**: Rab-interacting lysosomal protein
- **TBC1D15**: TBC1 domain family member 15
- **TBC1D17**: TBC1 domain family member 17
- **TBS**: Tris Buffer Saline
- **TGN**: Trans-Golgi Network
- **ULK1**: Unc-51 like autophagy activating kinase

1. Abstract/Resum

1.1. Abstract

Mitophagy is the mechanism developed by cells to degrade dysfunctional mitochondria. Alterations of this process have been found in several diseases such as cancer and Alzheimer's disease. Therefore, it is fundamental to study the proteins with a role in mitophagy. So far, proteins that intervene in the biosynthesis of the autophagosome that will engulf defective mitochondria, such as TBC1D15, and others, as RILP, in the transport of the vesicle to the lysosome for degradation, have been described.

Gq is a heterotrimeric G protein known to regulate the physiology of mitochondria, though its function in mitophagy is not well-known. The aim of the present work has been to analyze if Gq may inhibit this process by altering the location of the proteins TBC1D15, TBC1D17 and RILP. Also, it has been compared the effect of wild-type Gq and a constitutively active mutant, Gq(R183C).

Herein, transfection of HEK293 cells followed by staining of mitophagy proteins was used to analyze the effect of Gq on their location. The results presented here are useful to propose a model of how inactive Gq may be displaced from mitochondria by TBC1D15/17 upon induction of mitophagy. In contrast, its activation impedes this displacement and as a consequence TBC1D15 is sent to the periphery, thus impairing this process.

1.2. Resum

La mitofàgia és el mecanisme desenvolupat per les cèl·lules per degradar mitocòndries disfuncionals. S'han observat alteracions d'aquest procés en malalties com el càncer i l'Alzheimer. Per aquest motiu, és fonamental estudiar les proteïnes que regulen la mitofàgia. S'han descrit proteïnes que intervenen en la biosíntesi de l'autofagosoma que posteriorment englobarà la mitocòndria defectuosa, com TBC1D15 i FIS1. Altres proteïnes, com RILP, són bàsiques pel transport de la vesícula cap al lisosoma, on serà degradada.

La proteïna Gq és una proteïna G heterotrimèrica que intervé en la regulació de la fisiologia mitocondrial, però no es coneix bé la seva funció a la mitofàgia. L'objectiu d'aquest treball ha sigut analitzar si pot inhibir-la mitjançant l'alteració de la localització de les proteïnes mitofàgiques TBC1D15, TBC1D17 i RILP. A més, s'han comparat els efectes sobre aquest procés de la forma wild-type i una mutant constitutivament activa (Gq(R183C).

En aquest projecte la transfecció de cèl·lules HEK293 i posterior marcatge de les proteïnes mitofàgiques ha permès analitzar l'efecte de Gq en la seva localització. Els resultats permeten proposar un model en què, en induir-se la mitofàgia, TBC1D15/17 desplaça la Gq inactiva de la mitocòndria; en canvi, la forma activada no pot ser

desplaçada i, conseqüentment, TBC1D15 i RILP són enviats a la perifèria, alterant el procés de mitofàgia.

2. Integration of the different scopes

The development of this experimental work has required the integration of different educational fields, of which the main one has been Biochemistry and Molecular Biology. In order to study the role of Gq protein in mitophagy, it has been essential to perform molecular biology techniques to analyse its effects a cellular and subcellular levels.

Cellular Biology, as well as Genetic Engineering, have also been included as secondary fields. The former has been of great importance to understand the whole process of Mitophagy and its significance in cellular homeostasis. The latter has been useful for the techniques applied, as they have allowed to know the exact sequence of the fusion protein we created, as well as to obtain enough quantity of a specific DNA.

3. Introduction

Mitochondria have been demonstrated to play central roles in several cellular processes including energy production through oxidative phosphorylation, regulation of metabolism and even the response to viral infections (1). Besides, mitochondria are highly dynamic organelles and are characterized by frequent processes of fission and fusion. It is currently believed that these processes function as a quality control of the mitochondrial network (2), which is crucial to maintain a healthy pool of mitochondria. Fission may lead to asymmetric mitochondria: a healthy mitochondrion and one with damaged mitochondrial DNA or low membrane potential. Regarding fusion, it may allow the spreading of metabolites and it is found more frequently among healthy mitochondria.

Due to the importance of this organelle, cells have developed a mechanism to eliminate dysfunctional mitochondria by autophagy, named mitophagy. Mitophagy is tightly regulated and alterations in its ratio or pathways could lead to dysfunctional cells or cell death. Mitophagy is especially important in cells that do not divide, as is the case of neurons. In fact, an accumulation of damaged mitochondria can be especially harmful in neurons, since their energy production is mainly mitochondria-dependant (3). Therefore, this research has focused on analysing the mechanism of regulation of the mitophagy process.

3.1. Induction of mitophagy: PINK1 and Parkin

It has been already proved that in mammals the PTEN-induced kinase 1 (PINK1) and Parkin have a crucial role in triggering mitophagy in mammals (4). PINK1 is a ubiquitin kinase located in the outer membrane of mitochondria (OMM) that under basal

conditions is rapidly imported and cleaved under basal conditions. However, it accumulates upon loss of mitochondrial membrane potential, which leads to Parkin recruitment. Parkin is a cytosolic ubiquitin ligase that, once in the mitochondria, it becomes fully active trough phosphorylation. Then it starts a cascade of mitochondrial proteins ubiquitination; specifically, Parkin adds K48- or K63-linked ubiquitin chains. Some of these ubiquitinated proteins undergo proteasomal degradation (5) or act as signals that lead to lysosomal degradation in an autophagy-dependent manner (6). It has been demonstrated that both PINK1 and Parkin are mutated in Parkinson's disease (3).

The induction of mitophagy through ubiquitination of mitochondrial proteins is possible due to the recruitment of several proteins, including Autophagy-related protein 9A (Atg9A, an autophagosome unit), Unc-51 Like autophagy activating Kinase (ULK1) and Microtubule-associated protein 1A/1B-light chain 3 (LC3); both ULK1 and LC3 are involved in autophagosome biosynthesis and the latter is used as a marker for autophagy.

3.2. Formation of the autophagosome

3.2.1. Rab7

Several proteins are involved in the formation of the autophagosome. The Ras-related protein (Rab7) is a GTPase involved in different cellular processes, and particularly in autophagy and mitophagy. Rab7 is necessary for the maturation of the endosomes and intracellular vesicle transport in eukaryotic cells (7). It is also important in the fusion between the autophagosome and lysosomes. Besides, it has been proved that it acts in a more upstream level, participating in the biogenesis of autophagosomes; more specifically, it directs Atg9A vesicles from the Trans-Golgi Network (TGN) to damaged mitochondria, where Atg9A fusions with LC3-labeled pre-autophagosomes and the expansion of the membrane takes place (8).

In basal conditions, Rab7 is mainly located in lysosomes, whereas induction of mitophagy causes its recruitment to the mitochondria in a GTP-bound-dependent manner. Its activity is tightly regulated by GTPase activating proteins (GAPs, such as TBC1 domain family member 15 or TBC1D15), that catalyse the hydrolysis of GTP, and Guanine Nucleotide Exchange Factors (GEFs) that mediate GDP/GTP exchange.

It has been hypothesized that Parkin recruitment to mitochondria recruits RABGEF1 (a Rab GEF), which in turn activates Rab5 (8). As a consequence, it activates the vacuolar fusion complex MON1-CCZ1, which has been proved to interact mainly with the GDP-bound form of Rab7. The active complex triggers the GDP/GTP exchange of Rab7, leading to its recruitment to mitochondria. This process is impaired by MON1 or CCZ1 siRNA. Figure 1 summarizes the role of Rab7 in the biosynthesis of the autophagosome.

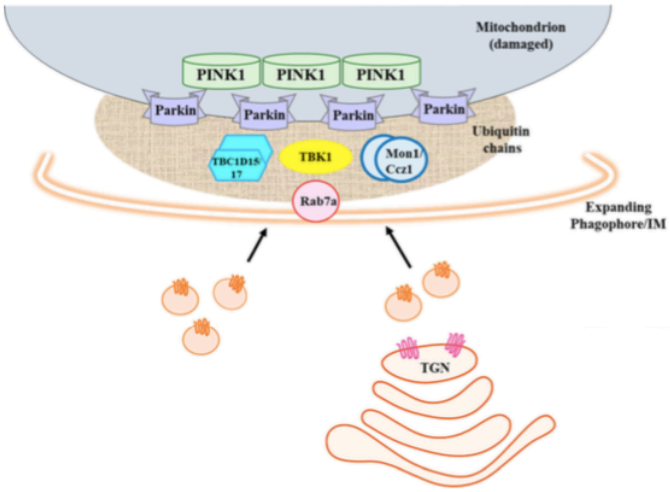

Figure 1. ***Role of Rab7 in mitophagy****. Damaged mitochondria become coated with ubiquitin chains due to the activation of the proteins PINK1 and Parkin. This recruits Rab7 to the mitochondria, where its activity is regulated by GEF MON1-CCZ1 and GAP TBC1D15. In this context, Rab7 promotes the input of ATG9a containing vesicles from the TGN, so that the membrane of the autophagosome can keep growing (9).*

3.2.2. FIS1 and TBC1D15/17

Mitochondrial fission 1 protein (FIS1) is a transmembrane protein located in the OMM; FIS1 may be important for the formation of the autophagosome, and its loss inhibits the degradation of damaged mitochondria, both in vitro and in vivo (4). FIS1 is anchored to the OMM, but has got cytosolic tetratricopeptide-repeat (TPR)-like domains at the N-terminus (10), through which is capable of interacting with TBC1D15 (4). As mentioned before, TBC1D15 is a Rab-GAP for Rab7 that participates in the autophagosome biogenesis through inhibition of Rab7 activity and binding to both the mitochondria (through association with FIS1) and the autophagosome (through LC3) (4). Exogenously expressed TBC1D15 is mainly located in cytoplasm, whereas along with co-expression with FIS1 it is recruited to the mitochondria (9,10). TBC1D15 and FIS1 act co-ordinately to control the autophagosome formation during Parkin-mediated mitophagy but not in starvation-induced autophagy. Moreover, it is thought that, through binding with LC3, they orient the growing isolation membrane to adequately form the autophagosome (9).

These two bound proteins regulate Rab7 activity; in fact, in both FIS1 knock-out and TBC1D15 knock-out mammalian cells, stress induced by mitochondrial inhibitors causes an accumulation of LC3 in a PINK1-dependent way (11) and prevents Atg9A from being assembled. The excess of LC3 colocalizes less with the mitochondria, which indicates that in these conditions damaged mitochondria cannot be efficiently engulfed (8). This accumulation is suppressed by Rab7 siRNA, indicating that it is due to an excessive activation of Rab7. Besides, inhibition of Rab7 leads to mitochondria-lysosome contacts untethering (12); broadly, all these data suggest that degradation of mitochondria is impaired in the absence of Rab7 (4) and that mitophagy is blocked not only in autophagosome biogenesis but also in later steps.

TBC1D17 (TBC1 domain family member 17) is another member of the TBC family that also acts through inhibition of Rab proteins such as Rab8; it has been hypothesized that TBC1D17 is capable of regulating both autophagy (13) and mitophagy (4). Furthermore, it can interact with FIS1 and ATG9A and both TBC1D15 and TBC1D17 form homodimers and heterodimers to each other (4). A proposed model of how mitophagy is induced and the autophagosome is formed is shown in Figure 2.

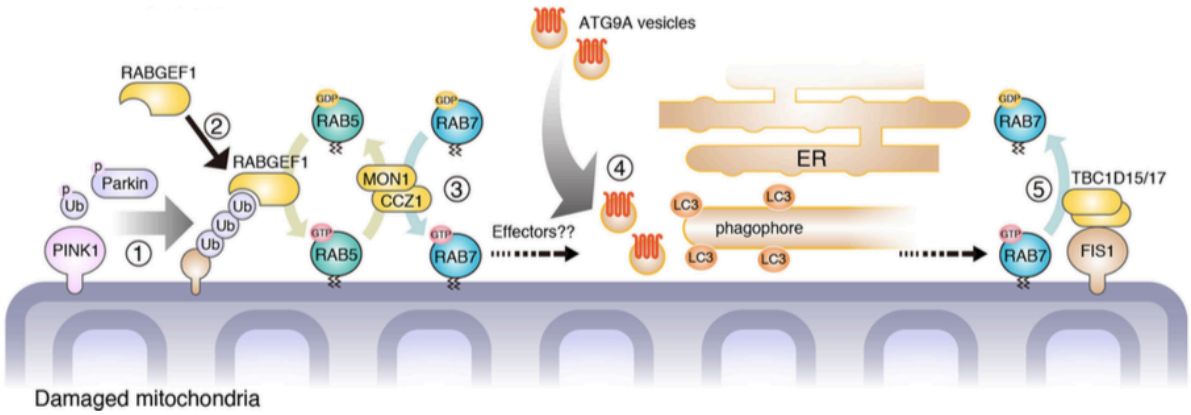

Figure 2. ***Proposed model of Parkin-induced mitophagy regulated by endosomal Rab cycles*** *(8).*

3.3. Transport and degradation of the autophagosome

Once the autophagosome is formed, transport proteins come into play. Dynein and kinesin are two families of cytoskeletal motor proteins that move along microtubules. The former moves toward the microtubule organizing centre MTOC (named the minus-end), whereas the latter is sent to the periphery (plus-end). RILP (Rab-interacting lysosomal protein) is a Rab7 adaptor protein that links Rab7-containing vesicles to dynein (Figure 3) (14,15). Through this interaction RILP allows the transport of several Rab7-containing vesicles including phagosomes and melanosomes (16,17) to the microtubule organizing centre (MTOC) (15). In fact, silencing of RILP has been demonstrated to impair autophagosome fusion to lysosomes (14).

In basal conditions, it has been found a certain percentage of cleaved RILP, which has been suggested to be a consequence of endogenous stress response (18). It generates a C-terminal fragment (containing amino acids 80 to 360) responsible for its interaction with Rab7 but not capable of mediating inward trafficking. This cleaved RILP, which misses the 80 N-terminal amino acids, is mainly found in the periphery, whereas full-length RILP is in the perinuclear region. Nevertheless, deletion of amino acids 1-80 promotes transport towards the plus-end (cell periphery) (18).

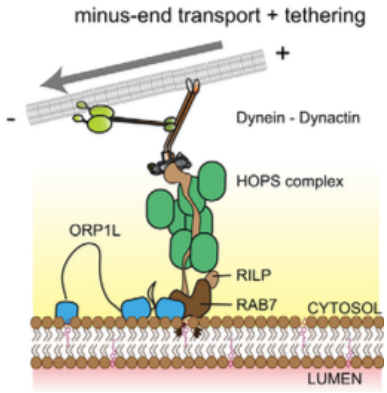

Figure 3. ***RILP acts as a linker between Rab7 and Dynein to transport Rab7-containing vesicles to the MTOC****. Adapted from Janssen H et al (14).*

The autophagosome finally fusions with a lysosome. The hydrolytic enzymes of this organelle degrade the components that have been engulfed by the autophagosome.

3.4. Gq

The heterotrimeric Gq protein is located both in membranes and at the cytosol and has an important role in several physiological processes. Downstream of several GPCRs, its stimulation induces the downstream activation of the phosphoinositol phospholipase C ζ (PLC ζ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol trisphosphate (IP3), both of which act as second messengers.

Some G-protein coupled receptors (GPCRs), including GPCRs coupled to Gq, have been demonstrated to regulate autophagy through its capacity to detect nutrients (19). The group of Dr. Aragay is currently studying its role in the regulation of mitophagy (20). Preliminary results show that it can interact with proteins (FIS1 as well as TBC1D15/17 and RILP) involved in mitophagy.

The group has identified what could be a Phox and Bem1 (PB1) domain in TBC1D15/17. A PB1 domain had been previously identified in the G α q (21). These domains are known for their capacity to induce protein-protein interaction among proteins carrying those domains. Depending on the amino acids that form this domain, it can be acidic or basic; TBC1D15 seems to have the basic PB1 domain while Gq has got the acidic one; these domains would allow their interaction to form a macromolecular complex.

These domains are known for having lysine residues that are crucial for the interaction; therefore, taking this into account, I. Izquierdo (doctoral student from the group) produced two mutants in two lysine residues: K136A and K142A; he demonstrated that TBC1D15-K136A tends to bind more tightly to Gq than the wild-type form, whereas the latter is less bound to Gq. To be able to study the role of this protein in mitophagy and autophagy it has also been developed a constitutively active mutant of Gq, Gq(R183C) (22).

Figure 4 summarizes how Gq is thought to allow mitophagy upon Parkin induction of this process.

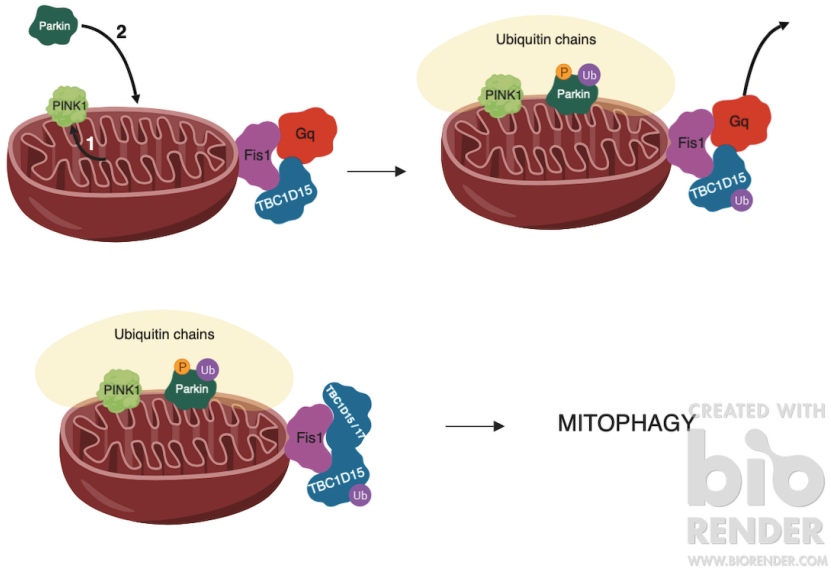

Figure 4. ***Role of Gq in mitophagy****. In basal conditions both TBC1D15 and inactive Gq interact with FIS1. It has been hypothesized that when Parkin is recruited to the mitochondria by PINK1, inactive Gq leaves the mitochondria. Then, TBC1D15 can form homo or heterodimers and, together with other proteins such as FIS1, regulate the biosynthesis of the autophagosome. Created with BioRender.*

4. Hypothesis and objectives

Preliminary results obtained in the laboratory suggested that Gq is capable of altering the location and function of certain mitophagic proteins. The aim of this project was to gain a better knowledge of how Gq regulates mitophagy and study if can inhibit this process by displacing TBC1D15 and RILP. Related to this, the objectives of this project were:

- Determine how activation of $G\alpha_q$ affects the location of TBC1D15, comparing the expression of $G\alpha_q$ with its permanently activated form Gq(R183C).
- Determine if $G\alpha_q$ co-localizes with TBC1D15.
- Study how two different mutations in TBC1D15 affect Gq interactions and its localization.
- Determine how Gq affects the location of TBC1D17, comparing the expression of $G\alpha_q$ with its permanently activated form Gq(R183C).
- Design a strategy to obtain a fusion protein of RILP that allows to differentiate between its location at the lysosomes and at the cell periphery.

5. Materials and methods

5.1. Materials

Cellular lines. HEK293 are derived from human embryonic kidney cells; they were purchased from ATCC.
For heat shock transformation, DH5α competent cells have been used. They were available and have been obtained from the research group.

Plasmids. The plasmids that have been used are: pCMV3-HA-TBC1D15, pCMV3-TBC1D15-K142A, pCMV3-TBC1D15-K136A, pCMV3-Flag-TBC1D17, pcDNA3-$G\alpha_q$ -WT, pcDNA3-$G\alpha_q$ -R183C, pcDNAI-$G\alpha_q$-GFP, pcDNAI-$G\alpha_q$-R183C-GFP and pmCherry-N1. They were available and have been obtained from the research group.

Antibodies

- Anti-HA High Affinity Monoclonal Antibody, from rat (ThermoFisher).
- Anti-Sodium Potassium ATPase Monoclonal Antibody (Plasma Membrane Loading Control), from rabbit (Abcam).
- Anti $G\alpha_q$ Monoclonal Antibody, from mouse (Santa Cruz).
- Monoclonal-Anti-Flag M2-FITC Monoclonal Antibody, from mouse (Sigma-Aldrich).
- Alexa Fluor 647 anti-Mouse IgG (H+L) Polyclonal Secondary Antibody, from goat (ThermoFisher).
- Alexa Fluor 488 anti-Rat IgG (H+L) Polyclonal Secondary Antibody, from donkey (ThermoFisher).
- Alexa Fluor 568 anti-Rabbit IgG (H+L) Polyclonal Secondary Antibody, from goat (ThermoFisher).
- Alexa Fluor 568 anti-Rat IgG (H+L) Polyclonal Secondary Antibody, from goat (Abcam).

Solutions

- Agarose gel: composed of 1% (W/v) agarose, 1:10000 SYBR Safe DNA gel stain and TAE.
- Bovine Serum Albumin (BSA) 10% IgG free**:** purchased from Sigma-Aldrich.
- Cell culture medium (DMEM-10%FBS): composed of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of inactivated FBS (Foetal Bovine Serum), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Inactivation of FBS consists on maintaining FBS at 37 °C for 30 min.
- Formaldehid 4%: composed of 4% formaldehid and PBS 1x.
- Lysogeny Broth (LB): nutrient-rich broth composed of 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl.

- PCR Purification kit: purchased from Qiagen. It includes a Binding buffer (to adjust DNA binding condition), Wash Buffer (to wash silica membrane) and Elution Buffer (to elute DNA).
- PBS: composed of 137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4.
- TAE: a buffer solution that contains 40 mM Tris-acetate (ph 7,6) and 1 mM EDTA.
- Tris Buffer Solution (TBS): an isotonic non-toxic buffer that maintains pH at 7-9,2 range. Composed of 120 mM NaCl and 50 mM Tris-HCl.
- TBS-NP-40: composed of 0,5% NP-40, 1% BSA IgG free and TBS 1x.
- TBS Tween 1x: composed of 1% (v/v) Tween-20 and TBS 1x.
- 6X loading buffer (6XDNA): composed of 10 mM Tris-HCl (pH 7,6), 0,25% bromophenol blue, 0,25% xylene cyanol FF and 60% glycerol 60 mM EDTA.

Reagents

- FuGENE® HD Transfection Reagent: used to transfect cells; purchased from Promega.
- Prolong Gold mounting medium: to mount the slides; purchased from Thermofisher Scientific.
- Trypsin-0,25%-EDTA: purchased from Biowest.

Primers for PCR

The two pairs of primers that have been used in this work are shown in Table 1.

Name	Sequence
RILP Forward	CGAATTCTGATGGAGCCCAGGAGGGCGGCG
RILP Reverse	CGGTACAGTGGCCTCTGGGGCGGCTGA
GFP Forward	GCTGAAGCTTATGGTGAGCAAGGGCGAGGAGC
GFP Reverse	GCGAAAGCTTCGAACTCCAGCAGGACCATG

Table 1. ***Primers used for PCR***

5.2. Methods

Cell culture

Cells were cultured in plaques in DMEM supplemented with 10% FBS in a humidified atmosphere at 37 °C and 5% CO2 and type 2 laminar flux cabin. When there was a 70-80% confluency, cells were transferred to a new plaque through a 1/10 dilution in complete DMEM-10%FBS medium. For this purpose, cells were trypsinized; firstly, medium was aspirated and the plate was washed with 5 mL of PBS, which was then removed. 1-1,5 mL of a solution of trypsin-0,25%-EDTA was added and left for 1-2 min at room temperature for the cells to disassociate.

A rutine microscope was used to confirm dissociation of the cells from the plate, and then trypsin solution was inactivated with 10 mL of complete DMEM medium. The

medium was mixed with the help of a pipette, and finally 1 mL was added to a new plaque containing 10 ml of medium.

Transfection of cells using FuGENE

HEK293 cells were plated in p12 plates the day before transfection. Transfection was done with FuGENE® following manufacturer instructions. After trypsinization, cells were counted mixing 10 μl of cell solution with 10 μl of TrypanBlue and using the Countless II FL from Life technologies. The cell concentration was used to calculate the necessary volume to get to $8 \cdot 10^4$ cells per well, considering that the content of each well was 1 mL of cell medium. Cells were kept at 37 °C. After 24 h, cells were transfected. For this, two solutions were prepared for each well: a) 50 μl of cold DMEM medium (without antibiotics and FBS) was mixed with 3 μl of FuGENE®; and b) 50 μl of cold DMEM medium was mixed with 0,8 μg of total DNA. Both solutions were mixed and after 30 min were added to the cells drop by drop, making sure that it was distributed throughout all the surface of the well, as the transfection starts immediately.

Immunofluorescence

For immunofluorescence experiments, a coverslip was placed in each well before plating and transfection. A day after, cells were washed with 1 mL of PBS and then fixed with 4% formaldehyde in DMEM-10%FBS at 37 °C for 10-15 min. These conditions were used to preserve the morphology of mitochondria.

Once cells were fixed, they were washed three times with 1 mL of TBS. Then, a solution of TBS-0.1% Tween was added for permeabilization of the cells. Cells were left in a shaker for 10 min. The wash step was repeated twice. For blocking, cells were treated for 10-15 min with TBS 1% BSA and 0,5% NP-40 before the first antibody solution was added. The antibodies were dissolved in the same blocking solution (TBS with BSA and NP-40), with the recommended dilution.

For the incubation, the coverslips were moved to a hand-made humidifying chamber to avoid cells from drying. A solution of the primary antibody (60 μl) was added to each cover slip (as a bubble) and cells were left for 1 h at room temperature and in the dark to guarantee the binding of the antibody to the antigen. After that time, cells were moved back to the p12 plate, where they were rinsed three times with 1 ml of TBS-0,1%Tween followed by incubation with the secondary antibody. The secondary antibody was diluted at 1/200 (final concentration: 0,5%) in the blocking solution. In the cases where several antibodies had to be labelled, cells were treated first with one of the primary antibodies followed by its secondary antibody; the process was repeated with the other pair of antibodies, always washing the coverslips with TBS-0.1%Tween between each step.

To finalize, cells were rinsed three times with TBS. Slides were mounted with 5-10 μl of ProlongGold mounting medium at room temperature and kept 24 h at room temperature before storage at 4 °C or image acquisition.

Capture of images

To visualize images, a Zeiss LSM780 Confocal Microscope was used with 63x 1,2 NA oil immersion lens. Depending on the experiment, three or four lasers were used: blue (DAPI, for the nucleus), 568 nm (red) and 444 nm (green); and 647 nm (far red) when necessary.

Most images were taken with 5 Z-slices of 0,5 μm each, usually at 1,2 zoom. However, in some cases Tile scan mode was used, taking 3x3 images of the area, providing a bigger field of cells.

Data analysis

Images were processed using the image processing package Fiji with the corresponding adjustment of colour, contrast and brightness. Depending on the case a single slice was used or a z-project was performed to obtain a unique picture that contained the 5 original slices.

The fluorescence profile was also studied utilizing ImageG (Fiji) to analyse where each one was located within the cells.

To check and ensure significance of the results, each experiments was performed three times each in different days.

Obtaining the fusion protein

To study the effect of Gq on RILP location it was necessary to create a fusion protein: mCherry-RILP-GFP. The reason to utilize two fluorescent proteins was to be able to follow the location of RILP between lysosome and other vesicles. The rationel is: if RILP is present in a transport vesicle both green and red fluorophores will be active and the presence of RILP will be followed by a yellow colour in a micrograph. If RILP is present at lysosomes the acidic pH will kill the GFP fluorescence and therefore only red will appear. Moreover, RILP has been described to be cleaved in the N-terminal region; the C'-terminal peptide is transported to the lysosome, where it is degraded. As a consequence, it was necessary to analyse the location of both fragments separately. Two fluorescent proteins were chosen, of which mCherry, that was tagged to the N'-terminal fragment, resists degradation by lysosomal enzymes.

Once the strategy had been decided, vectors and appropriate restriction enzymes were chosen. N'-mCherry vector was used, and the enzymes were EcoRI and KpnI. Besides, a third enzyme (HindIII) was selected to be able to introduce GFP once the m-Cherry-RILP plasmid had been created at the C-terminal tail of RILP.

The protocol to obtain the fusion protein was as follows:

1. PCR with two primers containing the sequence recognized by EcoRI (Forward primer) and KpnI (Reverse primer), to obtain enough amount of RILP with both sequences.
2. Digest the vector and the insert with EcoRI and KpnI.
3. Ligate the insert with the vector (mCherry-RILP).
4. Transform DH5α bacteria by heat-shock.
5. Select the cultures with the right plasmid: through PCR mini-prep, which helps with the selection of the expected colonies, followed by agarose gel and finally sending it to sequence.
6. Digest the vector (pC'-EGFP) and perform a PCR of mCherry-Rilp using two primers that contain HindIII.
7. Digest the insert with HindII, purify it and ligate it with the vector.
8. Transform bacteria through Heat shock.
9. Select the cultures with the appropriate plasmid, as explained in step 5.

Figure 5 shows a scheme of the whole protocol.

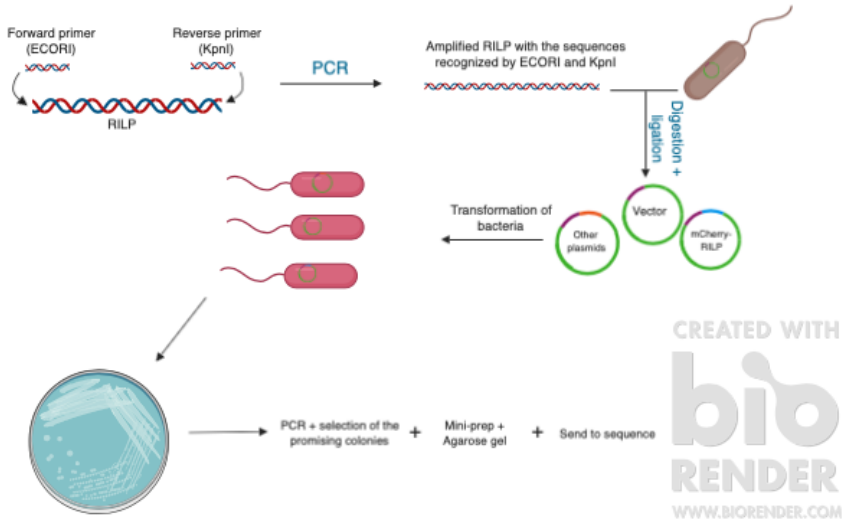

Figure 5. ***Protocol to obtain mCherry-RILP-GFP****. Created with BioRender.*

PCR (Polymerase Chain Reaction)

Once a forward and a reverse primer had been designed and ordered to Sigma-Aldrich, RILP was amplified by PCR. To perform a PCR, the reagents were unfrozen on ice and added in the following order:

1. Double distilled water (ddH_2O) to a final volume of 50 µl.
2. 10 µL buffer 5x HF Fusion.
3. 1,25 µl 200 µM dNTPs.

4. 1,5 μl DMSO. The significant percentage of G-C nucleotides made it necessary to add 1,5% DMSO to the PCR mixture, as it improves the reaction.
5. 1 μl of each primer (10 μM).
6. 1 μl of 1μg/μl DNA.
7. 0,5 μl Fusion High Fidelity DNA polymerase I.

The PCR thermocycling protocol had 8 steps (see Table 2):

Temperature	**Step**	**Time**
1. 96 °C	Breaking bacteria wall	10 min
2. 98 °C	Initial denaturation	30 s
3. 98 °C	Hydrogen bounds breakage	10 s
4. 58-62 °C	Primer hybridizing	30 s
5. 72 °C	DNA synthesis	30 s
6. Repeat steps 3-5 35 times		
7. 72 °C	Final extension	10 min
8. 4 °C	Hold	Up to 24 h

Table 2. **Steps followed to perform PCR**

After the PCR reaction, the obtained DNA was cleaned using the QIAquick PCR Purification Kit.

Once the PCR fragment was obtained it was digested following provider's instructions. Its website indicates which buffers are more suitable for each restriction enzyme. In this case, Buffer 1 + BSA was used for KpnI, and Buffer 2.1 for ECORI. However, in some experiments, buffer 2.1 was used with both enzymes (both digestions at the same time) as KpnI also works with this buffer although efficiency is slightly lower. Between each digestion, a PCR clean-up was performed to remove the buffer, as the mixture of buffers could cause precipitation or incompatibilities.

The proportion of the reagents where:

- 1 μg DNA (to have enough material for the necessary steps)
- 0,6 μl of each enzyme
- 10% Buffer
- 1% BSA (for Buffer 1)
- ddH_2O to get the desired final volume (usually 20 or 50 μl)

Obtaining the vector

The bacteria stocks with the different plasmids were kept frozen as glycerol stocks at -80 °C. The bacteria were recovered by using a pipette tip to scratch the frozen stock, add it to in bacteria culture tubs with 3 ml of LB and the appropriate antibiotic and

incubated at 37 °C over-day shaking (over-day culture). The over-day culture (1 ml) was added to a 200 ml of LB with the appropriate antibiotic in a 1 L erlenmeyer and left at 37 °C overnight. Finally, plasmids were purified following the PCR clean-up protocol previously mentioned.

Ligation

Once both the DNA and the vector were digested, ligation was performed, taking into account the proportion of base pairs (bp), which is proportional to the amount in mol. In this case, the vector had 4722 bb, whereas the insert 1206 bb; therefore, the proportion was 3,9. Besides, it was decided to increase the proportion of insert versus vector in order to increase the probability of ligation.

For the ligation, T4 ligase (purchased from Biolabs) was used. Insert and vector were mixed on ice with the Buffer ligase and ddH2O to get to 20 μl. Finally, the enzyme was added, and it was pipetted up and down and microfuged briefly. It was left overnight at 16 ºC.

6. RESULTS

6.1. Activated Gq sends TBC1D15 to the periphery

As mentioned before, previous experiments of the group suggest that Gq is present in a macromolecular complex with the GTPase protein TBC1D15 (20). In fact, TBC1D15 was fished out of a proteomic analysis searching for mitochondrial partners of Gq. It is still not known how this interaction affects the role of TBC1D15 in mitophagy and, specifically, whether Gq regulates the action of TBC1D15 or vice versa. In order to further study the effect of this interaction we sought to explore the cellular distribution of Gq or its constitutively active mutant Gq(R183C). For that, it was decided to express TBC1D15-HA in HEK293 cells in these two conditions.

The location of TBC1D15 was followed by staining with specific anti-HA antibodies. Figure 6 shows representative images of this experiment, where TBC1D15 can be observed in green and Gq in magenta. To label cytoplasmic membrane a specific antibody against the membrane ATPase protein (ATPase) was utilized, shown in red in the figure. As seen before (previous group experiments and (23)), overexpression of TBC1D15 alone in cells appears mainly in the cytoplasm (green staining). Co-expression with wild-type Gq did not change much the pattern of expression of TBC1D15 that was found at the cytoplasm of the cell. In contrast, in the presence of the constitutive active mutant, TBC1D15 was located at the periphery of the cell, close to the staining of the ATPase plasma membrane marker, but it did not completely co-localize.

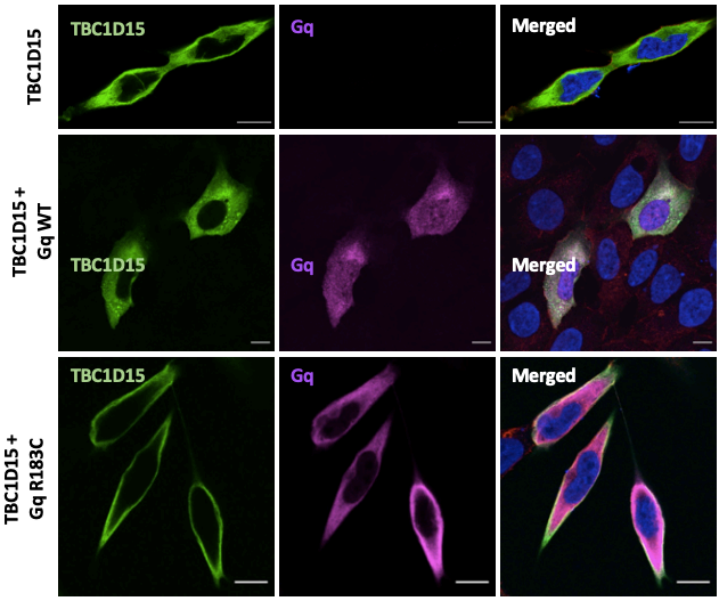

Figure 6. ***TBC1D15 is recruited to the periphery with constitutively activated Gq but not with wild-type Gq****. Confocal micrographs of HEK 293 cells were co-transfected with TBC1D15-HA alone or with Gq wild-type or Gq (R183C). Twenty-four hours after transfection cells were fixed and stained with two different antibodies: anti-HA (green), antiGq (magenta) and anti-ATPase (red). One layer of the stack is shown. Images are representative of the majority of the cells. Scale bars, 10μm.*

Figure 7 shows the fluorescence line profile of a cell transfected with TBC1D15 (green) and wild-type Gq (magenta) (as in Figure 6). It is clear that in these conditions TBC1D15 is located in the cytosol, as expected, which enforces the hypothesis that wild-type Gq is not capable of neither altering the location of TBC1D15 nor sending it to the periphery. Interestingly, Gq is located in the cytosol and aggregates ("dots") of this protein can be seen near the nucleus, probably in the mitochondria. This can be appreciated in Figure 8, that shows a cell transfected also with TBC1D15 and Gq WT; Gq colour has been changed to red to better image the colocalization. Part of its cytoplasm has been amplified so as to better observe Gq aggregates. It is possible to see that some of the dots, indicated by arrows, coincide with the aggregates formed by TBC1D15, suggesting that Gq colocalizes in aggregates or is close to the TBC1D15 protein.

Figure 9 shows the fluorescence line profile obtained from a cell that has been transfected with both TBC1D15 (green) and Gq(R183C) (magenta). In this case the line profile of TBC1D15 is high at the cell periphery and that Gq does not co-localize with TBC1D15. Altogether, these results corroborate that the activation of Gq induces a re-localization and concentration of TBC1D15 at the cell periphery.

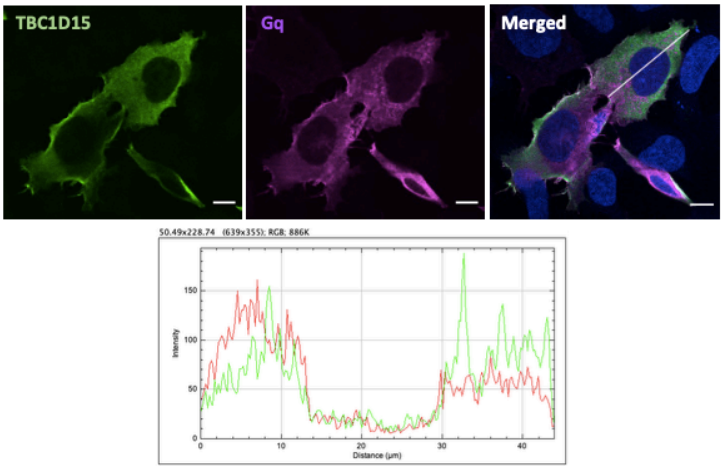

Figure 7. ***Fluorescence line profile proves that Gq wild-type is not capable of altering the location of TBC1D15****. Confocal micrographs of HEK 293 cells were transfected with TBC1D15-HA (green) and Gq wild-type (magenta). Twenty-four hours after the transfection cells were fixed and stained with two different antibodies: anti-HA (green) and antiGq (magenta). In Merged image, a white line displays where exactly the Fluorescence line profile has been analyzed (TBC1D15 in green, Gq in magenta). One layer of the stack is shown. Images are representative of the majority of the cells. Scale bars, 10µm.*

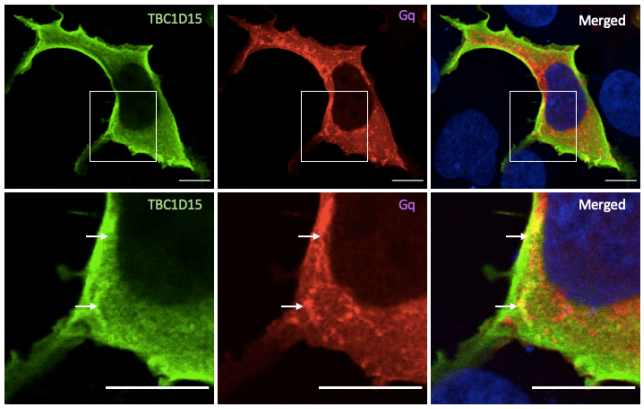

Figure 8. ***Gq forms aggregates that resemble the mitochondria and coincide with some of the dots of TBC1D15****. Confocal micrographs of HEK 293 cells were transfected with TBC1D15-HA (green) and Gq wild-type (red). Twenty-four hours after transfection cells were fixed and stained with two different antibodies: anti-HA (green) and antiGq (red). Pictures at the bottom are the magnified images of boxes in pictures at the top, respectively. The arrows indicate where the dots formed by TBC1D15 coincide with Gq aggregates (yellow). One layer of the stack is shownImages are representative of the majority of the cells. Scale bars, 10µm.*

In order to further study the effect of Gq on TBC1D15 cellular distribution, the expression of two Gq-chimeric proteins that are bound to GFP was analysed. For this, cells were transfected with TBC1D15 (green) and Gq-GFP WT (magenta) or TBC1D15 and Gq-GFP (R183C) (magenta) (Figure 10). The GFP-bound Gq form shows a cytoplasmic location similar to wild-type Gq. However, it presents a dotty pattern that resembles the mitochondria staining in agreement with previous observations from the group (24). What it is particularly interesting is that, when bound to GFP, both the wild type and the constitutively active mutant forms trigger the redistribution of TBC1D15 at the cell periphery. Moreover, again Gq remains in both cases at the cytoplasm. These

results together with the previous ones suggest that the presence and/or activation of Gq at the mitochondria, displaces TBC1D15 from it.

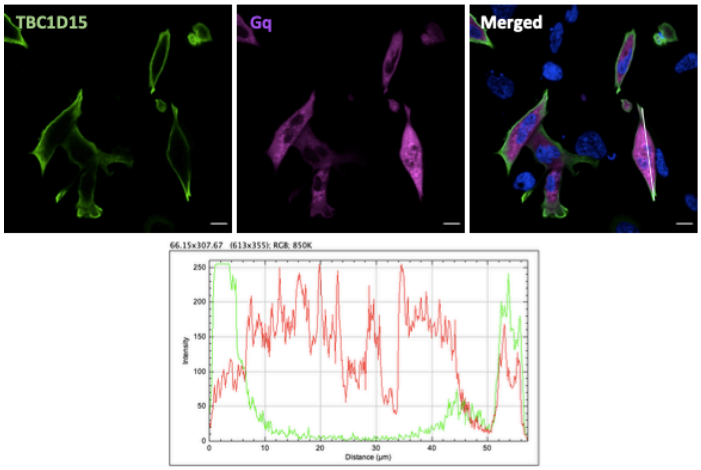

Figure 9. ***Fluorescence profile proves that Gq R(183C) alters the location of TBC1D15****. Confocal micrographs of HEK 293 cells were transfected with TBC1D15-HA (green) and Gq(R183C) (magenta). Twenty-four hours after transfection cells were fixed and stained with two different antibodies: anti-HA (green) and antiGq (magenta). In Merged image, a white line displays where exactly the Fluorescence line profile has been analyzed (TBC1D15 in green, Gq in red). One layer of the stack is shown. Images are representative of the majority of the cells. Scale bars, 10µm.*

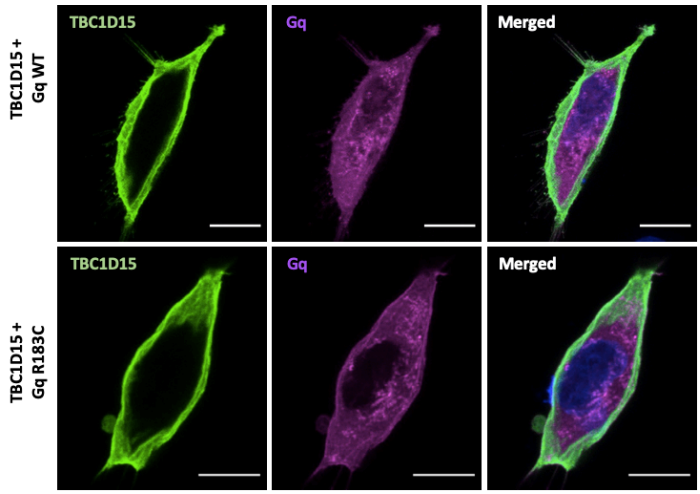

Figure 10. ***Expression of Gq-GF, both wild-type or R(183C) forms are capable of causing the transport of TBC1D15 to the periphery****. Confocal micrographs of HEK 293 cells were transfected with TBC1D15-HA (green) and Gq(R183C) (magenta). Twenty-four hours after transfection cells were fixed and stained with two different antibodies: anti-HA (green) and antiGq (magenta). The nucleus is shown in blue.The overlay of stacks is shown. Images are representative of the majority of the cells. Scale bars,10µm.*

6.2. TBC1D15 mutants with a lower affinity for Gq do not translocate to the cell periphery

Molecular biology studies of the interaction between TBC1D15 and Gq proof that Gthey interact through a region of 200 amino acids located at the N-terminal region of Gq.

Using homology studies, it has been identified a domain with close resemblance to PB1. A further mutagenesis analysis has found one lysine residue, K142, that upon mutated to alanine (K142A) decreases the TBC1D15 interaction with Gq. Therefore, it was decided to study the effect of this mutation on the expression pattern of TBC1D15. It also utilized a control mutation on K136 (K136A). Figure 11 displays the location of TBC1D15 in cells transfected with TBC1D15 wild-type, K136A and K142A (respectively) together with Gq(R183C). In order to visualize a broader field of cells, these images were taken as a Tile scan (3x3) with the same optical resolution. Observation of the pattern of TBC1D15-K142A mutant in the presence of active Gq denotes that this mutant concentrates less in the cell periphery, thus being more located in the cytosol. By contrast, the K136A mutant form of TBC1D15 is mostly found at the cell periphery in the presence of active Gq, as the wild-type TBC1D15. Therefore, these results suggest that the interaction of TBC1D15 with Gq is needed for the displacement of the protein to the cell periphery.

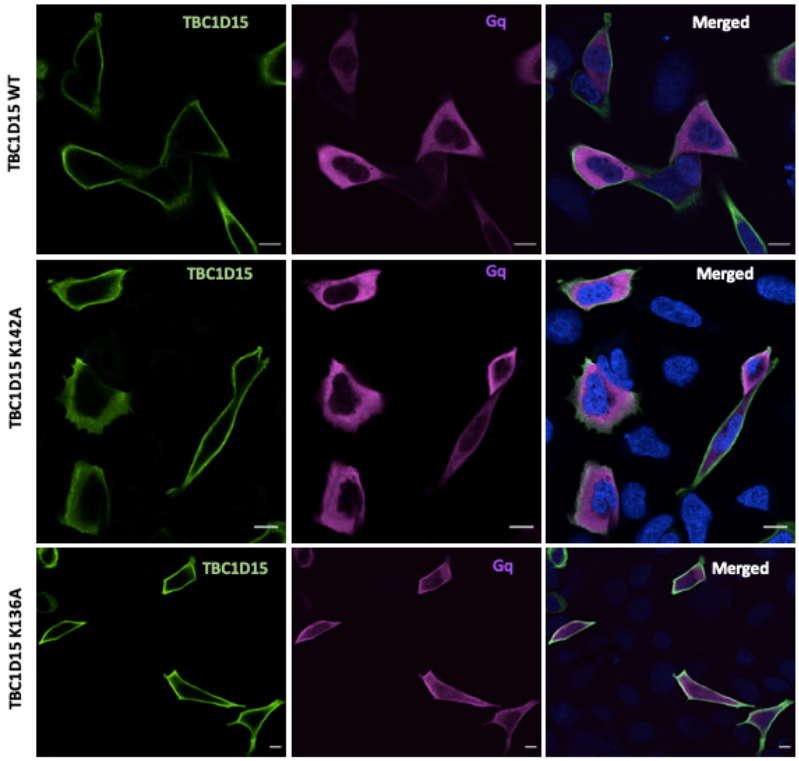

Figure 11. ***K136A TBC1D15 mutant is more directed to the periphery than K142A****. Confocal micrographs of HEK 293 cells were transfected with TBC1D15 WT or the mutants K136A or K142A (green) and Gq(R183C) (magenta). Twenty-four hours after the transfection cells were fixed and stained with two different antibodies: anti-HA (green) and antiGq (magenta). The nucleus is shown in blue. One layer of the stack is shown. Images are representative of the majority of the cells. Scale bars,10μm.*

6.3. Gq does not alter the location of TBC1D17

TBC1D15 is a GTPase for Rab7, as mentioned before (8,9), and it is known to dimerize with another GTPase named TBC1D17 (4). Both proteins are also known to be involved

in the process of mitophagy, but the reason why the heterodimer is needed is not known yet. Our group previous results have demonstrated that both proteins were found in immunoprecipitates of Gq and the presence of Gq in immunoprecipitates correlates with a decrease dimmer formation between TBC1D15 and TBC1D17. Little is known about the effect of Gq on the localization of TBC1D17. Therefore, it was decided to transfect 293 cells with TBC1D17-Flag in the presence or absence of wild-type Gq or its constitutively active mutant form (**Error! Reference source not found.** and Figure 13). Similar to the experiments with TBC1D15, the location of TBC1D17 can be followed by staining with specific anti-Flag antibodies.

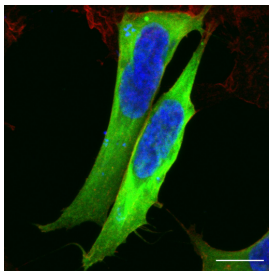

*Figure 12. **In basal conditions TBC1D17 is located in the cytosol**. Confocal micrographs of HEK 293 cells were transfected with TBC1D17 (green). Twenty-four hours after the transfection, cells were fixed and stained with anti-Flag (green). The overlay of stacks is shown. Images are representative of the majority of the cells. Scale bars,10μm*

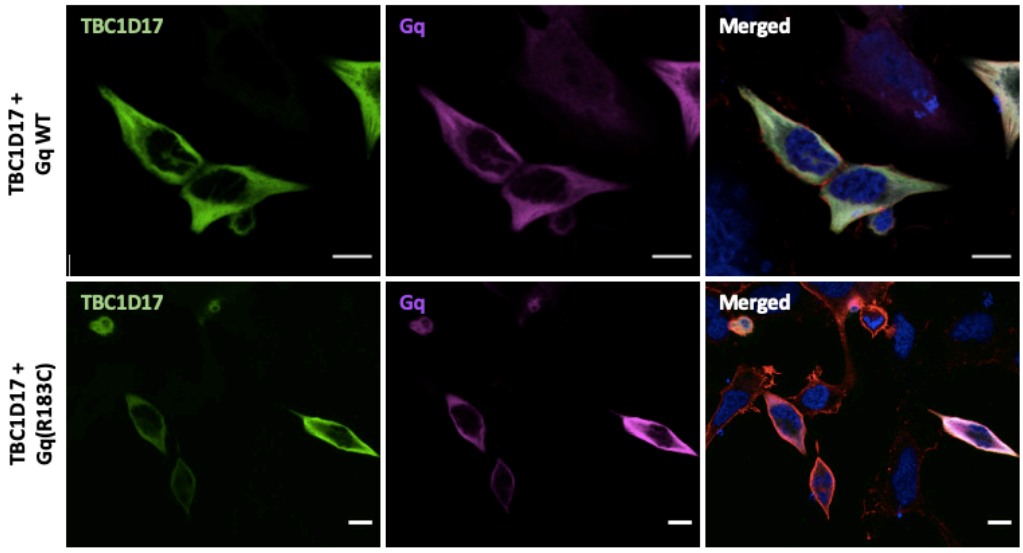

*Figure 13. **Neither Gq wild-type nor Gq (R183C) impair the location of TBC1D17**. Confocal micrographs of HEK 293 cells were transfected with TBC1D17 (green) and Gq WT or Gq(R183C) (magenta). Twenty-four hours after the transfection cells were fixed and stained with two different antibodies: anti-Flag-FITC (green) and anti-Gq (magenta). The nucleus is shown in blue and the membrane marker in red. One layer of the stack is shown. Images are representative of the majority of the cells. Scale bars, 10μm.*

In basal conditions TBC1D17 is located in the cytosol, resembling TBC1D15. Contrary to what was expected, upon Gq overexpression, the location of TBC1D17 does not seem to be altered, nor even with the constitutively active form of Gq. Thus, although Gq

immunoprecipitates with TBC1D17, it does not promote a change in its location. These results suggest that activation of Gq may disrupt the interaction between TBC1D15 and TBC1D17 by displacing TBC1D15 and inhibiting mitophagy.

6.4. Obtaining mCherry-RILP-GFP

As mentioned before, the induction of mitophagy promotes the contact between the complex TBC1D15, TBC1D17 and Fis1 with the Rab7-membrane, which contributes to the formation of the autophagosome. This vesicle is then transported by dynein through the microtubules to lysosomes. In this transport the interaction of Rab7 with several docking proteins, such as RILP protein, is crucial. Since Gq seems to alter the location of the TBC1D15 protein, it may inhibit mitophagy. It was suggested that Gq may also have an effect on the docking proteins. Preliminary results show that RILP is not present at the lysosome compartment in absence of Gq in Gq/11 Mouse Embryonic Fibroblasts (MEF) knockout cells. To facilitate the location of RILP and to be able to follow it in vivo it was decided to create a fluorescent-tagged RILP protein. Taking the advantage that the fluorescent protein GFP loses fluorescence in acid environments (such as the lysosome), a protein with two fluorescent proteins was designed; specifically, GFP and Cherry bound to RILP. Activation of mitophagy would lead to red vesicles (due to mCherry fluorescence), whereas vesicles would be yellow for the presence of both fluorophores. In this way, if activation of Gq inhibits mitophagy, cells will not show red dots. The fusion protein to be constructed was named mCherry-RILP-GFP (protocol explained in Materials and Methods, vector shown in Annex).

The agarose gel shown in Figure 14 represents the PCR amplification of RILP in different conditions. For the first five bands, only HF buffer was used, whereas for the bands 6-10, HF buffer together with DMSO were used. It is clear that the use of DMSO facilitated the reaction. Furthermore, the two bands with higher RILP amount (6 and 8), where the tubs that where at 60 and 62 °C during primer hybridization, respectively. The molecular weight of the bands, which was approximately 1200 pb, corresponded to the expected size for the amplification of RILP.

Once both were digested, they were ligated with N'-mCherry vector (sequence shown in Annex 1) and transformation of bacteria was performed. Bacteria cultures were obtained from the transformed bacteria; samples of these cultures were digested with ECORI and KpnI to analyse the number of obtained fragments as well as their length, and discard the incorrect ones. Figure 15 shows the result of the digestion.

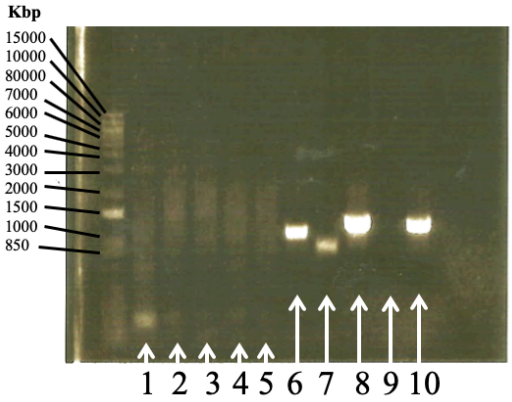

Figure 14. ***Amplification of RILP through PCR****. From left to right, in the first four bands HF buffer was used; for the bands 5 to 8, both HF buffer and DMSO were used.*

Bands 1, 3, 5 and 10-12 only present one band (do not have a smaller secondary band), which would indicate the presence of the insert. Also, bands 2 and 4 were discarded since they did not digest at all. Sample 9 has two bands but the smaller one, which would correspond to the insert, is too large. Finally, sample C2.1 (band 5) and sample C2.3 (7) show two bands each and with the adequate length: one the size of the vector and the smaller one approximately 1200 bp, which is the size of RILP protein.

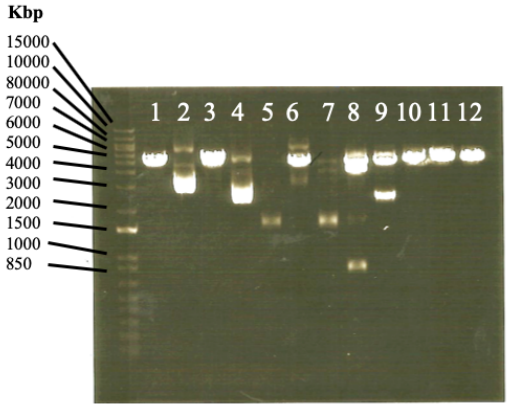

Figure 15. ***Digestion of the plasmids obtained through ligation of mCherry and RILP****. From left to right, bands come from plasmids C1.1-C1.4, C2.1-C2.5 and C3.1-C3.4. The first number in each plasmid refers to the proportion used for the ligation in each case.*

A PCR was performed with the chosen plasmids. RILP primers were used to ensure that the original RILP sequence had not been altered.

The amplified DNA was digested with KpnI, HindIII or both. None of the plasmids seemed to have the adequate sequence. Therefore, taking new transformed colonies and repeating the process is needed for future work.

7. Discussion

It is currently clear that autophagy plays a vital role in cell homeostasis. More concretely, autophagy of mitochondria (named mitophagy) is basic for the adequate performance of these organelles (3). Due to the crucial role of mitochondria, this process is now being extensively studied through several different points of view (2,3,6).

Recent studies have demonstrated that Gq regulates mitochondrial physiology (24). Although the authors suggested in that study that Gq knockout cells had increased autophagic ratio visualized by electron microscopy, little is known about the function of Gq in mitophagy. This project has been useful to gain a better understanding of how this heterotrimeric protein is capable of inhibiting mitophagy. It has been shown that Gq regulates the localization of the GTPase protein TBC1D15 but does not affect the localization of their partner protein TBC1D17.

TBC1D15 and its family member TBC1D17 have been recently proved to be implicated in autophagic process of mitochondria (4,8). Their role is linked to their regulation of the protein Rab7; TBC1D15 acts as a GTPase for Rab7 (23), but nothing is known about the activity of TBC1D17 towards Rab7. Results presented in this study suggest that activation of Gq protein would produce the translocation of the GTPase protein TBC1D15 to the cell periphery; this in turn would inhibit its role in the autophagic process, since TBC1D15 would not be able to regulate Rab7 membranes. It is worth mentioning that only the activated form of Gq produces this effect. Previous results from the group have demonstrated that TBC1D15 co-immunoprecipitated together with both the active and GDP forms of Gq, as well as with the mitochondrial protein Fis1. The results of this project indicate that TBC1D15 interaction with Gq would promote the dissociation of TBC1D15 from the mitochondria and therefore alter its location. Interestingly enough, utilizing a plasma membrane marker has been useful to notice that TBC1D15 is actually associated not in the plasma membrane but close to it, in an area that resembles the cortical actin structure.

In order to corroborate the hypothesis of this work it was decided to use two mutant forms of TBC1D15 that had an altered activity towards Gq. The TBC1D15-K142A mutant immunoprecipitates poorly with Gq, whereas TBC1D15-K136A strongly interacts with Gq. Interestingly, while the TBC1D15-K142A form does not change its location upon activation of Gq, TBC1D15-K136A is sent to the periphery in a fashion that resembles the wild-type TBC1D15 form. The fact that the TBC1D15-K142A mutant, which interacts less with Gq than the wild-type form, does not change is location, supports the hypothesis that Gq activation and interaction with TBC1D15 removes it from the mitochondria.

The results obtained with the construct of Gq-GFP, both the wild type and the constitutively active mutant, are in principle contradictory since the wild type form of Gq-GFP also induces the localization of TBC1D15 at the cell periphery, not being the case for the wild-type Gq form. However, it has to be taken into account that this chimeric

protein (GFP peptide is located in the middle of the N-terminal region of Gq), although considered functional, significantly concentrates in the mitochondria. In fact, a high proportion of the protein is found at the inter membrane space of this organelle. Therefore, if the presence and/or activity of Gq at the mitochondria is the reason to induce the dissociation of TBC1D15, it is logic that when Gq-GFP is transfected together with TBC1D15, even the wild-type form of Gq alters its translocation.

As mentioned before, TBC1D15 interacts with Fis1 and may form homodimers and heterodimers with TBC1D17 (4), which also can interact with Fis1 and has a role in both autophagy and mitophagy (13).

TBC1D17 is a Rab GAP protein for Rab8 (25), but it has not been described so far to have activity towards Rab7. It has been suggested that both TBC1D15 and TBC1D17 could form heterodimers and be present at the mitochondria (4). It is not clear, though, which is the role of TBC1D17 in this context. On the one hand, Gq has been found to immunoprecipitate with TBC1D17 in a proteomic analysis; from this, it emerged the hypothesis that Gq may regulate mitophagy through its interaction to TBC1D17. However, the results obtained indicate that neither Gq wild-type nor Gq(R183C) are capable of removing TBC1D17 from the mitochondria.

On the other hand, it is known that TBC1D15 and TBC1D17 bind to two TPR2 regions at the N-terminal domain of Fis1 (4,10), one of which is shared with Gq. Therefore, the results of this project suggest that Gq activation may remove TBC1D15 from Fis1 by competing for the TPR2 region. It is needed a dimmer of TBC1D15 and TBC1D15 or TBC1D17 to support mitophagy; Gq would displace one of the molecules of TBC1D15 and the dimmer of TBC1D17 would not be capable of supporting Rab7 activity and therefore mitophagy would be blocked. Previous results from the group show that TBC1D17 co-immunoprecipitates poorly with TBC1D15 when active Gq is present, which agrees with the present observations.

Another mechanism to inhibit mitophagy is at the level of transport of autophagosomes to lysosomes. The transport of lysosomes is regulated by the motor protein dynamin, that is linked to Rab7 through interaction with the protein RILP. Gq is thought to have no effect on the activity of neither Rab7 nor dynamin; however, these analyses indicate that Gq could interact with the protein RILP, opening the possibility that Gq could also affect the transport of the autophagosome particle. One of the goals of this project has been the design of a tool capable of following the location and activity of RILP. Currently, the fusion protein is still being synthesized; once obtained it will be useful to follow RILP expression with life microscopy after mitophagy induction by either CCCP or Valinomycin (4,8) and study the effect of Gq activation or depletion of its expression. Those experiments are part of future perspectives.

Altogether, these results suggest that active Gq negatively regulates mitophagy as shown in Figure 16.

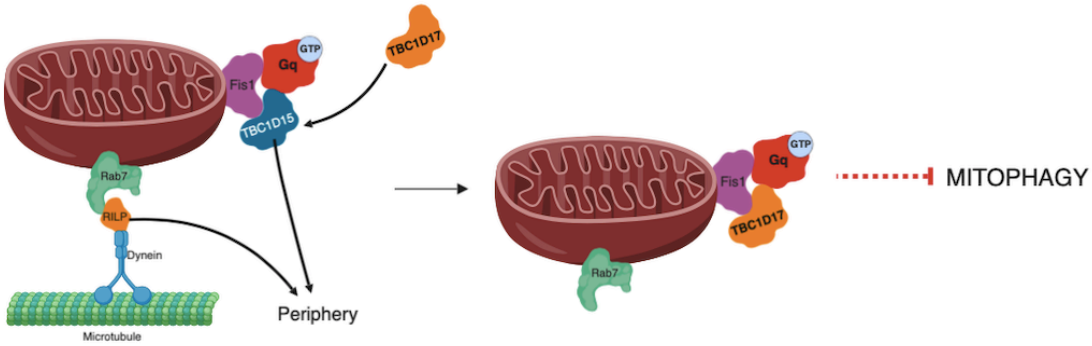

Figure 16. ***Gq probably inhibits mitophagy by disassembling of the protein complexes involved in this process****. Created with BioRender*

8. Conclusions

1. Active Gq sends TBC1D15 from the mitochondria to the periphery
2. TBC1D15 is not send to the plasmatic membrane but close to it
3. Gq stays in the mitochondria and, if it is more recruited to this organelle, wild-type Gq is also capable of sending TBC1D15 to the periphery
4. TBC1D17 displaces inactive Gq to interact with Fis1 and TBC1D15, but if Gq is activated it displaces TBC1D15, sending it to the periphery
5. A lower affinity for Gq causes a decrease in the transport of TBC1D15 to the periphery, even with overexpression of constitutively active Gq.

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11. Annex I. Sequence of the pmCherry-N1 vector

The enzymes that have been used for obtaining the fusion protein are circled in red.