Video Article

Study of Protein-protein Interactions in Autophagy Research

Secil Erbil-Bilir¹, Nur M. Kocaturk¹, Melih Yayli², Devrim Gozuacik^{1,3}

¹Molecular Biology, Genetics and Bioengineering Program, Sabanci University

²Information Center, Sabanci University

³EFSUN, Center of Excellence for Functional Surfaces and Interfaces for Nano Diagnostics, Sabanci University

Correspondence to: Devrim Gozuacik at dgozuacik@sabanciuniv.edu

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Abstract

Protein-protein interactions are important for understanding cellular signaling cascades and identifying novel pathway components and protein dynamics. The majority of cellular activities require physical interactions between proteins. To analyze and map these interactions, various experimental techniques as well as bioinformatics tools were developed. Autophagy is a cellular recycling mechanism that allows the cells to cope with different stressors, including nutrient deprivation, chemicals, and hypoxia. In order to better understand autophagy-related signaling events and to discover novel factors that regulate protein complexes in autophagy, we performed protein-protein interaction screens. Validation of these screening results requires the use of immunofluorescence and immunoprecipitation techniques. In this system, specific autophagy-related protein-protein interactions that we discovered were tested in Neuro2A (N2A) and HEK293T cell lines. Details of the technical procedures used are explained in this visualized experiment paper.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55881/>

Introduction

Macroautophagy (autophagy, herein) is a cellular stress mechanism that is characterized by the sequestration of bulk cytoplasm, proteins, and organelles in double membrane vesicles called autophagic vesicles. Through fusion of the outer layer of the double membrane, autophagic vesicles and their cargo are delivered to lysosomes and degraded therein¹. Autophagy occurs at low basal levels in all cell types and in all organisms, performing homeostatic functions such as protein degradation and organelle (e.g., mitochondria) turnover. Under conditions leading to cellular stress, such as starvation, autophagy is rapidly upregulated and allows the cell to maintain energy levels and basic metabolism^{1,2,3}.

Around 30 autophagy genes have been cloned from the yeast and their protein products were shown to play a role in various stages of the autophagic process, including vesicle nucleation, expansion, vesicle fusion to late endosome/lysosome, and cargo degradation^{4,5}. Orthologs of the majority of these genes have been identified and studies in various organisms confirmed preservation of their cellular functions⁶. Studies in the last decade showed that several autophagy-related protein complexes and protein-protein interactions exist and that they govern autophagy pathways in an intricate and controlled manner. Intersections, backups, feedback, and feedforward mechanisms exist, and they allow the cell to coordinate autophagy with other related events (such as vesicular secretion, lysosome biogenesis, endosomal sorting and transport⁷, etc.) In an unbiased yeast-two hybrid screen using the autophagy protein ATG5 as a bait, (ATG5 is a key autophagy protein involved in the E2-like conjugating system that mediates LC3 lipidation in starvation induced autophagy), we have identified Receptor Activated C-Kinase 1 (RACK1; GNB2L1) as a strong interactor and a novel autophagy component⁸. Importantly, the screen showed that the ATG5-RACK1 interaction was indispensable for autophagy induction by classical autophagy inducers (i.e., starvation and mTOR inhibition).

Immunofluorescence-based methods are commonly used to monitor protein-protein interactions. These techniques are mainly antibody-based, and help to visualize interactions and confirm cellular localizations. In this technique, fluorescent tag conjugated antibodies that are specific to proteins of interest are generally used for specific staining. Each protein may be labeled with antibodies coupled to different fluorescent dyes. Using protein-specific antibodies, an overlap in the signal when images are merged indicates the co-localization of proteins under confocal microscopy. The technique is applicable to cells or even tissues. Immunofluorescence techniques provide clues about interaction dynamics⁹, and help identify the size and distribution of protein complexes, while tracking general changes in cellular morphology under different conditions¹⁰. Immunoprecipitation is another commonly used antibody-based technique that allows for the analysis of interactions between given proteins¹⁰. Using this technique, proteins of interest are isolated from cells or tissue extracts using specific antibodies, resulting in the precipitation of proteins that are in a complex or in contact with a protein of interest. Co-immunoprecipitation, where the protein and its co-interactor are detected, reveals not only the interaction between the two proteins, but can measure its strength of interaction under different circumstances¹¹.

This protocol describes in detail key techniques that were used to confirm and characterize ATG5-RACK1 and RACK1-LC3 interaction. The focus is on immunofluorescence and immunoprecipitation techniques, with emphasis of critical steps and pitfalls for autophagy research, as well as troubleshooting suggestions.

Protocol

1. Immunofluorescence

1. Maintain HEK293T human embryonic kidney cells in DMEM high glucose medium, and N2A mouse neuroblast cells in DMEM low glucose medium, in a 5% CO₂-humidified incubator at 37 °C. Supplement the culture media with 10% heat-inactivated fetal bovine (FBS) serum, antibiotics (50 U/mL penicillin, 50 µg/mL streptomycin), and L-glutamine (2 mM).
2. Detach the cells by using 0.25% trypsin. First remove the media of the cell culture then wash the cells with 10 mL sterile phosphate-buffered saline (PBS) and incubate with 1 mL of trypsin at 37 °C for 5 min. Inactivate trypsin by adding 2 mL DMEM medium.
3. Wash the plate well by pipetting. Remove 10 µL of cell solution and mix it with 10 µL of trypan blue. Count the number of live cells using a hemocytometer.
4. **Both HEK293T and N2A are adherent cell lines, however, HEK293T cells detach from the plate easily. Therefore, if working with HEK293T cell line, coat the coverslips with sterile filtered 0.01% poly-L-lysine before seeding the cells.**
 1. Place sterile coverslips in a 10 cm Petri dish. Add the appropriate amount of poly-L-lysine solution onto each coverslide. Incubate the coverslips with poly-L-lysine solution for 10 min.
 2. Remove the poly-L-lysine solution.
Note: Poly-L-lysine is re-usable; it is optional to collect and reuse it for further experiments. Since poly-L-lysine is toxic, wait until all of the solution on the coverslips completely evaporates, and then recover the poly-L-lysine.
 3. Wash the coverslips with sterile PBS.
5. Add 1 mL DMEM into each well of a 12-well plate. Place a coverslide in each well.
6. **Seed 20,000 cells/well, drop by drop while shaking the plate at the same time for a homogenous distribution of cells on the coverslips. Keep cells in the CO₂ incubator at 37 °C and add the drug so that the total incubation time does not exceed 48 h, i.e., for 3 h Torin treatment, add the drug at 45 h post seeding.**
 1. For RACK1-LC3 interaction studies, Torin 1, rapamycin, and starvation were used as autophagy inducers. Cells were incubated with Torin 1 for 3 h (250 nM final concentration); with rapamycin for 16 h (200 nM final concentration); and with Earle's Balanced Salt Solution (EBSS) for 2 h in order to starve cells.
Note: Incubation in DMEM with 10% FBS was used as the control condition.
7. After 48 h of total incubation time, remove the media on the coverslips and wash them with 1 mL PBS.
8. To fix the cells, incubate the cells with 1 mL ice cold sterile filtered 4% paraformaldehyde (PFA) in 1x PBS (pH 7.4), for 20 min at room temperature without any agitation.
Since PFA is sensitive to light, keep (important) the plate in the dark during the incubation period; covering the plate with aluminum foil may help. Moreover, PFA is toxic, therefore, work under a fume hood while fixing the cells.
Note: Keep in mind that the fixation step in immunofluorescence experiments should be optimized, as specific antibodies may work best using different fixation agents and conditions.
9. After 20 min of PFA incubation, first remove the PFA solution on the coverslips and then wash each sample 3 times with 1 mL sterile filtered PBS. At this step, (important) wash the wells one by one so as not to let them dry. Avoid drying since it will interfere with subsequent reactions of the cells, change the cell morphology, and increase non-specific signals.
10. When the wash is complete, keep the samples in 1 mL PBS on ice.
11. Prepare the blocking solution: PBS with 0.1% Bovine Serum Albumin (BSA) and 0.1% saponin.
12. Cover a 6-well plate with paraffin film. Ensure that the plate bottom is smooth after the paraffin film covering. Label the 6-well plate.
13. Using tweezers, transfer the coverslips from the 12-well plates to the 6-well plates covered with paraffin film.
14. Discard the PBS on coverslips and add 100 µL of blocking solution onto each coverslide for permeabilization. Incubate the samples on ice for 30 min.
15. Prepare the primary antibody in blocking solution. Prior to the experiments, optimize the antibody dilutions to define the best working concentrations for staining.
NOTE: For example, LC3 antibody is used at a 1:100 dilution in blocking solution.
16. After the 30 min incubation, discard the blocking solution and wash the samples gently with 1 mL of PBS once.
17. Add 100 µL of primary antibody solution to each coverslide. Incubate the samples at room temperature for 1 h, on a shaker.
NOTE: Incubation time with the primary antibody should be optimized. Depending on the antibody, an overnight incubation at 4 °C may be preferable. It is best to incubate on a shaker gently (100 rotations/min) to equally distribute the antibody on the coverslips.
18. After incubation with the primary antibody, wash samples individually, 3 times with 1 mL PBS.
19. **Prepare 1:500 secondary antibody in blocking solution (optimizations of dilutions of the secondary antibody may be necessary). Here, anti-rabbit IgG Alexa Fluor 568 was used as a secondary anti-rabbit antibody.**
 1. Incubate the samples with the 100 µL of secondary antibody solution for 1 h at room temperature on a shaker. Since the fluorophore-labeled secondary antibodies are sensitive to light, (important) keep the solution in the dark. Place the samples in the dark during and after the secondary antibody incubation.
20. **After the secondary antibody incubation, wash the plate 3 times with PBS.**
NOTE: Based upon experience, some antibodies have high background signals that cannot be eliminated by the PBS washes. If dealing with such an antibody, after step 1.21, keeping the samples in PBS at 4 °C for 6 h may help.
 1. If needed, stain the cells with a DNA-binding dye, such as Hoechst, to mark the nuclei.
21. **To stain more than one protein, apply the same procedure starting from step 1.16 for the second protein.**

1. Make sure that there is no species reactivity between the antibodies when performing staining with more than one antibody (e.g., use mouse and rabbit antibodies).
Note: Here, RACK1 is stained as the second protein by using anti-RACK1 primary antibodies and anti-mouse IgG Alexa Fluor 488 secondary antibodies using the same concentrations mentioned above.
22. Prepare the mounting solution: 50% Glycerol in 1x PBS. Filter through a 22 μ m filter.
23. Wipe the slides with ethanol using a lint-free wipe and add 10 μ L mounting solution to each slide. Using a tweezer, place the coverslips on each drop so that the side with the cells is in direct contact with the mounting medium. Discard excess mounting solution gently using lint-free wipes.
24. Seal the coverslips with a transparent nail polish. First, stabilize the coverslips by adding drops of nail polish on the 4 edges and then seal them completely.
25. Analyze the samples under a fluorescent or confocal microscope as soon as possible, since the fluorescent signal may bleach after a few hours.

2. Immunoprecipitation

1. Detach and count the cells as described in steps 1.2 and 1.3.
2. If working with HEK293T cells, seed 4 million cells for each condition in 15-cm Petri dishes. If working with N2A cells, seed 5 million cells in 15 cm Petri dishes. Incubate the cells in a CO₂ incubator at 37 °C until the end of the experiment.
3. Treat the cells with the relevant drugs or conditions for a defined duration so that the total incubation time of the cells does not exceed 48 h.
Note: For the immunoprecipitation tests in this study, cells were treated for 3 h with Torin 1 (250 nM), 16 h with rapamycin (200 nM), and 2 h with EBSS. DMEM with 10% FBS was added to the cells as the control.
4. **After 48 h of total incubation time, remove the media and harvest the cells. Since HEK293T cells detach easily from the plate, washing the plate with ice cold PBS may be adequate for harvesting. However, N2A cells are more resistant to detachment; use cell scrapers to help harvest the cells.**
 1. Collect all cells in microcentrifuge tubes.
5. Centrifuge samples at 16,000 x g for 15 min at 4 °C. Discard the supernatant and wash the pellet with 1 mL PBS by re-suspending it by pipetting.
6. Repeat centrifuge step, but at the highest centrifuge speed at 4 °C for 15 min. Discard the supernatant.
7. **Prepare Radio Immuno-Precipitation Assay (RIPA) buffer before starting the experiment.**
NOTE: There are 3 types of RIPA buffer; choose according to the interaction intensity of the proteins being investigated (see formulas below).
 1. If there is a strong protein-protein interaction, choose the regular RIPA buffer.
 2. If the interaction is weak, choose either mild RIPA or milder RIPA.
Note: The recipes of these buffers are in the following steps. All buffers that are used in immunoprecipitation tests should be supplemented with protease inhibitors.
 1. Prepare regular RIPA Buffer using 1% NP-40, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate in 50 mM Tris pH 8.
 2. Prepare mild RIPA Buffer using 1% NP-40, 150 mM NaCl, and 0.25% sodium deoxycholate in 50 mM Tris pH 7.5.
 3. Prepare the milder RIPA Buffer using 1% Triton-X 100, 137 mM NaCl, 1% glycerol, and 1 mM sodium orthovanadate in 50 mM Tris pH 7.4.
8. After choosing the appropriate RIPA buffer, supplement the buffer with protease inhibitors (RIPA+). Then, lyse the cells with 3x the volume of the cell pellet in RIPA+.
9. Vortex the suspension for 15 s. Keep it on ice for 5 min. Repeat vortex and icing 5 times and then centrifuge the samples at 16,000 x g for 15 min at 4 °C.
10. Transfer the supernatant to a clean microcentrifuge tube and centrifuge to remove remnant cell debris. Transfer supernatant to a clean tube.
11. **Dilute the protein lysates 1:50 and prepare a 96-well plate with blank and standards. Dilute the samples.**
 1. Mix samples with Bradford solution 1:20. Incubate for 15 min in the dark.
 2. Measure the optical density at 595 nm wavelength. Calculate the protein concentration using absorbance values for each sample.
12. **The day before harvesting the cells, couple protein A Plus beads with antibodies that are specific to the protein of interest.**
 1. Cut the edge of a 200 μ L tip using scissors and use 25 μ L from the bead slurry for each condition.
 2. Wash the beads once with 1 mL PBS, once with 1 mL RIPA buffer, and once with 1 mL RIPA+. Carry out the wash steps at 3,300 x g, 4 °C for 1 min.
 3. After the last wash, add 300 μ L of RIPA+ on the beads and add 1.5 μ g antibody that is specific to the protein to be pulled down.
 4. Incubate the tubes overnight at 4 °C on a rotator (20 rotations/min).
Note: Here, ATG5 was immunoprecipitated using specific anti-ATG5 antibodies.
13. After the overnight incubation of beads with the antibody, wash the beads once with PBS, once with RIPA, and once with RIPA+ as described in step 2.12.2.
14. Add 2 mg of protein lysate on the beads for each condition and complete the total volume to 300 μ L with RIPA+ buffer/tube. Incubate the tubes overnight on a rotator at 4 °C.
15. **After the incubation, wash the beads as previously described (step 2.12.2).**
 1. After the last wash, use a smaller pipet tip to remove all the supernatant without touching the beads.
 2. Add 10 μ L of 3x loading dye: 6% SDS, 30% Glycerol, 16% β -Mercaptoethanol, and 0.1% Bromophenol blue in 1M Tris-HCl pH 6.8.
16. Prepare the input controls using at least 100 μ g protein lysate for each condition. Equalize the sample volumes using water and add the appropriate volume of 3x loading dye.

17. Boil samples at 95 °C for 10 min.
Note: Usually when boiling the samples, the caps of the tubes can open spontaneously and samples are lost. Avoid this by holding the caps closed with a specific cap blocker.
18. Spin down the samples and load onto SDS-PAGE gels to separate the proteins according to their sizes using standard protocols.
Note: In this study on RACK1 and ATG5, samples were run through 12% polyacrylamide gels at 80 V for 30 min, and then at 120 V for approximately 90 min. However, for smaller proteins such as LC3, using 15% polyacrylamide gels is more suitable.
19. After the gel running is complete, transfer the proteins to nitrocellulose or PVDF membranes using either wet or semi-dry transfer methods. Then, block the membranes by incubation in 5% non-fat milk in PBS with 0.05% Tween 20 (PBST) at room temperature for 1 h. Wash membranes 3 times with PBST for 5 min.
20. **Incubate the membranes with primary antibodies at working concentrations for 1 h at room temperature. For primary antibody dilutions, use BSA containing red solutions (5% BSA Cohn V Fraction, 0.02% sodium azide in PBST, pH 7.5; add Phenol red added as a pH marker) to permit the reuse of antibodies.**
NOTE: For example, dilute ATG5 antibody 2,000x in the red solution.
 1. At the end of the incubation time, wash the membranes 3 times with PBST.
21. Incubate the membranes with HRP-conjugated secondary antibodies that were prepared in 5% non-fat milk solution (here, anti-rabbit IgG at 1:10,000 was used). Then wash the membranes 3x with PBST for 5 min.
22. **Make the ECL solution: 25 mM luminol, 9 mM coumaric acid, 3×10^{-4} % H_2O_2 in 70 mM Tris-HCl pH 8.8. Use fresh H_2O_2 for each experiment.**
NOTE: The reaction starts after H_2O_2 addition and it continues for approximately 20 min.
 1. Incubate the membranes and X-ray films for 20 min in ECL. Develop and fix them in a dark room.
23. To check the co-immunoprecipitation of proteins with close or overlapping molecular weights, it may be necessary to strip the antibodies off the membranes by incubating the membranes at 60 °C for 30 min in the stripping buffer: (25 mM Tris-HCl pH 2 and 1% SDS).
24. Repeat steps 2.20 to 2.23 to check for co-immunoprecipitation using different antibodies. For example, following ATG5 detection and stripping of the membranes, RACK1 protein was tested on the same membranes using an anti-RACK1 primary antibody (1:1,000) and secondary antibodies (1:10,000). β -Actin was used as the loading control; and IgG signal was used as the loading control for immunoprecipitated samples.

Representative Results

In **Figure 1** an example of a co-localization result obtained using this protocol is shown. A figure from our recent paper is presented⁸. Here, endogenous RACK1 protein was stained in green, while endogenous LC3 was stained in red. The yellow dots that are observed in the merged pictures indicate sites of overlap between the green and red signals. Thus the yellow dots represent the partial co-localization of these two proteins.

Figure 1: Representative immunofluorescence results. HEK293T cells were cultured on coverslips. Cells were treated or not treated with rapamycin (Rapa, 200 nM, 16 h) or Torin 1 (Torin, 250 nM, 3 h), or starved in EBSS (Stv, 2 h). Then endogenous proteins were immunostained by using anti-RACK1 and anti-LC3 primary antibodies. Cells were analyzed under a confocal microscope. CNT, non-treated cells; Merge, overlay of green and red signals. White arrows show yellow cytoplasmic dots with RACK1 and LC3 co-localization. This research was originally published by Erbil *et al.*⁸ [Please click here to view a larger version of this figure.](#)

In **Figure 2**, an example of an endogenous immunoprecipitation result is presented. In this figure, ATG5 protein was precipitated and RACK1 co-immunoprecipitation was detected under different conditions. Normal rabbit serum was used as a negative control.

Figure 2: Representative immunoprecipitation results. HEK293T cells were treated or not treated with rapamycin (Rapa ,200 nM, 16 h) or Torin 1 (Torin, 250 nM, 3 h), or starved in EBSS (2 h). Endogenous ATG5 protein was immunoprecipitated from cell extracts using anti-ATG5 antibodies that were coupled to protein A Plus beads. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Serum, control rabbit serum. This research was originally published by Erbil *et al.*⁸. [Please click here to view a larger version of this figure.](#)

These results indicate that RACK1 protein is involved in autophagy pathways. RACK1 protein co-localized with the LC3 protein on the autophagosome-like structures during autophagy induction. Moreover, co-immunoprecipitation results showed that RACK1 and ATG5 proteins were interacting even at basal conditions, and the interaction levels increased under autophagy activating conditions. We previously confirmed using p62 degradation and LC3-II accumulation tests in the presence or absence of lysosomal inhibitors (Bafilomycin A, E64D/Pepstatin A) that autophagic flux was not inhibited under the experimental conditions⁸. Therefore, the results presented here and elsewhere showed that RACK1-ATG5 interaction is important for the initial stages of autophagy.

Discussion

Immunoprecipitation and immunofluorescence techniques are crucial for the study of protein-protein interactions. Although these two techniques are commonly used and well-established, several criteria should be considered to define the quality of experiments while using these techniques.

First, primary antibodies that are used in these tests should be specific to the proteins of interest. To ensure this, use shRNA knockdowns or knockout cells to test the specificity of the antibody in question. Additionally, use positive controls, or treat cells with a known inducer of the protein of interest. Batch to batch variations of antibodies exist as well. Moreover, some polyclonal antibodies or sera might have high background and non-specific bands. Although some of these bands may be alternative forms of the protein of interest, in general they result from cross-reactivity of polyclonal antibodies with related proteins or they may be non-specific interactors. Monoclonal antibodies are in general more specific in this sense, although signals generated can be weaker. The confirmation of the antibody specificity with tagged proteins and established anti-tag antibodies may be useful, but endogenous interactions are still more valuable and essential.

Co-localization or co-immunoprecipitation tests will not definitively establish whether protein-protein interactions are direct. It is possible that additional partners can mediate the observed interactions. Indeed, in **Figure 1**, a partial co-localization of RACK1 and LC3 proteins was observed, which could be a sign of their interaction; however, direct binding tests such as *in vitro* pull-down assays using recombinant proteins or Fluorescence Resonance Energy Transfer (FRET) microscopy may be used to establish whether the observed interactions are direct or not. On the other hand, techniques such as gel filtration will help to uncover the complex protein interactions involving more than two proteins in a more convincing manner. A more rigorous analysis of the immunofluorescence results from an autophagy perspective would be to monitor autophagy induction by counting the number of dots that are positive for the LC3 autophagy marker in every cell, since an increase of the number of LC3 positive dots correlates with the number of autophagosomes. Usage of the Pearson's coefficient, Li's method, or Manders' coefficients tests provide better quantitative and comparative assessment of the experimental results.

Another important point is the validation of interactions using independent techniques and different cell lines. Some interactions may be artifacts that are related to the stickiness of proteins or protein overexpression. If not carefully washed, the ATG5 protein is also prone to stick to the beads that are used in immunoprecipitations (both agarose or sepharose beads). Additionally, in immunofluorescence tests, protein overexpression can form aggregates that look like autophagosomes or autolysosomes. Therefore, while performing transient transfections using co-transfected fluorescent proteins as the transfection efficacy control, in immunoblots, expression levels of proteins of interest can be compared to fluorescent protein levels in the extracts using specific antibodies, providing a method of normalization.

In all these tests, reproducibility of results is of utmost importance. We prefer to repeat each experiment at least 3-4 times to be convinced of obtained results. We also perform similar experiments in at least two different cell lines to prove that our observations are not cell type-specific. Additionally, correct positive and negative controls (e.g., beads alone or control antibodies plus serum for immunoprecipitation tests) should be planned to reach correct conclusions. During optimization of immunofluorescence techniques, it is useful to have a secondary antibody only control where primary antibody is omitted. This control make sure that observed signals originate from the primary antibody binding to the protein of interest, and not from a non-specific binding of the secondary antibody.

The number of protein complexes regulating autophagy and other biological events are increasing in discovery, yet the research is far from having a complete picture. Although high-throughput techniques such as mass spectrometry or bioinformatics-based predictions continue to reveal networks of interaction for several autophagy-related proteins, confirmation of these interactions using classical biochemistry and microscopy methods is important in order to uncover functional and dynamic properties of this basic and important cellular pathway.

Disclosures

The authors declare that they have no competing financial interests.

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