RACK1 is an Interaction Partner of ATG5 and a Novel Regulator of Autophagy

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Autophagy is a highly conserved biological mechanism that is responsible for lysosome-dependent recycling of long-lived, abnormal or misfolded proteins as well as dysfunctional or unnecessary organelles (such as depolarized mitochondria) (1). Under normal conditions, basal autophagy helps maintain cellular homeostasis. Autophagy is rapidly upregulated following stress, including nutrient deprivation, accumulation of misfolded proteins, mitochondrial depolarization or exposure to toxic chemicals (2). Autophagy malfunctions were shown to contribute to several pathologies, such as neurodegenerative diseases, lysosomal storage disorders and cancer (3).

The process starts with the nucleation and elongation of double membrane structures called "autophagosomes" or "autophagic vesicles". As they mature through fusion with late endosomes or lysosomes, vesicles give rise to "autolysosomes", a hybrid compartment in which vesicle contents are degraded by the action of lysosomal hydrolases (4). So far, around 33 different core autophagy proteins (ATGs) were described (5). Among them, two ubiquitination-like reactions are key to autophagic vesicle membrane elongation: ATG12-ATG5-ATG16L1 and ATG8 (MAP1LC3 or shortly LC3 in mammals). The first ubiquitination-like reaction results in the covalent conjugation of a K130 residue of the ATG5 protein to a ubiquitin-like protein, ATG12. Following addition of ATG16L1 to the ATG12-conjugated ATG5, a larger complex of around 669-800 kDa forms (6). ATG12-ATG5-ATG16L1 complex serves as an E3-like enzyme for the second ubiquitination-like reaction. Here, the LC3 protein is covalently attached to a lipid molecule, generally to a phosphatidylethanolamine (PE), contributing to the elongation of autophagic membranes (7,8). Conversion of the free, cytosolic form of LC3 (LC3-I) to the lipid conjugated form (LC3-II) leads to its localization to dot-like autophagosome structures in the cytosol and changes its mobility in SDS-PAGE gels (9). ATG5 and LC3 conjugation systems are required for the canonical autophagy pathway to proceed, and abnormalities result in autophagy defects (10).

mTOR serine/threonine kinase is an important upstream regulator of the autophagy pathway. mTOR containing protein complexes (namely, mTORC1 and mTORC2) sense and integrate signals, including amino acid, growth factor and ATP availability, and regulate cellular responses. mTORC1 complex regulates cellular growth and protein synthesis through activation of its downstream targets, including p70 S6 kinase (p70S6K) and 4EBP protein. mTORC1 activity directly inhibits the autophagy pathway through its effect on the ULK1/2 autophagy protein complex (11). In case of deprivation of above-mentioned factors, mTOR activity is blocked, allowing ULK1/2 complex to function. Hence, chemical inhibitors of mTOR activity, such as rapamycin or torin 1, strongly activate autophagy.

Recent studies showed that, WD domain-containing proteins, such as ATG16L1, ALFY and WIPII play significant roles in different stages of the autophagy pathway (12-16). In this study, we...
describe RACK1 (Receptor Activated C-Kinase 1, GNB2L1), a WD40 repeat protein with a seven-bladed β-propeller structure, as an interaction partner of ATG5 and a novel autophagy protein. RACK1 was originally characterized as a scaffold/adaptor protein that binds to protein kinase C (PKC) isoforms (17). Since then, several non-PKC partners of RACK1 were described as well, and the protein was involved in the regulation of a variety of cellular functions, including proliferation, migration, apoptosis, angiogenesis and protein translation (18).

Here, we showed that RACK1-ATG5 interaction was regulated by autophagy-inducing signals, namely starvation and mTOR inhibition. Importantly, we provided evidence that RACK1 protein itself and RACK1-ATG5 interaction were indispensable for autophagy induction. Using chemical inhibitors and genetic approaches, we mapped signaling events regulating RACK1-ATG5 interaction downstream to the mTOR/p70 S6 kinase pathway. Therefore, our data shows that RACK1 is a new and dynamic component of the core autophagy machinery.

Results

RACK1 is a Novel ATG5 Interaction Partner—To discover novel ATG5 interactors and autophagy-related proteins, we performed a yeast two-hybrid (Y2H) screen using ATG5 as a bait. At least 3 different clones of RACK1 (GNB2L1) were found to interact with the autophagy protein. The results of the Y2H screen will be described in detail elsewhere (Erbil S and Gozuacik D, manuscript in preparation).

To confirm the interaction, we cloned full length RACK1 cDNA into a mammalian expression vector. Following overexpression experiments in HEK293T cells, we could show that immunoprecipitated ATG5 could pull-down RACK1 protein (Fig. 1A). Conversely, RACK1 immunoprecipitation resulted in the pull-down of ATG5 (Fig. 1B). We could also confirm the interaction between the endogenous proteins in mouse embryonic fibroblast cells (MEFs) (Fig. 1C and 1D). Moreover, we could validate that the two proteins interacted directly in GST pull-down in-vitro binding assays using recombinant proteins (Fig. 1E). Finally, examination of cells using confocal microscopy under basal fed conditions (culture in DMEM plus 10% FBS) showed that both proteins were partially colocalized in cytoplasmic dots (Fig. 1F and 1G). All these data indicated that, RACK1 is a novel ATG5-interacting protein.

Dynamic Nature of ATG5-RACK1 Interaction under Autophagy-inducing Conditions—To further confirm ATG5-RACK1 interaction, we performed gel filtration experiments with endogenous protein extracts using a gel filtration column with a separation range between 5-5000 kDa. In line with the published data (12,19), endogenous ATG12-5-16 complex was eluted from the column at 669-800 kDa fractions. Endogenous RACK1 protein was mostly absent in ATG12-5-16 containing fractions under fed conditions in HEK293T (Fig. 2A, CNT) and Neuro2A (N2A) (Fig. 2B, CNT) cells. Importantly, upon autophagy activation using an mTOR inhibitor, torin 1 (Fig. 2A and 2B), the amount of RACK1 protein that was recovered in fractions containing ATG12-5-16 complex was significantly increased.

Tri-SILAC labeling and LC-MS/MS analysis was performed following immunoprecipitation of a FLAG-tagged ATG5 protein from cells. Using this technique, we could confirm ATG5-RACK1 interaction under autophagy stimulating conditions (Fig. 2I).

Immunoprecipitation assays supported the data as well. Indeed, RACK1-ATG5 interaction was prominently increased following treatment of cells with mTOR inhibitors rapamycin or torin 1 (Rapa, Torin, Fig. 3A and 3C). Starvation of cells stimulated the interaction between two proteins as well (Stv, Fig. 3B and 3C).

To check the intracellular localization of RACK1-ATG5 interaction following autophagy activation, we performed confocal microscopy analyses. Treatment of cells with rapamycin or torin 1, or starvation led to an increase in the number of dot-like structures where ATG5 and RACK1 colocalized (Fig. 3D). Moreover, RACK1 positive dots colocalized with the autophagosome marker LC3 (Fig. 3E), indicating that these structures truly represent autophagosomes. (20).

RACK1 is Required for Autophagy Activation Following mTOR Inhibition and Starvation—Interaction of RACK1 with an
autophagy-related protein complex and in an autophagy stimulus-dependent manner indicated that RACK1 could be an important component of autophagy regulation. Alternatively, RACK1 could be an autophagy target that was recruited to autophagosomes through its interaction with autophagy proteins.

In order to determine the importance of RACK1 in autophagy mechanisms, we knocked down RACK1 using specific siRNAs, and quantified autophagy activation levels using LC3 dot quantification analyses. In cells transfected with a control siRNA (CNT siRNA), mTOR inhibition or starvation strongly induced autophagy (around 4-5 times basal levels). Strikingly, knockdown of RACK1 under the same conditions significantly attenuated autophagy induction by these stimuli (Fig. 4A and 4B). Moreover knockdown of RACK1 prevented LC3-II formation and led to an accumulation LC3-I in immunoblots. In line with this, p62 degradation that was observed in extracts from CNT siRNA transfected cells was abolished following RACK1 downregulation (Fig. 4A). Under these conditions, addition of bafilomycin A (an inhibitor of autophagosome-lysosome fusion blocking autolysosomal degradation) led to LC3-II accumulation in controls and could block p62 degradation. In contrast, bafilomycin A did not cause further p62 or LC3-II accumulation in extracts from cells with RACK1 knockdown (Fig. 4A). All these results indicate that RACK1 is involved in upstream stages before autophagosome-lysosome fusion and autolysosome formation.

To detect whether RACK1 could be an autophagy target, we stimulated autophagy using rapamycin or starvation in the absence or presence of translation inhibitor cycloheximide or bafilomycin A, and checked endogenous RACK1 protein levels (Fig. 4C and 4D). None of these treatments resulted in RACK1 degradation even at longer incubation times (24 h rapamycin treatment or 8 h starvation), and independent of cycloheximide treatment, bafilomycin A treatment did not result in significant RACK1 accumulation. Therefore, RACK1 itself is not degraded by autophagy, but it is required for autophagy induction by classical autophagy stimuli, i.e. mTOR inhibition and starvation.

mTOR and p70S6K regulate RACK1-ATG5 interaction—Since inhibition of mTOR resulted in an increase in RACK1-ATG5 interaction, we wondered whether RACK1-ATG5 interaction was regulated by mTOR itself or proteins that function downstream to mTOR.

Firstly, we overexpressed mTOR protein and checked RACK1-ATG5 interaction in co-immunoprecipitation assays. Under these conditions, we observed that overexpression of the mTOR kinase resulted in a decrease in RACK1-ATG5 interaction (Fig. 5A). In line with data obtained using chemical inhibitors, knockdown of mTOR using specific shRNAs resulted in an increase in RACK1-ATG5 interaction (Fig. 5B).

p70S6 kinase is a major serine/threonine kinase that functions downstream to mTOR. To check whether p70S6K played a role in the regulation of RACK1-ATG5 interaction, we first overexpressed a wild-type kinase (21). Indeed, overexpression of the kinase led to the dissociation of RACK1 from ATG5 (Fig. 5C). To further confirm that p70S6K protein itself was necessary for the observed effects on the interaction, we transfected cells with specific siRNAs. Indeed, knockdown of p70S6K promoted association of RACK1 with its partner ATG5 (Fig. 5D). All these results showed that RACK1-ATG5 interaction is regulated by mTOR, in a p70S6K-dependent manner.

Determination of RACK1 amino acid residues that are critical for the interaction—In a study searching for S6 kinase targets using a phospho-proteomics screen, Jastrzebski et al. found that 4 serine or threonine (S or T) residues in RxxS/T consensus sequences on RACK1 could be phosphorylated in a rapamycin-sensitive and S6 kinase-dependent manner (22). When we compared RACK1 amino acid sequences from different organisms from yeast to man, we observed that 3 of these consensus sequences were highly conserved (Consensus sequences containing residues T39, S63, T128) (Fig. 6A). We have mutated these S/T residues to alanine (A) mimicking the dephosphorylated state (Fig. 6B), or to aspartic acid (D) mimicking the phosphorylated state. We then performed ATG5 immunoprecipitation assays and checked its interaction with wild-type (wt) or alanine mutant RACK1 proteins (T39A, S63A or T128A
mutants). As shown in Fig. 6C, while the interaction of ATG5 was prominently increased with RACK1 T39A and T128A mutants, S63A mutation completely abolished RACK1-ATG5 binding.

RACK1 S63 residue is located in the WD40 repeat 2 region (Fig. 7A). We used docking and MD simulations to decipher molecular mechanisms of the interaction between RACK1 and ATG5, and particularly the role of S63 in this interaction. Haddock (High Ambiguity Driven Protein-Protein Docking, http://haddocking.org) is among the most widely used protein-protein docking algorithms. The tool proved itself for prediction of structures of unknown protein-protein complexes. Using the tool, RACK1-ATG5 was modeled and residues found within 3 Å of the other subunit were selected as the binding interface (ATG5 residues, green and RACK1 yellow) (Fig. 7B). The predicted protein-protein complex structure was found to possess a network of salt bridge interactions that were formed by D4-H62, D6-K38, D163-H64, of which negatively charged aspartic acids (D) belong to ATG5 while positively charged residues (K and H) to RACK1 (Fig. 7C). Moreover the backbone amide group of S63 in RACK1 forms a hydrogen bond with one of the Oδ of D6 in ATG5 (Fig. 7C). Along with this direct involvement of S63 in RACK1-ATG5 interaction, S63 might indirectly contribute to salt-bridge interactions through taking part in positioning the histidines (62 and 64) of RACK1. Overall, data presented here unveils a network of interactions in which a pivotal role for S63 might be suggested.

Further structural information about the role of S63 of RACK1 was collected from MD simulations of the native S63 or S63A and S63D mutant complexes. RMSD of backbone atoms of the complex converged to a plateau and oscillated less than 2 Å range, suggesting equilibration of the protein complexes (Fig. 7G). Moreover, the simulation data showed that S63A mutation led to the loss of D6-K38 interaction while the native and S63D complexes possessed the D6-K38 interaction (Fig. 7D-F). In line with these observations, fluctuations of ATG5 in the S63A complex have increased, particularly for the regions 32-36 and 50-54 that were also found at the binding interface (Fig. 7H). Increased mobility of the interface in S63A mutant indicated destabilization of the complex compared to native S63 and S63D complexes (Fig. 7H). Overall computational analyses confirmed the role played by S63, and also provided insights into the molecular machinery of RACK1-ATG5 interaction.

**RACK1-ATG5 Interaction was Necessary for mTOR Inhibition- or Starvation-induced Autophagy**—In order to check whether RACK1-ATG5 interaction is important for autophagy activation or not, we expressed wild-type, S63A or S63D mutant RACK1 N2A (Fig. 8A-D) cells, and quantified autophagy activation by torin 1 and starvation in the absence or presence of Bafilomycin A. LC3 dot quantification under control conditions (pcDNA3 expression) showed that torin 1 and starvation led to a strong and clear autophagy activation (2 to 3 times basal level) (Fig. 8A-D). Interestingly, while wild-type RACK1 or S63D mutant expression further increased the amplitude of torin 1 or starvation-induced autophagy and LC3-II formation on immunoblots, expression of the S63A mutant significantly and reproducibly inhibited LC3 dot numbers in cells and LC3-II formation in blots (Fig. 8A-D). Bafilomycin A treatment under these conditions did not prominently lead to accumulation of LC3 dots, yet in blots an increased in LC3-II levels were observed in wild-type RACK1 or S63D mutant expressing cell extracts. No significant LC3-II accumulation was observed in S63A mutant expressing cells following bafilomycin A treatment, underlining the fact that autophagosome formation was perturbed by this mutant.

We also tested whether wild-type RACK1, or its mutants could rescue autophagic activity in cells with RACK1 knockdown. While, the wild-type protein or the S63D mutant could reverse autophagy inhibition that was observed following RACK1 knockdown, S63A mutant did not show the same effect (Fig. 9B and 9C). Inhibition of lysosomal protease activity with E64d/pepstatin A led to an accumulation of LC3-II in wild-type RACK1 or S63D mutant expressing cell extracts. No significant LC3-II accumulation was observed in S63A mutant expressing cells following bafilomycin A treatment, underlining the fact that autophagosome formation was perturbed by this mutant.
These results clearly showed that, RACK1-ATG5 interaction is required for autophagy induction by classical autophagy inducing stimuli, i.e. mTOR inhibition and starvation.

Discussion

In this article, we showed that RACK1 protein is an interaction partner of the key autophagy protein ATG5. We confirmed this observation using various independent techniques, i.e. yeast two-hybrid, co-immunoprecipitation tests, confocal microscopy, SILAC analyses and gel filtration assays, and validated our results in 3 different cell lines (HEK293T, MEF, Neuro2A). In fact, we showed that endogenous RACK1 was included in a previously reported autophagy-related 669-800 kDa protein complex containing ATG12-5-16 (19).

Importantly, RACK1-ATG5 interaction was a dynamic one. Treatment of cells with classical autophagy inducers rapamycin and torin 1 (mTOR inhibitors) and starvation stimulated RACK1-ATG5 interaction in cytoplasmic dot-like structures, indicating that under autophagy-inducing conditions, the interaction occurred on ATG5 containing isolation membranes. Indeed in previous proteomics analyses, RACK1 was detected in isolated LC3 positive autophagic vesicles (23). To rule out the possibility that RACK1 protein itself could be an autophagy target, we performed kinetic autophagy experiments in the absence or presence of the protein translation inhibitor cycloheximide and bafilomycin A. We observed no significant degradation even at latest time points. Therefore, RACK1 is not an autophagy target protein and its association with the autophagy machinery should have a functional role.

In line with this idea, we observed that RACK1 was required for autophagic vesicle formation, and knockdown of RACK1 attenuated formation of LC3-positive autophagosomes. Interaction of RACK1 with ATG5 was a key event in autophagy regulation. A mutant RACK1 that could no longer interact with ATG5 (the S63A mutant), blocked autophagy-induced by mTOR inhibitors or starvation. Data presented here showed that RACK1 is an essential component of the autophagy machinery and its interaction with ATG5 is a critical event that recruits it to the autophagy pathway.

mTOR signaling pathway is one of the major regulators of autophagy. mTOR kinase complexes sense various upstream stimuli (ATP or amino acid depletion, growth factor deprivation etc.) and serve as master regulators of cellular anabolic and catabolic pathways. For example, under nutrient- and growth factor-rich conditions, activation of mTOR complex 1 (mTORC1) result in the phosphorylation and activation of the p70S6 kinase and eIF4BP (eukaryotic translation initiation factor-4E binding protein), stimulating protein synthesis and cell survival (24). Conversely, inhibition of mTOR strongly activates autophagic protein degradation. In our system, overexpression of the mTOR kinase or p70S6 kinase led to the dissociation the RACK1-ATG5 complex. In addition to data obtained using mTOR inhibitors rapamycin and torin 1, knockdown of the mTOR kinase itself or p70S6K stimulated RACK1-ATG5 association. Our results indicate that RACK1-ATG5 association control is one of the mechanisms used by mTORC1-p70S6 kinase pathway to regulate autophagy.

In fact, RACK1 S63 and other residues including T39 and T128 were previously suggested to be targets of S6 kinase isoforms (22). Conversion of S63 to alanine (S63A mutation) on RACK1, mimicking a non-phosphorylated form, was sufficient to abolish RACK1-ATG5 interaction and inhibit mTOR-related or starvation-induced autophagy. Moreover, the S63A mutant could not rescue autophagy inhibition following RACK1 knockdown. On the other hand, T39A/D or T128A/D mutants did not dissolve the RACK1-ATG5 complex. On the contrary, RACK1 T39A and T128A mutations did result in an increased interaction. Yet, different from the S63A mutant, expression of T39A and T128A mutants or their phospho mimicking aspartic acid (T39D and T128D) forms, did not lead to any change in the autophagic activity (Erbil S and Gozuacik D, unpublished results). These results indicated that, an optimum level of RACK1-ATG5 interaction is necessary and sufficient for autophagy.

Our results showed that both mTOR and p70S6K proteins were necessary for the regulation
of RACK1-ATG5 interaction, and overexpression of kinase active mTOR or p70S6K proteins had an inhibitory effect on complex formation. Nevertheless, our studies using mutants did not confirm autophagy-related relevance of a direct phosphorylation of RACK1 on previously reported p70S6K putative target residues (22). On the contrary, modification of a critical serine to a non-phosphorylatable form (S63A) was the only mutation that had a significant effect on autophagy activation. Therefore, further studies are required to determine kinase(s) or phosphate(s) that are downstream to mTOR and that regulate autophagy through modulation of this interaction. Overall, we showed that RACK1-ATG5 interaction is indispensable for autophagic activity.

Another question is about the role of RACK1 in the autophagy pathway. RACK1 is a scaffold/adaptor protein containing WD40 repeats that are important for protein-protein interactions. Of note, RACK1 was not required for ATG12-ATG5 or ATG12-5-16 protein complex formation (Erbil S and Gozuacik D, unpublished results). Therefore, RACK1 possibly functions in the recruitment of other relevant proteins to autophagic isolation membranes near ATG12-5-16 complexes. Future analyses, including SILAC assays, will allow the discovery of proteins whose interaction increase with RACK1 under autophagy-inducing conditions.

In fact, involvement of RACK1 was suggested in *Drosophila* studies where loss of RACK1 was associated with attenuated autophagy responses and glycogen storage defects (14). While, our manuscript was in preparation, Zhao et al. showed that RACK1 was required for hepatocyte autophagy and lipophagy (15). They also provided data about the contribution of RACK1 as scaffold to VPS34 PI3-kinase complex formation. It is possible that RACK1 plays a role at different stages of autophagosome biogenesis. In our hands, an ATG5-binding deficient mutant of RACK1 was able to block autophagy, indicating that ATG5-related functions of the scaffold protein are also rate-limiting for autophagy activation.

In this study, we characterized RACK1 as a new ATG12-5-16 complex component and a novel mTOR-dependent hub in the regulation of basic autophagy pathways. Overall, we believe that our results will open the way to a more comprehensive understanding of autophagy pathway and its molecular control mechanisms.

**Experimental Procedures**

*Plasmid Constructs, siRNAs—* To create the pGBK77-ATG5 yeast two-hybrid bait construct, full-length human ATG5 cDNA insert in the pCI-ATG5 construct was cut with Smal enzyme, blunted and blunt ligated into the pGBK77 Y2H bait vector that was previously cut with NcoI and Klenow filled. Flag tagged human ATG5 (RC235557), Human RACK1 (SC116322) and Flag tagged human RACK1 plasmids (RC505092) were purchased from Origene. Wildtype (wt) p70S6K plasmid (8984) (21), wt mTOR plasmid (26036) (25), shmTOR plasmid (1855) (26) were all provided through Addgene. siRNA RACK1 (M-006876-01) and CNT-siRNA (D-001210-01-20) were purchased from Dharmaco.

*Site-directed in vitro Mutagenesis—* Site-directed mutagenesis of T39, S63, and T128 residues of RACK1 was performed using Quickchange site-directed mutagenesis kit according to manufacturer's instructions (Stratagene, 200519-5). Following primers were used for mutagenesis: S63D forward: ctgcggggtacagacactttgatag; S63D reverse: actaacaaagtggtcgtgaccccgcag; S63A forward: ctgcggggtacagacactttgatag; S63A reverse: actaacaaagtggtcgtgaccccgcag; T39D forward: ctctccgcctctcgagataaggacatcatcatgtgg; T39D reverse: ccacatgatgatgatgtccttatctcgagaggcggagag; T39D forward: atcctctcgctctcgagataaagccatcatcatgtggaa; T39A reverse: ttccacatgatgatgatgtccttatctcgagaggcggagagat; T128D forward: attgtctctggatctcgagataaagacatcaagctatggaat; T128D reverse: attccatagcttgatgtctttatctcgagatccagagacaat; T128A forward: attgtctctggatctctgagataaagccatcatcatgtggaa; T128A reverse: attccatagcttgatgtctttatctcgagatccagagacaat

*Yeast Two-Hybrid (Y2H) Screen—* For screening, pGBK77-ATG5 construct was used as bait and a thymus cDNA library in the pACT2
vector was used as prey (Clontech). Screens were performed according to the protocols provided by the manufacturer. Briefly, constructs were transformed into the AH109 yeast strain, and interactions were monitored on selection plates lacking leucine, tryptophan, histidine and/or adenine. After 3–5 days of selection, plasmid inserts in surviving colonies were amplified using colony PCR, sequenced and characterized.

**Cell Culture**—HEK293T human embryonic kidney cells and mouse embryonic fibroblasts (MEFs) were maintained in DMEM high glucose medium (Sigma, 5671) and Neuro2A (N2A) mouse neuroblast cells were maintained in DMEM low glucose medium (Sigma, 5546) in a 5% CO2- humidified incubator at 37°C. Culture media were supplemented with 10% heat-inactivated fetal bovine serum (Biowest, S1810-500), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Biological Industries, 03-031-1B) and L-glutamine (2 mM, Biological Industries, B103-020-1B). In starvation experiments, cells were grown in Earl’s balanced salt solution (EBSS; Biological Industries, B102-010-1A). For chemical induction of autophagy, cells were incubated in media containing torin 1 (250 nM, Tocris, 4247) or rapamycin (200 nM, Sigma, R0395) that were dissolved in DMSO (Sigma, VWRSD2650). Cycloheximide (0.5 µg/ml, Sigma, 01810) was used in degradation tests.

**Gene Transfer**—Calcium phosphate transfection method was used for gene transfer into HEK293T cells according to standard protocols. N2A cells were transfected using Polyethyleneimine (PEI; PolySciences Inc., 23966) according to protocols that were described elsewhere (27).

**Cell Lysate Preparation and Immunoblotting**—Cells were washed in PBS, collected by centrifugation and lysates were prepared in RIPA buffer (25 mM Tris, 125 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.004% sodium azide, pH 8.0) containing complete protease inhibitors (Sigma, P8340) and 1 mM PMSF (Sigma, P7626). Protein concentrations were determined using Bradford assay (Sigma, B6916). Immunoblotting procedure was previously described (28). Immunoblots were performed using antibodies against ATG5 (Sigma, A0856), RACK1 (Santa Cruz, sc-17754), ATG16L1 (MBL, PM040), LC3B (Novus, NB100-2331), mTOR (Cell Signalling, 2972), p70S6K (Cell Signalling, 9206S), p62 (Abnova, H0000878), LC3 (Novus, 2331), and β-Actin (Sigma, A5441). ImageJ software was used to quantify protein band intensities (29).

**Immunoprecipitation Tests**—For the immunoprecipitation of Flag tagged proteins, whole cell lysates (1 mg) were incubated with an anti-Flag M2 affinity gel (Sigma #A2220). For the immunoprecipitation of endogenous RACK1 or ATG5, Protein G Plus Agarose Beads (Santa Cruz, sc-2002) or Protein A Plus Agarose Beads (Santa Cruz, sc-2001) were coupled to 10 µg/ml anti-RACK1 or anti-ATG5 antibodies respectively. Normal rabbit serum (Santa Cruz, sc-2027) or normal mouse serum (Santa Cruz, sc-45051) was used as negative control. Samples were analyzed by immunoblotting as described.

**GST Pull-Down Assay**—For the pull-down assays, 25 µl Glutathione Sepharose 48 beads (GE Healthcare, 17-0756-01) in Tris-HCl (20 mM, pH 7.5) were initially incubated (o/n, +4°C) with 5 ng of GST-tagged ATG5 recombinant protein (Abnova, H00009474). Following 3 washes in the same buffer, beads were incubated for 8 h at +4°C with 5 ng His-tagged RACK1 recombinant protein (Abcam, ab119442). Samples were analyzed by immunoblotting as described.

**Immunofluorescence Analyses**—2 x10⁵ cells were cultured on poly-L-lysine (Sigma, P8920) coated glass coverslides. Following 2 hours of starvation in the EBSS medium, incubation for 3 h with torin 1 (250 nM, Tocris, 4247) or 16 h with rapamycin (200 nM, Sigma, R0395), cells were fixed in ice cold 4% paraformaldehyde-PBS and permeabilized in PBS with 0.1% BSA (Sigma, A4503) and 0.1% saponin (Sigma, 84510). Immunostaining was performed using anti-RACK1 antibody (Santa Cruz, sc-17754), anti-ATG5 antibody (Sigma, A0856) or anti-LC3 antibody (Sigma, P7626). Samples were analyzed using a Carl Zeiss LSM 710 confocal microscope (Zeiss, Germany).

**Quantitative LC3 Analyses**—LC3 dot positivity was quantified following 2 hours of
starvation in the EBSS medium or 3 hours incubation with torin 1 (250 nM, Tocris, 4247) or 16 hours incubation with rapamycin (200 nM, Sigma, R0395), in the presence or absence of 1h incubation with E64D (10 µg/ml, Santa Cruz, 201280), Pepstatin A (10 µg/ml, Sigma, p5318) or Bafilomycin A (100 nM, Sigma, B1793). 10 LC3 dots per cell were considered as the threshold for basal autophagy. Minimum 150 cells were counted for each condition, and results were expressed as percentage of LC3 dot positive cells in total cell population (30).

**Gel Filtration Tests**—For separation of proteins, a Superose™ 6 10/300 GL column (separation range 5 to 5000 kDa) was used (GE-Healthcare, 17-5172-01). Sigma molecular weight kit was used for the calibration of the system (Sigma, MWGF-1000). Chromatography analyses were performed using an AKTA Prime FPLC system (AKTA FPLC UPC900 / P920 System / Frac 900 fraction collector, GE Amersham Pharmacia, US). For separation of proteins, a modification of a previously reported protocol was used (12). Briefly, chromatography column was calibrated with sonicated balancing buffer (1:1, 0.05% glycerol PBS: RIPA buffer) to optimize flux, absorbance and pressure parameters (Pressure: 1.5 MPa; Flow velocity: 0.5 ml/min.; Fraction volume: 0.5 ml; Loop volume: 500µl.) Protein extracts from HEK293T or N2A cells (5-7.5 mg protein) were loaded in a 500 µl volume, and 500 µl fractions were collected. The column was washed with 36 ml deionized water (3x column volume), and re-calibrated with sonicated balancing buffer after each sample. Immunoblotting of collected fractions were performed as described.

**SILAC Labelling**—Tri-SILAC labelling of Flag-tagged ATG5 expressing HEK293T cells was carried out using SILAC medium (DMEM without L-lysine and L-arginine (Thermo Fisher Scientific, 89985) supplemented with penicillin/streptomycin (100 units/ml, 100 µg/ml; PAN Biotech, 06-07100), L-glutamine (PAN Biotech, P04-80100), and 10 % dialyzed fetal calf serum (Gibco, 26400-044)). Heavy labelling was carried out in the addition of 42 µg/mL L-arginine-13C6,15N4-hydrochloride (Arg10) (Sigma-Isotec, St. Louis, MO; cat.no. 608033) and 40 µg/mL L-lysine-13C6,15N2-hydrochloride (Lys8) (Sigma-Isotec, 608041). Medium labelling was performed following addition of 42 µg/mL L-arginine-13C6-hydrochloride (Arg6) (Cambridge Isotope laboratories Inc, Andover MA., CLM-2265) and 40 µg/m L-lysine-4,4,5,5-d4 hydrochloride (Lys4) (Sigma-Isotec; 616192). Light labelling was achieved using corresponding non-labeled amino acids L-arginine (Sigma, 11039) and L-lysine (Sigma, L8662). All three types of SILAC media were supplemented with 112 µg/mL L-proline (Fluka 81709) (31). Labelling was continued for 5 cell doublings. Labels were swapped in independent experiments.

**Liquid Chromatography (LC)-Tandem Mass Spectrometry (MS/MS) Sample Preparation**—Labeled cells were collected by centrifugation, washed in PBS and lysed in a modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40 and 0.25% Sodium deoxycholate) with complete protease inhibitors (Roche, 10269700). Protein concentrations were determined using BCA assay (BCA Kit, Pierce, 23225). Overexpressed Flag-tagged ATG5 was immunoprecipitated from 2.5 mg of whole cell lysates using an anti-Flag M2 affinity gel (Sigma #A2220). Beads were incubated at 75°C for 30 min in Laemmli buffer (70% Tris/SDS pH 6.8, 30% Glycerol, 10% SDS and Bromophenol blue) with 1 mM DTT, then 5.5 mM iodoacetamide was added and samples were further incubated at RT for 30 min., allowing reduction and alkylation. Samples were separated by electrophoresis in 12% Bis-Tris gels (NuPAGE®Novex, NP0335) and using 2x MOPS-SDS running buffer (NuPAGE®Novex, NP001) with 0.07% antioxidant supplement (NuPAGE®Novex, NP0005). The gel was incubated for 10 min. in fixing solution (40% ddH2O, 50% methanol, 10% acetic acid) and protein bands were visualized using NuPAGE®Novex stainer solutions (NuPAGE®Novex, 46-7015 and 46-7016). Protein bands were cut from gels and washed in ABC buffer (100mM ammonium bicarbonate, pH:7.5) followed by incubation with ethanol (HPLC grade). Then, cold trypsin solution (PROMega, V511C) was added onto dried gel cubes followed by 20 min. incubation at RT and 0/n incubation at 37°C. Trypsin activity was blocked by adding 2% Trifluoroacetic acid (TFA). Following ethanol washes, supernatants were
concentrated using SpeedVac (ThermoScientific). Then Buffer A (0.5% acetic acid) and Buffer A/A* (75% / 25%; Buffer A*: 3% acetonitrile, 0.3% TFA) was added at a 3:1 ratio. Peptides were eluted from StageTips with C18 material (3M Empore 2215) using Buffer B (0.5% acetic acid, 80% acetonitrile) (32). Eluted samples were concentrated using SpeedVac, and ready-to-inject peptide samples were obtained following addition of 10 µl Buffer A/A*.

**LC-MS/MS—**Nanoscale HPLC was performed using either an Agilent 1200 or an Eksigent NanoLCultra connected online to a LTQ-Orbitrap XL (Thermo Scientific) set-up and a 15 cm in-house-packed fused-silica emitter microcolumn (SilicaTip PicoTip; New Objective) filled with reverse-phase ReproSil-Pur C18-AQ beads with the size of 3.5 µm and inner diameter of 20 µm (Dr. Maisch, GmbH, Germany). Peptides were separated over a linear gradient of 10-30% acetonitrile in 0.5% acetic acid with a flow rate of 250 nl/min. All full-scan acquisition was carried out in the FT-MS part of the mass spectrometer in the range of m/z 350–2000 with an automatic gain control target value of 106 and at a resolution of 60,000 at m/z 400. AGC target value was set to 5,000, ion selection thresholds were set to 1000 counts and a maximum fill time of 100 ms in order to sequentially perform MS/MS on the five most intense ions in the full scan (Top5) in the LTQ in a data-dependent mode. Wide-band activation was enabled with an activation q=0.25 applied for 30 ms at a normalized collision energy of 35%. Unassigned or single charged ions were excluded from MS/MS. Dynamic exclusion was applied to reject ions from repeated MS/MS selection for 45s.

**MS Data Analysis—**LC-MS/MS raw files, belonging to three biological replicates were processed all together in MaxQuant version 1.4.0.8 with default parameters using UniProt human database (33). MaxQuant was used to filter the identifications for a FDR below 1% for peptides, sites and proteins using forward-decoy searching. Match between runs was enabled.

**Molecular Dynamics Simulations—** Molecular dynamics (MD) simulations were performed using the predicted complex structure of RACK1-ATG5 obtained from docking step and its RACK1 mutants, S63A and S63D. The protein complexes were placed in water boxes with dimensions of 115x75x80 Å³, then the systems were ionized with a 100 mM NaCl solution to a neutral state. The resulting systems composed of ~63,000 atoms were used in MD simulations using the NAMD program (34) with the CHARMM22 parameters (35,36) which included correction map (CMAP) for backbone atoms (37,38). Water molecules within the system were treated explicitly using the TIP3P model (39). An NpT ensemble was used in MD simulations with periodic boundary conditions, and the long-range Coulomb interactions were computed using the particle-mesh Ewald algorithm. Pressure was maintained at 1 atm and temperature was maintained at 310°K using the Langevin pressure and temperature coupling. A time step of 2 femtoseconds was used in all MD simulations. The systems are fully energy minimized in 20,000 steps and equilibrated under constant temperature and volume for 5 nanoseconds. Visual molecular dynamics (VMD) was used for the analysis of trajectories and visualization of all of the structures. Root mean square displacements (RMSD) for the backbone atoms (C, N and Ca) and residue-wise root mean square fluctuations (RMSF) of Ca atoms were calculated as measures of equilibration and flexibility, respectively.
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Conflict of Interests

No potential conflicts of interest were disclosed.

Author Contributions

DG supervised and coordinated the study and wrote the paper with SE. SE performed the experiments shown in Figures 1-6 and 8,9. OO and GM contributed to the experiments shown in Figure 1C, 1D. CK contributed to the optimization of gel filtration-FPLC tests. EDT and OUS carried out 3D modeling and docking studies. EGM contributed to 3D modeling studies. GG and FG performed Y2H screens and initial co-IP confirmations. JD supervised SILAC analyses that were performed by SE. All authors reviewed the results and approved the final version of the manuscript.
RACK1 and autophagy

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Figure legends

FIGURE 1. RACK1 is a novel ATG5 interactor. A, HEK293T cells were co-transfected with plasmids encoding Flag-tagged ATG5 and/or non-tagged full length RACK1 proteins. 48h after transfection, immunoprecipitations (IP) were performed using Flag beads. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Input, total cell extract. IgG, Immunoglobulin G. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. B, HEK293T cells were co-transfected with Flag-RACK1 and/or non-tagged ATG5 constructs and immunoprecipitations (IP) were performed using Flag beads. C, Endogenous ATG5 protein was immunoprecipitated from wild-type MEF 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. D, Endogenous RACK1 protein was immunoprecipitated from wild-type MEF cell extracts using anti-RACK1 antibodies that were coupled to Protein G Plus beads. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Serum, control mouse serum. E, GST pull-down assay. Glutathione Sepharose beads that were bound to GST-ATG5 recombinant protein or not, were incubated with His-RACK1 recombinant protein and washed. Input, immunoblotting of recombinant proteins. GST pull-down, proteins after pull-down. Note that His-RACK1 did not bind to beads alone. F, HEK293T cells were cultured on coverslides and co-transfected with GFP-tagged RACK1 (green) and Cherry-tagged ATG5 (red) constructs. 48h post transfection, cells were fixed and analyzed under a confocal microscope. Merge, overlay of green and red signals. White arrows show yellow cytoplasmic dots formed by RACK1 and ATG5 colocalization. G, Non-transfected HEK293T cells were cultured on coverslides. After 72h of incubation, cells were fixed and endogenous RACK1 and ATG5 proteins were immunostained using anti-RACK1 and anti-ATG5 primary antibodies. Anti-mouse IgG Alexa Flour 488 (green) or anti-rabbit IgG Alexa Flour 568 (red) were used as secondary antibodies, respectively. Cells were analyzed under confocal microscope. Merge, overlay of green and red signals. White arrows show yellow cytoplasmic dots formed by RACK1 and ATG5 colocalization.

FIGURE 2. RACK1 is a novel component of a large ATG12-5-16 protein complex. A, Non-transfected HEK293T cell were treated with torin 1 or DMSO carrier control, and total cell lysates were fractioned in a gel filtration column. Chromatography fractions (F1-13) were separated in SDS-PAGE gels and immunoblotted using anti-ATG16, anti-ATG5 and anti-RACK1 antibodies. CNT, DMSO carrier control; TORIN, torin 1 treatment (250 nM, 3h); ATG16, ATG16L1; L, total cell lysate; F1 and F2, >800 kDa fractions; F3-6, 800-669 kDa fractions; F7-F10, 669-443 kDa fractions; F11 and F12, 443-200 kDa fractions; F13, 200-150 kDa fraction. No protein complexes were detected in lower molecular weight fractions. B, N2A cell were treated with torin 1 or DMSO carrier control, and total cell lysates were fractioned in a gel filtration column as in A. C, The chromatogram showing peaks of the molecular weight marker mix (Sigma, MWGF1000), Vₑ, elution volume. D, OD₅₉₅ absorbance confirmation of the peaks. E, Standardization of the gel filtration column by Vₑ/Vₒ, Vₒ, void volume. F, The curve showing correlation of fractions with protein sizes in kDa. G and H, representative chromatograms obtained for HEK293T (G), and N2A (H) cell lines. I, Tri-SILAC-LC-MS/MS analyses. ATG5 enrichment compared to beads alone (upper panel); enrichment of RACK-ATG5 complex under torin-treated conditions compared to DMSO-treated control (lower panel) (mean ± S.D of independent experiments, n=3, *p<0.05).

FIGURE 3. Dynamic nature of RACK1-ATG5 interaction under autophagy inducing conditions. A, HEK293T cells were co-transfected with Flag-ATG5 and/or non-tagged RACK1 constructs, and treated or not with rapamycin (Rapa, 200 nM, 16 h) or torin 1 (Torin, 250 nM, 3h). Immunoprecipitations (IP) were performed using Flag beads. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Input, total cell extract. IgG, Immunoglobulin G. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. Band intensities were quantified using the Image J
tool. B, HEK293T cells were co-transfected with Flag-RACK1 and/or non-tagged ATG5 constructs, and starved in EBSS (2 h) or not. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. C, HEK293T cells were treated or not with rapamycin (Rapa, 200 nM, 16 h) or torin 1 (Torin, 250 nM, 3h), 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. D, HEK293T cells were cultured on coverslides. They were treated or not with rapamycin (Rapa, 200 nM, 16 h) or torin 1 (Torin, 250 nM, 3h) or starved in EBSS (Stv, 2 h) or not. Then, endogenous proteins were immunostained using anti-RACK1 and anti-ATG5 primary antibodies. Anti-mouse IgG Alexa Flour 488 (green); and Anti-rabbit IgG Alexa Flour 568 (red) were used as secondary antibodies, respectively. Cells were analyzed under confocal microscope. CNT, non-treated cells. Merge, overlay of green and red signals. White arrows show yellow cytoplasmic dots with RACK1 and ATG5 colocalization.

FIGURE 4. RACK1 is required for mTOR inhibition and starvation-induced autophagy but it is not an autophagy target. A, HEK293T cells were cultured on coverslides and transfected with siRACK1 or control siRNA (CNT siRNA). 48 h post transfection, cells were treated or not (-) with rapamycin (rapa, 200 nM, 16h) or starved in EBSS (2 h) in the presence or absence of bafilomycin A (BafA, 100 nM,1 h). Endogenous LC3 proteins were immunostained using anti-LC3 primary antibodies and anti-rabbit IgG Alexa Flour 488 secondary antibodies. LC3 dot positive cells were quantified as percentage of autophagic cells in total cell population (mean ± S.D of independent experiments, n=3, *p<0.05, **p<0.01). Endogenous protein expression levels were checked in cell extracts from the same experiments using anti-p62, anti-LC3 and anti-RACK1 antibodies. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. Band intensities were quantified using the Image J tool. B, Representative immunofluorescence pictures of LC3 quantification experiments in A. (-), non-treated cells. White arrows show LC3 dots. C, HEK293T cells were treated with rapamycin (200 nM) for 12 or 24 h or with carrier DMSO (D, 24 h); or starved for 2, 4 or 8 h in EBSS or cultured in full medium (CNT) with or without of BafA (BafilomycinA, 100nM,1h) in the presence of translation inhibitor cycloheximide (0.5 µg/ml). Immunoblots were performed using anti-p62, anti-RACK1 or anti-LC3 antibodies. β-Actin was used as loading control. D, HEK293T cells were treated with rapamycin (200 nM) for 12 or 24 h or with carrier DMSO (D, 24 h); or starved for 2, 4 or 8 h in EBSS or cultured in full medium (CNT) with or without of BafA (BafilomycinA, 100 nM,1h) in the absence of translation inhibitor cycloheximide. Immunoblots were performed using anti-p62, anti-RACK1 or anti-LC3 antibodies. β-Actin was used as loading control.

FIGURE 5. Role of mTOR-p70S6K pathway in the regulation of RACK1-ATG5 interaction. A, HEK293T cells were co-transfected with Flag-ATG5 and/or non-tagged RACK1 constructs and/or an mTOR construct. Immunoprecipitations (IP) were performed using Flag beads. Anti-mTOR, anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Input, total cell extract. IgG, Immunoglobulin G. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. Band intensities were quantified using the Image J tool. B, HEK293T cells were co-transfected with Flag-RACK1 and/or non-tagged ATG5 constructs and/or an shmTOR construct. Immunoprecipitations (IP) were performed using Flag beads. Anti-mTOR, anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. C, HEK293T cells were co-transfected with Flag-RACK1 and/or non-tagged ATG5 and/or p70S6K wild-type (wt) constructs. Immunoprecipitations (IP) were performed using Flag beads. Anti-p70S6K, anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. D, HEK293T cells
were co-transfected with Flag-ATG5 and non-tagged RACK1 constructs and/or sip70S6 RNAi. Anti-p70S6K, anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting.

**FIGURE 6.** Determination of RACK1 amino acid residues that are critical for the interaction. A, Clustal Omega alignments of RACK1 protein sequences. Putative p70S6K target RxxS/T consensus sequences were highlighted in black boxes. S/T residue numbers were marked according to H.sapiens protein sequences. RACK1 Genbank reference sequences were: Homo sapiens, NP_006089; Mus musculus, NP_032169; Danio rerio, NP_571519; Drosophila melanogaster, AAB72148; Caenorhabditis elegans, NP_501859; Saccharomyces cerevisiae, NP_013834. B, Schematic depiction of RACK1 constructs. WD1-7, WD40 domains 1 to 7. wt RACK1, wild-type RACK1. T39A, S63A or T128A, mutant RACK1 constructs. Mutated residues were marked. C, HEK293T cells were co-transfected with Flag-ATG5, non-tagged wt RACK1 or T39A, S63A, T128A RACK1 mutant constructs. Immunoprecipitations (IP) were performed using Flag beads. Anti-ATG5 and anti-RACK1 antibodies were used for immunoblotting. Input, total cell extract. IgG, Immunoglobulin G. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. Band intensities were quantified using the Image J tool.

**FIGURE 7.** RACK1-ATG5 interaction model. A, A cartoon representation of human RACK1 (PDB number 4AOW). Each WD40 domain (WD1-7) is in a different color. The seven-bladed β-propeller structure is shown. Location of the S63 residue is marked in a square. B, A cartoon model of RACK1 (PDB number 4AOW, Silver color) and ATG5 (PDB number 4GDK, tan color) interaction. Residues found within 3 Å of the other subunit are selected as binding interface that was rendered in the wireframe surface model (probe radius: 1.4 Å) by coloring ATG5 residues in green and RACK1 in yellow. The region around the S63 residue of RACK1 is shown in a red wireframe and encircled. C, The interaction network of predicted RACK1-ATG5 model. D-F, Native and mutated RACK1 (S63A and S63D) were energy-minimized and equilibrated in molecular dynamics (MD) simulations. Snapshots of the binding interface are shown with S63, D6 and K38 in licorice models (C: cyan, O: red, N: blue). Structural integrity of binding interface was probed by the distance of the ionic interaction between D6 of ATG5 and K38 of RACK1. While the wild-type (native) and S63D complexes possessed an intact binding surface with D6-K38 ionic pairing (D and E), S63A showed an extension in D6-K38 distance (F), implying a weakened interaction of RACK1 and ATG5. G, RMSD of the backbone atoms C, N and Cα. Native, wild type RACK1; S63A, S63A RACK1; S63D, S63D RACK1. H, Fluctuations of ATG5 during 5 ns of MD simulations. S63A RACK1 mutant displayed increased fluctuations at two distinct regions (residues from 32 to 36 and from 50 to 54) which are found at the binding interface (right panel), the observation which suggests that S63A RACK1 destabilizes the ATG5-RACK1 complex.

**FIGURE 8.** RACK1-ATG5 interaction is necessary for mTOR inhibition- or starvation- induced autophagy in Neuro2A cells. Cells were cultured on coverslides and transfected with the empty control vector pcDNA3 or wild-type (wt) or mutant RACK1 (S63A or S63D) constructs. A, 48h post transfection, Neuro2A cells were treated or not with torin 1 (Torin, 250 nM, 3h) with or without bafilomycin A (BafA, 100 nM, 1 h). Endogenous LC3 proteins were immunostained using anti-LC3 primary antibodies and anti-rabbit IgG Alexa Flour 488 secondary antibodies. LC3 dot positive cells were quantified as percentage of autophagic cells in total cell population (mean ± S.D of independent experiments, n=3, *p<0.05). LC3 and RACK1 protein expression levels in cell lysates were checked in immunoblots using anti-LC3 and anti-RACK1 antibodies. β-Actin was used as loading control. B, Representative immunofluorescence pictures of LC3 quantification experiments in A. White arrows show LC3 dots. C, 48h post transfection, Neuro2A cells were cultured in full medium (non-STV) or starved in EBSS (STV, 2 h) with or without BafA (BafilomycinA, 100nM,1h). Endogenous LC3 proteins were immunostained using anti-LC3 primary antibodies and anti-rabbit IgG Alexa Flour 488 secondary antibodies. LC3 dot positive cells were quantified (mean ± S.D of independent experiments, n=3,
**p<0.01, *p<0.05). LC3 and RACK1 protein expression levels in cell lysates were checked in immunoblots using anti-LC3 and anti-RACK1 antibodies. β-Actin was used as loading control. D, Representative immunofluorescence pictures of LC3 quantification experiments in C. White arrows show LC3 dots.

**FIGURE 9.** Rescue experiments with RACK1 mutant constructs following siRNA knockdown of RACK1. A, HEK293T cells were transfected with siRACK1 or control siRNA (CNT siRNA). The effect of siRNAs was checked by immunoblotting using anti-RACK1 antibodies. Molecular weights were marked in kilo Daltons (kDa). β-Actin was used as loading control. Band intensities were quantified using the Image J tool. B, HEK293T cells were grown onto coverslides and transfected with the empty control vector pcDNA3 or wild-type (wt) or mutant RACK1 (S63A or S63D) constructs. 48 h post transfection, HEK293T cells were treated or not with torin 1 (Torin, 250 nM, 3 h) with or without E64D (E64D, 10 µg/ml, 1 h) and PepA (Pepstatin A, 10 µg/ml, 1h). Endogenous LC3 proteins were immunostained using anti-LC3 primary antibodies and anti-rabbit IgG Alexa Flour 488 secondary antibodies. LC3 dot positive cells were quantified as percentage of autophagic cells in total cell population (mean ± S.D of independent experiments, n=3, **p<0.01, *p<0.05). LC3 and RACK1 protein expression levels were detected by immunoblotting using anti-LC3 and anti-RACK1 antibodies. β-Actin was used as loading control. C, Representative immunofluorescence pictures of LC3 quantification experiments in A. White arrows show LC3 dots.
Fig. 2

A. CNT

| kDa | >800 | 800-669 | 669-443 | 443-200 | >150 |
|-----|------|---------|---------|---------|------|
| L   | F1   | F2      | F3      | F4      | F5   |
|     | F6   | F7      | F8      | F9      | F10  |
|     | F11  | F12     | F13     |         |      |

ATG16
ATG5-12
RACK1

B. TORIN

| kDa | >800 | 800-669 | 669-443 | 443-200 | >150 |
|-----|------|---------|---------|---------|------|
| L   | F1   | F2      | F3      | F4      | F5   |
|     | F6   | F7      | F8      | F9      | F10  |
|     | F11  | F12     | F13     |         |      |

ATG16
ATG5-12
RACK1

C. D. E. F. G. H. I.

C. Tyrosinase (569 kDa)
Apoptosis (443 kDa)
β-amylose (200 kDa)
Alcohol dehydrogenase (150 kDa)
Carbonic anhydrase (29 kDa)

E. Molecular weight (kDa)

F. Molecular weight (kDa)

G. mAU

H. mAU

I. Fold Change

- ATG5 Enrichment
- RACK1-ATG5 Complex Enrichment

* Significant difference

ATG5
Beads alone

TORIN
DMSO
RACK1 is an Interaction Partner of ATG5 and a Novel Regulator of Autophagy
Secil Erbil, Ozlem Oral, Geraldine Mitou, Cenk Kig, Emel Durmaz-Timucin, Emine Guven-Maiorov, Ferah Gulacti, Gokcen Gokce, Jörn Dengjel, Osman Ugur Sezerman and Devrim Gozuacik

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