Received: 30 November 2015 | Revised and accepted: 17 August 2016 | Uncorrected manuscript published: 23 August 2016 | Published on: 3 October 2016

DOI 10.1111/tra.12431

WILEY Traffic

ORIGINAL ARTICLE

Rab24 interacts with the Rab7/Rab interacting lysosomal protein complex to regulate endosomal degradation

Celina Amaya[1] | Rodrigo D. Militello[1] | Sebastián D. Calligaris[2] | María I. Colombo[1]

[1]Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM)-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina

[2]Centro de Medicina Regenerativa, Facultad de Medicina, Universidad del Desarrollo Clínica Alemana, Santiago, Chile

Corresponding Author: María I. Colombo, Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM)-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Casilla de Correo 56, Centro Universitario, Parque General San Martín, 5500 Mendoza, Argentina (mcolombo@fcm.uncu.edu.ar).

Funding information
This work was supported by the grants PICT 2011-0455, PICT 2013-0305 and Sectyp to María Isabel Colombo.

Endocytosis is a multistep process engaged in extracellular molecules internalization. Several proteins including the Rab GTPases family coordinate the endocytic pathway. The small GTPase Rab7 is present in late endosome (LE) compartments being a marker of endosome maturation. The Rab interacting lysosomal protein (RILP) is a downstream effector of Rab7 that recruits the functional dynein/dynactin motor complex to late compartments. In the present study, we have found Rab24 as a component of the endosome-lysosome degradative pathway. Rab24 is an atypical protein of the Rab GTPase family, which has been attributed a function in vesicle trafficking and autophagosome maturation. Using a model of transiently expressed proteins in K562 cells, we found that Rab24 co-localizes in vesicular structures labeled with Rab7 and LAMP1. Moreover, using a dominant negative mutant of Rab24 or a siRNA-Rab24 we showed that the distribution of Rab7 in vesicles depends on a functional Rab24 to allow DQ-BSA protein degradation. Additionally, by immunoprecipitation and pull down assays, we have demonstrated that Rab24 interacts with Rab7 and RILP. Interestingly, overexpression of the Vps41 subunit from the homotypic fusion and protein-sorting (HOPS) complex hampered the co-localization of Rab24 with RILP or with the lysosomal GTPase Arl8b, suggesting that Vps41 would affect the Rab24/RILP association. In summary, our data strongly support the hypothesis that Rab24 forms a complex with Rab7 and RILP on the membranes of late compartments. Our work provides new insights into the molecular function of Rab24 in the last steps of the endosomal degradative pathway.

KEYWORDS

Arl8b, endocytic pathway, late endosomes, lysosomes, Rab24, Rab7, RILP, Vps41

1 | INTRODUCTION

Endocytosis is a multistep process for extracellular solutes or particles internalization. Even though endocytosis can be achieved through phagocytosis, macropinocytosis, clathrin-coated vesicles or caveolaes, the final destiny of some of the internalized molecules is their recycling while others are degraded. Initially, the endocytosed cargo localizes in a vesicle called early endosome (EE) where molecules are sorted. Then, some molecules take the recycling pathway toward the plasma membrane, via recycling compartments. Others proteins follow the degradation process through vesicle maturation to late endosomes (LE).[1,2] Finally, LE endosomes fuse with lysosomes where cargo degradation takes place.[3]

Several proteins including the Rab GTPases family coordinate the endocytic pathway. Rabs are considered molecular switches that organize membrane traffic. These proteins cycle between a GDP-bound to a GTP-bound state controlling vesicles formation, movement, tethering, docking and fusion to the target compartment.[4]

In EE, Rab5 is the small GTPase that regulates the kinetics of both, lateral fusion of EE and fusion of newly formed vesicles, derived from the plasma membrane, with EE.[5] On the other hand, Rab11 is known to be associated with recycling endosomes and regulates the recycling

Abbreviations: ^{125}I-LDL, ^{125}I-labeled low-density lipoproteins; EE, early endosome; EGFR, epidermal growth factor receptor; FYCO1, FYVE and coiled-coil domain containing 1; HOPS, homotypic fusion and protein-sorting; HRP, horseradish peroxidase; KD, knockdown; LE, late endosome; RILP, Rab interacting lysosomal protein; Wt, wild type

© 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

process of endocytosed proteins. Rab11 is transported to the cell periphery through association with recycling carriers, and directly regulates vesicle exocytosis at the plasma membrane.[6] The small GTPase Rab7 is localized in LE compartments, where it plays a fundamental role controlling late endocytic membrane traffic.[7] Rab7 defines the maturation of endosomes, directing the trafficking of cargos along microtubules, participating in the final fusion step with lysosomes.[8] Through interaction with its partners (including upstream regulators and downstream effectors), Rab7 participates in multiple regulation mechanisms of endosomal sorting, lysosome biogenesis, phagocytosis and autophagy.[8–10]

RILP (Rab interacting lysosomal protein) is a 45 kDa protein acting as a downstream effector of Rab7 that specifically binds Rab7-GTP at its C-terminus.[11] RILP recruits the functional dynein/dynactin motor complex to Rab7-containing LE and lysosomes. Consequently, these compartments are transported by the mentioned motor toward the minus end of microtubules, effectively inhibiting their transport toward the cell periphery.[12]

Although the participation of many proteins has been fully characterized, the complete set of molecules involved in LE and lysosome fusion remains to be elucidated. In the present study, we have found that Rab24 is a component of the endosome-lysosome degradative pathway. Rab24 is an atypical protein of the Rab GTPase family which has been proposed to function in vesicle trafficking and autophagosome maturation;[13] however, its exact role remains poorly defined.

Rab24 differs from other Rab proteins in that it has low intrinsic GTPase activity and cannot be efficiently prenylated.[14,15] Besides, very little is known about Rab24 post translational modifications, regulation, interacting proteins and targets. Olkkonen and collaborators found that Rab24 was distributed at the endoplasmic reticulum/*cis*-Golgi region and on LE structures.[16] In a subsequent study, we demonstrated a change in the distribution of Rab24 upon starvation co-localizing with the autophagosomal protein LC3, suggesting that this Rab protein is involved in the autophagic pathway.[13] Other studies have demonstrated that Rab24 may have a role in the degradation of misfolded proteins and trafficking of molecules to the nuclear envelope.[17] We have also demonstrated that Rab24 is recruited to *Coxiella burnetii*-containing compartments,[18] a phagolysosomal-like compartment that is also labeled by the autophagic proteins LC3 and Beclin1.[19] More recently, we also analyzed the distribution of Rab24 during cell division and its interaction with microtubules, showing that similarly to Rab5, Rab24 is involved in chromosome congression.[20]

To date, it is unclear whether Rab24 has a specific participation in vesicle trafficking. In this work we present evidence indicating that Rab24 has a role in the endosome/lysosome degradation route. Using K562 cells as a model, we have found that Rab24 interacts with Rab7 and its effector RILP in late endocytic compartments, a process that is necessary for vesicle trafficking to the microtubules organizing center to allow protein degradation. Strikingly, either the expression of a dominant negative mutant of Rab24 or the knockdown (KD) of the protein caused a dramatic change in Rab7 distribution becoming mostly cytosolic, indicating that the activity of Rab24 is critical for Rab7 association with vesicular structures. Our present data indicate that Rab24 form a complex with Rab7 and RILP, allowing endosome-lysosome fusion. In this event the role of Rab24 seems to be crucial for Rab7 activation, anchoring to the membrane and functioning of downstream effectors. Thus, our work highlights novel insights into the function of Rab24 in the last steps of the endosomal degradative pathway by interacting with critical molecular participants.

2 | RESULTS

2.1 | Rab24 associates to LE/lysosomal structures labeled with Rab7

Rab24 is an atypical Rab GTPase whose function is mostly unknown. We have previously demonstrated that Rab24 is involved in autophagy; however, the specific steps in which this factor plays a role remain to be determined.[13] It is well known that Rab GTPases are molecular switches whose conformation alternates between GTP-bound active and a GDP-bound inactive.[4] In general, the GTP active form anchors to membranes whereas the GDP-bound form remains mostly as a soluble protein in the cytoplasm. In order to get further insights into Rab24 functions, we assessed the distribution of this protein in K562 cells. For this purpose, either a wild type (wt) Rab24 or a GDP-bound dominant negative mutant (Rab24 T21N) tagged with GFP or RFP were employed. When either GFP- or RFP- Rab24 wt were overexpressed in K562 cells, the protein presented mostly a reticular pattern, though many vesicular structures were also found. In contrast, and as expected, the Rab24 T21N mutant presented a soluble diffuse distribution similar to that presented by GFP or RFP vectors (Figure S1A, Supporting Information).

Since we observed a partial vesicular distribution of Rab24 wt, it was of interest to determine whether these vesicles corresponded to early, recycling or LEs/lysosomes. Hence, Rab24 wt was co-expressed with markers for those compartments such as Rab5, Rab11 and Rab7, respectively, and the proteins distribution was analyzed by confocal microscopy. As shown in Figure 1A a-f, no major co-localization of Rab24 with Rab5 or Rab11 was observed. Besides, Rab24 maintained both its reticular and vesicular distribution patterns. Surprisingly, when cells were co-transfected with Rab7, the Rab24-reticular distribution changed and most of the Rab24 markedly co-localized with Rab7 in vesicular structures (Figure 1A g-i). A similar change in distribution was observed in cells co-transfected with LAMP-1 (Figure 1A j-l). In addition, the Rab24-positive vesicular structures were also labeled with LysoTracker, a marker of acidic compartments (m-o). The degree of co-localization between the markers studied is shown in Figure 1B where the strong association of Rab24 to Rab7-positive vesicles can be observed.

These results indicate that a fraction of Rab24 co-localizes in vesicular structures labeled with late endosomal/lysosomal markers. Furthermore, it could be observed that the overexpression of Rab7 or LAMP1 markedly changes Rab24 distribution towards a vesicular pattern, where the proteins strongly co-localize.

2.2 | Rab7 distribution in vesicular structures requires a functional Rab24

As indicated above, the GTP active forms of Rab proteins anchor to membranes whereas the GDP-bound forms remain mostly as soluble

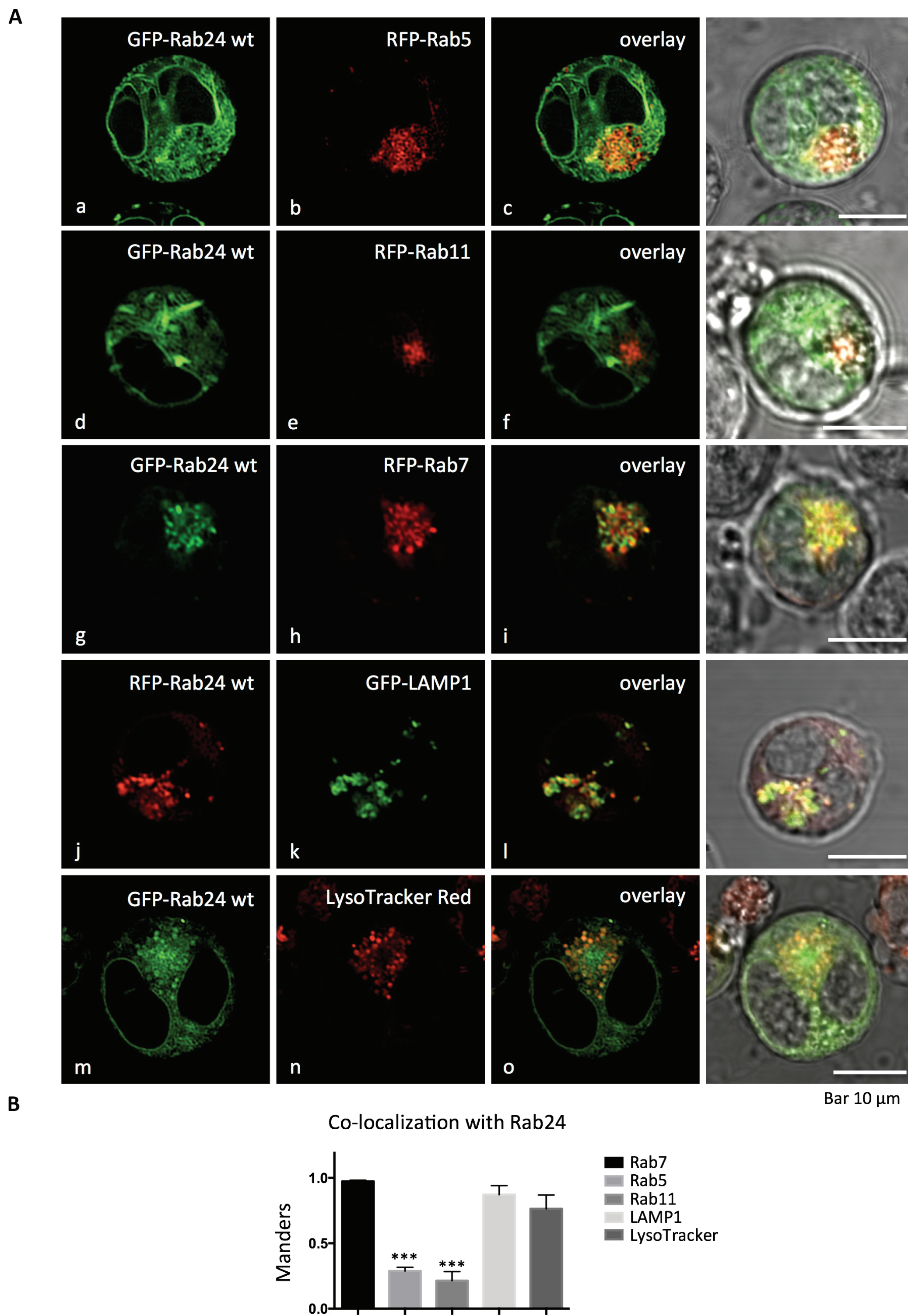


FIGURE 1 Rab24 associates to Rab7-labeled late endosomes and lysosomes. A, K562 cells were co-transfected with GFP- or RFP-Rab24 and RFP-Rab5, RFP-Rab11, RFP-Rab7 or GFP-LAMP1 constructs by electroporation as indicated in materials and methods. In another set of experiments with Rab24 transfected cells, acidic compartments were labeled with LysoTracker red. After 24 h transfection, cells were analyzed by confocal microscopy. Representative images are depicted. Scale bar: 10 μm. B, Manders´ coefficients of Rab24 co-localization with Rab7, Rab5, Rab11, LAMP1 and LysoTracker. Forty to sixty cells per condition were analyzed in at least 3 independent experiments. Results are expressed as means ± SEM. ***P < .001 compared to Rab24-Rab7 co-localization (ANOVA).

proteins in the cytoplasm. To study the effect of Rab24 activity in the localization of other Rab GTPases, we co-expressed the dominant negative mutant Rab24 T21N with Rab5, Rab11 or Rab7. Rab24 T21N showed a typical cytosolic distribution and no changes in the vesicular distribution of Rab5 and Rab11 were observed (Figure 2A a-f) as compared to cells transfected with GFP or RFP vectors alone (Figure S1B). In contrast, the co-expression of Rab24 T21N with Rab7 caused a dramatic change in Rab7 distribution

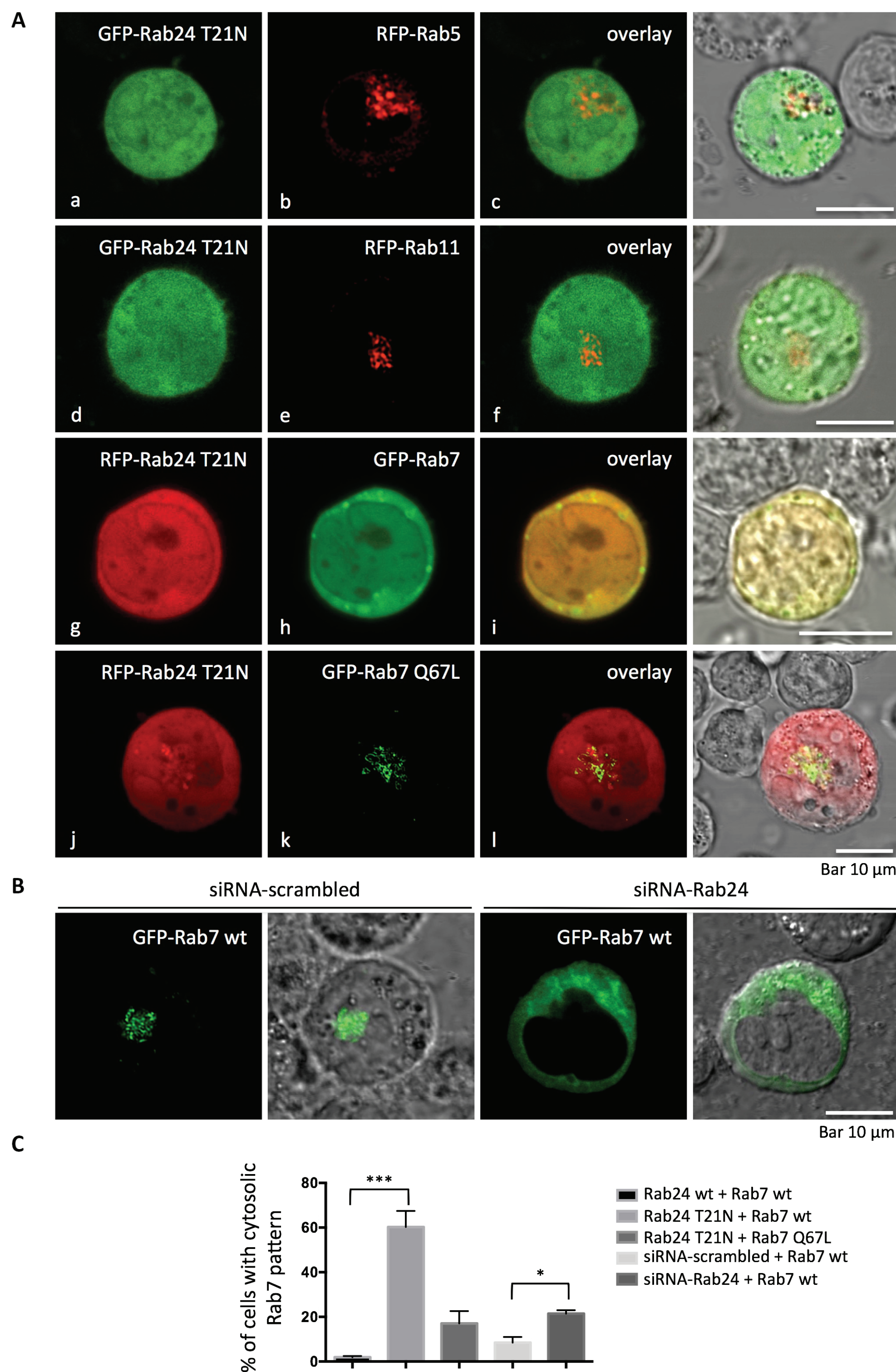


FIGURE 2 Rab7 distribution depends on Rab24 activity. A, Representative confocal images of K562 cells transiently co-expressing GFP- or RFP-Rab24 T21N and RFP-Rab5, RFP-Rab11, GFP-Rab7 or GFP-Rab7 Q67L. B, Cells transiently expressing GFP-Rab7 wt and silenced with a siRNA against Rab24. A scrambled siRNA was used as control. Representative confocal images are depicted. Scale bar: 10 μm. C, Percentage of cells with a cytosolic distribution of Rab7. Approximately 50 cells per condition were analyzed in at least 3 independent experiments. Results are expressed as means ± SEM, ****P* < .001 and **P* < .05 compared to Rab24 wt + Rab7 wt condition (ANOVA).

because the latter became mostly cytosolic (Figure 2A g-i). However, the negative form of Rab24 did not significantly affect the distribution of LAMP1 or LysoTracker-labeled vesicles (Figure S2A), indicating that the effect was specific for the distribution of Rab7 and not for the whole LE/lysosomal compartment. Interestingly, when Rab24 T21N was co-expressed with the active GTPase deficient mutant of Rab7 (ie, Rab7 Q76L), this Rab7 mutant maintained its normal vesicular distribution (Figure 2A j-l).

To confirm the requirement for Rab24 in Rab7 attachment to vesicular membranes, we silenced the expression of Rab24 with a specific siRNA to KD this protein, and the Rab7 distribution was then evaluated. As a control, we used a scrambled siRNA. The knocking down of Rab24 was assessed by Western blot and the decrease in Rab24 levels is shown in Figure S2B,C. When GFP-Rab7 was overexpressed in Rab24 KD cells, a similar effect to that achieved upon co-expression with the dominant negative mutant Rab24 T21N was observed. As shown in Figure 2B, in siRNA-scrambled cells, Rab7 was localized in typical vesicles whereas in Rab24 KD cells, Rab7 presented a more cytosolic distribution, indicating that critical levels of Rab24 are required for Rab7 association

to vesicular structures. The quantification of these observations is presented in Figure 2C.

Taking together, these observations suggest that the distribution of Rab7 wt depends on the presence of a functional Rab24 to allow the activation of Rab7, whereas when the latter protein is already in its GTP conformation it is not affected by the absence of an active Rab24.

2.3 | Rab24 activity is required for endo/lysosomal degradation

It is well known that Rab7 is necessary for fusion of LEs with lysosomes and cargo degradation.[21] To test if Rab24 participates in this process, we overexpressed Rab24 wt or Rab24 T21N and evaluated the degradation of DQ-BSA, a self-quenched fluorogenic substrate for lysosomal proteases, which requires an enzymatic cleavage in an acidic degradative compartment to emit fluorescence. As shown in Figure 3A panels a-c, cells expressing Rab24 wt were able to break down DQ-BSA as indicated by the presence of punctate fluorescent structures whereas, in cells expressing Rab24 T21N a non-fluorescent DQ-BSA was observed (Figure 3A d-f). The z-stack images showed that none, or only a few vesicles labeled with fluorescent DQ-BSA were visualized in Rab24 T21N cells (Figure S3A panels a), strongly suggesting that lysosomal degradation is hampered in cells overexpressing the Rab24 dominant negative mutant.

In order to confirm the requirement of Rab24 for the normal functioning of the late endosomal/lysosomal pathway, we assessed DQ-BSA degradation in Rab24 KD cells. Similarly to the effect of the overexpressed Rab24 T21N, Rab24 KD impaired DQ-BSA degradation (Figure 3A panels g-l) and this inhibitory effect was not overcome by co-expression with Rab7 wt (Figure 3A panels m-r and quantification in Figure 3B). As shown in the z-stack images of Rab24 KD cells, no DQ-BSA degradation was observed (Figure S3 panels b and c). Furthermore, the hampering of DQ-BSA proteolysis due to possible inhibition of internalization of the probe was ruled out, since no alterations in dextran or transferrin incorporation in cells expressing Rab24 wt or Rab24 T21N, as well as in Rab24 KD cells was observed (Figure S3B and C). Thus, we conclude that a functional Rab24 is essential for endo-lysosomal degradation.

2.4 | Rab24 interacts with Rab7 and its effector RILP

Given that Rab24 activity seems to be required for Rab7 association with vesicles, it was of interest to analyze the potential connection of Rab24 with Rab7 effectors. RILP is a downstream effector that specifically binds to GTP-bound Rab7 via its C-terminal domain.[11] Overexpression of RILP causes the clustering of the endo/lysosomal compartment in the perinuclear region by interacting with dynein/dynactin motor proteins that transport vesicles to minus-end microtubules toward the microtubule organizing center.[12]

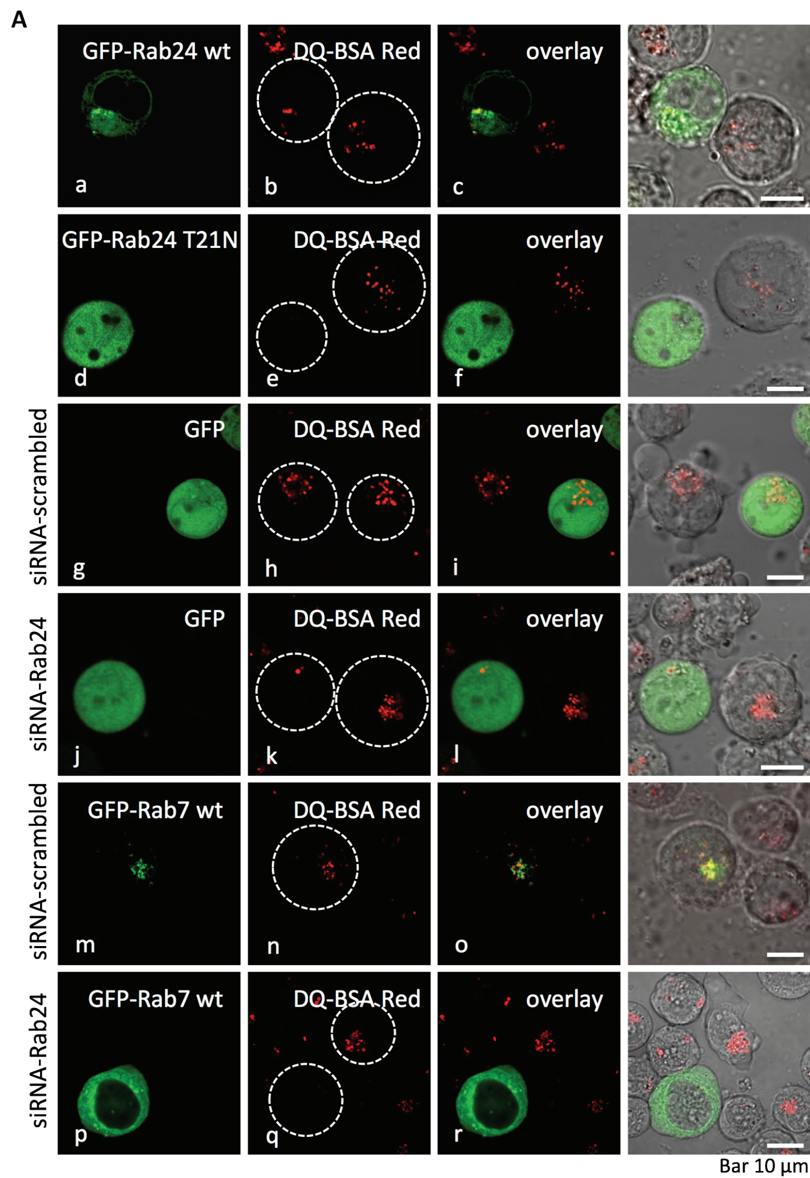
To determine whether the distribution of RILP was affected by overexpression of Rab24, K562 cells were co-transfected with either GFP- Rab24 wt or GFP/RFP -Rab24 T21N, and RFP- RILP wt or RFP-RILP ΔN, a truncated form of the protein lacking the N-terminal domain of RILP (Δ199). The truncated RILP mutant is able to bind Rab7 but interacts deficiently with the microtubule motors, therefore, the transport toward the cell center is hampered.[12] Firstly, we analyzed the distribution of Rab7 in K562 cells overexpressing RILP wt or the truncate mutant. In agreement with previous results,[12] Rab7 strongly co-localized with either RILP wt or the RILP ΔN, which is in line with the ability of Rab7 to associate with both, RILP wt or with the C-terminus truncated mutant of RILP (see Figure S4B).

We next analyzed the distribution of the proteins in cells co-expressing Rab24. Interestingly, when co-expressed with RILP wt most of the reticular distribution of Rab24 wt changed to a vesicular pattern strongly co-localizing with RILP in the perinuclear region (Figure 4A a-c), indicating that the overexpression of RILP favors the vesicular association of Rab24 and drives the Rab24/RILP-positive vesicles toward the cell center. Similarly, we observed a marked co-localization of Rab24 with the dynactin subunit p150Glued in vesicular structures (Figure S7A, a-c). In contrast, in cells overexpressing the truncated mutant RILP ΔN, the majority of Rab24 wt remained reticular and no vesicular co-localization with the more dispersed (and sometimes enlarged) vesicles generated by this RILP mutant was observed (Figure 4A, panels d-f). The degree of co-localization of Rab24 wt and RILP wt or RILP ΔN is clearly depicted in Figure 4B. These results suggest that the N-terminal domain of RILP is important for its association with Rab24. Interestingly, in cells overexpressing the dominant negative mutant Rab24 T21N, RILP wt -labeled vesicles clustered in the cell center (panels g-i), whereas the vesicles labeled with the RILP ΔN truncated mutant presented the expected more disperse distribution, but no co-localization with the Rab24 mutant was observed since the Rab showed the usual cytosolic distribution (panels j-l).

We next analyzed the Rab24 distribution when the protein was co-expressed with another microtubule-related protein. FYVE and coiled-coil domain containing 1 (FYCO1) is a Rab7 effector that interacts with the motor proteins kinesins to transport vesicles to the plus-end microtubules.[22] Unlike RILP, no co-localization between FYCO1 and Rab24 was observed. Even though vesicles were labeled with FYCO1, Rab24 acquired only a reticular distribution (Figure S7A d-f), indicating that Rab24 does not associate with the vesicles recruiting FYCO1.

Altogether, these data indicate that the overexpression of RILP wt induces the recruitment of Rab24 to vesicular structures, most probably to exert its function in the late Rab7-positive endo/lysosomal compartment. Besides, given that the difference between RILP wt and RILP ΔN is restricted to amino acids 1-199, we speculate that this region of RILP might be implicated in the interaction with Rab24. In addition, the lack of co-localization with FYCO1 suggests that Rab24 associates with vesicles trafficking toward the minus-end microtubules.

Given the strong relationship between Rab24 and Rab7, and its effector RILP, we then evaluated if these 2 Rabs are part of the same protein complex. To this end, we studied the possible interaction of these proteins by immunoprecipitation. The K562 cells were co-transfected with constructs expressing myc-Rab24 and GFP-Rab7 or GFP-RILP and immunoprecipitations were performed using an anti-myc resin. Interestingly, we found that Rab7 and RILP co-



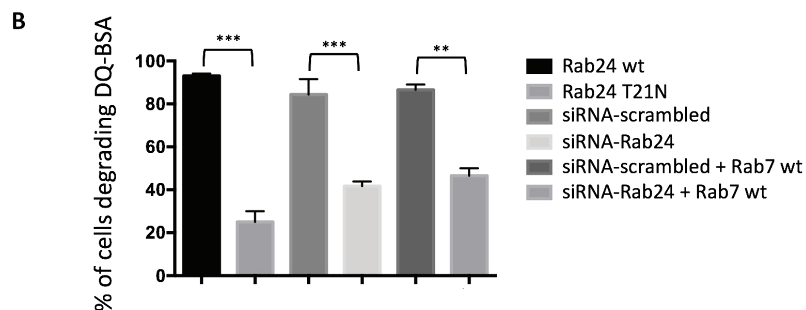


FIGURE 3 Rab24 activity is required for endo-lysosomal degradation. A, Endo-lysosomal degradation was assessed with DQ-BSA red (see material and methods) in cells expressing GFP-Rab24 wt, GFP-Rab24 T21N or in cells silenced with a siRNA against Rab24 or with a control scrambled siRNA, with or without GFP-Rab7 wt co-expression. The images depicted are representative of at least 3 independent experiments. Scale bar: 10 μm. B, Percentage of cells degrading DQ-BSA. Results are expressed as means ± SEM, ***P < .001, **P < .01 (ANOVA). More than 200 cells per condition were analyzed.

immunoprecipitated with Rab24 whereas as a control, immunoprecipitation of the GFP vector alone with Rab24 was not observed (Figure 5A). Next, in order to address the possible direct interaction between the proteins we carried out several pull down assays using purified recombinant proteins. As shown in Figure 5B Rab24 was able to bind RILP wt (lane 2) but not to the truncated mutants indicated as RILP1 (GST-RILP1-198) or RILP2 (GST-RILP199-401) corresponding to the amino- and carboxi-terminal half of the proteins, respectively, indicating that the full length protein is required for this interaction. In addition, Rab24 directly interacted with Rab7 (lane 8) but not with Rab3, Rab6 or just GST (control) indicating the specificity of this association. Moreover, in order to determine if the binding of Rab24 and Rab7 was dependent on the active state of both GTPases a pull down assay using the wt proteins or the corresponding dominant negative mutants was performed. The results depicted in Figure 5C clearly indicate that the Rab24/Rab7 interaction depends on the active state of both proteins (ie, GTP conformation). Thus, taken together these findings allow concluding that Rab24

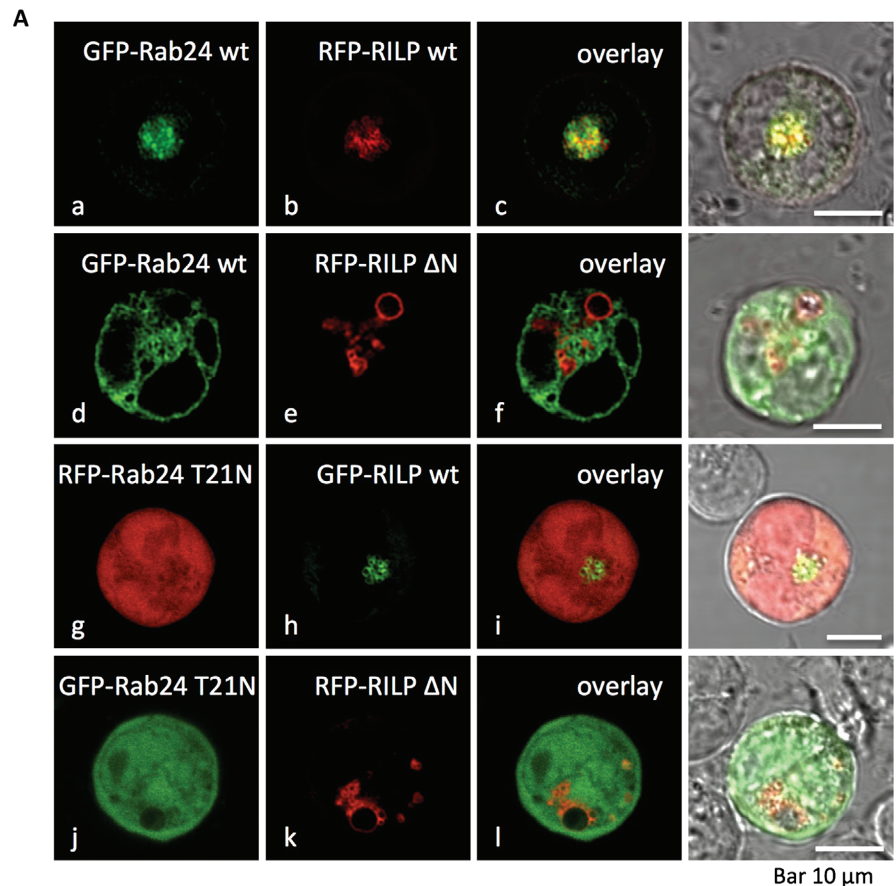


FIGURE 4 Rab24 is recruited to Rab interacting lysosomal protein (RILP)-associated vesicles involved in transport toward the perinuclear region. A, Confocal images of K562 transiently expressing RFP- or GFP- tagged Rab24 wt or the T21N dominant negative mutant. Cells were co-transfected with GFP/RFP-RILP wt or RFP-RILP ΔN. The images depicted are representative of at least 3 independent experiments. Scale bar: 10 μm. B, Manders´ coefficients of co-localization. Results are expressed as means ± SEM, ***P < .001 compared to Rab24 wt + RILP wt condition (ANOVA).

forms a complex with Rab7 and RILP, likely participating in the transport of vesicles toward the minus-end microtubules.

2.5 | The HOPS subunit Vps41 hinder the Rab24-RILP association

The homotypic fusion and protein-sorting (HOPS) complex is a multi-subunit tethering complex that regulates membrane fusion events with the vacuole in yeasts. Mammalian homologs of all HOPS components have been described, with Vps41 and Vps39 being required for late endosomal-lysosomal fusion events and for the delivery of the endocytic cargo to lysosomes in human cells.[23] Furthermore, the Vps41 subunit of the HOPS complex has been described as the major partner interacting with RILP. Besides, this interaction is independent of Rab7.[24] Since Rab24 seems to participate in the endosome-lysosome fusion process and forms a complex with RILP/Rab7, we assessed whether this Rab is also connected with Vps41. To this end, we used K562 cells to co-express HA-Vps41 with GFP-Rab24, GFP-Rab7, GFP-LAMP1, RFP-RILP wt, RFP-RILP ΔN, or the GFP vector alone. Then, the HA-Vps41 was stained by immunofluorescence and the proteins distribution was analyzed by confocal microscopy. As shown in Figure 6A a-c, Vps41 co-expressed with the GFP vector had a vesicular pattern distributed throughout the cytoplasm and this pattern did not undergo important alterations when Vps41 was co-expressed with the other constructs (Figure 6A d-r). Specifically, in the Vps41 with Rab24 co-expression, the last 1 maintained a reticular distribution with a few Rab24-labeled vesicles (Figure 6A d-f). The number of Vps41 vesicles co-localizing with every protein was similar for Rab24, Rab7, LAMP1 and RILP wt, with the exception of RILP ΔN which the number of co-localizing vesicles was low (Figure 6B).

There is evidence indicating that in mammals, HOPS subunits are not recruited by Rab7 but by its effector RILP. Van der Kant et al have demonstrated that Vps41 exhibits a low affinity for Rab7 alone, a 10–15-fold higher affinity for RILP and even higher affinity

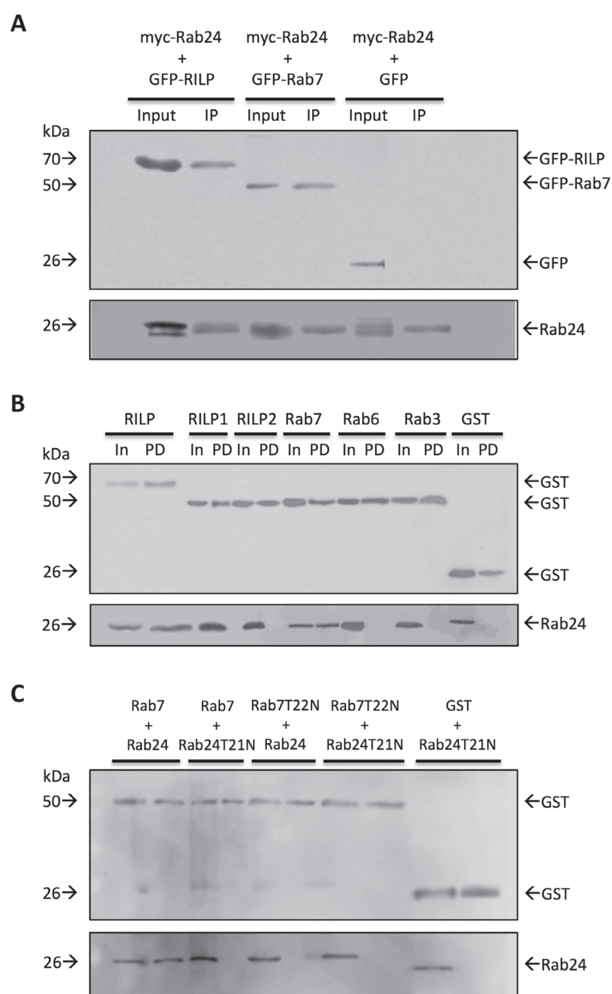


FIGURE 5 Rab24 interacts with Rab7 and Rab interacting lysosomal protein (RILP). A, Protein lysates of K562 cells co-expressing myc-Rab24 wt with GFP-Rab7 wt, GFP-RILP wt or GFP vector alone were subjected to immunoprecipitation with a rabbit anti-myc antibody and subsequently processed for immunoblotting. Overexpressed Rab24 was detected with a mouse anti-Rab24 antibody and GFP-Rab7, GFP-RILP or GFP vector with a mouse anti-GFP antibody, following incubation with appropriated HRP-secondary antibodies. B and C, The pull-down assays were done with the purified recombinant proteins Rab24, Rab24 T21N, GST-Rab7, GST-Rab7 T22N, GST-RILP, GST-RILP1 (1-198), GST-RILP2 (199-401), GST-Rab6, GST-Rab3 and GST as a control. Rab24 was detected with a mouse anti-Rab24 antibody and the GST-proteins with a rabbit anti-GST antibody following incubation with appropriated HRP-secondary antibodies. Western blots are representative of 3 independent experiments. In: input, corresponding to 10% of the whole lysate, S: supernatant, IP: immunoprecipitation and PD: pull-down.

for RILP associated with Rab7.[25] Since Rab24 co-localizes with RILP wt, but not with RILP ΔN (Figure 4), and knowing that the difference between those RILPs is related to amino acids involved in the Vps41 recruitment,[25] we performed triple co-expressions to analyze the Vps41, RILP and Rab24 distribution by confocal microscopy. As shown in Figure 7A, co-localizations of RFP-Rab24 with GFP-Rab7 or GFP-LAMP1 were maintained in cells co-expressing HA-Vps41 (panels a-h), suggesting that Vps41 does not compete with Rab24 for Rab7 and LAMP1 association because the co-localization levels were high (Figure 7B), similarly to the degree of co-localization observed in Figure 1. In contrast, when HA-Vps41 was co-expressed with GFP-Rab24 and RFP-RILP wt, no co-localization of these proteins was detected. In this case RILP maintained its typical vesicular pattern while Rab24 was lead to a more reticular distribution (Figure 7A panels i-l). However, the co-expression of HA-Vps41 did not affect the co-localization of GFP-Rab7 and RFP-RILP wt (Figure 7A m-p and B). Taking into account previous data on RILP and Vps41 interaction,[24] our present results suggest that Vps41 would be affecting the Rab24/RILP association.

Recently, Khatter et al have demonstrated that the small GTPase Arl8b, but not Rab7, is essential for membrane localization of the human Vps41 subunit.[26] Since Arl8b interacts directly with Vps41 and since we have shown that Vps41 hinders the Rab24/RILP association and induces a reticular pattern of Rab24, we studied the effect of Arl8b in the Rab24/RILP association. For this purpose, we co-expressed GFP-Arl8b with HA-Vps41, RFP-RILP and RFP- or myc-Rab24 in K562 cells and analyzed the proteins distribution by confocal microscopy. In this case, we evinced some co-localization between GFP-Arl8b and HA-Vps41 and a marked co-localization with RFP-RILP (Figure 8A d-i). In addition, a high co-localization between RFP-Rab24 and GFP-Arl8b was also observed, indicating that the proteins were recruited to the same vesicular compartments (Figure 8A j-l). On the other hand, in triple transfection experiments, the expression of HA-Vps41 caused the dissociation of RFP-Rab24 and GFP-Arl8b (Figure 8B a-d), similarly to the disrupting effects caused by Vps41 in the RILP/Rab24 association (see Figure 7). Interestingly, the co-expression of GFP-Rab24 did not appear to prevent the association between RFP-RILP and myc-Arl8b; however, Rab24 maintained a reticular pattern (Figure 8B e-h). Considering these results, it would seem that the elevated levels of Vps41 achieved either through the protein overexpression or through the induction of its recruitment via overexpression of Arl8b, disrupt the association of Rab24 from RILP-positive vesicular structures.

3 | DISCUSSION

Central molecules in ensuring that cargos are delivered to their correct destinations are the Rab GTPases, that control membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of effector proteins.[27] Many cascade mechanisms define directionality in traffic and guarantee that different Rabs do not overlap in the pathways that they regulate.[28]

In the early endocytic pathway, Rab5 regulates the homotypic fusion of EEs, and fusion of plasma membrane-derived endocytic vesicles with EEs.[29] While the degradative cargo concentrates in progressively fewer and larger endosomes that migrate from the cell periphery to the center, Rab5 is rapidly replaced with Rab7 as the mechanism of cargo progression between early and LEs.[30] Afterwards, in the late endosomal compartment, Rab7 and its effector RILP play a fundamental role in the transport of the endocytic cargo to the lysosome.[11]

Many functions and interactions of members involved in endosome maturation and cargo degradation remain to be elucidated. In this regard, Rab24 is a poorly characterized GTPase of the Rab family

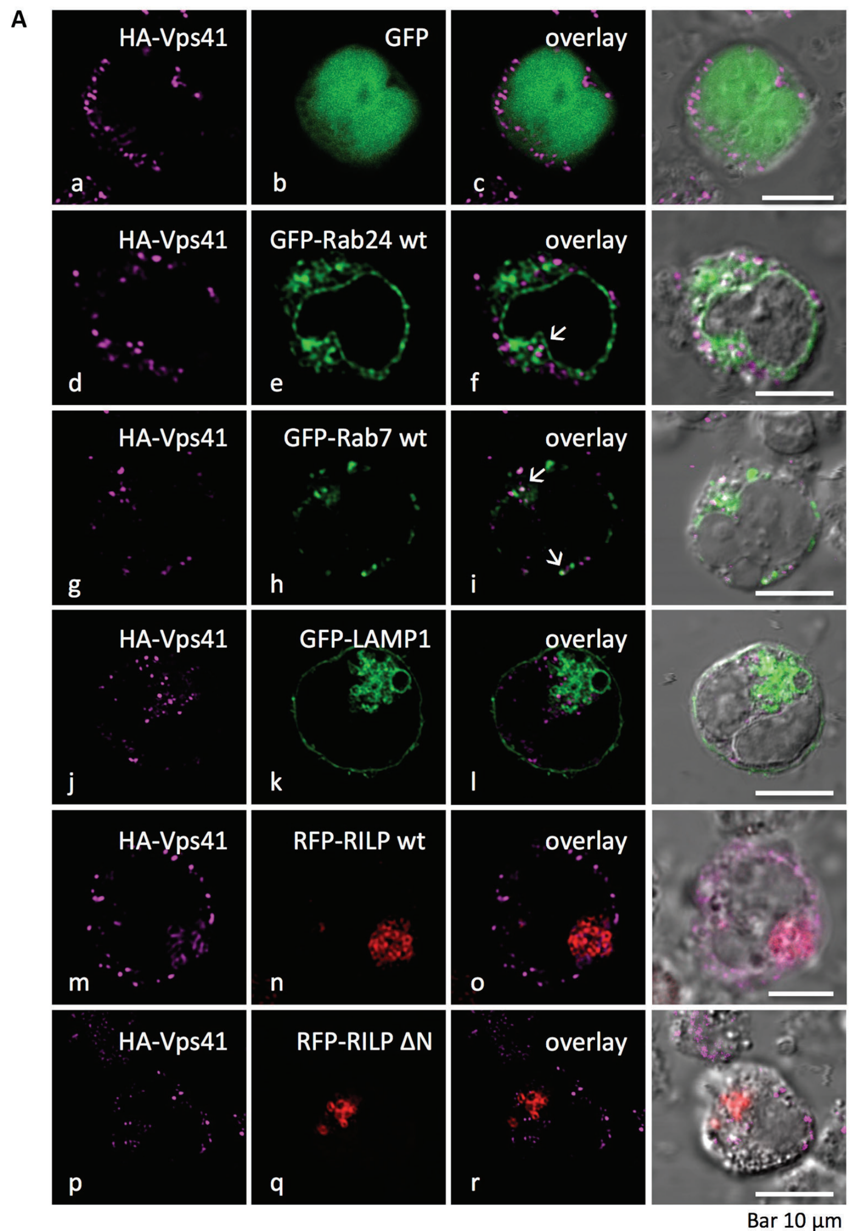
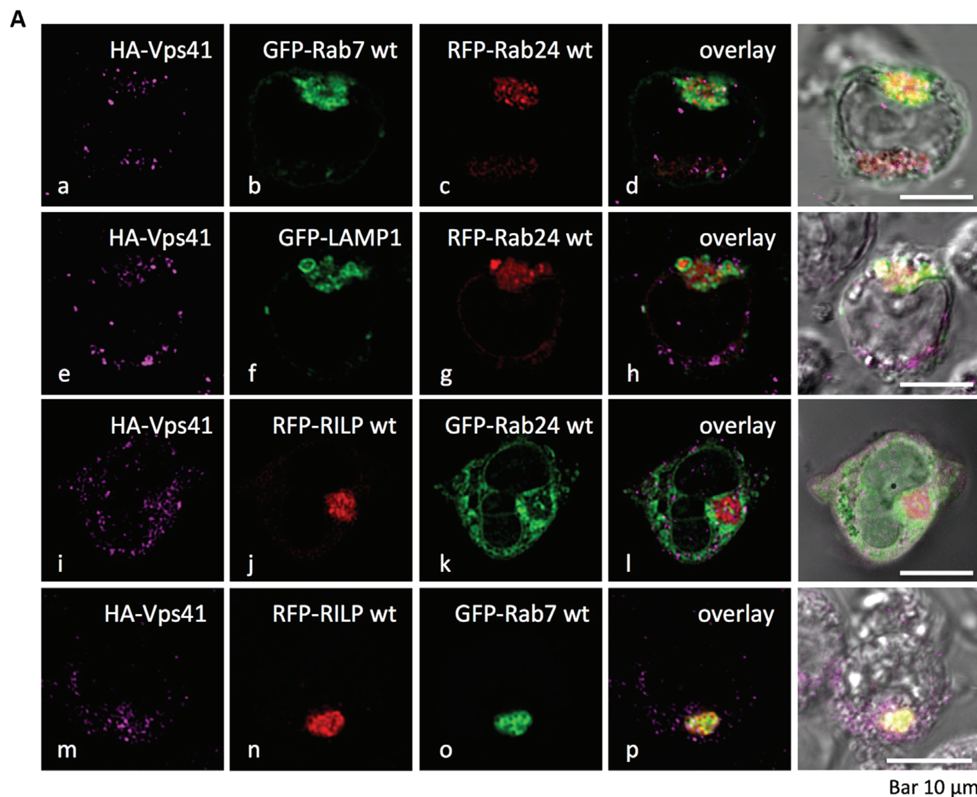


FIGURE 6 Co-expression of the HOPS subunit Vps41 with late endosomal/lysosomal components. HA-Vps41 was co-expressed with GFP vector alone, GFP-Rab24, GFP-Rab7, GFP-LAMP1, RFP-Rab interacting lysosomal protein (RILP) wt and RFP-RILP ΔN. HA-Vps41 was stained for immunofluorescence with a mouse anti-HA antibody and an appropriated secondary antibody. A, Representative confocal images of at least 3 experiments. Scale bar: 10 μm. Arrows show points of co-localization. B, Number of Vps41-labeled vesicles co-localizing with GFP- or RFP-tagged proteins. Results are expressed as means ± SEM, $^{**}P$ < .01 compared to Vps41/Rab24 wt condition (ANOVA).

that has been found in the endoplasmic reticulum/cis-Golgi region and on late endosomal structures.[16] Under normal nutrient conditions, Rab24 presents a reticular distribution around the nucleus in CHO cells. In contrast, in amino-acid starvation-induced autophagy, the distribution of Rab24 shifts towards punctate structures that co-localize with markers for autophagic vacuoles, such as LC3 and monodansylcadaverine.[13] Other studies carried out with the *drs* gene product suggest that drs interacts with Rab24 in the autophagosome and that it may regulate the fusion of autophagosomes with lysosomes.[31] The participation of Rab24 in the final stages of autophagy was recently demonstrated in HeLa cells under nutrient-rich conditions, showing the relevance of this rab during the clearance of the degradative autophagic compartments.[32]

Considering the scanty information about the role of Rab24 in vesicle trafficking, we analyzed the function of this Rab in endosome maturation in a K562 cell model. The K562 is a chronic myeloid leukemia cell line that has long been used as a substrate to perform studies on vesicle trafficking, Rabs[33–36] and autophagy.[37–39] In addition, we considered this cell line as an appropriated model for our studies since we found by Western blot that it presents high levels of



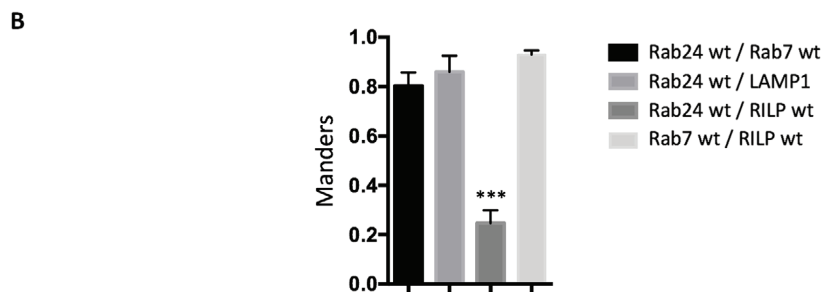


FIGURE 7 High levels of Vps41 affect the Rab24/Rab interacting lysosomal protein (RILP) association. Triple co-expression of HA-Vps41 with GFP/RFP-Rab24, GFP-Rab7, GFP-LAMP1 and RFP-RILP were performed in K562 cells. HA-Vps41 was stained for immunofluorescence with an anti-HA antibody and a specific secondary antibody. A, Representative confocal images of 3 independent experiments. Scale bar: 10 μm. B, Manders' coefficients of co-localization. Results are expressed as means ± SEM, ANOVA $^{***}P < .001$ compared to Rab24 wt + Rab7 wt condition (ANOVA).

endogenous Rab24 compared to other cell lines such as HeLa, CHO and B16F0 cells (Figure S7B,C).

It has been demonstrated that Rab24 is distributed in the cytoplasm, mainly in the ER /cis-Golgi region and on late endosomal structures in several cell lines as BHK, HeLa, MDCK, NmuLi, CHO, HEK293 and NIH3T3 mouse fibroblasts.[40] In K562 cells, as well as in other cell lines as HeLa, CHO, B16F10 and COS-7 we found that the endogenous Rab24 (Figure 7D) and the overexpressed Rab24 presents a reticular-ER like distribution with some vesicular formations. When Rab24 was co-expressed in K562 cells with markers of early endosomes (Rab5) or recycling endosomes (Rab11), Rab24 maintained a reticular pattern and only a few vesicles co-localized with Rab5 or Rab11. In contrast, co-expression of Rab24 with markers of LEs/lysosomes (ie, Rab7 and LAMP1) caused dramatic changes in Rab24, becoming more vesicular and showing a strong co-localization with both proteins. Furthermore, a marked co-localization with the acidotropic probe LysoTracker was also observed, strongly supporting that Rab24 is related to late endosomal/lysosomal vesicles. The co-localization between overexpressed Rab24 and Rab7 was also observed in other cell lines including B16F10 and COS-7 cells. This co-localization was also confirmed, analyzing the distribution of overexpressed Rab7 and the endogenous Rab24 in K562, B16F10 and COS-7 cells (see Figures S5 and S6). These findings suggest that the overexpression of Rab7 proteins favors Rab24 association to late endocytic compartments.

Co-expression of Rab5, Rab11 or Rab7 with the dominant negative mutant of Rab24 (Rab24 T21N, the GDP-bound form) in K562 cells showed that Rab24 T21N maintained a normal cytosolic distribution and no evident alterations in Rab5 or Rab11 vesicles were observed but, surprisingly, when we analyzed the Rab7-Rab24 T21N co-expression the Rab7-labeled vesicles practically disappeared and Rab7 became cytosolic with a homogeneous distribution. Then, similar results were obtained when Rab24 was depleted with a siRNA-Rab24, a treatment that induced the cytoplasmic dispersion of Rab7. The effect of Rab24 T21N overexpression in Rab7 distribution was also analyzed in other cells lines such as HeLa, CHO and B16F0 showing the same effect: the cytosolic dispersal of Rab7 (data not shown) indicating that the Rab24 dominant negative mutant effect was independent on the cell type used. It has previously been reported that Rab7 is associated with late endocytic structures, mainly lysosomes, which aggregate and fuse in the perinuclear region. Upon expression of the dominant-negative mutant Rab7 T22N, which is localized mainly to the cytosol, the perinuclear lysosome aggregate disappeared and lysosomes, identified by LAMP1, became dispersed

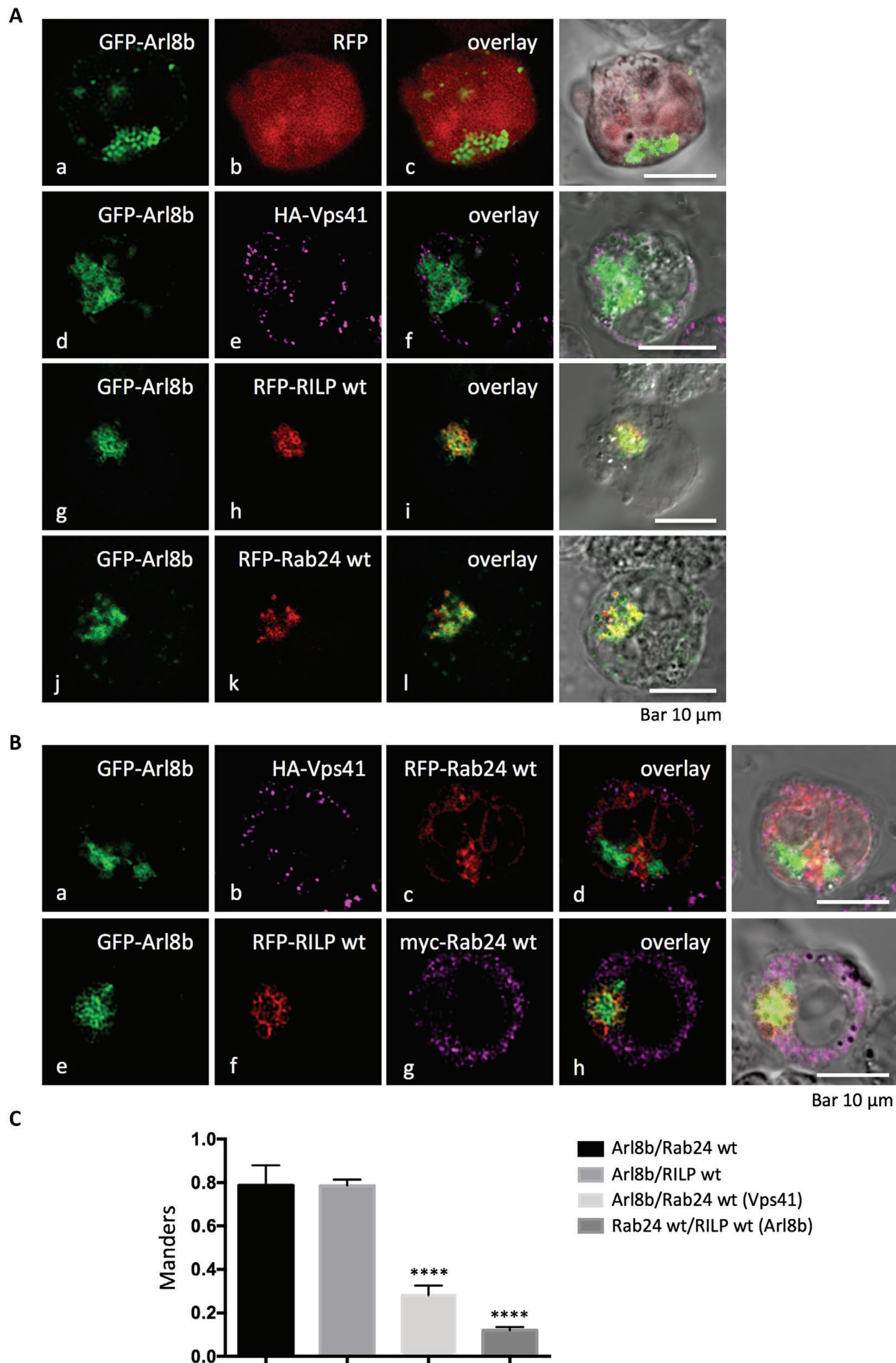


FIGURE 8 Effect of Arl8b in the Rab24/Rab interacting lysosomal protein (RILP) association. A, Representative images of K562 cells co-expressing GFP-Arl8b with HA-Vps41, RFP-Rab24 wt or RFP-RILP wt. The HA-Vps41 was immunostained with an anti-HA antibody. B, Triple co-expressions of GFP-Arl8b, HA-Vps41 and RFP-Rab24 (a-d), and GFP-Arl8b, RFP-RILP and myc-Rab24 (e-h). Myc-Rab24 was immunostained with an anti-myc antibody and an appropriated secondary antibody. Scale bar: 10 µm. C, Manders' coefficients of co-localization of Arl8b/Rab24 and Arl8b/RILP, and Arl8b/Rab24 or Rab24/RILP in triple co-expression with Vps41 and Arl8b, respectively. Results are expressed as means ± SEM, *****P* < .0001 compared to Arl8b/Rab24 wt condition (ANOVA).

throughout the cytoplasm, with a marked reduced acidity determined by the LysoTracker probe.[41] However, according to our present results neither Rab24 T21N nor Rab24 KD seemed to alter GFP-LAMP1 and LysoTracker-labeled vesicles, suggesting a normal lysosome biogenesis while the activity of Rab24 is affected. Although this contradictory last issue has not been studied intensively our results indicate that in K562 cells the activity of Rab24 is necessary for the recruitment of Rab7 to vesicles.

Since Rab7 controls aggregation and fusion of late endocytic structures and degradation of the endocytosed cargo,[41] we evaluated whether Rab24 was required for this process. Using the fluorogenic substrate DQ-BSA we observed a hampering of BSA degradation in cells expressing Rab24 T21N as well as in Rab24 KD cells. The latter effect could not be reverted by the co-expression with Rab7 wt, indicating the requirement of Rab24 in this degradative process. Despite the fact that BSA degradation was abrogated in cells expressing Rab24 T21N or in Rab24 KD cells, its endocytic capacity seemed not to be altered because they were able to take up dextran and to internalize transferrin. Our results are consistent with the reported inhibition of the degradation of ^{125}I-labeled low density lipoproteins (^{125}I-LDL) in cells transfected with the Rab7 dominant negative mutant, whereas in these cells, the internalization of ^{125}I-LDL was not affected either.[7] More recently, Vanlandingham and Ceresa studied the role of Rab7 in trafficking between LEs and lysosomes and showed that Rab7 is dispensable for delivery of cargo (as the epidermal growth factor receptor [EGFR]) to the LE, but is required for efficient fusion of the LE to the lysosome.[42] In the present report we have observed that the lack of activity of Rab24 affects the recruitment of Rab7 to vesicles and abrogates the cargo degradation. Thus, our findings strongly support the idea that Rab24 would play a fundamental role in controlling late endocytic membrane trafficking and that this protein is required for endosomal/lysosomal degradation.

RILP has been identified as an interacting partner of the GTPases Rab7, Rab12, Rab34 and Rab36.[43–45] Active Rab7 recruits RILP on the late endosomal/lysosomal vesicles; therefore, RILP plays a role as a Rab7 effector controlling the transport to degradative compartments. Indeed, RILP induces recruitment of dynein-dynactin motor complexes to Rab7-containing LEs and lysosomes.[46] In our experiments, RILP wt recruited Rab24 wt in vesicles, but the RILP ΔN mutant (which maintains the interaction with Rab7 but does not interact with the dynein-dynactin motor complex)[25,46] did not recruit Rab24, maintaining Rab24 its reticular form. The fact that Rab24 co-localizes with RILP is consistent with our results showing Rab24-Rab7 co-localization, but in the case of RILP ΔN the lack of co-localization led us to hypothesize that the N-terminal extension of RILP could be implicated in the Rab24 recruitment. The direct association between RILP and different Rabs (Rab24 among them) has been studied by yeast 2-hybrid screening and the authors have only found interaction of RILP with Rab7, Rab34 and Rab36.[45] Nevertheless, we assessed the ability of Rab24 to associate with RILP by performing immunoprecipitation and pull-down assays, finding that only the wt form of Rab24 interacted with Rab7 wt and RILP wt, and there was no interaction with the GDP form of Rab7 or the truncated forms of RILP. Therefore, the confocal microscopy analysis, the immunoprecipitation and pull-down experiments indicated that it is likely that Rab24 forms a complex with Rab7 and RILP, contributing to regulate late endocytic traffic.

The HOPS complex is a multisubunit tethering conglomerate that regulates membrane fusion events with the lysosome. The complex consists of the class C core complex supplemented with Vps39 and Vps41, and both are required for late endosomal-lysosomal fusion events and the delivery of endocytic cargo to lysosomes in human cells.[23] It is known that RILP interacts with HOPS, recruiting their subunits to the late endosomal compartment. Structurally, the N-terminal extension of RILP interacts with the HOPS complex, and the Vps41 subunit has been identified as the major partner in this interaction.[24] In the present study we have analyzed the effect of Vps41 overexpression on Rab24-RILP association and we observed that Vps41 partly co-localized with Rab24, Rab7, LAMP1 and RILP wt but the levels of co-localization were very low with the truncated mutant RILP ΔN. In addition, in triple co-expression experiments with Vps41, the co-localization of Rab24 and Rab7 or LAMP1 was maintained but the co-localization of Rab24 with RILP wt was affected, leading to a Rab24 reticular distribution, exposing that high levels of Vps41 interfere with the association of Rab24-RILP in vesicles. Further, we also observed these results in the B16F10 cells (data not shown). Taking into account the work of Van der Kant[25] and Lin[24] who have demonstrated that Vps41 interacts with the N-terminal extension of RILP, it is likely that high levels of Vps41 would be competing for the interaction of Rab24 with RILP. Although, in our pull-down assays there was no direct interaction between Rab24 and the N-terminal truncated form of RILP, it may be possible an indirect interaction with this fragment. Alternatively, since the full length RILP is necessary for the Rab24/RILP association, it is likely that overexpression of Vps41 which binds with high affinity to the N-terminal domain may interfere with the stable interaction of Rab24/RILP. It is also possible that the excess of Vps41 may sequester Rab24, hampering its interaction with the late vesicular compartment. Further experiments are necessary to demonstrate the specific regions of Vps41 and Rab24 involved in this association.

The Arf-like GTPase Arl8b has been identified as a critical regulator of cargo delivery to lysosomes.[47] The HOPS complex subunits are effectors of Arl8b and have been found to be dependent on it for recruitment to lysosomes, suggesting that Arl8b-HOPS plays a role in directing the traffic to lysosomes.[47] It has recently found that Arl8b is essential for the membrane localization of Vps41 and that the assembly of the core HOPS subunits to Arl8b- and Vps41-positive lysosomes is guided by their subunit-subunit interactions.[26] Since our results indicate that Vps41 strongly affects the Rab24-RILP co-localization, we also assessed the role of Arl8b in this association. Consequently, we found that Arl8b markedly co-localized with Rab24 when both proteins were co-expressed. However, the Vps41 co-expression abrogates the Arl8b-Rab24 co-localization. Likewise, when Arl8b was co-expressed with RILP and Rab24, Arl8b and RILP maintained their co-localization in vesicles but Rab24 redistributed to a reticular pattern. We also settled these experiments in B16F10 cells showing similar consequences. Taking together our results, we propose a model depicted in Figure 9 in which excess Vps41 either by overexpressing the protein or by increasing its recruitment via Arl8b would interfere with the association of Rab24 from the Rab7/RILP complex. Taking

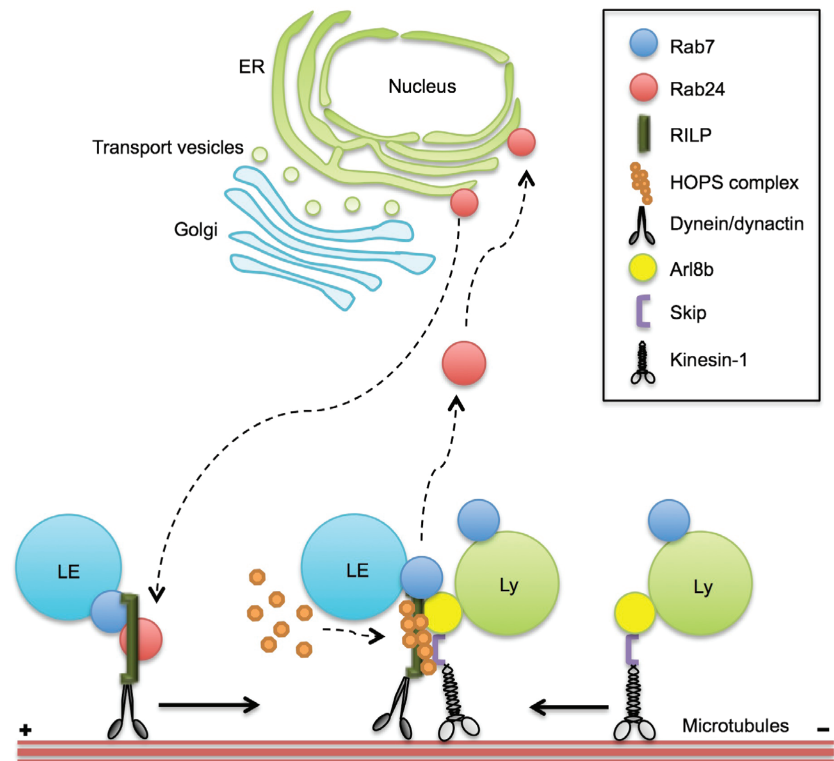


FIGURE 9 Model of the complex constituted by Rab24 and molecular components of late endocytic/lysosomal vesicles. Based on the literature and our present findings, we suggest that when a degradative process is stimulated an undetermined signal induce the interaction of Rab24 with Rab7 and Rab interacting lysosomal protein (RILP) in late endosomes. Then, when the Vps41 subunit of the HOPS complex is recruited to RILP and Arl8b, Rab24 is released from the complex adopting a reticular pattern.

into account previous works indicating that the Vps41 interaction with RILP and Arl8b is necessary for LE and lysosome fusion[24,26] we believe that Rab24 dissociates from the Rab7/RILP complex after the Vps41 recruitment to LE/lysosome vesicles.

In summary, we have demonstrated, using a model of transiently expressed proteins in K562 cells that Rab24 participates in the late steps of the endocytic pathway contributing to the late endosomal/lysosomal degradative process. In addition, our data strongly support a model in which Rab24 forms a complex with Rab7 and RILP at the membranes of LEs and lysosomes and that the activity of Rab24 is essential for Rab7 membrane recruitment. Then, when the HOPS complex is engaged to RILP and Arl8b, the Rab24 protein would no longer be necessary, adopting a reticular pattern. It is evident that further experiments will be necessary to determine the specific domains of Rab24 involved in these complex interactions and to fully understand the intricate relationship among these molecular associates.

In the last few years many studies have found the gen expression of Rab24 as a risk factor for some illnesses such as multiple sclerosis,[48] tuberculosis,[49,50] hereditary ataxia,[51] and ischemic events.[52] Since these findings suggest the involvement of Rab24 in pathological processes, it seems crucial to study the role of Rab24 and its interacting partners in the vesicular transport, at the level of the degradation steps, in order to find biomarkers for diagnosis or for the improvement of treatments.

4 | MATERIALS AND METHODS

4.1 | Antibodies, reagents, plasmids and siRNA

For immunofluorescence microscopy and Western blot, the following antibodies were used: mouse anti-HA (Sigma, Buenos Aires, Argentina), rabbit anti-myc (Santa Cruz, Buenos Aires, Argentina), mouse anti-Rab24 (BD Bioscience, Buenos Aires, Argentina), mouse anti-GFP (Abcam, Buenos Aires, Argentina), Cy3 anti-rabbit, Cy5 anti-mouse, rabbit anti-GST (Abcam, Buenos Aires, Argentina) and HRP-anti-mouse (Jackson, Georgia). Another mouse anti-Rab24 antibody was generated for our lab as previously reported.[20] To label endocytic compartments, cells were incubated overnight with 70 kDa dextran-texas red (Molecular probes, Buenos Aires, Argentina) and with transferrin alexa fluor 633 (Invitrogen, Buenos Aires, Argentina) for 15 min, and for labeling acidic organelles the incubation was done with LysoTracker red (Molecular probes, Buenos Aires, Argentina) for 1 h. To test lysosomal degradation of the endocytosed cargo, cells were incubated overnight with DQ-BSA (Invitrogen). siRNA-mediated Rab24 KD was performed using a siRNA against Rab24 obtained form Integrated DNA Technologies Inc. (Coralville, Iowa) and the scrambled siRNA from Bioneer (Buenos Aires, Argentina). The generation of the GFP-Rab24 and GFP-Rab24 T21N constructs has been described elsewhere[13] and the RFP-Rab24, RFP-Rab24 T21N, GST-Rab24 and GST-Rab24 T21N constructs were obtained using standard subcloning procedures. The myc-Rab24 plasmid was kindly provided by William A. Maltese (Medical College of Ohio, USA). GFP-RILP and RFP-Rab7 was kindly provided by Mauricio Terebiznik (University of Toronto, Toronto, Canada). RFP-RILP, RFP-RILP ΔN, GFP-p150Glued, GST-RILP wt, GST-RILP1(1-198) and GST-RILP2 (199-401) were generously given by Jacques Neefjes (The Netherlands Cancer Institute, Amsterdam, The Netherlands). GFP-Rab7 and GFP-Rab7 Q67L were kindly provided by Bo van Deurs (University of Copenhagen, Copenhagen, Denmark). GST-Rab7, GST-Rab7 T22N and GST-Rab6 were generously supplied by Bruno Goud (Curie Institute, France). mCherry-FYCO1 was provided by Terje Johansen (Institute of Medical Biology, University of Tromsø, Norway). HA-

Vps41 was given by Corlinda ten Brink (University Medical Centre Utrecht, Utrecht, The Netherlands). The generation of RFP-Rab11 construct has previously been described.[53] GFP-Arl8b was generously provided by Roberto Botelho (Department of Chemistry and Biology, Ryerson University, Toronto, ON).

4.2 | Cell culture

K562 cells (ATCC) were cultured in T25 flasks and 6 well dishes in RPMI medium (Life Technologies, Buenos Aires, Argentina) supplemented with 10% fetal bovine serum (PAA, Buenos Aires, Argentina), 50 IU/mL penicillin and 50 μg/mL streptomycin, at 37°C, in a 5% CO_2 humidified culture hood. HeLa, CHO, B16F0, B16F10, and COS-7 cells were incubated in the same conditions in RPMI, D-MEM or alfa-MEM media according to the ATCC recommendations.

4.3 | Transfection and immunofluorescence

DNA constructs, scrambled siRNA and Rab24-siRNA were transfected into K562 cells by electroporation following standard procedures. Briefly, for every condition, 4×10^6 cells were suspended in 250 μL RPMI with 20 mM HEPES pH 7, 40 mM NaCl and 10 μg of plasmid or 100 nM siRNA. Then, electroporation was performed in a Bio-Rad electroporator through 1 pulse at 230 V and 975 μF. Subsequently; cells were placed in complete RPMI medium and incubated for 24 h (plasmids) or 72 h (siRNA). HeLa, CHO, B16F0, B16F10, and COS-7 were transfected by similar procedures. For immunofluorescence, cells were fixed in 4% paraformaldehyde/PBS, quenched with 50 mM ammonium chloride/PBS and permeabilized with 0.5% BSA, 0.1% saponin in PBS, at room temperature for 15 min every step. Samples were incubated with primary antibodies overnight and with the corresponding secondary antibody for 2 h. After 3 washes with PBS, cell suspensions were mounted and observed in an Olympus FluoView TM FV1000 confocal microscopy (Olympus, Buenos Aires, Argentina). The 2D image spectral deconvolutions, as well as co-localization analysis were performed with the ImageJ software (ImageJ 1.48v, National Institute of Health, USA). The quantification of the degree of co-localization of 2 channels reflecting the fraction of 2 fluorophores co-localizing was performed by Manders' M1/M2 co-localization coefficients.[54]

4.4 | Immunoprecipitation and immunoblot analysis

K562 cells transiently expressing myc-Rab24, GFP-Rab7, GFP-RILP, or GFP vector were harvested and placed in lysis buffer (Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100 and a protease inhibitor cocktail (Calbiochem, Buenos Aires, Argentina)). Lysates were subjected to immunoprecipitation with a polyclonal rabbit anti-myc antibody (Santa Cruz) and protein-A coupled agarose beads (Millipore, Buenos Aires, Argentina). Briefly, lysates were pre-cleared by incubation for 30 min with protein-A agarose beads. For the immunoprecipitation pre-cleared lysates were incubated with anti-myc protein-A agarose for 6 h at 4°C. Pull-down assays were performed with the purified recombinant proteins GST-RILP, GST-RILP1 (1-198), GST-RILP2 (199-401), GST-Rab7, GST-Rab7 T22N, GST-Rab6, GST-Rab3, Rab24, Rab24 T21N, and GST alone. Glutathione sepharose beads (GE Healthcare, Buenos Aires, Argentina) were pre-coated with the GST-proteins in PD buffer (25 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100 and protease inhibitor cocktail) at 4°C for 1 h, in a rotating shaker. Then, the purified Rab24 or Rab24 T21N was incubated with the beads at 4°C for 6 h in the presence of 0.5 mM GTPγS. A fraction corresponding to 10% of the mix was kept to run in a SDS-gel (input) and the 90% of the mix was washed 3 times with the PD buffer. Subsequently, the beads and the supernatant were re-suspended in DTT sample buffer, boiled and loaded onto the gel. For Western blot, lysates from cells equivalents/lane were made in DTT sample buffer, analyzed in 15% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Membranes were then incubated overnight with primary antibodies followed by 2 h with a secondary HRP antibody. Immunological reactions were revealed with ECL reagents (Millipore) and analyzed in an ImageQuant LAS 4000.

4.5 | Degradative compartments analysis

Lysosomal degradation was analyzed by confocal microscopy using DQ™ Red BSA (self-quenched red BODIPY dye conjugated to BSA. Red DQ-BSA requires enzymatic cleavage in acidic intracellular lysosomal compartments to generate a highly fluorescent product that can be monitored by confocal microscopy. K562 cells overexpressing Rab24 or silenced for this protein were incubated in RPMI media containing DQ-BSA (10 μg/mL) for 18 h and then washed twice with PBS. For confocal images analysis, cells were harvested, plated on coverslips and the red-fluorescence of DQ-BSA was immediately analyzed in an Olympus FV1000 confocal microscopy.

4.6 | Statistical analysis

Numerical data are presented as means ± SEM. Statistical comparisons were performed by the 2-tailed Student's *t*-test, or ANOVA followed by Dunnett's or Bonferroni's multiple comparisons test, using the GraphPad Prism version 6.0c.

ACKNOWLEDGMENTS

We thank Dr. Luis Mayorga for a helpful discussion about this manuscript. We also thank Dr. Bruno Goud for a critical reading and useful comments of the paper before submission. We are grateful to Jacques Neefjes, Mauricio Terebiznik, Roberto Botelho, Walter Berón, Diego Bustos, Marina Uhart and Bruno Goud for gifts of molecular constructs, reagents and antibodies. This work was supported by the grants PICT 2011-0455, PICT 2013-0305 and Sectyp to María Isabel Colombo.

CONFLICT OF INTEREST

We declare no competing or financial interests.

REFERENCES

1. McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*. 2011;12(8):517–533.
2. Grant BD, Donaldson JG. Pathways and mechanisms of endocytic recycling. *Nat Rev Mol Cell Biol*. 2009;10(9):597–608.
3. Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat Rev Mol Cell Biol*. 2009;10(9):623–635.
4. Aloisi AL, Bucci C. Rab GTPases-cargo direct interactions: fine modulators of intracellular trafficking. *Histol Histopathol*. 2013;28(7):839–849.
5. Zerial M, Stenmark H. Rab GTPases in vesicular transport. *Curr Opin Cell Biol*. 1993;5(4):613–620.
6. Takahashi S, Kubo K, Waguri S, et al. Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. *J Cell Sci*. 2012;125(Pt 17):4049–4057.
7. Vitelli R, Santillo M, Lattero D, et al. Role of the small GTPase Rab7 in the late endocytic pathway. *J Biol Chem*. 1997;272(7):4391–4397.
8. Hyttinen JMT, Niittykoski M, Salminen A, Kaarniranta K. Maturation of autophagosomes and endosomes: a key role for Rab7. *Biochim Biophys Acta Mol Cell Res*. 2013;1833(3):503–510.
9. Zhang M, Chen L, Wang S, Wang T. Rab7: roles in membrane trafficking and disease. *Biosci Rep*. 2009;29(3):193–209.
10. Gutierrez MG, Munafó DB, Berón W, Colombo MI. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J Cell Sci*. 2004;117(Pt 13):2687–2697.
11. Cantalupo G, Alifano P, Roberti V, Bruni CB, Bucci C. Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. *EMBO J*. 2001;20(4):683–693.
12. Jordens I, Fernandez-Borja M, Marsman M, et al. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol*. 2001;11(21):1680–1685.
13. Munafó DB, Colombo MI. Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic*. 2002;3(7):472–482.
14. Erdman RA. Rab24 Is an atypical member of the Rab GTPase family. *J Biol Chem*. 2000;275(6):3848–3856.
15. Ding J, Soule G, Overmeyer JH, Maltese WA. Tyrosine phosphorylation of the Rab24 GTPase in cultured mammalian cells. *Biochem Biophys Res Commun*. 2003;312(3):670–675.
16. Olkkonen VM, Dupree P, Killisch I, Lütcke A, Zerial M, Simons K. Molecular cloning and subcellular localization of three GTP-binding proteins of the rab subfamily. *J Cell Sci*. 1993;106(Pt 4):1249–1261.
17. Maltese WA, Soule G, Gunning W, Calomeni E, Alexander B. Mutant Rab24 GTPase is targeted to nuclear inclusions. *BMC Cell Biol*. 2002;3:25.
18. Gutierrez MG, Vázquez CL, Munafó DB, et al. Autophagy induction favours the generation and maturation of the Coxiella-replicative vacuoles. *Cell Microbiol*. 2005;7(7):981–993.
19. Berón W, Gutierrez MG, Rabinovitch M, Colombo MI. Coxiella burnetii localizes in a Rab7-labeled compartment with autophagic characteristics. *Infect Immun*. 2002;70(10):5816–5821.
20. Militello RD, Munafó DB, Berón W, et al. Rab24 is required for normal cell division. *Traffic*. 2013;14(5):502–518.
21. Kümmel D, Ungermann C. Principles of membrane tethering and fusion in endosome and lysosome biogenesis. *Curr Opin Cell Biol*. 2014;29(1):61–66.
22. Pankiv S, Alemu EA, Brech A, et al. FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end - directed vesicle transport. *J Cell Biol*. 2010;188(2):253–269.
23. Pols MS, Ten Brink C, Gosavi P, Oorschot V, Klumperman J. The HOPS proteins hVps41 and hVps39 are required for homotypic and heterotypic late endosome fusion. *Traffic*. 2013;14(2):219–232.
24. Lin X, Yang T, Wang S, et al. RILP interacts with HOPS complex via VPS41 subunit to regulate endocytic trafficking. *Sci Rep*. 2014;4:7282.
25. van der Kant R, Fish A, Janssen L, et al. Late endosomal transport and tethering are coupled processes controlled by RILP and the cholesterol sensor ORP1L. *J Cell Sci*. 2013;126(Pt 15):3462–3474.
26. Khatter D, Raina VB, Dwivedi D, Sindhwani A, Bahl S, Sharma M. The small GTPase Arl8b regulates assembly of the mammalian HOPS complex on lysosomes. *J Cell Sci*. 2015;128(9):1746–1761.
27. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*. 2009;10(8):513–525.
28. Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. *Physiol Rev*. 2011;91(1):119–149.
29. Bucci C, Parton RG, Mather IH, et al. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*. 1992;70(5):715–728.
30. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab conversion as a mechanism of progression from early to late endosomes. *Cell*. 2005;122(5):735–749.
31. Tambe Y, Yamamoto A, Isono T, Chano T, Fukuda M, Inoue H. The drs tumor suppressor is involved in the maturation process of autophagy induced by low serum. *Cancer Lett*. 2009;283(1):74–83.
32. Ylä-Anttila P, Mikkonen E, Happonen KE, et al. RAB24 facilitates clearance of autophagic compartments during basal conditions. *Autophagy*. 2015;11(10):1833–1848.
33. Green EG, Ramm E, Riley NM, Spiro DJ, Goldenring JR, Wessling-Resnick M. Rab11 is associated with transferrin-containing recycling compartments in K562 cells. *Biochem Biophys Res Commun*. 1997;239(2):612–616.
34. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci*. 2002;115(Pt 12):2505–2515.
35. Feng D, Zhao W-L, Ye Y-Y, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic*. 2010;11(5):675–687.
36. Ferrándiz-Huertas C, Fernández-Carvajal A, Ferrer-Montiel A. Rab4 interacts with the human P-glycoprotein and modulates its surface expression in multidrug resistant K562 cells. *Int J Cancer*. 2011;128(1):192–205.
37. Drullion C, Lagarde V, Gioia R, et al. Mycophenolic Acid overcomes imatinib and nilotinib resistance of chronic myeloid leukemia cells by apoptosis or a senescent-like cell cycle arrest. *Leuk Res Treat*. 2012;2012:861301.
38. Yan H-W, Hu W-X, Zhang J-Y, et al. Resveratrol induces human K562 cell apoptosis, erythroid differentiation, and autophagy. *Tumour Biol*. 2014;35(6):5381–5388.
39. Zhu J, Zhou Y, Wang G-N, Tai G, Ye X-S. Cell cycle arrest, apoptosis and autophagy induced by iminosugars on K562 cells. *Eur J Pharmacol*. 2014;731:65–72.
40. Wu M, Yin G, Zhao X, et al. Human RAB24, interestingly and predominantly distributed in the nuclei of COS-7 cells, is colocalized with cyclophilin a and GABARAP. *Int J Mol Med*. 2006;17(5):749–754.
41. Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: a key to lysosome biogenesis. *Mol Biol Cell*. 2000;11(2):467–480.
42. Vanlandingham PA, Ceresa BP. Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. *J Biol Chem*. 2009;284(18):12110–12124.
43. Johansson M, Rocha N, Zwart W, et al. Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor βIII spectrin. *J Cell Biol*. 2007;176(4):459–471.
44. Efergan A, Azouz NP, Klein O, et al. Rab12 regulates retrograde transport of mast cell secretory granules by interacting with the RILP-dynein complex. *J Immunol*. 2016;196(3):1091–1101.
45. Matsui T, Ohbayashi N, Fukuda M. The Rab interacting lysosomal protein (RILP) homology domain functions as a novel effector domain for small GTPase Rab36: Rab36 regulates retrograde melanosome transport in melanocytes. *J Biol Chem*. 2012;287(34):28619–28631.
46. Colucci AMR, Campana MC, Bellopede M, Bucci C. The Rab-interacting lysosomal protein, a Rab7 and Rab34 effector, is capable of self-interaction. *Biochem Biophys Res Commun*. 2005;334(1):128–133.
47. Garg S, Sharma M, Ung C, et al. Lysosomal trafficking, antigen presentation, and microbial killing are controlled by the Arf-like GTPase Arl8b. *Immunity*. 2011;35(2):182–193.
48. Igci M, Baysan M, Yigiter R, et al. Gene expression profiles of autophagy-related genes in multiple sclerosis. *Gene*. 2016;588(1):38–46.
49. Jacobsen M, Repsilber D, Gutschmidt A, et al. Ras-associated small GTPase 33A, a novel T cell factor, is down-regulated in patients with tuberculosis. *J Infect Dis*. 2005;192:1211–1218.

50. Jenum S, Dhanasekaran S, Lodha R, et al. Approaching a diagnostic point-of-care test for pediatric tuberculosis through evaluation of immune biomarkers across the clinical disease spectrum. *Sci Rep*. 2016;6:18520.

51. Agler C, Nielsen DM, Urkasemsin G, et al. Canine hereditary ataxia in old english sheepdogs and gordon setters is associated with a defect in the autophagy gene encoding RAB24. *PLoS Genet*. 2014;10(2):e1003991.

52. Swaminathan B, Goikuria H, Vega R, et al. Autophagic marker MAP1LC3B expression levels are associated with carotid atherosclerosis symptomatology. *PLoS One*. 2014;9(12):e115176.

53. Fader CM, Sánchez D, Furlán M, Colombo MI. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in K562 cells. *Traffic*. 2008;9(2):230–250.

54. Manders EMM, Verbeek FJ, Aten JA. Measurement of colocalization of objects in dual-color confocal images. *J Microsc*. 1993;169:375–382.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Amaya C, Militello RD, Calligaris SD, Colombo MI. Rab24 interacts with the Rab7/Rab interacting lysosomal protein complex to regulate endosomal degradation, *Traffic*, 2016;17(11):1181–1196.