The role of the autophagy related protein ATG4C in association with the B-cell scaffold protein BANK1 in autoimmunity

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Summary

Systemic lupus erythematosus (SLE) is an autoimmune disease. The cause is still unknown but a genetic variation in several genes associated with immunity has been observed. The B-cell scaffold protein BANK1 has previously been reported as being a susceptibility gene for SLE. Possible binding and co-localization studies with BANK1 are therefore necessary for a deeper understanding of its role in the possible progression of the disease.

Autophagy related protein ATG4C is believed to be one of many autophagins responsible for the homeostasis in the cell. Autophagins are believed to assemble isolation membranes (e.g. ER) to encapsulate cytoplasmic material and organelles in autophagosomes which are then fused with the lysosomes and degraded. This process is now known as autophagy and may be used by the immune system to regulate autoimmunity.

In this study ATG4C and its possible correlation with BANK1 was explored. Previous genetic associative studies of SLE patients and controls indicate epistasis between BANK1 and ATG4C. The genetic sequence corresponding to ATG4C was PCR amplified from a B-cell cell line and inserted into a cloning plasmid. Plasmids carrying ATG4C and BANK1 were co-transfected into HEK 293 cells to investigate their possible physical interaction and co-localization. Immunoprecipitation was performed to analyze the physical interactions; no direct binding could be established between ATG4C and BANK1.

Confocal microscopy was performed with fluorescent tags inserted in frame with the polypeptide of ATG4C. Images taken with the confocal microscope indicate a co-localization of ATG4C and BANK1. The hypothetical conclusion is that co-localization occurs late in the autophagy pathway, when the autophagosomes have been formed. However more optimization and verification is necessary.
Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by loss of tolerance to nuclear antigens, immune complex deposition and tissue destruction. Multiple abnormalities within different components of the immune system have been described for the disease (Cheung Y-H. et al. 2009). For example, SLE causes abnormal autoantibody production of B-cells which leads to tissue damage in multiple organs. SLE has a high heritability and identification of the genetic determinants underlying genetic susceptibility is of great interest (Moser K.L. et al. 2009). There is currently no unifying model to fully explain the mechanisms underlying the development of SLE, however, genetic studies (e.g. GWA and genotyping of SNPs) have identified and robustly replicated more than 20 SLE susceptibility loci (Harley I.T. et al. 2009). These loci include the major histocompatibility complex (MHC) as well as interferon (IFN) and B-cell scaffold protein (BANK1)

The branch of the adaptive immune responses triggered by activation of B-cell receptors and the fine tuned production of B-cells are a significant part in regulating immunity (Kurosaki T. 2002). Understanding the mechanisms for hyperactivity and autoantibody-production of B-cells in SLE are important as it may help us unveil the causes of disease progression. BANK1 is involved in B-cell receptor signaling. Recent studies indicate that BANK1 may play an important role in B-cell activation, and has been indicated as a susceptibility gene for SLE. Identified variants of BANK1 associated with SLE are indicated to be involved in an altered threshold for B-cell activation (Kozyrev S.V. et al. 2007). Additional studies, including co-localization and interaction studies, are required to fully understand its involvement in SLE.

Genetic analysis of SLE affected individuals and controls revealed an epistatic interaction between BANK1 and ATG4C (unpublished results). Autophagy related 4 homolog C (ATG4C) gene codes for a cystein protease first described in Saccharomyces cerevisiae. It is believed to be a cytosolic enzyme and assemble late in the autophagy pathway. ATG4C has a conserved peptidase C54 domain with a catalytic cysteine residue, which can catalyze the hydrolysis of polypeptide chains and regulate autophagy (Mariño G. et al. 2003).
Autophagy is the mechanism where the cell under physiological conditions, as well as cell stress, can sustain homeostasis. Autophagy related proteins named ATG can form autophagosomes with isolation membranes (e.g. ER) which is used to encapsulate and clear the cytosole from excess cytoplasmic material and organelles. The autophagosomes then fuse with the lysosomes creating autolysosomes and the content is degraded (Mizushima N. et al. 2010).

The immune system can utilize autophagy degradation to regulate adaptive immunity. It has been shown to limit pathogen replication and also to deliver antigens for MHC presentation (Schmid D. et al. 2007). The degradation of proteins can be carried out by two major hydrolytic pathways: proteasomes and lysosomes. In this study the autophagy degradation pathway by lysosomes will be investigated.

There are some indications that ATG4C is involved in autophagy after stressful conditions as starvation (Mariño et al. 2007). However, more recent studies explain two separate autophagic responses: conventional macroautophagy and alternative macroautophagy.

The conventional macroautophagy which involve the autophagy-related proteins Atg5-Atg7 that initiates the degradation of sub cellular material by the formation of autophagosomes, and are mediated by the microtubule-associated protein light chain 3 (LC3) / autophagy-related gene 8 (Atg8) (Nishida Y. et al. 2009). LC3/Atg8 is an ubiquitin-like protein that is involved in the tethering and hemifusion of the double membrane-bound autophagosomes (Nakatogawa H. et al. 2007). Recent studies in Saccharomyces cerevisiae indicate LC3/Atg8 as being important in the autolysosome to vacuole pathway. This pathway is both activated and terminated by the cleavage of a C-terminal part in the polypeptide chain of LC3/Atg8 by ATG4C (Lynch-Day M.A. et al. 2010).

The alternative macroautophagy is thought to be mediated by the fusion of isolation membranes derived from the trans-Golgi and late endosome. This alternative macroautophagy is mediated by the Ras-related protein (Rab9), which regulates late endosome size and stability (Nishida Y. et al. 2009). Lysosome-associated membrane protein (LAMP1) is a membrane glycoprotein situated in the lysosomal membrane (Eskenilnen E-L. et al. 2003).

In this study, proteins involved in the two different pathways of autophagy as well as the lysosomal marker LAMP1 were used. The aim was to examine their possible physical co-localization and interaction with BANK1 and ATG4C.
Results

Cloning and transformation

As a first step to detecting interactions between BANK1 and ATG4C a plasmid with ATG4C was constructed. The open reading frame of ATG4C was PCR amplified from the B-cell cDNA cell line BjaB. The amplification product was analyzed on a 0.8% agarose gel (Figure 1). The band at 1370 corresponds to the coding sequence of ATG4C. A model showing the gene structure and the protein domains of ATG4C is showed in (Figure 2).

Figure 1. 0.8% agarose gel loaded with the PCR reaction. The PCR amplified band is shown at 1370bp for the coding sequence of ATG4C. The DNA ladder used was 2-Log DNALadder (Invitrogen). 1. PCR product using 1ul of the B-cell cDNA library as template; 2. PCR product using 0.1ul of the B-cell cDNA library as template.
Figure 2. Hypothetical model of coding (red) and non-coding (blue) regions of ATG4C from 5' to 3'. The translated protein at 458 amino acids (red) with the homology fitted conserved peptidase domain (grey) located approximately between amino acids 75-400. The conserved catalytic cysteine (black) is located at approximately amino acid 110.

The amplified band from the PCR reaction (the open reading frame of ATG4C) was then inserted in a cloning vector and transformed into chemically competent E. coli cells. To verify the construct, colony PCR was performed and analyzed on 0.8% agarose gel (Figure 3). Clones 1, 4, 6 and 8 showed a band at the expected size and were sent for sequencing at the Uppsala Genome Center. The results of the sequencing showed that the insert of plasmids 1 and 8 had a point mutation and the insert of plasmids 4 and 6 had a deletion. These plasmids were not used for subsequent experiments. Six new colonies were sequenced in the same way in order to identify an insert without any mutations. Of the 6 clones picked, two had the correct insert and were named ATG4C-p12 and ATG4C-p16. The plasmid with the correct insert was amplified in E. coli, purified and used for further experiments.
**Figure 3.** 0.8% agarose gel displaying the bands from the colony PCR screened with the primers CMVf and 4c817r generating fragment at 896bp. The DNA ladder used was 2-Log DNALadder (Invitrogen). Colonies 1, 4, 6 and 8 was used for the sequencing.

**Introduction of fluorescent tag**

In order to use the plasmid constructed with *ATG4C* for microscopy, a fluorescent tag was inserted. The fluorescent proteins cyan fluorescent protein (CFP) and green fluorescent protein (GFP) were introduced into the plasmid ATG4C-p12. The genes encoding the fluorescent proteins were extracted with restriction endonucleases and isolated in a 0.8% agarose gel (*Figure 4*). The plasmid and fluorescent protein insert fragment were ligated and transformed into chemically competent *E. coli* cells. To verify the insert, colony PCR was performed; positive clones were sent for sequencing at the Uppsala genome center. The sequenced plasmids all contained the correct construct two of them were amplified in *E. coli*, purified and used for further analysis, and were named ATG4C-p12+CFP and ATG4C-p12+GFP.
Figure 4. 0.8% agarose gel with 1xTBE displaying plasmids cleaved with restriction endonucleases NotI and ApaI. The ladder used was 2-Log DNA ladder (Invitrogen). 1. ATG4C plasmid cut with NotI and ApaI; 2. BANK1 plasmid cut with NotI and ApaI; 3. CFP insert fragment cut with NotI and ApaI; 4. BANK1 plasmid cut with NotI and ApaI; 5. GFP insert fragment cut with NotI and ApaI.

Reference plasmids

The expression plasmids with the reference proteins were prepared to be used to analyze interactions with BANK1 and ATG4C. The plasmid pHYA876, 7343bp carrying the gene *Rab9a* was verified by restriction endonucleases KpnI and NcoI. The restriction reaction was analyzed on agarose gel (Figure 5). The expression plasmids with reference proteins LAMP1, ATG5 and LC3 were not analyzed, and were directly used for further experiments.

Figure 5. 0.8% agarose gel with 1xTBE showing the bands from restriction reaction of plasmid pHYA876, 7343bp. With restriction endonucleases KpnI and NcoI generating bands for KpnI at 3600bp and 3700bp, seen as one band on the due to the poor resolution of the gel, band for NcoI at 6600bp and 700bp. The ladder used was 2-Log DNA ladder (Invitrogen). 1.; 2. pHYA876+KpnI; 3. pHYA876+KpnI; 4. pHYA876+NcoI; 5. pHYA876+NcoI.
Immunoblotting

Immunoprecipitation was used to see if BANK1 and ATG4C interact directly by binding. A Flag-BANK1 plasmid was co-transfected with ATG4C-p12 plasmid carrying a V5 epitope into human embryonic kidney 293 (HEK 293) cells. Immunoprecipitation of the ATG4C-V5 epitope was carried out with anti-V5 antibodies.

Antibodies specific for BANK1 and V5 was used respectively (Figure 6).

The protein ATG4C is 458 amino acids corresponding to a molecular weight of 53kDa, BANK1 has a protein molecular weight of 110kDa. In this experiment BANK1 and ATG4C is both expressed in the cells, but seem not to be a physically interacting by direct binding.

![Figure 6](image_url)

**Figure 6.** Immunoblotting of extract and immunoprecipitate of cells co-transfected with ATG4C and Flag-BANK1, antibodies specific for BANK1 and ATG4C-V5 epitope was used. Marker used was PageRuler Plus Prestained protein ladder (Invitrogen) indicate protein sizes in kDa. When primary antibody anti-V5 was used a band in 53kDa is visible representing ATG4C. No band in the immunoprecipitates are visible when primary antibody anti BANK1 was used. BANK1 is not binding to ATG4C and are therefore not precipitated out and detected.
Confocal microscopy

In order to examine the physical co-localization of ATG4C and BANK1, BANK1 was co-transfected with ATG4C as well as different reference plasmids expressing proteins acting in the autophagy pathway. BANK1-YFP plasmid was used, which was the same plasmid as explained in materials and methods (Figure 10) with Yellow fluorescent protein inserted in frame with BANK1. BANK1-YFP was co-transfected with ATG4C-p12+CFP plasmid into HEK 293 cells. The protein expression and physical localization were analyzed with a confocal microscope (Figure 7). In this experiment there seem to be some co-localization between BANK1 and ATG4C, Indicated by the arrow.

Figure 7. Confocal images of a HEK 293 cell co-transfected with BANK1-YFP and ATG4C-p12+CFP. The image was magnified with 63X oil objective; colors are changed for a better contrast. From left to right 1, cell visualized with Nomanski channel; 2, cell with BANK1-YFP visualized; 3, cell with ATG4C-CFP visualized; 4, cell with BANK1 and ATG4C visualized yellow and cyan channel merged, some physical co-localization is indicated in the fourth picture with an arrow.

BANK1 was also co-transfected with reference proteins ATG5, LAMP1, Rab9a and LC3. The reliability and quality of the images were not good, so the image of LAMP1 is only included (Figure 8).
There seem to be some co-localization between BANK1 and LAMP1; additional experiments need to be performed to verify this pattern. BANK1 may be interacting with LAMP1 when the lysosomes are fusing with the autophagosomes.

**Figure 8.** HEK 293 cell co-transfected with BANK1-CFP and LAMP1-RFP. The image was magnified with 63x oil objective; colors are changed for a better contrast. From left to right 1, cell with BANK1-CFP visualized; 2, cell with LAMP1-RFP visualized; 3, cell with BANK1-CFP and LAMP-RFP visualized cyan and red channel merged. Some co-localization indicated and zoomed, this co-localization may occur when autophagosomes fuse with the lysosomes.
Discussion

In these preliminary immunoprecipitation experiments using the anti-V5 antibody, there did not seem to be a direct physical interaction between ATG4C and BANK1. The ATG4C-V5 epitope binds to the Dynabeads in the immunoprecipitation experiment, ATG4C is then precipitated from the protein extract. If BANK1 binds to ATG4C, it will also be precipitated, hence there was no signal in the immunoprecipitates when antibody anti-BANK1 was used targeted towards detecting BANK1 (Figure 6). However, to reach more definite results, the experiments need to be further optimized. In immunoprecipitation the primary inaccuracy lies in the transfection, in order to compare results the expression level of the proteins need to be in parallel. The possibility of additional proteins interacting may also be necessary for the binding of BANK1 and ATG4C. These preliminary results also indicate that ATG4C is not post-transcriptionaly spliced at the 3’ end; hence the V5 epitope is detected, so a fluorescent tag could be placed there.

The physical localization analyzed with confocal microscopy indicates some co-localization of ATG4C and BANK1. BANK1 is present in the cytosole and when expressed in access it assembles in the autophagosomes (seen as brighter dots, Figure 8). ATG4C is expressed in the cytosole; earlier studies in *S. cerevisiae* indicate that ATG4C is associated with autophagosome modification and interaction with LC3 (seen as a brighter dot, Figure 8).

The general hypothesis is that BANK1 and ATG4C assemble late in the autophagy pathway, when the autophagosomes are formed. The pattern of the proteins in the image from the confocal microscopy (Figure 8) demonstrates this preliminary conclusion.

Results obtained from additional co-tranfections with protein LAMP1 show some co-localization patterns indicating that BANK1 may be interacting with LAMP1 when the lysosomes are fusing with the autophagosomes (Figure 9) however, further experiments are necessary.

The selection of different fluorescent tags is depending on the excitation wavelengths. When fluorescent proteins with excitation wavelengths close in proximity are used the specificity of excitation/emission might be mixed.
After excitation the amount of light emitted from the sample can be narrowed so that the detected light visualized by the confocal microscope is limited for a higher contrast. The methods used in this study may be further optimized to gain a more reliable result.

Future research to further understand the association between BANK1 and ATG4C may be to induce stress to the transfected cells before confocal microscopy, such as nutrient starvation. The resolution should also be increased in order to see cell structures more clearly and determine localization in the cell. The transfection into different cell types such as B-cells would also be of interest to see if the observed pattern is consistent.

Additional interaction studies are also of interest to analyze potential secondary binding.
Materials and Methods

Cloning and transformation

The gene ATG4C was PCR amplified from a human cell line from B-cell (BjaB) cell line. The primers used were forward (4Cf) and reverse (4ATGer) (*Table 1*) designed to amplify the target gene.

4ng DNA was used for the PCR with 2.5µl 10x PCR Buffer with MgCl₂ (50mM), 1.5µl dNTP (5mM), 0.5µl of each of the primers (10mM), 0.3 µl DNA polymerase (PfuUltra™ II Fusion HS DNA polymerase). The PCR reaction was performed with the following temperatures 95°C 3min; 95°C 20 sec, 55°C 20sec, 72°C 30sec this cycle was run 30x; the elongation was run at 72°C for 10min; 4°C∞.

The PCR amplification generated a fragment 1370bp long; the product was analyzed on 0.8% Agarose gel with Ethidium bromide. The PCR product was purified using a small size exclusion chromatography column kit ILLusra™ DNA and gel band purification kit (GE Healthcare), the protocol was followed, and the DNA was eluted in 30µl TE buffer pH 8.0.

The amplified ATG4C was cloned into a TOPO® Cloning vector, pcDNA 3.1 D/V5-His-TOPO 5514bp (*Figure 10*) The TOPO® Cloning reaction was transformed into one shot® TOP10 chemically competent *E. coli* cells. The cloning and transformation kit from Invitrogen and the provided protocol was followed. The cells were then plated on selective LB agar plates with 100µg/ml ampicillin and grown over night.

Single colonies were picked and analyzed with colony PCR. The primers used were forward (CMV) targeted to the vector and reverse (4c817r) targeted to the inserted gene fragment (*Table 1, Figure 9*).

The 11 colonies picked were resuspended in 1µl 10x PCR Buffer with 0.4µl MgCl₂ (50mM), 0.4µl dNTP (5mM), 0.24µl of each of the primers (10mM), 0.08 µl DNA polymerase (Taq DNA polymerase). The PCR reaction was performed with the following temperatures 95°C 3min; 95°C 15 sec, 56°C 15sec, 72°C 30sec this cycle was run 31x; the elongation was run at 72°C for 10min; 4°C∞.
Overnight cultures with the 11 colonies were also started 3ml LB media with 50μg/ml ampicillin. Plasmids from the positive colonies were prepared using GeneElute™ HP plasmid miniprep kit (Sigma-Aldrich), the provided protocol was followed. The prepared plasmids were sequenced at the Uppsala genome center using primers (BGH) and (4c817r) (Table 1, Figure 9). Out of the 11 colonies picked 4 were positive in the colony PCR screening and were sent for sequencing, all of the constructs were having mutations or deletions so 6 more colonies were amplified, prepared and sequenced in the same way described previously. Out of the 6, two positive plasmids were identified with the correct construct and were named ATG4C-p12 and ATG4C-p16.

The cultures carrying the correct construct were scaled up; 50ml LB media with 50μg/ml ampicillin was grown over night at 37ºC. The plasmids were prepared using GeneElute™ HP plasmid minidiprep kit (Sigma-Aldrich), the provided protocol was followed.

Table 1. Primers used for the PCR and sequencing of ATG4C

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence</th>
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<tbody>
<tr>
<td>4Cf</td>
<td>CACCATGGAGGCTACAGGAACAGATG</td>
</tr>
<tr>
<td>4ATGcr</td>
<td>AAGCAAGACAAACTCTTCCGTGC</td>
</tr>
<tr>
<td>CMV-forw</td>
<td>GCAATGGGCGGTAGGCGTG</td>
</tr>
<tr>
<td>4c817r</td>
<td>CAATTACATCAGAATTGTAAAC</td>
</tr>
<tr>
<td>BGH</td>
<td>TAGAAGGCACAGTCGAGG</td>
</tr>
<tr>
<td>Not-EYFP</td>
<td>GGGCGGCGCGCattgtagcaagggcgag</td>
</tr>
<tr>
<td>V5cR</td>
<td>ACCGAGGAGGGTTAGGGAT</td>
</tr>
</tbody>
</table>

1, 2. Oligos for amplification of ATG4C open reading frame from cell line.
3. Oligo targeted for the CMV promoter in expression vector pcDNA3.1.
4. Oligo used for sequencing of ATG4C targeted in the middle of the nucleotide sequence.
5. Oligo targeted for the BGH pA region in the expression vector.
6. Oligo used for the verification of insert in frame with ATG4C in the expression vector, NotI restriction site indicated.
7. Oligo targeted for the V5 epitope in the cloning plasmid.
Figure 9. Representing pcDNA3.1Atg4C-V5 expression vector with open reading frame of ATG4C inserted. The sites of the amplification primers from table 1 are indicated for a better understanding. 4Cf and 4ATGcr amplifying the open reading frame of ATG4C. 4c817r amplifying the middle section of Atg4C that is useful for sequencing. CMV-forw, Not-EYFP, V5cR and BGH in the expression vector.

Figure 10. pcDNA 3.1 D/V5-His-TOPO cloning vector used for the cloning of ATG4C, restriction sties NotI and ApaI are indicated. Vector is equipped with Ampicillin resistance for transformation in E.coli and neomycin resistance for transfection into eukaryotic cells, CMV promoter and TOPO cloning site, V5 epitope for antibody recognition, BGH pA to protect mRNA from hydrolysis and as a termination of transcript and His Tag for protein purification.
Introduction of fluorescent tag

In order to use the cloned plasmid for microscopy a fluorescent tag was introduced. In the plasmid two restriction sites were used NotI and ApaI to introduce the fluorescent tag in frame with ATG4C.

The plasmid (5µg) was cleaved with NotI and ApaI (Fermentas) for 3 h respectively, the provided protocol was followed. Plasmids (5µg) with fluorescent tag were also cleaved in the same way to produce the correct ligation ends. Cyan fluorescent protein (CFP) and Green fluorescent protein (GFP) were used.

Restriction digest were purified with gel band purification kit (GE Healthcare), the protocol was followed, and the DNA was eluted in 50µl sterile H2O. The cleaved plasmids were run on 0.8% agarose gel with 1x TBE. The bands were isolated from the gel and purified with gel band purification kit (GE Healthcare), the provided protocol was followed, and the DNA was eluted in 30µl TE Buffer.

The isolated and purified fluorescent proteins and plasmids were ligated using T4 DNA ligase (Invitrogen). Ligation mix was made with 42ng fluorescent insert (~0.7kbp) and 57ng plasmid (~5kbp) with the molar ratio 1:3, 0.2µl T4 DNA ligase, and 1 x ligase buffer and diluted with H2O to 10µl. The ligase mix was incubated for 1h at room temperature.

The ligation mix was then transformed into TOP 10 chemically competent E. coli cells according to the protocol. The E. coli cells were the spread on LB agar plates with 100µg/ml ampicillin and incubated at 37ºC over night. Colonies were then picked and incubated in a 96well plate with LB media and 100µg/ml ampicillin for 1.5h. The plate was then analyzed in fluorescent microscope to differentiate between positive and negative colonies.

The fluorescence could not be distinguished so colony PCR was also performed to screen for positive colonies. The primers used were forward (Not-EYFP) and reverse (V5cR) (Table 1, Figure 9). 1µl culture was used for the PCR with 1µl 10x PCR Buffer with 0.4µl MgCl2 (50mM), 0.4µl dNTP (5mM), 0.24µl of each of the primers (10mM), 0.08 µl DNA polymerase (Taq DNA polymerase). The PCR reaction was performed with the following temperatures 95ºC 3min; 95ºC 15 sec, 56ºC 15sec, 72ºC 30sec this cycle was run 31x; the elongation was run at 72ºC for 10min; 4ºC ∞.
Overnight cultures with the selected colonies, 3ml LB media with 100µg/ml ampicillin were started. Plasmids from the positive colonies from the colony PCR screening were prepared using GeneElute™ HP plasmid miniprep kit (Sigma-Aldrich), the provided protocol was followed. The prepared plasmids were sequenced at the Uppsala genome center using primers forward (CMV) and reverse (V5cR) (Table 1, Figure 9) the clones sequenced were verified. The plasmids carrying the correct construct were scaled up; 50ml LB media 50µg/ml ampicillin was grown at 37°C over night. The plasmids were prepared using GeneElute™ HP plasmid minidiprep kit (Sigma-Aldrich), the provided protocol was followed.

Reference expression plasmids

To analyze the localization of ATG4C four possible associated proteins Rab9a, ATG5, LAMP1 and LC3 were prepared as reference proteins with known location and properties. The plasmid pHYA876 7343bp carrying the gene Rab9a was transformed into one shot® TOP10 chemically competent E. coli cells (Invitrogen), the provided protocol was followed. The cells were then plated on selective LB agar plates with 100µg/ml ampicillin and incubated over night at 37°C. Two single colonies were picked and incubated over night at 37°C in 3ml LB media with 50µg/ml ampicillin. The plasmids were then prepared using GeneElute™ HP plasmid minidiprep kit (Sigma-Aldrich), the provided protocol was followed. The transformed plasmids were analyzed using restriction endonucleases KpnI and NcoI. The restriction analysis was analyzed on 0.8% Agarose gel.

Expression plasmids pmCherry-Atg5, 5517bp, carrying the gene Atg5 (with Cherry fluorescent protein), Lamp1-RFP, 5906bp carrying the gene Lamp1 (with Red fluorescent protein) and EGFP-LC3, 5113bp, carrying the gene LC3 (with Green fluorescent protein) was purchased from AddGene. The three expression plasmids carrying kanamycin resistance had been transformed into E. coli cells prior to purchase. The pre-transformed cells were plated on selective LB agar plates with 50µg/ml kanamycin and incubated at 37°C over night. Two single colonies were picked incubated over night at 37°C in 3ml LB media with 50µg/ml kanamycin.
The plasmids were then prepared using GeneElute™ HP plasmid miniprep kit (Sigma-Aldrich), the provided protocol was followed.

**Transfection of HEK 293 cells in 6-well plate**

400,000 Human Embryonic Kidney 293 cells (HEK 293) cells were seeded in 6-well plates (surface area 10cm²) and incubated over night at 37°C, to 70-80% confluency in 2ml complete media, containing Dulbecco’s modified eagle’s media (DMEM, Sigma) with 10% of fetal bovine serum. Lipofectamin 2000 (Invitrogen) diluted 1:25 in Opti-Mem medium (Gibco) was mixed with 2 µg plasmid diluted in 50µl Opti-Mem medium. Both dilutions were made separately and incubated for 10 min at RT respectively. After mixing the solutions were incubated for 20 min more at RT. The Lipofectamin–plasmid complexes were then added to the cells carefully, and the plates were incubated for 48h at 37°C.

**Protein preparation and Immunoprecipitation**

The proteins from the cells in the 6-well plate were then prepared. From now on the reactions were kept on ice. Lysis buffer A 250µl was added to the cells, containing HEPES pH7.1 (50mM), NaCl (150 mM), EDTA (1mM), Na₃VO₄ (2mM), 1% Triton X-100, 10% Glycerol and 1.0% SDS. The lysis mixture was incubated for 20 min on ice. The cells were then scraped from the surface of the wells and mechanically disrupted, then transferred to a 2ml microcentrifuge tube and centrifuged 15 min at 1300rpm, 4°C. A sample of 20µl crude protein extract was taken to be used as input control. The supernatant containing the protein extract was used for Immunoprecipitation.

The immunoprecipitation was carried out using Immunoprecipitation kit-Dynabeads ProteinG (Invitrogen) incubated with antibody anti-V5 specific for the V5 epitope present in the transfected plasmid used, the provided protocol was followed.
Western blot

The two samples, crude protein input control (lysates) and immunoprecipitation reaction (IP) were prepared for SDS-PAGE electrophoresis. Lysates were mixed with 20µl 4x NuPAGE sample buffer, IP were mixed with 10µl Lysis buffer A and 10µl 4x NuPAGE sample buffer (Invitrogen).

The samples were boiled at 100°C for 4 min before loaded on 4-12% BisTriss NuPAGE gels. PageRuler Ladder and MOPS NuPAGE SDS running buffer (Invitrogen) was used. The SDS-PAGE electrophoresis was run for 1.5 h at 150V.

The proteins were then transferred from the NuPAGE gel to a prepared sheet of hydrophobic polyvinylidene difluoride (PVDF) Hybond-P membrane (GE Healthcare), the provided protocol was followed. NuPAGE transfer buffer was used and the transfer was made at 400mA for 2h or 15V overnight at 4°C.

Immunoblotting

The Hybond-P membrane from the western blot was incubated for 45 min in 5ml TBS, 0.05%Tween20 (TBS-T) with 5% no-fat milk powder at room temp. The membrane was then washed in TBS-T and incubated overnight at 4°C in diluted primary antibody in TBS-T with 5% no-fat milk powder (Table 2). The membrane was then washed 3 x 5 min with TBS-T and incubated for 1h at room temperature in diluted secondary antibody in TBS-T with 5% no-fat milk powder, for antibody list (Table 2). The membrane was washed 3 x 10 min in TBS-T.

The following reactions were performed in a dark room. The membrane was assembled in a cassette for developing, 1ml chemiluminecence luminal reagent ECL western blotting analysis system (Amersham) was added to the membrane, a hyper film ECL high performance chemiluminescence film (Amersham) was then placed on top and the cassette was closed. The film and membrane was incubated for different time frames to capture the signal on the film. The film was then developed in a CEAPRO processing machine (AGFA-Healthcare).
Table 2. Antibodies used for the immunoblotting

<table>
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<th>Target</th>
<th>Primary antibody</th>
<th>dilution</th>
<th>Secondary antibody</th>
<th>dilution</th>
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<tr>
<td>Bank1</td>
<td>Bank1-ET52</td>
<td>1:250</td>
<td>anti-rabbit HRP- Goat</td>
<td>1:10000</td>
</tr>
<tr>
<td>V5</td>
<td>Anti-V5</td>
<td>1:5000</td>
<td>anti-mouse HRP-Goat</td>
<td>1:10000</td>
</tr>
</tbody>
</table>

1. Primary and secondary antibody diluted in TBS-T with 5% no-fat milk powder

Transfection of HEK 293 cells 8-well plate, used in microscopy

50,000 HEK 293 cells were seeded in 8-well plates (0.69cm² surface area) and incubated for 24h at 37°C, to 70-80% confluency with 400µl complete media. Lipofectamin 2000 diluted 1:12.5 in Opti-Mem medium was mixed with 0.8 µg plasmid (carrying a fluorescent tag) diluted in 50µl Opti-Mem medium. Both dilutions were made separately and incubated for 10 min at room temperature respectively. After mixing the solutions were incubated for 20 min more at room temperature. The Lipofectamin–plasmid complexes were then added to the cells carefully, and the plates were incubated for 48h at 37°C.

The media was removed and the cells were carefully washed with PBS. The cells were then fixed by the addition of 8% formaldehyde solution diluted 1:1 in PBS the plates were incubated for 20 min at room temperature. The cells were then washed with PBS and quenched with 0.1M Glycine in PBS; the cells were washed again with PBS, anti-fading solution was added. The wells were removed leaving the object glass, and a glass cover slip was placed on top, the cover glass was fixed with nail polish.
Confocal microscopy

The confocal microscope used was a Zeiss LSM 510 microscope with four working lasers. The selection of laser is depending on the fluorescent tag used. The detected light was narrowed by the beam emission filter used (Table 3). The microscope was set at 63x oil object magnification and pictures were taken of the cells transfected with the plasmids carrying the fluorescent tags.

Table 3. Settings for the confocal microscope

<table>
<thead>
<tr>
<th>Fluorescent protein</th>
<th>excitation</th>
<th>beam splitter$^1$</th>
<th>emission</th>
<th>emission filter$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green fluorescent protein</td>
<td>488nm</td>
<td>488</td>
<td>507nm</td>
<td>BP 505-550$^5$</td>
</tr>
<tr>
<td>Cyan fluorescent protein</td>
<td>458nm</td>
<td>458-514</td>
<td>480nm</td>
<td>BP 470-500$^4$</td>
</tr>
<tr>
<td>Yellow fluorescent protein</td>
<td>514nm</td>
<td>405/514</td>
<td>527nm</td>
<td>LP 530-600$^5$</td>
</tr>
<tr>
<td>Cherry fluorescent protein</td>
<td>543nm</td>
<td>488/543</td>
<td>610nm</td>
<td>LP 560$^6$</td>
</tr>
<tr>
<td>Red fluorescent protein</td>
<td>543nm</td>
<td>488/543</td>
<td>583nm</td>
<td>LP 560$^7$</td>
</tr>
</tbody>
</table>

1. Filter the emitted light
2. Deflect indicated laser lines onto specimen; allow emitted fluorescent light to pass
3. BP 505-550 transmits emission light within the indicated wavelength band
4. BP 470-500 transmits emission light within the indicated wavelength band
5. LP 530-600 transmits emission light with wavelengths longer than the indicated threshold value
6. 7. LP 560 transmits emission light with wavelengths longer than the indicated threshold value
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References


