INRS
Institut-Armand-Frappier

THE ROLE OF POST TRANSLATIONAL MODIFICATIONS IN MODULATING RAB7 FUNCTIONS

Graziana Modica

A thesis submitted for the degree of
Doctor of Philosophy (Ph.D.)
in Biology

Evaluation Committee Members

Committee Chair and Internal Examiner	Dr. Daniel G. Cyr INRS-Institut Armand-Frappier
External Member	Dr. Jean-François Côté Institut de recherches clinique de Montreal
External Member	Dr. Christian Rocheleau McGill University Health Centre
Supervisor	Stéphane Lefrançois INRS-Institut Arman-Frappier
Co-supervisor	Hugo Wurtele Université de Montreal

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ACKNOWLEDGMENT

This journey has not been always easy but it has been totally worth it. I am really grateful for all the people that have been supporting me during the ups and downs of my research.

I am extremely thankful to my supervisor Dr. Stéphane Lefrançois who gave me the opportunity to pursue this project in his lab. He has been an amazing supervisor and mentor, he taught me what a good scientist and person should be.

I want to thank my co-supervisor Hugo Wurtele for his helpful advice and practical suggestions. A special thanks goes to the jury members Dr. Daniel G. Cyr, Dr. Jean-François Côté and Dr. Christian Rocheleau for their constructive criticism and valuable advice.

Thank you to my lab mates for their continuous support and help, it has been great working with them.

Many thanks to the founding agencies that have been supporting my salary making this work possible: Ministère de l'Éducation, du Loisir et du Sport (MELS), Fondation de l'Hôpital Maisonneuve-Rosemont, Fondation Armand-Frappier and Fond de recherche du Québec – Santé (FRQS). Thanks to the founding agencies that have been supporting the work in the lab: EU Joint Programme-Neurodegenerative Disease Research (JPND), Canada Foundation for Innovation, Natural Sciences and Engineering Research Counsil (NSERC), Canadian Institute of Health and Research (CIHR).

Thank you to my family for being always supportive and ready to help when I was in need. I am particularly grateful to Claudio for his willingness to proof read the French part of this work.

I cannot begin to express my thanks to my amazing husband for believing in me and for always supporting me. It has been hard sometimes but he has always been there to encourage me, I could not have made it without him. I wish to thank my wonderful children Orazio and Alessandro for inspiring me every day.

Thanks to my mom, I wish you could see it.

RÉSUMÉ

La petite GTPase Rab7 cordonne le trafic des membranes dans les endosomes tardifs (LE). À travers l'interaction avec plusieurs effecteurs différentes, Rab7 est capable de régler les événements de fusion entre les LE et les lysosomes et entre les autophagosomes et les lysosomes, elle contrôle le mouvement et le positionnement des LE et elle est aussi responsable du trafic vers le Trans-Golgi-Network pour le recyclage des récepteurs cargos. Il n'est pas clair comment une soule protéine peut diriger toutes ces routes de trafic.

Des travails publiés récemment ont souligné l'importance des modifications post-traductionnelles (PTM) dans la régulation des fonctions de Rab7. En fait il a été montré que Rab7 peut être ubiquitinée, phosphorylée et avec mon travail, nous avons montré que Rab7 peut être aussi palmitoylée. Comment chaque PTM contribue à régler les activités de la petite GTPase n'est pas clair. Avec mon projet j'ai apporté des nouvelles informations sur ces mécanismes de régulation. Avec mon premier travail, j'ai montré que Rab7 est palmitoylée sur les cystéines C83 et C84 et que cette modification est nécessaire pour recruter efficacement le complexe du retromer dans les membranes endosomales et pour le correct fonctionnement de ce complexe dans le trafic vers le TGN. Aussi, il est intéressant de noter qui la palmitoylation n'affect pas la fonction de Rab7 de régler la dégradation des protéines transmembranaires, comment le récepteur EGFR. Avec mon deuxième travail, nous avons analysé le rôle de la phosphorylation sur la serine 72 et sur la tyrosine 183 dans le contrôle des fonctions de Rab7. Nous avons montré qui la phosphorylation S72 est nécessaire pour recruter le retromer dans la membrane. Nous avons aussi trouvé qui l'absence de cette modification cause la réduction de la palmitoylation de Rab7, en suggérant qui les deux PTMs sont connexes.

La fonction de la phosphorylation Y183 il n'était pas clair, certaines donnés suggéraient qui ce PTM était nécessaire pour la dégradation du récepteur EGFR, autres travails montraient le contraire. Dans notre travail, en utilisant cellules Rab7-KO, nous montrons qui la phosphorylation Y183 est nécessaire pour ce processus. En plus, nous suggérons un nouveau modèle où la phosphorylation Y183 agit comment un interrupteur moléculaire qui inhibe l'interaction entre Rab7 et RILP et favorise l'interaction entre ce dernier et le complexe ESCRT-II. Cette interaction est nécessaire pour le tri efficace des protéines destinées à la dégradation dans les lysosomes.

Donc mon travail a identifié et caractérisé nouveaux mécanismes de régulation de la petite GTPase Rab7.

Mots clés : Rab7 ; modifications post-traductionnelles; palmitoylation; phosphorylation; retromer; trafic intracellulaire; endosome; ESCRT-II; TBK1; RILP

ABSTRACT

The small GTPase Rab7 coordinates membrane trafficking pathways at the late endosome. By interacting with and engaging different effectors, Rab7 is able to coordinates late endosome- and autophagosome-Lysosome fusion events, to control late endosome movement and positioning and to regulate late endosome-to-Trans Golgi Network (TGN) trafficking for the retrieval of cargo-receptors. How the same protein can orchestrate such variety of events is not completely understood.

Recent data are revealing the key role of post-translational modification (PTM) in customizing Rab7 functions. Indeed it has been shown that Rab7 can be ubiquitinated, phosphorylated and with my work, we have shown that Rab7 is also palmitoylated. The contribution of these PTMs in the regulation of the small GTPase functions is not completely understood. With my project I contributed to clarify some of the mechanisms by which PTMs regulate Rab7 function. With my first paper, we have shown that Rab7 is palmitoylated on cysteine 83 and 84 and that palmitoylation is required for efficient retromer recruitment and function at the endosome. Interestingly, it is dispensable for the degradation of integral membrane protein, such as EGFR. With my second paper, we investigated the impact of phosphorylation on serine 72 and tyrosine 183 on Rab7 function. We show that S72 phosphorylation is required for the recruitment of retromer onto membranes. We also show that in the absence of S72 phosphorylation, Rab7 is less palmitoylated, suggesting interplay between phosphorylation and palmitoylation.

The role of Rab7 Y183 phosphorylation was not clear, with data suggesting that this modification is required for EGFR degradation, while other work showing it was not required. Here we show that Y183 phosphorylation is required for EGF degradation using Rab7-KO HEK293 cells. Moreover we suggest a new model where phosphorylation on Y183 acts as a molecular switch by blocking the interaction between Rab7 and its effector RILP, and favouring the interaction of the latter with the ESCRT-II complex. This interaction is necessary for the efficient sorting of cargos into luminal vesicles (ILVs), resulting in their lysosomal degradation.

Overall my work has identified and characterized novel mechanisms of regulation of the small GTPase Rab7.

Key words: Rab7; post-translational modifications; palmitoylation; phosphorylation; retromer; intracellular trafficking; endosome; ESCRT-II; TBK1; RILP

SOMMAIRE RÉCAPITULATIF

La biogenèse des lysosomes

Les lysosomes ont été décrits pour la première fois par de Duve. Ce dernier les a nommés lysosome pour décrire leur capacité à dégrader les produits cellulaires qui ne sont plus nécessaires. Cependant, cette première classification des lysosomes comme sac à déchets cellulaires a été progressivement abandonnée. En effet, les lysosomes sont non seulement responsables de la dégradation des déchets cellulaires, mais ils jouent également un rôle crucial dans la régulation de plusieurs fonctions cellulaires.

La biogenèse et les fonctions des lysosomes sont globalement contrôlées par le facteur de transcription EB (TFEB), qui régule l'expression du groupe des gènes CLEAR (Coordinated Lysosomal Expression and Regulation). Les gènes CLEAR agissent comme codeurs pour les enzymes lysosomaux solubles, les protéines de membrane ainsi que l'ensemble des facteurs et des protéines accessoires qui régulent la physiologie des lysosomes.

Le tri des protéines du lysosome

Les enzymes lysosomales, aussi connues sous le nom de cargo, sont synthétisés dans le réticulum endoplasmique (ER) sous la forme de pro-enzymes. Dans l'appareil de Golgi, les enzymes lysosomales sont glycosylés et modifiés par l'ajout du Mannose-6-phosphate (M6P) qui est chargé d'identifier les protéines qui doivent être transportées vers les lysosomes. Ce signal est reconnu par le récepteur du M6P: cation dependent Mannose Phosphate Receptor (CD-MPR) et cation independent MPR (CI-MPR). Au-delà du M6P, il existe aussi une autre voie indépendante pour le transport des cargos vers les lysosomes. Cette route est contrôlée par le récepteur sortilin. Bien que certains cargos, tel que cathepsine D, peuvent être transportés en utilisant les deux routes, d'autres sont transportés exclusivement par un ou l'autre récepteur. Notamment, prosaposin est transportée vers les lysosomes uniquement par sortilin.

Les enzymes liés aux récepteurs sont transportés vers les endosomes dans des vésicules recouvertes de clathrine. Ce processus dépend des facteurs AP-1 (Adaptor protein 1) et GGAs (Golgi-localized, γ-ear containing, ADP-ribosylation factor binding proteins). Ces facteurs reconnaissent des séquences d'aminoacides pour le tri situées dans la portion cytosolique des récepteurs cargo. Lorsque les vésicules arrivent aux endosomes, le pH acide favorise la dissociation du cargo au récepteur. Cette dissociation permet au cargo d'être transporté vers les lysosomes et au récepteur d'être recyclé vers le TGN pour un autre cycle de transport.

Le retromer est responsable de l'identification des récepteurs cargo et de leur transport vers le TGN. Le retromer est un complexe conservé au cours de l'évolution qui a été décrit pour la première fois dans la levure. Dans cet organisme, le retromer se compose de trois sous-unités : Vacuolar Protein Sorting (Vps)26, Vps29 et Vps35. Il est également composé par un dimère formé par les sorting nexin (SNX) Vps5 et Vps17. Dans les organismes plus complexes, il y a une diversification du dimère de SNX, mais le trimère est conservé. Par ailleurs, il est important de noter que le trimère est aussi connu sous le nom de Cargo Selective Complex (CSC) afin de souligner son importance dans l'identification et dans la reliure des récepteurs cargo tels que sortilin et CI-MPR. Le mauvais fonctionnement du retromer provoque une réduction du transport des enzymes lysosomes et, par le fait même, la réduction de la capacité de dégradation des lysosomes.

Les membranes des lysosomes contiennent de nombreuses protéines transmembranaires. Ces dernières sont synthétisés dans le ER et elles sont très glycosylées. Cette modification est nécessaire car elle permet la formation du glycocalyx dans la lumière du lysosome, ce qui protège la partie luminale de la membrane contre les dommages causés par le pH acide du milieu lysosomal. Dans le TGN, les protéines de la membrane lysosomale sont reconnues par AP-1 et transportées vers la membrane plasmique, où ils sont reconnues par AP-2 et internalisées par endocytose dans des vésicules recouverts de clathrin. Ces vésicules sont ensuite transportées vers les endosomes où les protéines transmembranaires sont finalement triées vers les lysosomes.

La contribution de l'endocytose à la biogenèse des lysosomes

L'endocytose est un processus à travers lequel une partie de la membrane plasmique et les protéines qui lui sont associées sont internalisées et transportées vers les endosomes précoces. Le pH de cette organelle est faiblement acide est favorise la dissociation des ligands associés aux récepteurs. Lors de cette dissociation, les protéines et les lipides qui doivent être réutilisés sont recyclés vers la membrane plasmique, tandis que le matériel à éliminer est trié vers les lysosomes. À cet égard, la première étape dans ce processus de dégradation consiste à identifier les protéines à éliminer. Cette identification, se fait généralement en modifiant les protéines par le processus d'ubiquitination. Les protéines ubiquitinisées sont reconnues par le complexe de tri endosomal requis pour le transport (Endosomal Sorting Complex Required for Transport - ESCRT). L'ESCRT séquestre les protéines destinées aux lysosomes dans les sous-régions d'endosomes recouverts de clathrine. L'accumulation des protéines à éliminer dans ces

sous-régions endosomales entraîne la formation des vésicules intra-luminales (ILV). Ces ILV finissent par se détacher et vont se transformer en corps multivésiculaires (MVB). Les MVBs sont transportées sur des microtubules et se fusionnent avec les endosomes tardifs (Late endosome - LE), le dernier compartiment endosomal qui précède les lysosome. L'échange de matériel entre ces compartiments s'effectue par étapes de fusion et fission. Un premier modèle pour l'échange de matériel entre LE et les lysosomes est appelé « kiss-and-run ». Selon ce modèle, les LE et les lysosomes se fusionnent partiellement et temporairement en générant un pore qui permet le transfert du matériel (Kiss) pour ensuite se séparer (Run) et reconstituer les deux compartiments d'origine. Le second modèle prévoit la fusion complète entre les deux compartiments avec la formation d'une organelle hybride appelé endolysosome. Ces deux modèles ne sont pas mutuellement exclusifs, mais tous deux contribuent à la maturation des lysosomes.

L'autophagie

L'autophagie, qui signifie "se manger soi-même", est le processus par lequel les cellules digèrent leur propre cytoplasme en formant des structures multi-membranaires appelées autophagosomes qui se fusionnent avec les lysosomes. L'autophagie a été initialement décrite comme un mécanisme de protection des cellules activé par carence. Toutefois, il est maintenant évident que l'autophagie a une fonction beaucoup plus complexe dans la cellule. Elle est en effet impliquée dans la rotation des organelles endommagées, dans la dégradation des agrégats de protéines et dans l'élimination des pathogènes. Les autophagosomes et les lysosomes sont fortement liés et il a été démontré que les lysosomes peuvent contrôler l'activation de l'autophagie. Ce processus, est important pour l'élimination des lysosomes endommagés.

Les lysosomes contrôlent l'activation de l'autophagie à travers le complexe des protéines LYNUS (lysosomal nutrient sensing) qui est situé sur le côté cytosolique de la membrane lysosomal. Une protéine très importante dans ce complexe est la protéine kinase mTORC1 (mechanistic target of rapamycin 1). Lorsque le niveau des nutriments dans les cellules est élevé, mTORC1 est associé au complexe LYNUS et phosphorylera le facteur de transcription TFEB, qui dans cet état ne peut être localisé dans le noyau. Au contraire, lorsque le niveau des nutriments est faible, mTORC1 se dissocie du complexe LYNUS et le niveau de phosphorylation du facteur TFEB est réduit. Cela permet à la protéine de se déplacer vers le noyau. Une fois la protéine dans le noyau, TFEB peut activer la transcription des gènes CLEAR

pour la biogenèse des lysosomes ainsi que les gènes nécessaires au processus d'autophagie. La réduction de l'activité de mTORC1 entraîne également la réduction des niveaux de phosphorylation du complexe ULK, qui est formé par les kinases unc51-like (ULK) 1, les protéines ATG (autophagy-related gene) 13 et ATG101, et la protéine FIP200 (focal adhesion kinase family interacting protein of 200kDa, aussi appelée ATG17). La réduction de phosphorylation de ULK1 active ce complexe qui phosphoryle et active à son tour le complexe Beclin-1. Ce dernier est formé par la kinase de l'inositol-3-phopshate (PI(3)) Vps34, Beclin1/ATG6, Barkor/ATG4 et Vps15/p150. Les complexes ULK et Beclin-1 sont nécessaires pour l'induction de l'autophagie. La deuxième étape de ce processus est appelée nucléation et implique le recrutement de plusieurs protéines, telles que WIPI (WD-repeated-domain phosphoinositide interacting proteins) qui lie le PI(3)P produite par Vps34 (WIPI), ATG18 et DFCP1 (double FYVE containing protein 1). Ces protéines recrutent à leur tour les facteurs d'élongation pour la formation et fermeture de l'autophagosome. Au cours de cette dernière étape, la protéine ATG12 est conjuguée à ATG5 et les protéines de la famille ATG8 (LC3, GABARAP or GATE-16) sont conjuguées à la phosphatidylethanolammine (PtdEtn). En particulier, la formation de LC3-II, la forme de LC3 conjuguée à la PtdEtn, est le marqueur le plus utilisé pour identifier les autophagosomes.

Dans les cellules de mammifères, des sites d'assemblage du phagosome (PAS) peuvent se former dans plusieurs régions du cytoplasme. Par exemple, dans les neurones, les phagosomes peuvent provenir des régions plus distales de l'axone et ensuite être transportés vers le soma à l'aide des microtubules pour se fusionner avec les lysosomes.

La petite GTPase Rab7 dans l'organisation du trafic des membranes dans le compartiment LE-lysosomal

Le transport des membranes dans les cellules est coordonné par des membres de la famille des petites GTPases Ras. En particulier, les mouvements des membranes dans le compartiment endolysosomal sont contrôlés par la petite GTPase Rab7. La famille des Rabs (Ras-like in the brain) a un rôle crucial dans la définition de l'identité des membranes. Elle a également un rôle primordial dans la régulation des événements de fusion dans la membrane plasmique, dans le compartiment endolysosomal, dans le Golgi et dans le réticulum endoplasmique.

Les petites GTPases sont caractérisées par leur capacité de changer entre un état actif lorsqu'elles sont liées au GTP et à un état inactif lorsqu'elles sont liées au GDP. La transition entre leur état actif et inactif est accélérée par les GAPs (GTPase activating protein) qui favorisent l'hydrolyse du GTP à GDP. Par contre, les GEFs (Guanine Exchange Factors) favorisent la libération du GDP et l'association avec une autre molécule du GTP.

Dans leur état actif, les Rabs peuvent interagir avec des effecteurs au fin de diriger des fonctionnes situées en aval. Dans le compartiment endolysosomal, Rab7 a plusieurs fonctions. Elle contrôle la fusion des endosomes et des autophagosomes avec les lysosomes, dirige le mouvement et la position des LEs et des lysosomes et est aussi responsable du recrutement du retromer dans les endolysosomes. De plus, Rab7 a également une fonction importante dans la régulation de la mitophagie. Ce processus permet l'élimination des mitochondries endommagés par l'autophagie. Dans ce contexte Rab7 est importante pour les premières étapes de formation et d'expansion du mitophagosome.

Les événements de fusion dans les LE-lysosomes sont régulés par l'interaction de Rab7 avec plusieurs effecteurs. En particulier l'interaction entre Rab7 et RILP (Rab7 lysosomal interacting protein) et entre Rab7 et PLEKHM1 (Pleckstrin homology domain containing protein family member 1). Ces interactions sont nécessaires pour le recrutement du complexe d'attache (tethering complex) HOPS (Homotypic fusion and vacuolar protein sorting), un complexe des protéines nécessaires pour l'approche des membranes destinées à la fusion. Le recrutement du HOPS à travers l'action combinée de Rab7, RILP et PLEKHM1 est crucial pour une fusion efficace dans ce compartiment. En outre, l'interaction entre Rab7 et PLEKHM1 est très importante pour la fusion entre les autophagosomes et les lysosomes. PLEKHM1 est en effet capable de lier les autophagosomes grâce à une interaction directe avec la protéine LC3, localisée sur la membrane des autophagosomes.

Les mouvements des LEs sont contrôlés par Rab7 à travers des interactions avec différents effecteurs en fonction de la direction du mouvement. Le mouvement en direction du noyau (retrograde trafficking) dépend de l'interaction entre Rab7, RILP et ORP1L (oxysterol-binding protein–related protein 1L). La formation de ce complexe est nécessaire pour le recrutement de la dyneine qui est le moteur moléculaire nécessaire pour le transport des vésicules en direction du noyau sur les microtubules. Par contre, le transport des LE en direction de la périphérie cellulaire est atteinte avec l'interaction entre Rab7 et FYCO-1 (FYVE and Coiled-Coil-(CC)-domain containing 1). Cette protéine est capable de lier la kinesine qui est le moteur moléculaire nécessaire pour le transport antérograde sur les microtubules.

Le recrutement par Rab7 du retromer est nécessaire pour le transport des membranes des LE au trans-Golgi-Network (TGN). Cette route est très importante pour le recyclage des récepteurs de cargos, tel que sortilin et CI-MPR, qui sont impliqués dans le transport des enzymes lysosomal.

Les modifications post-traductionnelles de Rab7

Ces dernières années, plusieurs travaux ont souligné l'importance des modifications post-traductionnels dans la régulation de l'activité de Rab7. En effet, il a été démontré qui cette petite GTPase peut être ubiquitinée et phosphorylée. La combinaison de ces modifications est nécessaire pour régler finement l'habilité de Rab7 à interagir et à recruter ses effecteurs.

Phosphorylation

Rab7 peut être phosphorylé dans au moins deux positions, la serine 72 (S72) et la tyrosine 183 (Y183). Il a été démontré que la phosphorylation de la S72 est effectuée par la kinase Tank Binding Kinase (TBK)1 lorsque que la mitophagie est activé. La phosphorylation de la serine 72 permet à Rab7 de recruter les effecteurs nécessaires pour réaliser ce processus. Une autre kinase, la Leucine-Reach Repeat kinase (LRRK)1 est aussi capable de phosphoryler Rab7 sur la serine 72. Dans ce contexte, cette modification favorise l'interaction entre Rab7 et RILP. La phosphorylation de la Y183 est effectuée par la kinase c-Src et régule négativement l'interaction avec RILP. La fonction de cette modification n'est pas très claire. En effet l'expression dans les cellules du mutant de Rab7 qui ne peut pas être phosphorylé (Rab7 Y183F) ou de le mutant qui imite une version constamment phosphorylée de la petite GTPase (Rab7 Y183E) les deux entraînent un ralentissement de la dégradation du récepteur EGFR.

Ubiquitination

L'ubiquitination de Rab7 est effectuée par l'enzyme ubiquitine-ligase PARKIN. Cette modification consiste à ajouter une molécule d'ubiquitine aux résidus de lysine K38, K191 et probablement K194 de la petite GTPase. Cette modification est nécessaire pour localiser Rab7 dans la membrane et augmente également l'interaction entre Rab7 et RILP. L'élimination de l'ubiquitine est effectuée par des enzymes deubiquitinant (DUB). En particulier, le DUB USP32 élimine les molécules d'ubiquitine des lysine situées dans la partie c-terminale de Rab7.

Premier article : La palmitoylation de Rab7 est nécessaire pour le trafic efficient à partir des endosomes vers le TGN

Dans les endosomes, Rab7 contrôle plusieurs voies de trafic des membranes. Elle contrôle le trafic à partir des endosomes vers les lysosomes. Ce contrôle est très important pour la dégradation dans les lysosomes des protéines ubiquitinées et triées dans les MVB. Le contrôle est aussi important pour le trafic vers le TGN, qui est fondamental pour le recyclage des récepteurs cargo. La première route prévoit l'interaction de Rab7 avec RILP et PLEKHM1 ainsi que le recrutement du complexe HOPS pour la fusion avec les lysosomes. Dans la seconde voie, Rab7 doit recruter le retromer capable de reconnaitre les récepteurs cargo qui doivent être transportés vers le TGN. Selon les recherches, il n'est pas clair comment Rab7 est capable d'interagir et recruter ses effecteurs pour remplir ces fonctions qui semblent être en opposition.

Des études publiées précédemment ont identifié la palmitoyl-transferase (PAT) DHHC5 dans l'ensemble des protéines nécessaires au trafic des endosomes vers le TGN. Les PAT sont des enzymes qui lient, d'une manière réversible, le palmitate au résidu de cystéine dans les protéines ciblées. Cette modification post-traductionnelle peut modifier la fonction des protéines de différentes manières. Par exemple, elle peut réguler la localisation des protéines, favoriser les itérations intermoléculaires ou encore stabiliser les protéines en prévenant leur dégradation. De plus, dans la levure, deux palmitoyl-transferases, Swf1 et Akr1 ont été impliquées dans le tri de la protéine vacuolaire (la vacuole est la contrepartie des lysosomes dans la levure) CPY (Carboxy peptidase Y). Ces résultats suggèrent que la palmitoylation peut jouer un rôle important dans la régulation du trafic entre les endosomes et le TGN.

Pour comprendre le fonctionnement de la palmitoylation dans ce processus, nous avons d'abord vérifié si les sous-unités du retromer ou Rab7 sont palmitoylées. Pour ce faire, nous avons utilisé la technique Acyl-RAC (Resin Assisted Capture), que nous avons adapté à partir des autres études déjà publiées. Nos recherches nous ont permis de trouver que Rab7 est palmitoylée contrairement à toutes les sous-unités du CSC de retromer (Figure 3.1A). Dans le but d'identifier le ou les résidus ciblés par cette modification, nous avons généré des protéines mutées de Rab7 où les cystéines sont remplacées par la serine. Nous avons ensuite analysé leur niveau de palmitoylation avec Acyl-RAC. Finalement, nous avons observé que la mutation individuelle de la cystéine 83 (Rab7C83S) ou 84 (Rab7C84S) provoque la réduction du niveau de palmitoylation de Rab7. Cependant, la protéine n'est pas palmitoylée lorsque les deux cystéine 83 et 84 (Rab7C,83,84S) sont mutées (Figure 3.1B).

Pour vérifier si la palmitoylation a un rôle dans la localisation de Rab7 vers la membrane, nous avons utilisé la technique de séparation de membrane, qui permet de séparer les membranes de la fraction soluble de la cellule. Rab7 ainsi que Rab7C83S, Rab7C84S et Rab7C83,84S ont été trouvés dans la fraction contenant les membranes (Figure 3.1C). Nous avons également analysé l'emplacement de Rab7 et de ses protéines mutées par immunofluorescence. Ceci, nous a permis de trouver que, comme Rab7, les protéines Rab7C83S, Rab7C84S et Rab7C83,84S se localisent dans les membranes des endosomes (Figure 3.2). Ces résultats suggèrent qui la palmitoylation de Rab7 n'a pas la fonction de localiser la petite GTPase dans la membrane.

Nous avons considéré que la palmitoylation de Rab7 peut avoir un rôle dans le recrutement de retromer vers les endosomes. Afin de vérifier notre hypothèse, nous avons utilisé la technique de CRISPR/Ca9 pour générer des cellules qui n'expriment pas la protéine Rab7 (Rab7-KO, Figure 3.3A). En confirmant les résultats déjà publiés sur des cellules où l'expression de Rab7 a été réduite par siRNA, dans la cellule Rab7-KO, l'association du retromer avec les membranes est significativement réduite (Figure 3.3B, 3.4B). Ce phénotype est reversé par l'expression dans les cellules Rab7-KO de la protéine Rab7 wild-type, mais pas par celle de Rab7C83,84S (Figure 3.3B, 3.4). Ces observations suggèrent que la palmitoylation de Rab7 est nécessaire pour le recrutement de retromer vers les endosomes.

Pour comprendre les raisons pour lesquelles Rab7C83,84S n'est pas capable de recruter efficacement le retromer, nous avons effectué des expériences en utilisant BRET (Bioluminescence Resonance Energy Transfer). Cette technique nous permet d'analyser les interactions entre protéines dans les cellules vivantes et de maintenir l'emplacement cellulaire physiologique des protéines d'intérêt. Dans le BRET, il existe une molécule, que l'on appelle "donneur". Cette molécule est capable d'émettre de l'énergie après l'ajout du substrat. L'énergie émise par le donneur est transférée à une deuxième molécule, "accepteur" qui à son tour émet de l'énergie que nous pouvons détecter. Dans notre expérience, la molécule donneuse, Renilla Luciferase II (RlucII) a été clonée dans la portion N-terminale de Rab7 (RlucII-Rab7) et la molécule acceptrice,GFP10, a été clonée dans la portion C-terminale de la sous-unité du retromer Vps26A. À travers l'expression d'une quantité constante de RlucII-Rab7 et des concentrations croissantes de Vps26A-GFP10, il est possible d'obtenir des courbes de saturation. À partir de cette courbe, nous pouvons calculer la valeur BRET50, qui représente la concentration de la molécule acceptrice nécessaire pour obtenir la moitié de la BRETmax. BRET50 fournit une indication de la capacité d'interaction des deux protéines: plus cette valeur

est faible, plus l'interaction des deux protéines est importante. Lorsque nous avons testé l'interaction de RlucII-Rab7 et RlucII-Rab7C83,84S avec Vps26A-GFP10, nous avons observé que la BRET50 pour le couple RlucII-Rab7C83,84S/Vps26A-GFP10 est trois fois supérieur à celle de RlucII-Rab7/Vps26A-GFP10, en indiquant une capacité d'interaction réduite (Figure 3.5A,3.5B). De plus, en utilisant la microscopie illumination structurée (SIM), nous avons analysé le niveau de colocalisation de myc-Rab7 et myc-Rab7C83,84S avec Vps26A endogène. Le niveau de colocalisation de Rab7C83,84S et Vps26A est significativement réduit par rapport à celui observé entre Rab7 et Vps26A (Figure 3.5C, 3.5G). Ceci, confirme nos observations précédentes.

Le retromer coordonne le recyclage de récepteurs de cargo, qui transportent les enzymes qui résident dans les lysosomes. Si ce recyclage ne fonctionne pas correctement, les enzymes du lysosome s'accumulent dans le TGN et sont finalement sécrétés dans le milieu extracellulaire. Donc, pour analyser la fonctionnalité du retromer, nous avons examiné la présence de la protéine lysosomal Cathepsine-D dans les cellules Rab7-KO et Rab7-KO exprimant Rab7wt ou le mutant Rab7C83,84S. Comme nous l'avons supposé, nous avons trouvé que les cellules Rab7-KO et Rab7-KO exprimant Rab7C83,84S sécrètent beaucoup plus Cathepsine-D que les cellules Rab7-KO exprimant Rab7. Ces résultats suggérant encore une fois un fonctionnement réduit de retromer (Figure 3.6A).

Pour vérifier si la palmitoylation de Rab7 a un effet spécifique sur le retromer et non sur la capacité de Rab7 de contrôler la route de dégradation des protéines, nous avons étudié la dynamique de dégradation du récepteur du facteur de croissance épidermique (EGFR) dans les cellules Rab7-KO et Rab7-KO exprimant Rab7wt ou le mutant Rab7C83,84S. Bien que les cellules Rab7-KO ne sont pas capables de dégrader EGFR, les cellules Rab7-KO exprimant Rab7 ou Rab7C83,84S ont la même capacité de dégrader le récepteur. Ceci montre que la palmitoylation de la petite GTPase n'est pas nécessaire pour la route de dégradation lysosomal (Figure 3.6B, 3.7).

Avec ce travaille, nous avons démontré que la petit GTPase Rab7 est palmitoylée et que cette modification post-traductionnelle est nécessaire pour le fonctionnement de retromer. En effet, l'expression de la protéine Rab7, qui ne peut être palmitoylée (Rab7C83,84S) dans les cellules où la protéine Rab7 endogène a été supprimée, ne rétablit pas la correcte activité de retromer. Si Rab7 n'est pas palmitoylée, le retromer ne peut pas être efficacement recruté vers les membranes des endosomes. En conséquence, il n'est pas capable de recycler les récepteurs cargo. L'altération de ce processus détermine le changement de localisation des

enzymes du lysosome qui ne sont pas triés dans les lysosomes mais sécrétés dans le milieu extracellulaire. De plus, nous avons démontré que la palmitoylation n'affecte pas toutes les fonctions de Rab7, alors que la petite GTPase est capable de coordonner la dégradation du récepteur EGFR en l'absence de palmitoylation. Ces observations suggèrent qu'à travers la palmitoylation les cellules sont capables de modifier l'activité de Rab7.

Des travaux récemment publies ont identifié d'autres modifications post-traductionnelles à la charge de Rab7 : la phosphorylation de la serine 72 et de la tyrosine 183, et l'ubiquitination de la lysine 38. Ces modifications modulent l'activité de Rab7 de plusieurs façons, en changeant la capacité de la petite GTPase d'interagir avec ses effecteurs. Les détails sur ces mécanismes et la façon dont les différents changements interagiront les uns avec les autres ne sont pas clairs.

Deuxième article : Le rôle de la phosphorylation dans la régulation de la fonction Rab7

Les endosomes tardifs (Late Endosome - LE) sont un compartiment cellulaire très dynamique, où le tri des nombreuses protéines provenant de différentes régions cellulaires est décidé. La petite GTPase Rab7 est responsable des événements liés au tri et au trafic des protéines dans les LE. Elle dirige le mouvement et le positionnement des LE, est responsable de la fusion entre le LE et les lysosome ainsi que la fusion entre les autophagosomes et le lysosome. La petite GTPase Rab7 cordonne aussi le trafic des récepteurs cargo vers le trans Golgi-network (TGN). Plusieurs études récemment publiées ont souligné l'importance des modifications post-traductionnelles dans la régulation des fonctions de Rab7. Cette protéine est prenylée après sa translation dans le cytosol. La prenylation est importante pour la localisation de la petite GTPase vers la membrane. De nombreuses autres modifications ont été identifiées qui contribuent à réguler la fonction de Rab7 dans la cellule. En effet, Rab7 peut être ubiquitinée, phosphorylée et avec mes travaux précédents, j'ai démontré qui Rab7 est aussi palmitoylée.

Rab7 peut être phosphorylée dans au moins deux positions; en correspondance de la serine 72 et de la tyrosine 183. La kinase LRRK1 peut phosphorylée Rab7 ce qui augmente l'interaction avec RILP. Une autre kinase, Tank Binding Kinase 1 (TBK1) peut phosphoryleré Rab7 dans le cadre de l'activation de la mitophagie.

La palmitoylation contrôle la capacité de Rab7 à interagir et à recruter le retromer vers les membranes des LE. Le retromer est un complexe de protéines constitué par le trimer

Vps26, Vps29 et Vps35. L'activité du retromer est très importante pour le fonctionnement des lysosomes. En effet, ce complexe s'occupe du recyclage des récepteurs de cargo à partir des endosomes vers le TGN. Les récepteurs de cargo, comme sortilin et CI-MPR, s'occupent du transport des protéines solubles présentes dans le lysosome. Ces protéines sont synthétisées dans le Golgi et reconnues par les récepteurs de cargo, ce qui favorise leur transport vers les endosomes. Dans le milieu endosomal, le pH acide favorise la dissociation des cargos de son récepteur. Les cargos sont transportés dans le lysosome mais le récepteur est recyclé vers le TGN à travers le retromer. Si le retromer ne fonctionne pas correctement, les récepteurs de cargo sont dégradés dans les lysosomes et le tri des protéines du lysosome solubles est réduit, ce qui entraîne au mauvais fonctionnement des lysosomes. La palmitoylation de Rab7 est très importante pour réguler l'activité du retromer. En effet, dans les cellules Rab7-KO qui n'expriment que le mutant Rab7 qui peut ne pas être palmitoylé, la fonction du retromer est très affectée.

La phosphorylation de Rab7 sur le résidu Y183 est effectuée par la kinase Src. Cette modification prévient l'interaction entre Rab7 et RILP, ce qui est important pour le positionnement et le mouvement des LE et aussi que pour les événements de fusion entre LE et les lysosomes. Le positionnement et le mouvement des lysosomes dépend de l'interaction entre RILP, Rab7 et ORP1L (oxysterol-binding protein–related protein (ORP)1L). Ce complexe est responsable du recrutement de la dynéine, le moteur qui permet le mouvement rétrograde sur les microtubules. Les événements de fusion sont contrôlés par l'interaction entre Rab7\RILP, et Rab7\PLEKHM1 avec le complexe HOPS. La phosphorylation de Y183 de Rab7 bloque l'interaction entre Rab7 et RILP, mais les conséquences de cette modification dans le processus de fusion ne sont pas claires. Des études ont montré que la dégradation du récepteur EGFR est bloquée en exprimant la forme mutant de Rab7 qui ne peut pas être phosphorylé (Rab7Y183F) ou avec le mutant qui imite une version constamment phosphorylée de la petite GTPase (Rab7Y183E).

Dans ce travail nous analysons comment la phosphorylation sur la serine 72 et la palmitoylation sur les cystéines 83 et 84 travaillant ensemble pour moduler la capacité de Rab7 d'interagir avec le retromer. Nous démontrons que l'expression de Rab7, qui ne peut pas être phosphorylée dans la serine 72 (Rab7S72A) dans les cellules Rab7-KO, n'est pas capable d'interagir avec le retromer et de le recruter dans les endosomes. Ce comportement est pareil à celui observé avec l'expression du mutant non palmitoylé (Rab7C83,84) dans les cellules Rab7-KO.

Dans ce travail, nous démontrons également que la phosphorylation du résidu Y183 est nécessaire à la dégradation du récepteur EGFR. De plus, nous fournissons des évidences qui suggèrent que ce processus est indépendant de l'interaction entre Rab7 et RILP.

Pour comprendre le rôle de la phosphorylation de la serine 72 dans la régulation du retromer, nous avons généré des mutants qui imitent la version constamment phosphorylée de Rab7 (Rab7Y183E et Rab7S72E) et mutants qui ne peuvent être phosphorylés (Rab7S72A et Rab7Y183F). Des résultats récemment publiés montrent que le mutant RabS72E n'est pas efficacement prenylée et qu'il n'est donc pas capable de se localiser dans la membrane. Les résultats obtenus par l'analyse de ce mutant peuvent être dus à l'absence de prenylation et non à la présence permanente de la phosphorylation. Pour ces raisons, nous avons décidé de ne pas utiliser ce mutant dans notre analyse.

Pour analyser si la phosphorylation influence l'interaction entre Rab7 et le retromer, nous avons utilisé la technique BRET, qui a déjà été utilisée dans notre travail précédent. Pour tester la validité de cette technique, nous avons d'abord évalué l'interaction entre RILP-GFP10 et RlucII-Rab7 et ses mutants RlucII-Rab7S72A, RlucII-Rab7Y183F, et RlucII-Rab7Y183E. Nous avons trouvé que le mutant RlucII-Rab7Y183E n'est pas capable d'interagir avec RILP, alors que d'autres mutants peuvent interagir avec RILP de la même manière que Rab7 (Figure 5.1 A et B). Après avoir vérifié la fiabilité de cette technique pour ce type d'analyse, nous avons testé l'interaction entre Vps26A-GFP10 et les mêmes mutants. Nous avons constaté que seulement RlucII-Rab7S72A n'est pas capable d'interagir avec Vps26A, alors d'autres protéines mutantes se comportent comment Rab7 (Figure 5.1C et D).

Afin d'exclure que l'incapacité de Rab7S72A à interagir avec le retromer soit due à un changement dans l'emplacement cellulaire de ce mutant, nous avons évalué la capacité des mutants de la phosphorylation de co-localiser avec le marquer des LE-Lysosome Lamp1. Nous avons exprimé cerulean-Lamp1 et myc-Rab7 et ses mutants dans les cellules U2OS et avons examiné à travers immune-fluorescence la position des mutants. Nous n'avons observé aucune différence dans la localisation des mutants par rapport au marquer Lamp1. Par conséquent, le défaut dans l'interaction entre Rab7S72A et le retromer n'est pas causé par une altération de l'emplacement du mutant (Figure 5.2).

Nous avons donc supposé que la phosphorylation de la serine 72 est nécessaire pour le recrutement du retromer vers les membranes des endosomes. Pour tester notre hypothèse, nous avons exprimé myc-Rab7 et ses mutants dans les cellules Rab7-KO que nous avions générées précédemment. Dans ces cellules, le retromer n'est pas associé aux membranes,

mais l'expression de myc-Rab7 rétablit l'emplacement du retromer dans la membrane. Nous avons observé que les mutants de la tyrosine sont capables, comme Rab7, de rétablir la localisation du retromer, mais ce n'est pas le cas du mutant Rab7S72A (Figure 5.3). Ces données suggèrent que la phosphorylation de la serine 72 de Rab7 est nécessaire à l'interaction avec le retromer et au recrutement dans la membrane des endosomes.

Nous avons démontré que la palmitoylation de Rab7 est nécessaire pour le recrutement du retromer, . Ainsi, puisque le mutant Rab7S72A n'est pas capable d'interagir avec cette combinaison, nous avons décidé de tester la palmitoylation de Rab7S72A. Nous avons trouvé que ce mutant est moins palmitoylé que Rab7 (Figure 5.4A et B). Cette information suggère qu'il existe une relation entre la phosphorylation et la palmitoylation de Rab7. Nous avons aussi essayé de déterminer si l'augmentation de la phosphorylation de Rab7 est corrélée à l'augmentation de la palmitoylation de Rab7. Nous avons donc exprimé une quantité croissante de la kinase TBK1 et analysé la palmitoylation de Rab7 à travers la technique de l'Acyl-RAC. Nous n'avons pas détecté aucun changement dans le niveau de palmitoylation de la petite GTPase (Figure 5.4C et D). De plus, nous avons constaté que la surexpression de TBK1 n'affecte pas beaucoup la localisation de Rab7 ou du retromer (Figure 5.4E, G, H). Nous avons également observé qui l'inhibition de TBK1 avec la molécule MRT67307 est capable de réduire le niveau de palmitoylation de Rab7, mais que cette réduction n'entraîne pas de changement important dans la distribution du retromer dans les cellules (Figure 5.5). Par conséquent, ces résultats suggèrent qui la phosphorylation de Rab7 par la kinase TBK1 ne module pas la capacité de la petite GTPase à interagir et de recruter le retromer, du moins dans des conditions stables.

La phosphorylation de Rab7 du résidu Y183 bloque l'interaction entre Rab7 et RILP (Figure 5.1A et B). Plusieurs études ont analysées la fonction de cette modification dans le processus de dégradation lysosomal, mais les résultats qui sont publiés ne sont pas clairs. En effet, certaines données suggèrent que la phosphorylation de la Y183 est nécessaire à la dégradation du récepteur EGFR, mais d'autres suggèrent que cette modification n'est pas impliquée dans ce processus. Toutes ces études ont été réalisées en exprimant des mutants de Rab7 dans cellules qui expriment aussi des protéines endogènes. Cette condition peut altérer les résultats des expériences. Pour comprendre comment la phosphorylation de la tyrosine module la fonction de Rab7 pendant la dégradation du récepteur EGFR, nous avons exprimé myc-Rab7 et ses mutants dans les cellules Rab7-KO, afin d'éliminer la contribution des protéines endogènes à ce processus. Nous avons donc stimulé ces cellules avec EGF-488

pendant 30 minutes et après une heure, nous avons analysé la quantité d'EGF-488 présente dans les cellules. En une heure, les cellules HEK293 ont dégradé la quasi-totalité l'EGF-488, mais les cellules Rab7-KO ne sont pas capable d'éliminer l'EGF-488. En effet, nous avons trouvé la molécule EGF-488 accumulé dans la cellule. Les cellules Rab7-KO expriment myc-Rab7, myc-Rab7S72A or myc-Rab7Y183E sont capables de dégrader l'EGF-488 de la même manière que les cellules HEK293. L'expression du mutant myc-Rab7Y183F n'a pas été capable de rétablir la capacité de dégradation des cellules HEK293. Nous avons trouvé une quantité d'EGF-488 dans la cellule qui est similaire à celle des cellules Rab7-KO. Ces résultats confirment que la phosphorylation de Rab7 est nécessaire à la dégradation du EGF dans les cellules (Figure 5.6A et B).

Après la stimulation par EGF, Rab7 est très rapidement phosphorylée sur le résidu Y183. Cette modification est importante pour le tri du récepteur EGFR dans la route de dégradation lysosomal. Cette fonction est assurée par le complexe de tri Endosomal Sorting Complex Required for Transport (ESCRT)-II, capable de reconnaitre les protéines destinées à la dégradation vers les lysosomes et de les trier dans les vésicules intraluminales (intraluminal vesicles - ILVs). RILP régule la fonction du complexe ESCRT-II par l'interaction avec les sous-unités Vps22 et Vps36. De plus, RILP a été identifié comme acteur clé dans la dégradation du récepteur EGFR. Nous avons donc considéré que la phosphorylation du résidu Y183 de Rab7 est un interrupteur moléculaire (molecular switch) qui provoque la dissociation de RILP de Rab7 et favorise l'interaction de RILP avec ESCRT-II pour le tri efficace du récepteur EGFR. Pour tester cette hypothèse, nous avons utilisée le BRET pour étudier les interactions Rab7/RILP, RILP/Vps22 et RILP/Vps36 en "état-stable" ou en exprimant la kinase Src, qui phosphoryle Rab7 dans la tyrosine 183. Nous avons observé que si Src est exprimé dans les cellules, l'interaction entre Rab7 et RILP est réduite, ce qui confirme les travaux publiés précédemment. De plus nous avons trouvé que si Src est exprimé l'interaction entre RILP et Vps22 et entre RILP et Vps36 est augmentée (Figure 5.6). Bien que des expériences supplémentaires sont nécessaires pour mieux caractériser ces mécanismes, nos observations préliminaires suggèrent que la phosphorylation de Rab7 peut agir comme un interrupteur moléculaire pour favoriser la fonction de RILP dans le tri du récepteur EGFR.

Pour une fusion correcte des membranes entre les LE et les lysosomes, plusieurs protéines sont recrutées. L'interaction entre Rab7 et RILP est nécessaire pour recruter le complexe « homotypic fusion and vacuole protein sorting (HOPS) ». HOPS est nécessaire pour l'approche des membranes destinées à la fusion. Pour stabiliser ce complexe, PLEKHM1 est

aussi recrutée par Rab7 vers la membrane. Nos résultats sur la dégradation du EGF-488 suggèrent qui l'interaction entre Rab7 et RILP n'est pas nécessaire pour compléter la fusion. En effet, la protéine mutante Rab7Y183E, qui n'interagit pas avec RILP, est capable de rétablir la dégradation du EGF dans les cellules Rab7-KO. Nous avons utilisé BRET pour tester l'interaction RILP/Vps39 (une sous-unité du complexe HOPS) et RILP/PLEKHM1 dans les cellules Rab7-KO et nous avons trouvé que toutes ces interactions sont dépendantes de la présence de Rab7 (Figure 5.7). Plus, nous avons effectué des expériences pour établir que la phosphorylation de Rab7 a un rôle dans la régulation de ces interactions. Ainsi, nous avons testé avec BRET, les interactions RILP/PLEKHM1 dans les cellule Rab7-KO, et Rab7-KO exprimant myc-Rab7 et myc-Rab7S72A, myc-Rab7Y183E et myc-Rab7Y183F. À travers ces expériences, nous avons trouvé que seulement myc-Rab7Y183E n'est pas en mesure de de rétablir l'interaction RILP/PLEKHM1 (Figure 5.8A et B). Ces résultats sont en accord avec nos observations précédentes sur l'incapacité de Rab7Y183E d'interagir avec RILP.

Rab7 interagit avec plusieurs effecteurs pour la direction du trafic des membrane dans les LE. Nous nous sommes donc demandé si la phosphorylation de Rab7 contrôle l'interaction de la petite GTPase avec d'autres effecteurs. Nous avons constaté à travers BRET que l'interaction entre Rab7Y183F et oxysterol-binding protein–related protein (ORP)1L est supérieur de cela de Rab7 (Figure 5.9). ORP1L participe avec RILP et Rab7 au contrôle de la position et du mouvement des LE. Nos résultats suggèrent que la phosphorylation de Rab7 peut également être impliquée dans la régulation de ces mécanismes.

Avec cette recherche, nous avons montré qui la phosphorylation de Rab7 module la fonction de cette protéine dans plusieurs processus cellulaires. En particulier, nous avons constaté que la phosphorylation de la serine 72 module l'interaction entre Rab7 et le retromer. En effet la protéine mutante qui ne peut pas être phosphorylée sur la serine 72 (Rab7S72A) n'interagit pas avec le retromer et n'est donc pas capable de recruter ce complexe dans le membrane des LE. Il est intéressant de noter que le mutant Rab7S72A n'est pas palmitoylée efficacement. Ce résultat, confirme nos travaux antérieurs sur l'importance de la palmitoylation de Rab7 pour son interaction avec le retromer. De plus, puisque l'absence de phosphorylation entraîne la réduction du niveau de palmitoylation de Rab7, cela suggère que les deux modifications post-traductionnelles sont corrélées. On peut envisager que la phosphorylation est nécessaire pour favoriser l'interaction de Rab7 avec les protéines palmitoyltransferases, qui sont les enzymes responsables de la palmitoylation des protéines. Toutefois, des études supplémentaires sont nécessaires pour vérifier cette hypothèse. Dans le cadre de notre travail,

nous n'avons pas relevé une corrélation entre la kinase TBK1 et la capacité de Rab7 d'interagir avec le retromer. TBK1 phosphoryle Rab7 pendant la mitophagie, quand Rab7 a été recrutée sur la membrane des mitochondrie. Dans ce contexte, la phosphorylation de Rab7 est nécessaire à la maturation du mitophagosome pour l'élimination dans le lysosome des mitochondries endommagées. Pour la maturation du mitophagosome, il est nécessaire de recruter des membranes contenants la protéine ATG9. Ce processus est dépendant du retromer. Il est intéressant de noter que si la phosphorylation de Rab7 pendant la mitophagie est bloquée, le recrutement des membranes avec ATG9 est aussi bloqué. Il est donc plausible de considérer que la phosphorylation de Rab7 par TBK1 dans le cadre de la mitophagie est nécessaire pour recruter le retromer pour la mobilisation des membranes avec ATG9. D'autres études sont requises pour clarifier ces mécanismes.

Dans cette étude, nous avons également montré qui la phosphorylation de la tyrosine 183 de Rab7 contrôle la dégradation dans le lysosome du EGF. Plus précisément, nous avons trouvé que la protéine Rab7Y183F qui ne peut pas être phosphorylée n'est pas capable de restaurer la dégradation du EGF dans les cellules Rab7-KO. Aussi, nous avons fourni des données préliminaires sur la fonction de cette modification comme interrupteur moléculaire qui induit le dégagement de RILP et ne favorise l'interaction avec le complexe ESCRT-II. Cette dernière interaction est importante pour le tri des protéines internalisées par endocytose qui doivent être dégradées dans les lysosomes.

La fusion entre le LE et le lysosome est un processus complexe qui nécessite la participation de plusieurs protéines. Dans ce contexte, Rab7/RILP recrute HOPS vers les membranes des LE. Pour stabiliser ce complexe, Rab7 doit recruter PLEKHM1 qui est aussi capable d'interagir avec HOPS et de le recruter dans les membranes. L'action combinée de RILP et PLEKHM1 stabilise HOPS sur les membranes et favorise la fusion. Les résultats de nos travaux suggèrent que l'interaction entre RILP et Rab7 n'est pas nécessaire pour la fusion entre les LE et les lysosomes. En effet si l'interaction RILP/Rab7 est bloquée, les cellules sont encore capables de dégrader l'EGF. De plus, nos résultats préliminaires suggèrent que le recrutement du RILP dans la machine de fusion sur les LE dépend de Rab7, en particulier, il dépend fortement de la phosphorylation de la petite GTPase.

La phosphorylation de Rab7 sur Y183 peut aussi être impliquée dans le mouvement et dans le positionnement des LE. En effet, ces fonctions sont régulées par le complexe RILP/Rab7/ORP1L qui est en mesure de recruter la dynéine pour permettre son transport en

direction du noyau. Il est donc possible d'envisager que la phosphorylation de Rab7, en contrôlant l'interaction de la petite GTPase avec RILP, contrôle aussi le mouvement des LE.

Il est intéressant de noter que si Rab7 n'est pas phosphorylée sur la tyrosine 183, elle interagit mieux avec ORP1L. Ce dernier est également impliqué dans la formation des régions de contact entre le LE et le réticule endoplasmique (ER) par l'interaction avec la protéine VAMP- associated ER protein (VAP)-A. L'accroissement de l'interaction de Rab7Y183F avec ORP1L peut induire une interaction croissante entre ORP1L et VAP-A qui peut à son tour bloquer les LEs dans les régions de contact avec l'ER. La mobilité réduite des LE est peut-être la raison pour laquelle Rab7Y183F n'est pas en mesure de dégrader l'EGF. Des travaux futurs permettront de déterminer si cette hypothèse est valide.

En conclusion, avec ce travail, nous avons découvert des nouveaux niveaux de régulation de la petite GTPase Rab7 qui dépendent de la phosphorylation. En particulier, nous avons trouvé que la phosphorylation de la serine 72 est importante pour contrôler l'interaction avec le retromer. Nos observations préliminaires suggèrent que la phosphorylation sur la tyrosine 183 agit comme un interrupteur moléculaire pour favoriser le tri des protéines destinées à la dégradation dans les lysosomes. Nous avons trouvé des évidences préliminaires qui supportent le rôle de la phosphorylation de la tyrosine 183 dans la régulation du mouvement et du positionnement des LE.

Conclusion

Dans le cadre de mon projet de doctorat, j'ai contribué à clarifier les mécanismes que régulent la fonction de la petite GTPase Rab7 dans les LE. En particulier, j'ai contribué à comprendre le rôle des modifications post-traductionnelles, telles que la phosphorylation et la palmitoylation, dans la régulation des fonctions de Rab7.

J'ai recueilli des données qui suggèrent que la phosphorylation de la tyrosine 183 de Rab7 est importante pour le contrôle de la voie de dégradation dans les lysosomes et probablement dans le contrôle du mouvement et positionnement des LE. Dans la première situation, la phosphorylation de la Y183 agit comme un interrupteur moléculaire qui favorise le tri efficace des protéines destinées à la dégradation dans les lysosomes. Dans le cadre du mouvement des lysosomes, la phosphorylation sur Y183 pourrait favoriser la dissociation des LE des régions de contact avec le réticule endoplasmique. Toutefois, des analyses supplémentaires sont nécessaires pour vérifier cette hypothèse. Mes recherches ont aussi montré que Rab7 est

palmitoylée sur les cystéines 83 et 84. J'ai aussi découvert que cette modification est importante pour l'interaction efficace entre Rab7 et le retromer. Si Rab7 ne peut être pas palmitoylée, le retromer n'est pas recruté correctement dans la membrane des LE. J'ai également montré que l'interaction entre le retromer et Rab7 est aussi contrôlé par la phosphorylation sur la serine 72 de Rab7. De plus, ces modifications sont connexes. En effet, l'inhibition de la phosphorylation de S72 induit la réduction du niveau de palmitoylation de Rab7.

Le recrutement du retromer dans les membranes est important pour le trafic des LE vers le TGN. Ce processus est important pour le recyclage des récepteurs cargo qui s'occupent du transport des protéines du lysosome du TGN vers les endosomes. Par conséquence, l'altération fonctionnelle du retromer entraine la dégradation des récepteurs cargo qui à son tour, cause le mauvais trafic des protéines du lysosome. Ceux-ci s'accumulent dans le TGN et sont en fin secrétées dans le milieu extracellulaire. Le résultat final de cette altération fonctionnelle est une réduction des fonctions des lysosomes qui ont été associées avec plusieurs maladies neurodégénératives, comme la maladie d' Alzheimer et la maladie de Parkinson. Donc, l'identification de nouveaux niveaux de contrôle de l'activité du retromer, par exemple à travers la régulation des kinases et phosphatase ou des palmitoyltransferases et thioesterases qui modulent les modifications post-traductionnelles de Rab7, est très importante pour identifier des nouvelles cibles thérapeutiques.

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ABBREVIATIONS

Acyl-protein thioesterase (APT)

Acyl-Resin Assisted Capture (Acyl-RAC)

Adaptor protein (AP)

ADP-ribosilation factor (Arf)

Amyloid Precursor Protein (APP)

Associated molecule with the SH3 domain of STAM (AMSH)

Autophagy-related genes (ATG)

β-amyloid precursor protein-cleaving enzyme (BACE)

β-glucocerebrosidase (βGC)

Bioluminescence Resonance Energy Transfer (BRET)

Calcium caffeine zinc sensitivity (Ccz)

Carboxypeptidase Y (CPY)

Charged Multivesicular body protein 4 (CHMP4)

Class III phosphatidilinositol-3-phosphate kinase (PI(3)KIII)

Clathrin-mediated endoctytosis (CME)

Clathrin-independent endocytosis (CIE)

Coordinated lysosomal expression and regulation (CLEAR)

Class C core vacuole/endosome tethering (CORVET)

Cargo-selective complex (CSC)

Cation-depended mannose-phosphate receptor (CD-MPR)

Cation-independent mannose-phosphate receptor (CI-MPR)

Coiled-Coil (CC)

Dithiothreitol (DTT)

De-ubiquitylating enzymes (DUBs)

Detergent Resistant Microdomains (DRMs)

Divalent metal transporter (DMT)

Double FYVE containing protein (DFCP)

Early endosome (EE)

Endoplasmic reticulum (ER)

ER-Golgi intermediate compartment (ERGIC)

Endosomal Sorting Complex Required for Transport (ESCRT)

Epidermal Growth Factor Receptor (EGFR)

FYVE and Coiled-Coil - (CC)- domain containing 1 (FYCO1)

Focal adhesion kinase family interacting protein of 200kD (FIP200)

GAT (GGA and TOM)

Glycosaminoglycan (GAG)

Golgi-localized, γ-ear containing, ADP-ribosylation factor binding proteins (GGAs)

GRAM-like ubiquitin binding in EAP45 (GLUE)

GTPase activating proteins (GAPs)

Guanine-nucleotide dissociation inhibitor (GDI)

Guanine nucleotide exchange factor (GEF)

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)

α/β hydrolases domain-containing 17 proteins (ABHD17)

Homotypic fusion and vacuole protein sorting (HOPS)

Intraluminal vesicles (ILVs)

Late endosomes (LEs)

LC3 interacting region (LIR)

Leucine-reach repeat kinase 1 (LRRK1)

Light chain-2 of kinesin-1 (KCL2)

Lysosome-associated membrane protein (Lamp)

Lysosomal Membrane Proteins (LMPs)

Lysosomal integral membrane protein 2 (LIMP-2)

Lysosomal nutrient sensing (LYNUS)

Lysosomal storage disorders (LSDs)

Low density lipoproteins (LDL)

Mannose-6-phosphate (M6P)

Mechanistic target of rapamycin complex 1 (mTORC1)

Microtubule-associated light chain protein 3b (MAPLC3b or LC3)

Monensin sensitivity (Mon)

Multi-subunits tethering complex (MTC)

Multivesicular bodies (MVBs)

N-ethylmaleimide-sensitive fusion protein (NSF)

Neuronal Ceroid Lypofuscinosis (NCL)

Niemann-Pick C (NPC)

OSBP related domain (ORD)

Outer mitochondria membrane (OMM)

Oxysterol-binding protein–related protein (ORP)

Palmitoyl Transferase (PAT)

Palmitoyl protein thioesterase 1 (PPT1)

Parkinson’s disease (PD)

Palmitoyl protein thiesterase (PPT)1

Plasma membrane (PM)

Phagosome assembly site (PAS)

Phosphatidylethanolammine (PtdEtn)

Phosphatidylinositol-3-phosphate (PI(3)P)

Pleckstrin homology domain containing protein family member 1 (PLEKHM1)

Post-translational modification (PTM)

PTEN-induced putative kinase 1 (PINK1)

Rab Escort Protein (REP)

Rab7 lysosomal interacting protein (RILP)

Ras analog in the brain (Rab)

Recycling endosome (RE)

Signal transducing adapter molecule 1 (STAM)

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)

α-soluble NSF-attachment protein (SNAP)

Sortin nexins (SNX)

Sorting protein-related receptor with A-type repeat (SorLA)

Sucrose non-fermentating (SNF)

Synaptosomal associated protein (Snap)

Syntaxin (Stx)

Tank Binding Kinase (TBK1)

Trans-Golgi network (TGN)

Transcription factor EB (TFEB)

Transient Receptor Potential (TRP)

Tumor susceptibility gene (Tsg)

Two phenylalanines [FF] in an acidic tract (FFAT)

Two-pore Channel (TPC2)

Ubiquitin-associated protein 1 (UBAP1)

Ubiquitin-specific protease 8 (USP8)

UNC51 like kinase (ULK)

Vacuolar ATPase (v-ATPase)

Vacuolar protein sorting (Vps)

Vesicle associated membrane protein (VAMP)

Vesicle-associated membrane protein -VAMP- associated ER protein (VAP)

VHS (Vps27, Hrs, STAM)

WASH Wiskott-Aldrich Protein and SCAR Homolog

WD-repeated-domain phosphoinositide interacting proteins (WIPI)

1 INTRODUCTION

1.1 LYSOSOME BIOGENESIS

The serendipitous discovery of lysosome in 1955 by C. de Duve, for the first time, pointed out the presence inside the cell of membrane-bound organelle devoted to the degradation of cellular macromolecules (de Duve 2005). This initial view of lysosome as “cell waste bags” has progressively been set aside, and it is now clear that lysosomes are much more than a degradative compartment and play a crucial role in regulating cellular functions (Luzio 2003, Appelqvist 2013, Saffi, G. T.; Botelho, R. J. 2019).

1.1.1 Transcriptional regulation of lysosomal proteins

Lysosomal biogenesis and function are globally controlled by Transcription factor EB (TFEB), the master regulator of a group of lysosomal-related genes: the coordinated lysosomal expression and regulation (CLEAR) gene network (Sardiello 2009). TFEB regulates lysosome biogenesis by controlling the expression level of lysosomal hydrolases and accessory proteins, Lysosomal Membrane Proteins (LMPs), vacuolar ATPase (v-ATPase) pumps, and regulators of lysosomal biogenesis and autophagy (Palmieri 2011). In physiological conditions, TFEB is phosphorylated by, and kept in the cytosol by, the lysosomal nutrient sensing (LYNUS) machinery located at the lysosomal surface. LYNUS is a multimeric complex that includes among other proteins, the v-ATPase pump that senses the nutrient level inside the lysosome, and the kinase complex mechanistic target of rapamycin complex 1 (mTORC1) that phosphorylates TFEB. During starvation, mTORC1 is released by LYNUS and not able to phosphorylate TFEB, which can then translocate to the nucleus and induce the transcription of CLEAR genes (Settembre 2013, Settembre 2012).

1.1.1.1 Soluble lysosomal proteins

The acidic lumen of lysosomes contain more than 70 hydrolases, including but not limited to proteases, lipases and sulfatases, that together are able to degrade complex macromolecules into their basic constituents (Bainton 1981, Appelqvist 2013, Lawrence, R. E.; Zoncu, R. 2019).

Cathepsins are one of the best characterized lysosomal proteases family. They are classified in three main groups according to the amino acid present in their catalytic domain: cathepsin A and G are serine proteases, cathepsin D and G are aspartic proteases and cathepsins B, C, F, H, K, L, O, S, V, W, and X are cysteine proteases (Appelqvist, H.; Waster, P.; Kagedal, K.; Ollinger, K. 2013). These enzymes are synthesized in the endoplasmic reticulum (ER) as proenzymes, characterized by the presence of an inhibitory peptide that binds and blocks the protease catalytic domain inhibiting the enzymatic activity of the protein. Once the proenzyme reaches the acidic pH of the endolysosomal compartment, the peptide dissociates from the active domain and is cleaved or auto-cleaved releasing the active hydrolase (Stoka, V.; Turk, V.; Turk, B. 2016). Mutations associated with some of these proteins are linked to the development of Neuronal Ceroid Lipofuscinoses (NCLs), a group of early-onset, genetically inherited neurodegenerative diseases, characterized by the lysosomal accumulation of auto fluorescent material, lipofuscin. Thirteen genes have been associated with the development of these diseases, including cathepsin D (CLN10), cathepsin F (CLN13), the palmitoyl protein thioesterase 1 (PPT1), which is responsible for the cleavage of palmitate from palmitoylated proteins), tripeptidyl-peptidase 1 (TPP1), a serine protease and CLN5, a lysosome matrix protein whose function is not completely understood (Mole, S. E.; Cotman, S. L. 2015).

Lipases are responsible for the catabolism of complex glycolipids. The synthesis and degradation of these macromolecules is tightly regulated, and alteration in the degradative pathway of these lipids leads to the development of fatal neurodegenerative diseases. α- and β-galactosidases, hexosaminidases, sialidases, arylsulfatase A, β-galactosyl-ceramidase, β-glucocerebrosidase, ceramidases and sphingomyelinases, are some of the enzymes involved in the catabolism of gangliosides. Mutations associated to these enzymes or their activating proteins have been linked to a family of inherited diseases collectively identified as gangliosidosis, characterized by the lysosomal accumulation of gangliosides or their catabolic intermediates. Some of the lysosomal storage disorders (LSDs) associated respectively with the above mentioned lipase are: GM1-gangliosidosis, Fabry diseases, GM2 gangliosidosis, sialidosis, Metachromatic leucodystrophy, Krabbe disease, Gaucher disease, Farber disease and Niemann-Pick disease (Gieselmann, V. 1995, Sandhoff, K.; Harzer, K. 2013).

Another relevant group of lysosomal enzymes, collectively known as sulfatases, are responsible of the catabolism of glycosaminoglycan (GAG), such as heparan sulfate, dermatan sulfate and chondroitin sulfate. This family of lysosomal enzymes counts eleven members, mutations affecting these hydrolases lead to the development of mucopolysaccharidosis,

characterized by the lysosomal accumulation of non-degraded or partially degraded GAGs (Gieselmann, V. 1995, Kobayashi, H. 2019).

Trafficking of soluble lysosomal proteins

After their insertion into the lumen of the ER, the majority of the hydrolases is glycosylated and trafficked to the Golgi compartment. In the *cis*-Golgi, a mannose-6-phosphate (M6P) group is added to N-linked oligosaccharides. Moving on in the secretory pathway, M6P-tagged hydrolases are recognized and bound in the *trans*-Golgi network (TGN) by two dedicated type I transmembrane protein receptors: the cation-depended mannose-phosphate receptor (CD-MPR) and the cation-independent mannose-phosphate receptor (CI-MPR) (Coutinho 2012). Beyond MPRs, other cargo-receptors transport hydrolases to lysosome. Indeed, when the M6P route is destroyed, some hydrolases are still able to localize to lysosomes. In I-cell disease (also known as Mucolipidosis type II) cells, the activity of the enzyme responsible for the mannose group phosphorylation of newly synthesized hydrolases (N-acetylglucosaminyl phosphotransferase) is lost. In these cells, the majority of lysosomal hydrolases is not recognized by the MPRs and is misrouted to the extracellular space (Coutinho, M. F.; Prata, M. J.; Alves, S. 2012). However, a subset of lysosomal proteins including cathepsin D, are still found in lysosomes, indicating the existence of M6P independent trafficking route (Glickman 1993).

There are at least two other lysosomal cargo-sorting routes. The first is coordinated by lysosomal integral membrane protein 2 (LIMP-2) and the other by a member of the Vacuolar protein sorting (Vps)-10 family of receptors, sortilin (Coutinho 2012). LIMP-2 is a lysosomal integral membrane protein responsible for the lysosomal trafficking of β-glucocerebrosidase (β-GC). LIMP-2 binds β-GC in the ER, at neutral pH, and transports the protein through the secretory pathways. In the lysosome, the acidic pH allows the dissociation of the hydrolase from its receptor (Reczek 2007).

The Vps10 receptor family is a group of type I transmembrane proteins regulating protein trafficking and signal transduction in neuronal and non-neuronal cells. Members of this family present a Vps10 domain, a structure of 700 amino acids first identified in the yeast Vps10p protein, the cargo receptor responsible for the vacuole (yeast counterpart of mammalian lysosome) trafficking of carboxypeptidase Y (CPY). This domain was the first 10-bladed beta-propeller structure identified and thanks to the high number of blades, is capable of

accommodating large ligands. The Vps10 module is evolutionary conserved among species. In vertebrates, five proteins containing the Vps10 domain have been identified: sortilin, sorting protein-related receptor with A-type repeat (SorLA, known also as SorL1 or LR11), SorCS1, SorCS2, SorCS3. These proteins are synthesized in the ER as precursor proteins with a pro-peptide that is cleaved in the Golgi by the protease furin (Willnow 2008, Lane 2012). Sortilin is responsible for the lysosomal trafficking of several cargos, including prosaposin, GM2 activator protein, acid sphingomyelinase, Cathepsin D and H (Coutinho 2012, Lefrancois 2003, Ni 2006, Canuel 2008, Coutinho 2012) and the β-amyloid precursor protein-cleaving enzyme (BACE)-1 (Finan 2011). SorLA has a crucial role in controlling the sorting of Amyloid Precursor Protein (APP) and in preventing BACE-1 mediated cleavage of the protein and the subsequent generation of the pathogenetic Aβ fragment. SorLA binds APP at the endosome and favours its retrieval to the TGN, preventing BACE-1 mediated cleavage of the protein (Dumanis, S. B.; Burgert, T.; Caglayan, S.; Fuchtbauer, A.; Fuchtbauer, E. M.; Schmidt, V.; Willnow, T. E. 2015). Although these receptors are expressed in different cell types, their role appears to be particularly important in the central and peripheral nervous system. Indeed mutations altering their functions give rise to an ample spectrum of neurologic diseases, including Alzheimer's disease, age-related dementia and nervous system senescence. However, an increasing number of data highlights the importance of these receptors in the pathogenesis of non-neuronal diseases, among which type-2 diabetes, cardiovascular diseases and cancer (Westerterp 2015, Kjolby 2015, Roselli 2015).

The sorting of hydrolases to the lysosome is a tightly regulated mechanism not completely well defined. How the M6P dependent and independent route cooperate in regulating cargo sorting is not completely elucidated. Some hydrolase, such as cathepsin H, relies only on sortilin for their trafficking to the lysosome, while for others, such as cathepsin D and acid sphingomyelinase, MPR and sortilin cooperates to guarantee the efficient transport of the enzyme to the lysosome (Canuel et al., 2008a; Ni and Morales, 2006) (**Figure 1-1**).

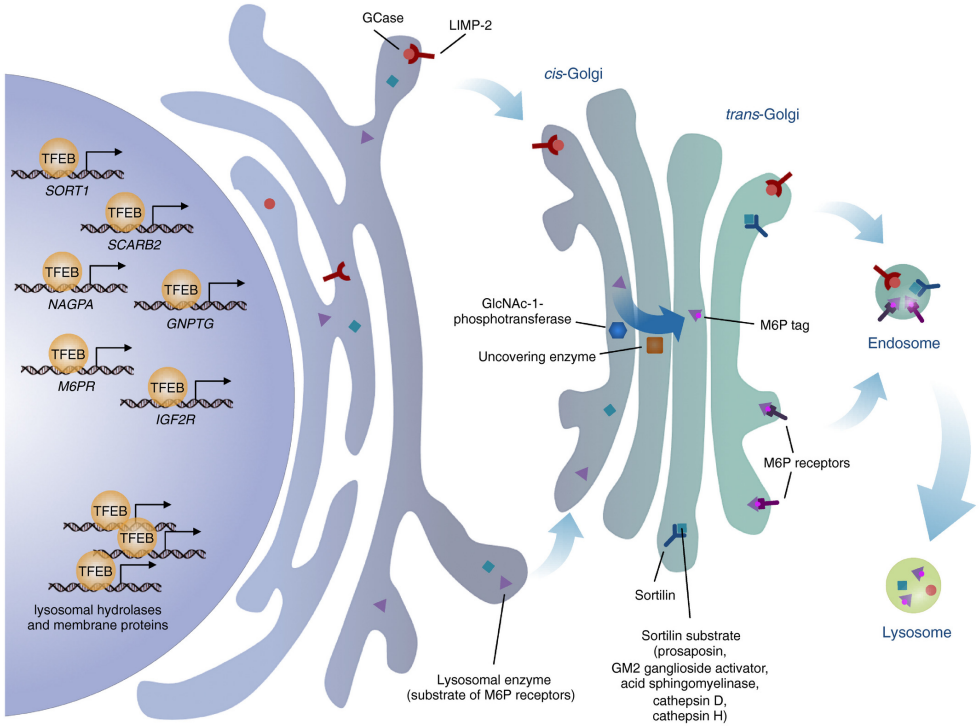

Figure 1-1 Synthesis and trafficking of lysosomal proteins

TFEB controls the expression of lysosomal enzymes and of the cargo receptors : LIMP-2 (*SCARB2*), sortilin (*SORT 1*), CI-MPR (*IGF2R*), CD-MPR (*M6PR*). TFEB also controls the translation of the enzymes responsible for the addiction of the M6P tag, GlcNac-1-phosphotransferases (*GNPTG*) and the uncovering enzyme - UCE (*NAPGPA*).

Adapted from Bajaj et al., J Neurochem, 2019

Trafficking from the TGN to the endosome

At the TGN, cargo-loaded receptors are packaged into clathrin-coated vesicles, a process mediated by the adaptor protein (AP)-1 and the Golgi-localized, γ-ear containing, ADP-ribosylation factor binding proteins (GGAs). AP-1 and GGAs recognize a sequence motif in the cytosolic tail of the cargo-receptors and simultaneously bind clathrin (Coutinho et al., 2012a; Luzio et al., 2014). Adaptor proteins are heterotetrameric protein complexes made of two large subunits of 100-160 kD, a medium subunit of 50 kD, and a small subunit of 30 kDa. Five AP complexes have been identified in higher eukaryotes, AP-1 through 5. In particular, AP-1 consists of two large subunits (γ and β1), the medium subunit μ1, and the small subunit σ1. The

μ1 subunit of AP-1 recognizes and binds a region in the cytosolic tail of CI-MPR and sortilin known as the tyrosine motif (YXXΦ) (Bonifacino and Traub, 2003). The binding of AP-1 to CI-MPR and sortilin can also occur via the dileucine based sorting signal ([DE]xxxL[LI]) which is located at the C-terminus of the receptor tail. The dileucine motif is recognized by the hemi-complex composed of the γ-σ1 subunits of AP-1 (Canuel et al., 2008b; Doray et al., 2007; Guo et al., 2014). GGAs are monomeric proteins ubiquitously expressed. In mammalians cells, three GGAs have been identified, GGA1-3. While the function of GGA1 and GGA3 appear to be redundant, the knockout of GGA2 is embryonic or neonatal lethal, suggesting a more specific function for this adaptor during development (Govero et al., 2012; Guo et al., 2014). GGAs share a common domain organization, with a N-terminal VHS (Vps27, Hrs, STAM) domain that recognizes the dileucine sorting motif of the cargo-receptors (Misra et al., 2002), a GAT (GGA and TOM) domain, a hinge domain and a C-terminal EAR domain. The GAT domain is folded into two subdomains where the N-terminal portion binds ADP-ribosilation factor (Arf) 1 and the C-terminal that can interact with different partners (Puertollano et al., 2001). The hinge domain recruits clathrin and the EAR domain is important for the engagement of coat proteins (Miller et al., 2003). The recruitment of GGAs to the Golgi membrane is dependent upon the activity of the small GTPase Arf1 (Puertollano et al., 2001) and of its guanine nucleotide exchange factor (GEF) GBF1 (Lefrancois and McCormick, 2007). GGAs and AP-1 are both important for the correct endosomal trafficking of CI-MPR and sortilin. Indeed siRNA mediated down-regulation of the μ subunit of AP-1 results in sortilin accumulation at the TGN. The same effect is obtained with the removal of the hinge and EAR domain of GGA3 as well as with the down-regulation of GBF1 (Canuel et al., 2008b; Lefrancois et al., 2003).

Trafficking from endosomes back to the TGN

Once the loaded cargo-receptor reaches the endosome, the acidic pH allows the release of the cargo: the latter follows its route to the lysosome, while the receptor is retrieved back to the TGN for another round of trafficking. A key component of the endosome-to-Golgi retrieval pathway is the retromer complex (Seaman, 2004). Retromer is an evolutionary conserved complex identified in yeast as responsible of the retrieval of Vps10p from the pre-vacuolar compartment to the TGN (Seaman et al., 1997; Seaman et al., 1998). In yeast, retromer is a heterotrimer of Vps-26, Vps35, and Vps29 and a dimer of the sorting nexins (SNX)-bin/amphiphysin/rvs (BAR) Vps5 and Vps17. The heterotrimer is evolutionary conserved and it is responsible for cargo recognition, indeed it is also referred to as the cargo-selective complex

(CSC) (Seaman et al., 1998). On the contrary, there is significant diversity in SNXs among species (Koumandou et al., 2011). In humans, SNX1/2 and SNX5/6 are the homologues of Vps5 and Vps17 respectively. While in yeast, the CSC and the SNX dimer are strongly associated, in humans this interaction is weak and transient (Chen et al., 2019). Furthermore, the CSC and SNXs seem to coordinate the formation of distinct CI-MPR containing ETC (Endosome Transport Carrier) that are recognized by different tethering complexes at the Golgi (Cui et al., 2019). Another member of SNX family, SNX3, has an established role as a retromer adaptor for the endosome-to-TGN transport of cargo receptors such as Transferrin (Chen et al., 2013), CI-MPR (Cui et al., 2019), Wnt sorting receptor Wntless (Harterink et al., 2011) and the iron transporter divalent metal transporter (DMT)1-II (Tabuchi et al., 2010). The ability of SNX3 to bind the phosphatidylinositol-3-phosphate (PI(3)P) on the early endosomal membrane is required for retromer membrane recruitment (Lucas et al., 2016). SNX27 is also involved in the membrane recruitment of retromer for endosome-to-plasma membrane recycling (Lauffer et al., 2010; Temkin et al., 2011).

For the efficient trafficking of cargos to endosome, retromer binds via its Vps35 subunit the Wiskott-Aldrich Protein and SCAR Homolog (WASH) complex (Gomez and Billadeau, 2009; Harbour et al., 2010). The latter is a pentameric complex (KIAA1033, Strumpellin, FAM21, WASH1 and CCD53) that activates actin nucleation and the formation of branched actin network. WASH activity favours endosomal membrane tubulation and eventually the fission of retromer coated endosomal tubular carrier directed to the TGN (Chen et al., 2019; Li et al., 2016).

At the late endosome, retromer is responsible for the late endosome-to-TGN retrieval of different cargo receptors, including CI-MPR, sortilin, SorLA and SorCS1 (Arighi et al., 2004; Canuel et al., 2008b; Fjorback et al., 2012; Lane et al., 2010). Cargo receptor recognition is dependent on the CSC, which recognizes a specific amino acid motif embedded in the tail of the receptors. For example, cargo-receptors such as sortilin and CI-MPR, have a conserved aromatic hydrophobic motif F/W-L-V/M required for the interaction with the Vps35 subunit of the CSC (Seaman, 2007), while SorLA is able to bind the Vps26 subunit via the FANSHY sequence in its cytoplasmic domain (Fjorback et al., 2012). However, it has been recently shown that a second recognition motif is required for cargo-receptor retrieval, suggesting that retromer recognize a bipartite signal on the tail of the substrates that must be retrieved (Suzuki et al., 2019). (**Figure 1-2**)

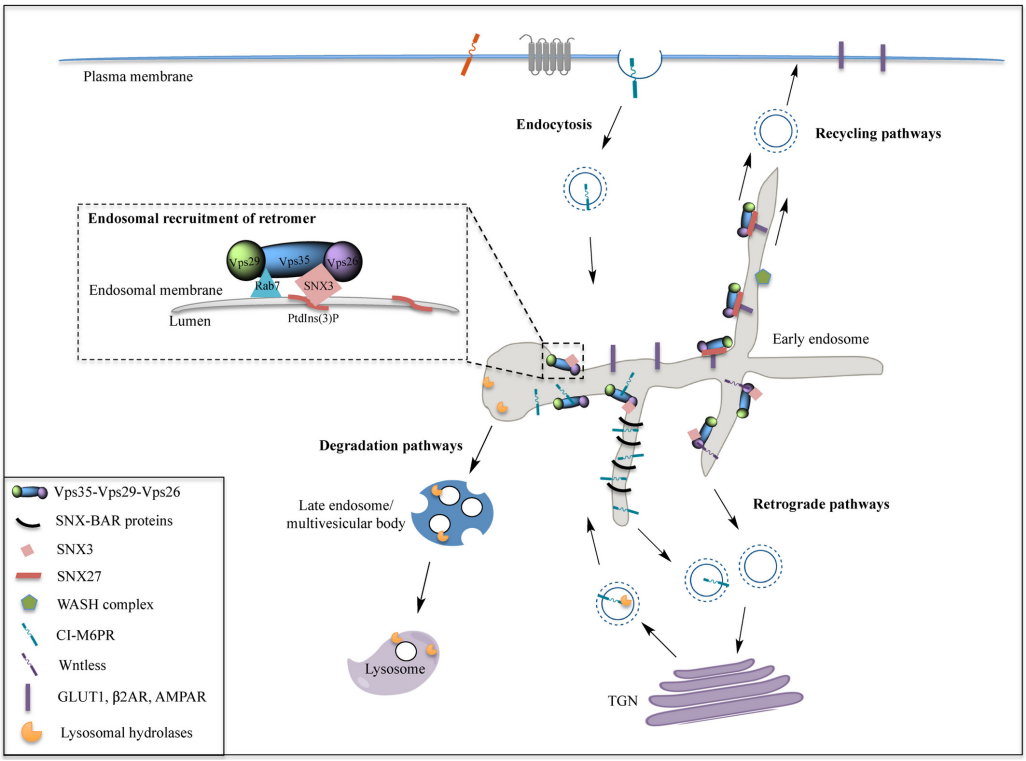

Figure 1-2. The function of retromer at the endosome.

Retromer is a tetrameric complex of Vps35, Vps29 and Vps26 that is recruited on the endosomal membrane by Rab7 and SNX3. At the endosome retromer can interact with SNX27 to mediate the recycling to the PM of internalized receptor (GLUT1, AMPAR, B2AR) or it can interact with SNX-BAR to coordinate the retrieval of cargo-receptor (CI-MPR) in a retrograde pathway. The interaction with WASH complex is required for the formation of actin filament to favour tubulation of endosomal membrane.

From Cui et al, FEBS Lett, 2018

Retromer in neurodegenerative diseases

Increasing evidence supports the importance of retromer to guarantee optimal lysosomal function. Mutations affecting retromer subunits or proteins involved in the regulation of this complex have been linked to the pathogenesis of several neurodegenerative diseases (Zhang et al., 2018). Autosomal dominant mutations in the Vps35 subunit of the CSC have been associated with the development of Parkinson's disease (PD). These mutations affect the endosome-to-TGN retrieval of CI-MPR, resulting in a reduction in the lysosomal levels of cathepsin D, the dominant proteases for the non pathogenetic processing of α-synuclein.

Altered or impaired processing of α-synuclein leads to the formation of neurotoxic aggregates, responsible for the pathogenesis of PD (Follett et al., 2014; Small and Petsko, 2015). Vps35 has also been involved in the lysosomal trafficking of the lysosome-associated membrane protein (Lamp)2, a lysosomal membrane protein important for the degradation of cytosolic proteins via chaperone-mediated autophagy, a pathway that contributes in the degradation of α-synuclein (Tang et al., 2015).

1.1.1.2 Lysosomal integral membrane proteins.

Proteomic analysis shows that the limiting lysosomal membrane contains more than 100 membrane proteins (LMPs) (Schroder et al., 2010; Schwake et al., 2013). Most of these proteins are highly glycosylated on their luminal domain forming a glycocalyx that prevents membrane damage due to the acidic lysosomal environment. The most abundant LMPs are Lamp1, Lamp2, LIMP-2 and CD63. Lamp1 and Lamp2 together, represent more that 50% of the LMPs. Lamps are involved in the fusion of lysosomes and phagosomes (Huynh et al., 2007). Lamp2 is also important for lysosome maturation and lysosomal cholesterol homeostasis. Mutations affecting Lamp2 are associated with Danon disease, an x-linked lysosomal disorder with symptoms including cardiomyopathy, muscle weakness and intellectual disability (Endo et al., 2015). Lamp2 is required for chaperone-mediated autophagy (CMA), a process that allows the degradation of selected cytosolic proteins (Kaushik and Cuervo, 2018). LIMP-2 is responsible for the trafficking of β-GC, but recent data showed an important role of this protein in mediating the lysosomal export of low-density lipoproteins (LDL) derived cholesterol via an ectodomain tunnel (Heybrock et al., 2019). Lysosomal cholesterol homeostasis is regulated also by the Niemann-Pick C (NPC)-1 and NPC-2 protein complex. NPC-2 is a small small soluble protein that binds LDL derived cholesterol and transfers it to NPC-1, a large transmembrane protein that controls lysosomal cholesterol export. Mutations in either NPC-1 or NPC-2 cause Niemann-Pick type C disease, which is characterized by the lysosomal accumulation of cholesterol and glycosphingolipids (Pfeffer, 2019). Another set of transmembrane lysosomal proteins is responsible for the acidification of the lysosomal lumen and the maintenance of the acidic pH. The v-ATPase proton pump drives protons derived from ATP hydrolysis into the lysosomal lumen establishing the acidic pH of the organelle. However, the maintenance of lysosomal pH is a complex process and the mechanisms as well as the players involved are not completely understood.

CLN3 and CLN7 are two other multispan transmembrane proteins localized at the late endosome-lysosome whose functions are not well defined. CLN3 contains six transmembrane domains and has proposed roles in several intracellular processes, including autophagy and maintenance of the lysosomal pH (Carcel-Trullols et al., 2015). Recently a role of CLN3 in regulating endosome-to-TGN trafficking of sortilin and CI-MPR has been identified (Yasa et al., 2019). CLN7 (also known as MFSD8), is a multispan transmembrane protein with 12 predicted transmembrane domains (Sharifi et al., 2010). It seems to be involved in autophagy and lysosomal motility (von Kleist et al., 2019), as well in the maintenance of adequate lysosomal level of cathepsin D and CLN5 (Danyukova et al., 2018), but the molecular mechanisms are not known.

Lysosomal targeting of LMPs

Newly synthesized LMPs are targeted to the lysosome via a direct TGN-to-endolysosome route, or in an indirect way, from the TGN to the plasma membrane (PM) and to the endosome via endocytosis (Luzio et al., 2014; Saftig and Klumperman, 2009; Settembre et al., 2013). LMPs that contain tyrosine-based or dileucine-based motifs follow the indirect route to reach the lysosome. At the TGN, they are recognized by AP-1 and packed into clathrin-coated vesicles that are trafficked to the PM where they are recognized by AP-2. This adaptor complex is composed of α, β2, μ2 and σ2 subunits (Robinson, 2004). The μ2 subunit recognizes and binds the YXXΦ signal in the cytosolic tails of the transmembrane proteins resulting in their packaging into clathrin-coated vesicles, followed by endocytosis to the endosomal system. As with AP-1, the recognition of the proteins by AP-2 that need to be internalized occurs via the hemi-complex α-σ2 that binds the ([DE]xxxL[LI]) motif (Doray et al., 2007). At the sorting endosome, the binding of AP-3 to the sorting signal allows the delivery of the LMPs to the lysosomes (Bonifacino and Traub, 2003; Luzio et al., 2014). AP-3 is composed of δ, β3, μ3 and σ3 subunits, with the μ3 subunit responsible for the recognition of the YXXΦ motif and the hemi-complex δ-σ3 recognizing the [DE]xxxL[LI] motif (Janvier et al., 2003). The direct TGN-lysosome transport of LMPs is mediated by non-clathrin coated carriers that contain the human vesicular protein sorting 41 (hVps41) and the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein vesicle associated membrane protein-(VAMP)-7 (Luzio et al., 2014; Pols et al., 2013).

1.1.2 Endocytosis: from the plasma membrane to the lysosome

Endocytosis is a process that leads to the internalization and trafficking to the endocytic compartment of a piece of the PM and its associated membrane proteins There are different forms of endocytosis, generally divided in macropinocytosis, clathrin-mediated endoctytosis (CME) and clathrin-independent endocytosis (CIE). Macropinocytosis involves the internalization of extracellular components as well as a large portion of the PM into a macropinosome. This organelle rapidly acquires markers of late endosomes and ultimately fuses with the lysosome. It is originated by external stimuli that converge on the actin cytoskeleton reorganization that is responsible for the formation of the macropinosome. Macropinocytosis is the main mechanism exploited by extracellular pathogens to gain access into the cell (Carvalho et al., 2015; Kerr and Teasdale, 2009). Clathrin mediated endocytosis allows the internalization of PM cargos into clathrin-coated vesicles that are eventually delivered to the early endosome. Some PM proteins that need to be internalized carry sorting signal in their intracellular tails recognized by cargo adaptor such as AP-2. However some post-translational modifications such as phosphorylation and ubiquitination can also function as internalization signals (Kaksonen and Roux, 2018; Traub, 2009).

CIE is a still not fully characterized pathway leading to the internalization of PM proteins in vesicles not coated with clathrin *en route* to the endocytic compartment. This mechanism appears to be a faster and more versatile way to respond to cellular endocytic needs (Hemalatha and Mayor, 2019).

Once the endocytic vesicle fuses with the EEs, the mildly acid pH (≈ 6.2) allows the separation of almost all the bound ligands from their receptors. Proteins and lipidic components that must be reused are recycled to the PM or retrieved to the TGN. Proteins can be directly recycled to the PM or moved to the recycling endosome first and then transported to the PM (Scott et al., 2014). Proteins that need to be retrieved to the TGN can follow different pathways: some cargos can be moved to the late endosomes first, and then retrieved to the TGN, while others can go to the recycling endosome and then to TGN (Taguchi, 2013).

The signal to discriminate among proteins that need to be recycled to the PM and the ones that are destined to lysosomal degradation is ubiquitination. As a general mechanism, PM receptors that are destined for lysosomal degradation are ubiquitylated at the PM. After endocytosis and trafficking to the early endosome, ubiquitylated receptors are recognized and

sorted through the degradative pathway by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery (Figure 1.1.3) (Katzmann et al., 2002; Scott et al., 2014). This machinery consists of four complexes, ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, plus accessory proteins that together are responsible for the sequestration of the ubiquitylated cargos and for the biogenesis of the intraluminal vesicles (ILVs) (Raiborg and Stenmark, 2009).

ESCRT-0 is a heterodimer of Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal transducing adapter molecule 1 (STAM) (Cullen and Steinberg, 2018). At steady state, ESCRT-0 is localized to early endosomes, thanks to the interaction of the FYVE domain of Hrs with phosphatidylinositol-3-phosphate (PI(3)P). ESCRT-0 subunits recognize and bind monoubiquitylated cargoes that reach the early endosomes as a result of endocytic events, and cluster the cargoes in clathrin coated subdomains (Raiborg et al., 2002; Raiborg et al., 2001). ESCRT-I is a heterotetramer consisting of tumor susceptibility gene (Tsg)-101, Vps28, Vps37 and ubiquitin-associated protein 1 (UBAP1). The interaction between Hrs and Tsg-101 is responsible for ESCRT-I recruitment. ESCRT-II is composed of Vps36, Vps22 and two subunits of Vps25 and is recruited to the endosomal membrane via an interaction with Vps28. The recruitment of ESCRT-I and ESCRT-II, which are both able to recognize and bind ubiquitylated cargoes, contributes to the maintenance of the subdomain. ESCRT-II via its Vps25 subunit, recruits ESCRT-III that surrounds the degradative subdomain forming filaments leading to membrane bending and ILVs budding (Cullen and Steinberg, 2018; McCullough et al., 2018). The ESCRT-III complex is composed in humans of a total of 12 subunits that can assemble in different complexes with a conserved core composed of Vps20/Charged Multivesicular body protein 6 (CHMP6), Sucrose non-fermentating (SNF7)/CHMP4, Vps2/CHMP2 and Vps24/CHMP3 that represent the necessary elements to form ESCRT-III filaments (Gatta and Carlton, 2019; Hurley and Hanson, 2010; McCullough et al., 2018). Before sorting into the ILVs, ubiquitin is removed by de-ubiquitylating enzymes (DUBs) such as ubiquitin-specific protease 8 (USP8) and associated molecule with the SH3 domain of STAM (AMSH), which are recruited by ESCRT-III and ESCRT-0, to maintain the cytosolic ubiquitin pool (Kyuuma et al., 2007; Ma et al., 2007; Row et al., 2007; Tomas et al., 2014). The deposition of ESCRT-III subunit into spiralizing filaments leads to the budding of ILVs. Finally, the action of the ATPase Vps4 induces the dissociation if ESCRT-III subunits and the scission of ILV (Cullen and Steinberg, 2018). Although ubiquitination is the main signal to target cargoes for degradation, ubiquitin-independent ILVs targeting routes have been described, mainly coordinated by the ESCRT accessory subunits ALIX (Dores et al., 2012; Dores et al., 2016). ILVs accumulate in the multivesicular region of the early endosome and eventually detach and

mature becoming multivesicular bodies (MVBs – also known as endosomal carrier vesicles) (Bissig and Gruenberg, 2013; Bissig and Gruenberg, 2014). (**Figure 1-3**)

MVBs are transported via microtubules toward the late endosome, with which they fuse. The maturation of early endosomes into late endosomes is accompanied by a decrease in luminal pH, due to the action of the V-ATPase pump, as well as to the change in the membrane composition. Indeed, while early endosomes are enriched in PI(3)P, the maturation into late endosomes is accompanied by a conversion of PI(3)P into PI(3,5)P2 mediated by the PIKFYVE kinase. The Vps24 subunit of ESCRT-III complex binds PI(3,5)P2 and this binding is important for an efficient sorting of protein into MVBs (Wang et al., 2019). The lysosomal membrane contains several species of phosphoinositides, possibly reflecting the heterogeneous origin of the membranes of this organelle. PI(3)P and PI(3,5)P2 are the major constituent of the lysosomal phopshoinositides pool, but a small amount of PI(4)P and PI(4,5)P, characteristic of Golgi and plasma membrane respectively, have been detected on lysosomal membranes (Ebner et al., 2019) . Together with a change in the composition of phosphoinositides, maturation into lysosomes is characterized by the acquisition of lysobisphosphatidic acid (LBPA), that is only detected in ILVs of late endosomes and in lysosomes (Bissig and Gruenberg, 2013).

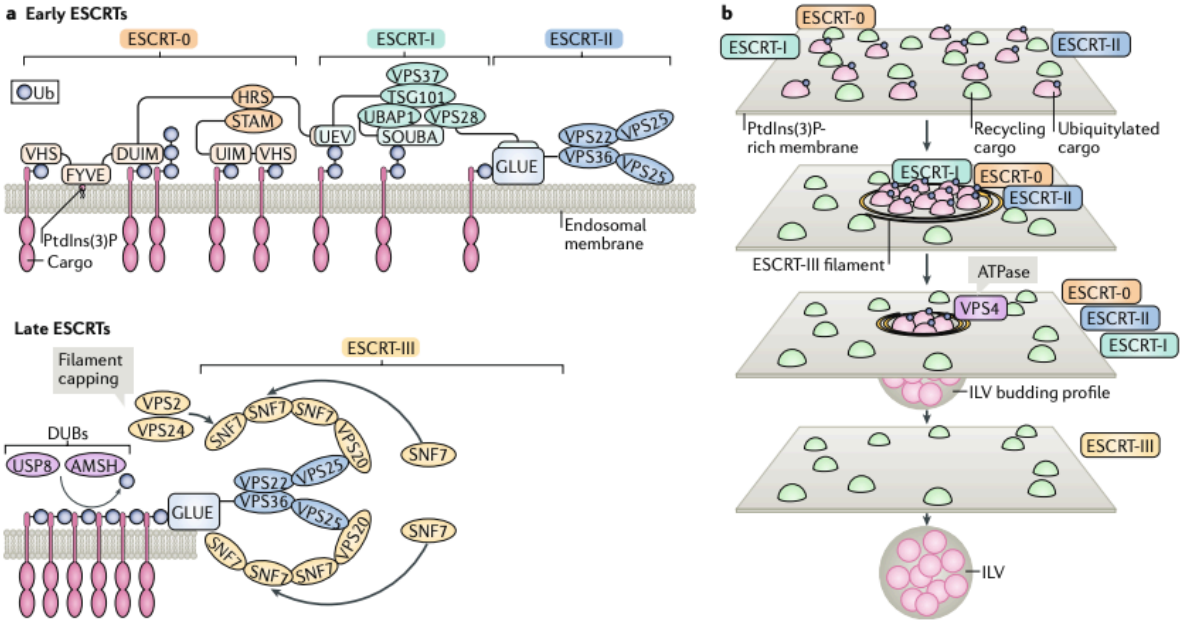

Figure 1-3. The ESCRT complex.

(A) Internalized cargo are first recognized by ESCRT-0 complex, composed by Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and Signal Transducing Adapter Molecule 1 (STAM). The FYVE domain of HRS binds to phosphatidylinositol-3-phosphate (PI(3)P) allowing the complex to associate to the membrane of Early Endosome (EE). The Vps-27, Hrs, STAM (VHS) domain, the ubiquitin-interaction motif (UIM) and the double-sided ubiquitin-interaction motif (DUIM) recognize and bind ubiquitin. ESCRT-I is a tetramer dimer of tumour susceptibility gene 101 protein (TSG101),ubiquitin-associated protein 1 (UBAP1), Vps37 and Vps28. This complex binds ubiquitinated proteins via the ubiquitin-conjugating enzyme E2 variant (UEV) and solenoid of overlapping ubiquitin-associated (SOUBA) domains.ESCRT-II is made of Vps36 (Eap45), Vps22 and two Vps25 subunits. The GRAM-like ubiquitin binding in EAP45 (GLUE) of Vps36 binds ubiquitinated cargos, PI(3)P and the Vps28 subunit of ESCRT-I. ESCRT-III complex is a tetramer of Vps20/CHMP6, SNF7/CHMP4, Vps2/CHMP2 and vps24/CHMP3 recruited to the endosomal membrane via the interaction with the Vps25 subunit of ESCRT-II. ESCRT-III recruits the deubiquitilating (DUB) enzymes Ubiquitin-specific protease 8 (USP8) and associated molecule with the SH3 domain of STAM (AMSH) which remove the ubiquitin from the cargos.

(B) The ordered assembly of the ESCRT complexes leads to the binding and clustering of ubiquitinated cargo into endosomal subregions that following the deposition of ESCRT-III subunits and the formation of spiral filaments leads to the budding of intra lumina vesicles (ILVs). The action of the ATPase Vps4 leads to ESCRT-III dissociation and ILV scission

From Cullen & Steinberg, Nat. Rev. Mol. Cell. Biol., 2018

Late endosomes are the last station of the endosomal compartment that precedes lysosomes (**Figure 1-4**). At the molecular level, it is difficult to distinguish between these two compartments since almost all the LMPs are present also in the late endosome membranes. The distinction can be established according to their structural and physical properties. Lysosomes are indeed spherical and electron-dense and present a high-dense core, while late endosomes can be round or oval and appear as low-dense core structures (Huotari and Helenius, 2011). Late endosomes can be separated from lysosome via ultracentrifugation, since they are less dense than lysosome, originally referred as light lysosomes.

The mechanisms governing the exchange of material between late endosomes and lysosomes are not completely defined. In mammalian cells, two mechanisms have been proposed, the kiss-and-run and the fusion-fission model. According the the kiss-and-run model, late endosomes and lysosomes transiently and partially fuse to create a pore to transfer material (kiss), followed by a scission event to reconstitute the two original compartments (run) (Saffi and Botelho, 2019; Storrie and Desjardins, 1996). According to the second model, late endosomes and lysosomes completely fuse resulting in the formation of a hybrid organelle defined as endolysosomes. These two mechanisms are not mutually exclusive indeed both contribute to the maturation of lysosomes (Bright et al., 2005). In contrast with the classical model proposing lysosomes as the last degradative station, it seems more likely that most of the degradative process occurs in the endolysosome. According to this model, the core-dense lysosomes behave more like a hydrolase storage compartment that is ready to be delivered when the fusion with late endosomes occurs (Luzio et al., 2014).

The homotypic fusion and vacuole protein sorting (HOPS) complex is a tethering complex involved in fusion events at the endolysosomal compartment. HOPS is an hexameric complex in which Vps8 and Vps11 act as a backbone, the trimer Vps41, Vps33 and Vps16 represents the head and Vps39 is the tail (Brocker et al., 2012). HOPS acts as a bridge between the vesicle and the target membrane, keeping the vesicle close enough to allow the formation of the SNAREs complex that is eventually responsible for the actual fusion event (Kuhlee et al., 2015).

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) represents a family of small proteins characterized by the presence of the SNARE domain that drive membrane fusion events between vesicle and the target membrane. Most of the SNAREs are transmembrane proteins, although a few of them are associated with the membrane through lipid modifications such as prenylation (McNew et al., 1997) and palmitoylation (Vogel and

Roche, 1999). According to the amino acids present in the central position of the SNARE motif, SNAREs are classified in Q and R SNAREs. To accomplish fusion events, one R-SNARE and three Q-SNARE motifs are required (Fasshauer et al., 1998). The formation of the fusion machinery requires the recognition between the vesicular (v)-SNAREs and the ones present on the target membrane (t)-SNARE (Sollner et al., 1993a). The formation of the *trans*-SNARE complex brings the membrane close enough and generates enough energy to accomplish the fusion of the lipid bilayer. After the fusion, the *trans*-SNARE complex changes into a cis-SNARE complex that is associated with the target membrane and binds α-soluble NSF-attachment protein (SNAP) and N-ethylmaleimide-sensitive fusion protein (NSF). The latter hydrolyses ATP leading to the dissociation of the SNARE complex that can at this point be re-used for further fusion events (Rothman, 2002; Sollner et al., 1993b; Wang et al., 2017). The SNARE complex coordinating the homotypic fusion events at the late endosome is composed by Syntaxin (Stx) 7, Stx8 and Vt1b and Vamp8 (Vesicle associated membrane protein) 8 (Antonin et al., 2000). During the late endosome-Lysosome fusion events, Vamp7 replaces Vamp8 in the complex (Luzio et al., 2014; Mullock et al., 2000; Pryor et al., 2004; Ward et al., 2000).

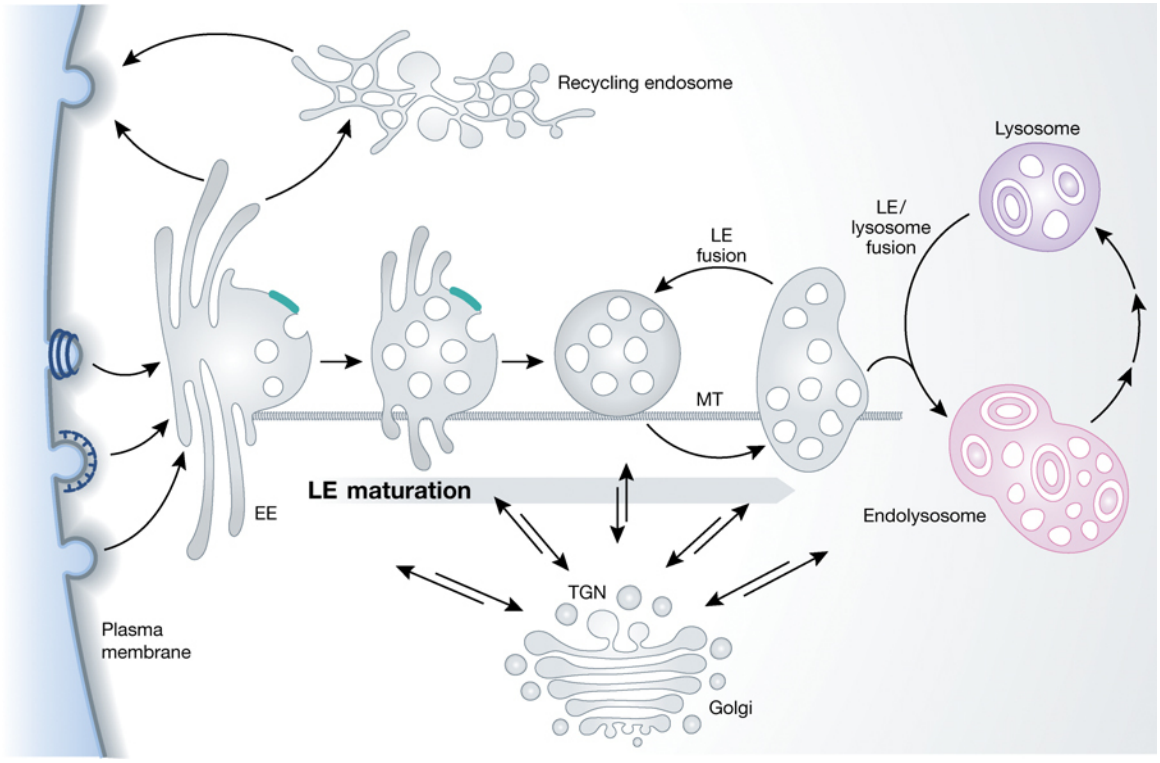

Figure 1-4. Representation of the endo-lysosomal system

Endocytic vesicles are rapidly trafficked to the Early Endosome (EE). Here they can be recycled directly form the EE to the Plasma Membrane (PM) or indirectly via recycling endosome. Alternatively, vesicles can progressively mature in LE while moving on the microtubule (MT) toward the perinuclear space. During this process homotypic fusion events take place and the vesicles progressively acquire markers of LE. Finally, LE fuses with the Lysosome originating the endolysosme where the degradation takes place.

From Houtari & Helenius, Embo J., 2011

1.1.3 Autophagy

Autophagy was initially described as a process by which the cell digests its own cytosolic components via lysosomes (Klionsky, 2014). It was originally described as a protective process induced by starvation conditions (i.e. nutrient deprivation), but it is now clear that autophagy plays a more complex role inside the cell. Indeed it is involved in the turnover of damaged

organelles, degradation of protein aggregates and the removal of cellular pathogens. Three main forms of autophagy have been described with different functions and mechanisms, but with the common goal of the lysosomal degradation of targeted substrates: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy.

During CMA, the HSC70 (Heat Shock Cognate 70 kDa protein) chaperone complex delivers proteins to be degraded to the lysosome surface from where they are translocated into the lysosomal lumen by Lamp2A. During this process, Lamp2A forms a trimer that creates a channel for the transport of proteins from the cytosol into the lumen of lysosomes to be degraded. Microautophagy is referred to as the process by which endolysosomes uptake cytosolic proteins by direct invagination of their membranes (Kaushik and Cuervo, 2018; Nixon, 2013; Settembre et al., 2013; Shen and Mizushima, 2014; Weckman et al., 2014). Macroautophagy (hereafter referred as autophagy) is the best characterized among these processes, and consists in the engulfment of cytosolic components in double-membrane vesicles (autophagosomes) that eventually fuses with the lysosome to achieve the degradation of their contents. Non-selective autophagy occurs during starvation, when cells digest their cytosolic components to produce new nutrients for survival. Selective autophagy is instead a form of quality control to eliminate protein aggregates (aggrephagy), damaged mitochondria (mitophagy), external pathogens (xenophagy), peroxisomes (pexophagy), ER (reticulophagy), lysosome (lysophagy) and ribosomes (ribophagy) (Hyttinen et al., 2013; Stolz et al., 2014).

A strong correlation exists between lysosomes and autophagy. Indeed, it is now evident that lysosomes control the induction of autophagy and that autophagy is involved in the turnover of damaged lysosomes as well as in the reformation of lysosome after autophagosome fusion (Settembre et al., 2013).

Autophagosome maturation can be divided into three steps: initiation, nucleation and expansion. During the initiation step, the autophagy activation signal is transduced to the membrane source where nucleation occurs. In the second phase, several molecular partners consecutively interact to isolate the membrane from the membrane source. Finally, during the expansion phase, the autophagosome forms and closes (Lamb et al., 2013). Unlike yeast cells where autophagosomes only originates near the vacuole, in mammalian cells, phagosome assembly site (PAS) can happen anywhere in the cytosol. In neurons autophagosomes form in the distal axons, and are transported in a microtubule-dependent way to the soma, where the majority of the lysosomes are located (Cheng et al., 2015a; Cheng et al., 2015b).

In physiological conditions, the autophagy inhibitor mTORC1 is associated to the LYNUS complex on the lysosomal membrane, where it can phosphorylate and inhibit the autophagy activator complex. Under condition of low nutrients, mTORC1 is released from LYNUS (Lim and Zoncu, 2016). This leads a decrease in the phosphorylation level and in the activation of two complexes required for the autophagy nucleation phase: the ULK1 and the Beclin-1 complexes. The ULK1 complex contains the UNC51 like kinase (ULK)1, the autophagy-related genes (ATG) 13 (ATGg13), ATG101 and the focal adhesion kinase family interacting protein of 200kD (FIP200)/ATG17. The loss of phosphorylation of ULK1 mediates in turn the phosphorylation of FIP200 to induce the formation of the autophagosome. Equally important to complete the nucleation phase is the action of the Beclin-1 complex, composed of the PI(3)-kinase VPS34, Beclin1/ATG6, Barkor/ATG4 and Vps15/p150. The activation of this complex via ULK1 mediated phosphorylation, induces the production of PI(3)P, which is required for the recruitment of the PI(3)P binding complex: WD-repeated-domain phosphoinositide interacting proteins (WIPI) (ATG18 in yeast) and double FYVE containing protein (DFCP)1. This complex in turn recruits the ATG12 conjugating system, where the ubiquitin-like protein ATG12 is conjugated to ATG5 via a reaction mediated by ATG7 (that works like an E1-ubiquitin activating enzyme – E1-like) and ATG10 (that acts like an E2-ubiquitin conjugating enzyme – E2-like). ATG5/ATG12 is then attached to ATG161L, recruited to the PAS via an interaction with WIPI/DFCP1. The ATG5/ATG12/ATG16L1 complex, which acts as an E3-ubiquitin ligase, together with ATG7 and ATG3 (an E2-like enzyme) mediates the conjugation of ATG8 family proteins to phosphatidylethanolamine (PtdEtn). Microtubule-associated light chain protein 3b (MAPLC3b or LC3) is the best-characterized member of this family. It is synthesized as a precursor that is cleaved at the C-terminus by ATG4B originating the LC3-I form. The latter is then conjugated to PtdEtn to become LC3-II, the form associated with the autophagosome (Bento et al., 2013; Hansen et al., 2018; Lamb et al., 2013).

The expansion of the autophagosome is not clearly understood. The transmembrane protein ATG9 is considered to be responsible for the recruitment of membranes to expand the autophagosome. In nutrient-rich conditions, ATG9 traffics between the TGN, PM and recycling endosomes. Upon autophagy induction, ATG9 is recruited to the PAS via mechanisms not completely well defined. Proceeding in its maturation, the autophagosome fuses with the endolysosomal compartment. Autophagosome can fuse with early endosomes, originating an amphisome or with the lysosome, originating an autolysosome (Shen and Mizushima, 2014). In neurons autophagosomes in the axon fuse with late endosomes forming autolysosomes to acquire dynein necessary to be trafficked toward the soma where most of the lysosomes are

localized (Cheng et al., 2015a). The fusion with the lysosomes leads to the formation of the autolysosome where phagocytosed cellular components are degraded and intracellular substances are released. This process requires the HOPS complex that favours the interaction between the autophagosomal SNAREs Stx17, synaptosomal associated protein (Snap) 29 and the late endosomal SNARE Vamp8 or the lysosomal SNARE Vamp7 (Itakura et al., 2012; Zhao and Zhang, 2019).

The increasing availability of nutrients leads to the reactivation of mTORC1 and its recruitment to the LYNUS machinery, where it phosphorylates TFEB, blocking its nuclear translocation. During the final stage of the autophagic process, tubular-like structures containing Lamp1 but not the autophagosome protein LC3 originate from the autolysosome. These tubular structures, that do not contain hydrolases, eventually detach as vesicles and mature into lysosomes (Yu et al., 2010). This process, named Autophagic Lysosomal Reformation (ARL), leads to the recovery of the original pool of lysosomes in the cell, that otherwise would be depleted (Shen and Mizushima, 2014; Yu et al., 2010). (**Figure 1-5**)

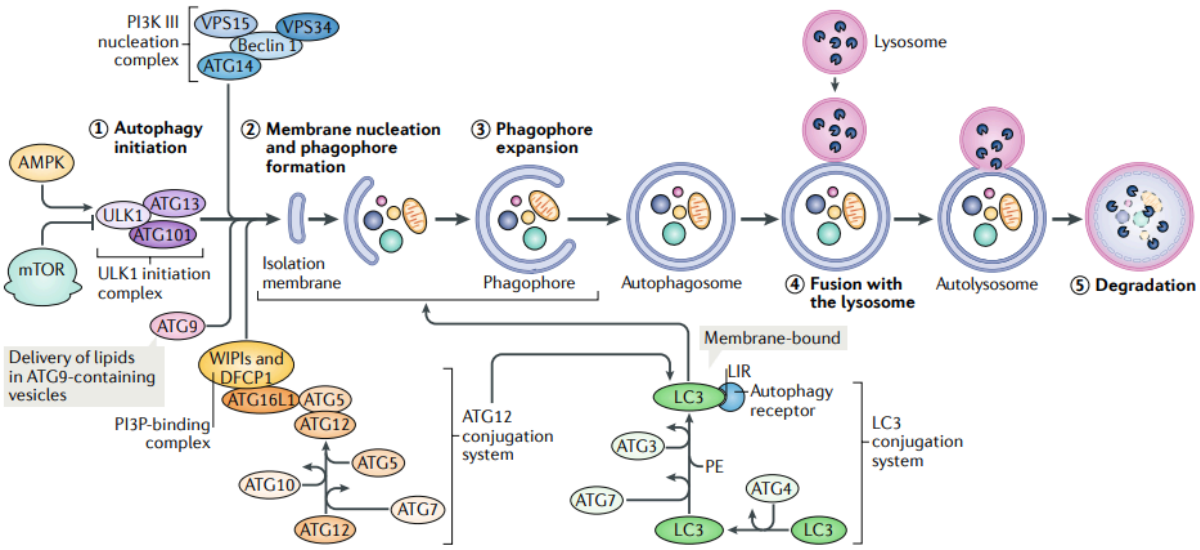

Figure 1-5. Autophagy activation cascade

(1) The release of mechanistic Target fo Rapamycin 1 (mTORC1) from the LYNUS complex on the lysosomal membrane due to lack of nutrients causes a reduction in the phosphorylation level of and the activation of unc-51-like kinase (ULK1) initiation complex made of ULK1, autophagy-related gene (ATG)13, ATG101 and ATG17 (not shown) that are responsible for autophagy initiation (1). ULK1-mediated phosphorylation activates the class III phosphatidilinositol-3-phosphate kinase (PI(3)KIII) nucleation complex, that contains Vps15, ATG14, Beclin-1 and the PI(3)-kinase Vps34. The activation of PI3KIII complex induces membrane nucleation and the formation of the phagophore (2) that contains lipids delivered by ATG9+ vesicles. The increased concentration of PI(3)P recruits the PI(3)P binding complex which includes WD-repeated-domain phosphoinositide interacting proteins (WIPI) and double FYVE containing protein (DFCP)1. This complex in turn recruits the machinery for the formation and closure of the autophagosome. These include the ATG12 conjugating system that includes the ubiquitin-like protein ATG12, ATG7 (that works like an E1-ubiquitin activating enzyme) and ATG10 (that acts like an E2-ubiquitin conjugating enzyme). ATG5/ATG12 is then attached to the E3-like ubiquitin ligase ATG16L1. ATG5/ATG12/ATG16L1 complex mediates the lipidation of LC3I (or GAPARAP), the product of the ATG4 mediated cleavage of LC3, via the action of ATG7 and ATG3 (E2-like enzyme). During phagophore formation (2) and expansion (3) LC3II is incorporated into the phagophore membrane and it is recognized by protein bearing a LC3-interacting (LIR) motif. This leads to the trafficking of the phagophore towards the endolysosomal compartment where it fuses with lysosomes (4) forming a hybrid organelle, the autolysosome, where the degradation (5) of the phagosome material take place.

Adapted from Hansen et al., Nat. Rev. Mol. Cell. Biol., 2018

1.2 Rab7 as a master regulator of the endolysosomal compartment

The family of Ras proteins in the brain (Rab) proteins includes approximately 70 members in humans. These proteins play crucial roles in defining the membrane identity of and coordinating fusion events on the PM, endolysosomal compartments, Golgi and ER (Zerial and McBride, 2001).

1.2.1 Rab GTPase cycle

Rabs are synthesized in the cytosol, but right after translation, they are bound by Rab Escort Protein (REP) (Alexandrov et al., 1994) and presented to the geranylgeranyltransferase (RabGGTase) (Andres et al., 1993) to be prenylated (geranylgeranylated) on their C-terminal cysteines (Joberty et al., 1993). This irreversible post-translational modification (PTM) provides Rabs with a lipid moiety required for membrane binding, indeed mutations affecting prenylated cysteines make the protein incapable of membrane localization resulting in a completely cytosolic protein (Modica et al., 2017).

Rabs are small GTPases characterized by the ability to cycle between active and inactive states depending on their association with GTP or GDP respectively. In the inactive-GDP bound state, Rab is recognized by the guanine-nucleotide dissociation inhibitor (GDI) (Soldati et al., 1993), that binds the inactive protein and by masking the prenylated tail keeps the Rab soluble in the cytosol (Muller and Goody, 2018). Guanine Exchange Factors (GEF) induces the release of Rab from GDI and enables GTP binding (Burton et al., 1994). The mechanisms by which cytosolic GDI-bound Rabs are recruited to their target membranes are not completely clear, but seem to be at least partially dependent upon the Rab GEFs (Blumer et al., 2013; Wu et al., 2010). Once on the membrane, in its active GTP-bound state, Rab can engage and interact with specific effectors to coordinate downstream membrane trafficking and fusion events. GTPase activating proteins (GAPs) trigger the hydrolysis of GTP to GDP to terminate Rab activity (Barr and Lambright, 2010). Once back in its inactive GDP-bound state, GDI extracts Rab from the membrane, translocates it to the cytosol, and the cycle can begin anew. (**Figure 1-6**)

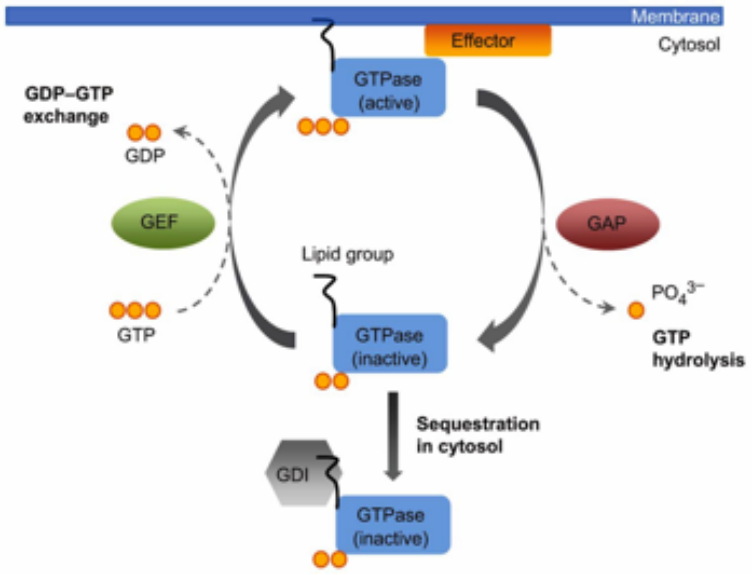

Figure 1-6. Rabs cycle

In its inactive state Rab is kept in the cytosol by GDI (GDP Dissociation inhibitor) that by masking the prenylated tail keeps the protein soluble in the cytosol. The interaction with Guanine Nucleotide Exchange Factor (GEF) activates the protein by favouring the exchange of GDP for GTP and the membrane localization of the protein. Once at the membrane Rab can interact with its effector and coordinate downstream pathways. The activity of the protein is terminated by the action of GTP-ase activations protein (GAP) that increase the rate of GTP to GDP hydrolysis. In its inactive, GDP-bound state Rab binds GDI and re-localize in the cytosol.

Adapted from Bento et al., JCS, 2013

1.2.2 Rab7 recruitment to the late endosome

Membrane trafficking at endosomes is regulated by different Rabs, each defining a specific compartment. For instance, Rab5 is responsible for the trafficking events at the EE, Rab4 coordinates the EE-to-PM and EE-to-sorting endosome trafficking of recycling receptors, and Rab11 is responsible for the sorting endosome-to-PM receptor recycling (Wandinger-Ness and Zerial, 2014).

The small GTPase Rab7 is responsible for vesicle trafficking and membrane fusion events at the late-endosome/lysosomal compartment. By interacting with different effectors, Rab7 coordinates several trafficking pathways such as LE-lysosome fusion during endocytic degradation, autophagosome-lysosome fusion during autophagy and mitophagy, cargo-

receptors retrieval from endosome-to-TGN and lysosome positioning (Guerra and Bucci, 2016; Tan and Tang, 2019).

The transition from EE to LE is characterized by the loss of Rab5 and the acquisition of Rab7 (Poteryaev et al., 2010), which is accompanied by the loss of the EE multi-subunit tethering complex (MTC) class C core vacuole/endosome tethering (CORVET) (Balderhaar et al., 2013) and the recruitment of HOPS, the MTC involved in fusion events at the lysosomes. The cascade of events that leads to Rab5-Rab7 switch is triggered by GTP-bound Rab5. Active Rab5 recruits its effector Rabaptin-5 that in turns recruits the Rab5 GEF Rabex-5 (also known as RABGEF1). This process establishes a positive feedback loop that increases the amount of active Rab5 at the EE (Zerial and McBride, 2001). GTP-bound Rab5 recruits another effector, the Monensin sensitivity (Mon)1/Calcium caffeine zinc sensitivity (Ccz)1 complex that is a GEF for Rab7. Mon1/Ccz1 is recruited to the membrane by Rab5 and once on the membrane, it displaces the Rab5 GEF Rabex-5 (Poteryaev et al., 2010) and activates Rab7 (Epp et al., 2011; Nordmann et al., 2010). Finally the Rab5 effector Vps34, a class III phosphoinositide 3-kinase (PI3K), induces the association to the membrane of the GAP Armus/TBC1D2A (Jaber et al., 2016; Law et al., 2017) that could potentially terminate the activity and induce the displacement of Rab5 from the maturing late endosome membranes (Law et al., 2017).

1.2.3 Rab7 functions at the Late Endosome

At the late endosome, Rab7 interacts with several effectors to coordinates different membrane trafficking pathways. Rab7 is indeed responsible for retromer recruitment for the retrieval trafficking pathways to the TGN, it is also responsible for the movement and positioning of late endosome via the interaction with RILP and ORP1L and FYCO-1, and it mediates the fusion events between late endosome and lysosome by recruiting HOPS.

1.2.3.1 Rab7 coordinates endosome-to-TGN trafficking

Cargo-receptor endosome-to-TGN retrieval is mediated by the retromer complex and is necessary for optimal lysosomal function. Rab7 coordinates the recruitment of retromer to the late endosome membrane (Rojas et al., 2008; Seaman et al., 2009) by directly interacting with the Vps35 and Vps26 subunits of the CSC (Priya et al., 2015; Seaman et al., 2009). It has also been proposed that a synergic action between SNX3 and Rab7 is required for the efficient recruitment of retromer on the membrane. According to this model, the simultaneous binding of Rab7 and SNX3 to the N-terminal region of Vps35 allows the recruitment of the complex on the

membrane (Harrison et al., 2014). Interestingly, the Rab7 GAP TBC1D5 is associated with retromer (Seaman et al., 2009) via interactions with the Vps29 and Vps35 CSC subunits (Jia et al., 2016). It could be speculated that the activity of the GAP through Rab7 is required for the release of retromer from the membrane and the recycling of the cargo receptor. However reduced expression of TBC1D5 does not leads to accumulation of retromer on the endosomal compartment but it is associated with an increased retromer function (Seaman et al., 2018).

Rab7-dependent retromer recruitment at the late endosome is controlled by the soluble lysosomal protein CLN5 (Jules et al., 2017; Mamo et al., 2012). CLN5 regulates Rab7 late endosome membrane localization and consequently is required for proper retromer function (Mamo et al., 2012). Indeed CLN5 mutants retained in the ER failed to properly localize cathepsin D, which is found in the extracellular media rather than in the lysosomal compartment, supporting a defect in retromer function (Qureshi et al., 2018). A second NCL-linked protein, the integral membrane protein CLN3 has been involved in the regulation of Rab7-retromer function. CLN3 is localized in the endolysosomal compartment, interacts with both Rab7 and retromer, and is required for a proper function of the latter. In the absence of functional CLN3, sortilin and CI-MPR are degraded, suggesting impaired lysosomal hydrolyses trafficking and consequently impaired lysosomal function (Yasa et al., 2019).

1.2.3.2 Rab7 regulation of LE movement

At the late endosomes, Rab7 engages its effector Rab7 lysosomal interacting protein (RILP) (Cantalupo et al., 2001; Jordens et al., 2001), and this interaction is required to coordinates vesicle movement and fusion events between late endosomes and lysosomes. RILP forms homodimers via its c-terminal domain and interacts simultaneously with two Rab7 molecules (Colucci et al., 2005; Wu et al., 2005). The Rab7-RILP dimer in turn recruits the Vps41 subunit of HOPS, the endolysosome tethering complex required for membrane fusion (van der Kant et al., 2013). The efficient LE-lysosome fusion requires the engagement of a second Rab7 effector, Pleckstrin homology domain containing protein family member 1 (PLEKHM1) (McEwan et al., 2015), which interacts with the Vps39 HOPS subunit, stabilizing the association of the tethering complex at the vesicle membrane (Wijdeven et al., 2016).

In addition to its role in HOPS recruitment, RILP coordinates vesicle trafficking by interacting directly via its N-terminal domain with the C-terminal domain of $p150^{Glued}$, a subunit of the dynein motor complex for retrograde microtubule transport (Johansson et al., 2007; Jordens et al., 2001). RILP recruitment of the $p150^{Glued}$ subunit is not sufficient for vesicle movement

(Johansson et al., 2007). Indeed for the actual transport, the RILP-Rab7 complex interacts with ORP1L, a member of the oxysterol-binding protein–related protein (ORP) family. ORP1L interacts with Rab7 at the endosome (Johansson et al., 2005) and links the Rab7-RILP complex to the βIII subunit of the αIβIII dynein receptor associated with late endosomes. Both the binding to βIII via ORP1L and the RILP-dependent recruitment of $p150^{Glued}$ are necessary for retrograde transport (Johansson et al., 2007). ORP1L controls late endosome positioning in a cholesterol-dependent way. Cholesterol present at the late endosomes is bound by the OSBP related domain (ORD) domain of ORP1L leading to a conformational rearrangement of the protein that favours the recruitment of the dynein motor and the microtubule minus end directed trafficking of the LEs. During low cholesterol condition, the ORD domain is free which translates in a conformation that allows the interaction of the two phenylalanines [FF] in an acidic tract (FFAT) domain of ORP1L with the ER transmembrane protein vesicle-associated membrane protein -VAMP- associated ER protein (VAP)-A. This interaction interferes with the ability of the Rab7-RILP complex to recruit $p150^{Glued}$ and establishes a LE-ER membrane contact site that locks LEs at the cell periphery (Rocha et al., 2009).

Rab7 also coordinates the trafficking of late endosomes towards the cell periphery by interacting with its effector FYVE and Coiled-Coil-(CC)-domain containing 1 (FYCO1). FYCO1 contains a LC3 interacting region (LIR) domain that allows the protein to bind LC3 on the autophagosome and a FYVE domain that binds PI(3)P. Thanks to its CC domain, FYCO1 works like an adaptor to recruit the light chain-2 of kinesin-1 (KCL2) for the microtubule plus-end trafficking of the autophagosome and LEs (Pankiv et al., 2010). In particular for late endosome trafficking, the engagement of FYCO1 and the kinesin motor is dependent upon the interaction of the ER protein protrudin with active Rab7 and PI(3)P. This interaction leads to the loading of FYCO1 with kinesin-1 enabling the anterograde trafficking of the late endosomes (Raiborg et al., 2015). Both protrudin and FYCO1 binding to the late endosome is dependent on the interaction with PI(3)P on the late endosome membrane. While PI(3)P is enriched in early endosomes, late endosomes are enriched in PI(3,5)P, therefore it is possible that dephosphorylation of PI(3,5)P has a role in the recruitment of FYCO1 and protruding to membranes of late endosomes (Velichkova et al., 2010; Wijdeven et al., 2015). It is still not clear if there is an interplay between VAP-A dependent dynein displacement from the RILP-Rab7-ORP1L complex and the protrudin-mediated kinesin loading of the Rab7-FYCO1 pair. Interestingly, protrudin has a FFAT domain that interacts with VAP-A (Saita et al., 2009). Moreover, VAP-A exist as a dimer on the ER membrane (Kaiser et al., 2005) and could

therefore bind both the FFAT domain of protrudin and ORP1L, regulating in this way the direction of the late endosome vesicle movement (Wijdeven et al., 2015). (**Figure 1-7**)

1.2.4 Rab7 in autophagy

Several Rabs are involved in different steps of autophagy (Ao et al., 2014). Rab7 in particular plays a role in the final step of maturation of autophagosome (Ganley et al., 2011; Gutierrez et al., 2004; Jager et al., 2004). Indeed the small GTPase orchestrates the retrograde trafficking of autophagosomes in a RILP dependent manner (Bains et al., 2011) towards the perinuclear region were the majority of lysosomes are located. Here Rab7 is still required to coordinate the fusion events between autophagosomes and lysosomes, in a process that requires the engagement of the HOPS complex in a RILP and PLEKHM1 dependent manner. Indeed the LIR domain in PLEKHM1 binds ATG8A/GABARAP on the autophagosome and it is also able to bind the HOPS subunit Vps39 with its c-terminal region (McEwan et al., 2015). According to the current model, the Rab7-dependent combined interaction of RILP and PLEKHM1 with Vps41 and Vps39 respectively ensure the efficient recruitment of HOPS complex and the fusion of the autophagosome with the lysosome (Wijdeven et al., 2016).

1.2.4.1 Rab7 in mitophagy

Mitophagy is a specific form of autophagy that allows the removal via lysosomal degradation of dysfunctional mitochondria. The PINK1/PARKIN axis is the best characterized signaling pathway that triggers mitophagy. During steady-state, PTEN-induced putative kinase 1 (PINK1) is normally imported into the mitochondria and rapidly degraded. In dysfunctional mitochondria, PINK1 degradation is impaired leading to the accumulation of the kinase on the outer mitochondria membrane (OMM). Increasing localization of this protein on the OMM causes its dimerization and activation via auto-phosphorylation (Jin et al., 2010). PINK1 recruits and activates the cytosolic E3-ubiquitin ligase Parkin (Matsuda et al., 2010; Narendra et al., 2010), that once on the mitochondrial membrane creates a platform of ubiquitinated proteins that recruits autophagy receptors and initiates the autophagy cascade (Rodger et al., 2018; Tan and Tang, 2019). In the context of mitophagy, Rab7 seems to be involved in the early stages of the mitophagosome formation and expansion. Indeed Rab7 is recruited on mitochondria membrane upon mitophagy stimulation where presumably coordinates $Atg9^{+}$ membrane recruitment. The cycling of the small GTPase from GTP- to GDP-bound state seems to be crucial for its activity in mitophagosome maturation although the mechanisms controlling this

pathway are not completely clear. The mitochondrial GAP complex TBC1D15/TBC1D17 appears to be important in containing Rab7 activity to limit the acquisition of autophagosomal membranes surrounding the targeted mitochondria (Yamano et al., 2014). The retromer CSC has been shown to have a key role during mitophagy. Indeed it seems to regulate Rab7 localization onto damaged mitochondria and its ability to induce $Atg9^{+}$ membrane mobilization from the TGN. Furthermore, retromer, via its binding with the GAP TBC1D5, controls the GTP-GDP Rab7 cycle, that appears to be crucial for mitophagic flux (Jimenez-Orgaz et al., 2018).

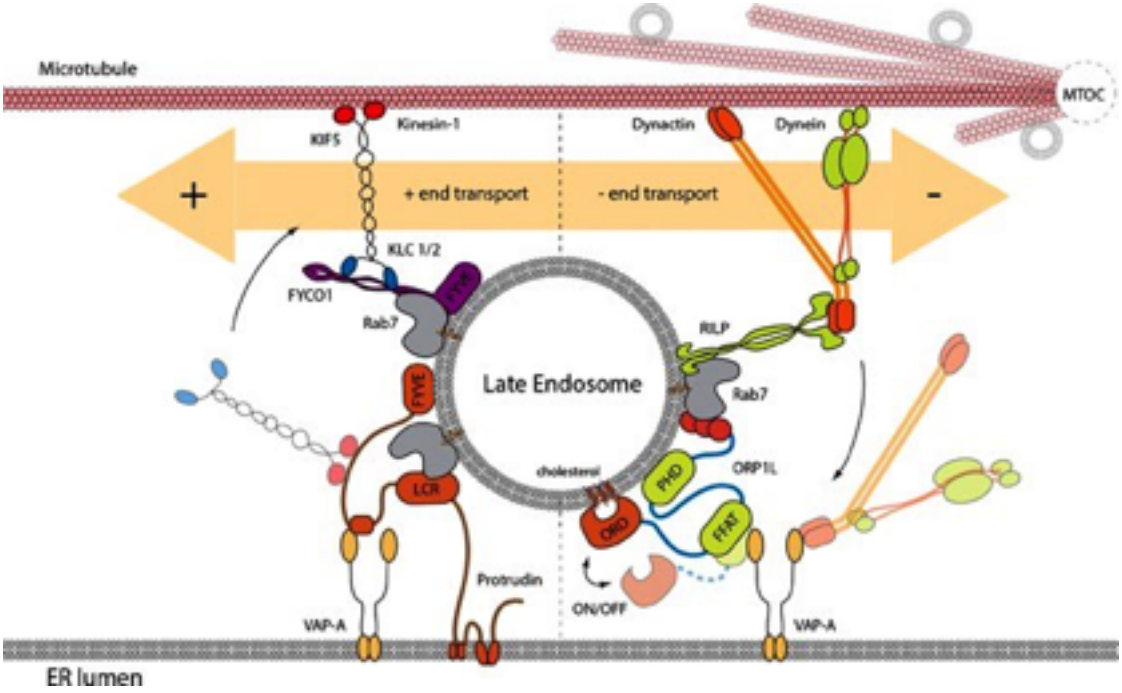

Figure 1-7 Rab7 dependent regulation of LE endosomal trafficking

Anterograde trafficking of the LE is controlled by the interaction between Rab7/FYCO1, the ER protein protrudin and the motor protein kinsein-1. At the ER protrudin binds VAP-A and the KIF-5 subunit of kinesin. The simultaneous binding of protrudin to phosphaditilinositol-3-phosphate via its FYVE domain and to Rab7 via its low complexity region (LCR) domain, leads to the transfer of Kinesin-1 to FYCO1, via the interaction of the latter with the light chain 2 of kinesin-1 (KLC1/2) for the microtubule plus-end directed transport. Retrograde trafficking is dependent upon the interaction between RAB7/RILP/ORP1L and dynein subunit $p150^{Glued}$ (not shown). Low cholesterol induces the binding of ORP1L to VAP-A, this inhibits the interaction between RILP and $p150^{Glued}$ locking the LE at the ER contact site. Upon high cholesterol, ORP1L binds the latter on the LE membrane, allowing the binding of RILP to $p150^{Glued}$ and the engagement of the dynein motor for the minus-end directed transport.

Adapted from: Wijdeven et al., Bioessays, 2015

1.2.5 Rab7 post-translational modifications

Increasing evidence suggests that post translational modifications (PTMs) are paramount to control Rab7 activity by preventing or favouring the interaction between the small GTPase and its numerous effectors. Beside the irreversible attachment of the prenyl tail mandatory for the ability of the protein to bind the membrane, other important PTMs that control Rab7 activity are: phosphorylation and ubiquitination (Modica and Lefrancois, 2017).

Phosphorylation

Rab7 has been shown to be phosphorylated on two sites, serine 72 (S72) and tyrosine 183 (Y183) (Shinde and Maddika, 2016). Serine 72 localizes in the switch II domain of Rab7, a region important not only to regulate the GTP-GDP activity of the small GTPase, but also to control protein-protein interactions. Upon mitophagy induction, activated Tank Binding Kinase (TBK1) phosphorylates mitochondrial localized Rab7 on S72. This phosphorylation is required for the recruitment of $Atg9^{+}$ membranes to the damaged mitochondria (Heo et al., 2018). The catalytically active form of leucine-reach repeat kinase 1 (LRRK1) is also able to phosphorylate Rab7 on S72 (pS72) at the endosome. In this context pS72 favour the binding of Rab7 with its effector RILP, promoting the lysosomal degradation of internalized Epidermal Growth Factor Receptor (EGFR) (Hanafusa et al., 2019).

Phosphorylation on tyrosine 183 (pY183) is mediated by Src kinase and negatively modulates the ability of Rab7 to interact with its effector RILP (Lin et al., 2017b). The role of Rab7 pY183 in the context of LE-Lysosome degradation is not clear. Both constitutive (Shinde and Maddika, 2016) or increased (Lin et al., 2017b) and deficient phosphorylation (Francavilla et al., 2016) of Y183 results delayed EGFR degradation, suggesting that Rab7 needs to cycle between phosphorylated and non-phosphorylated form to ensure efficient degradative pathway. Till now, only PTEN has been identified as Rab7 phosphatase acting on both tyrosine and serine residues (Shinde and Maddika, 2016).

Ubiquitination

Ubiquitination consists in the reversible attachment of ubiquitin, a small regulatory protein, to a lysine residue of a target protein by the coordinated action of three enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligases (Welchman et al., 2005). Parkin is responsible for Rab7 ubiquitination on K38, K191 and K194. In particular K38 ubiquitination seems to stabilize Rab7 membrane association and increases the interaction between the small GTPase and RILP (Song et al., 2016). Recently the

deubiquitylating enzyme USP32 has been identified as responsible of Rab7 deubiquitylation on K191 and possibly K194. The action of USP32 on Rab7 c-terminal lysines is required for a proper interaction with RILP and the minus-end directed movement of vesicles (Sapmaz et al., 2019). Furthermore USP32 associates with the Vps35 subunit of retromer (Sowa et al., 2009) and in this context, its action seem to be required for the release of retromer from late endosomes (Sapmaz et al., 2019).

1.3 Palmitoylation and small GTPases

S-palmitoylation (from now on palmitoylation) is a reversible post-translational modification consisting in the attachment of palmitate on a cysteine residue of the target protein (Linder and Deschenes, 2007). Palmitoylation can modify protein functions in several ways, such as preventing degradation, modifying the ability to interact with other proteins or changing membrane affinity (Chamberlain and Shipston, 2015) . *In silico* analysis predicts that approximately 10% of the human proteome is palmitoylated (Blanc M., 2015), pointing out the importance of this PTM in regulating protein functions.

In mammals palmitoylation is mediated by a family of 24 enzymes called protein acyltransferases (PATs). These enzymes are characterized by the presence of a cysteine-rich (CRD) domain with a conserved sequence Asp-His-His-Cys (DHHC) that is crucial for their enzymatic activity (Fukata et al., 2004). DHHCs are integral membrane proteins with four to six transmembrane domains localized in the Golgi, ER, endosomes and the PM (Ohno et al., 2006). These enzymes do not recognize conserved domains on target proteins, but analysis of identified PAT substrates lead to the identification of some shared common features. In the case of single span transmembrane proteins, palmitoylation occurs on cysteines close to the PM, while for soluble proteins, often the modification occurs on cysteines close to other lipidated residues (Daniotti et al., 2017).

The depalmitoylation of modified protein is performed by depalmitoylases (or protein thioesterase) that can be divided in three classes; acyl protein thioesterases (APT1 and APT2), protein palmitoyl thioesterases (PPT1), and the α/β hydrolases domain-containing 17 proteins (ABHD17A, ABHD17B, ABHD17C). PPT1 (also known as CLN1) is a lysosomal enzyme involved in the removal of palmitate from proteins destined for degradation (Verkruyse and Hofmann, 1996) whose mutations are associated with the development of NCL (Vesa et al., 1995). APT1 and APT2 are two cytosolic enzymes that control the depalmitoylation of several

peripheral proteins. Both the enzymes are palmitoylated on cysteine two, a modification required for the membrane localization of the enzymes (Daniotti et al., 2017). The ABHD17 class of depalmitoylases is the last class of depalmitoylase identified so far. These enzymes are cytosolic proteins that relies on palmitoylation for their activity and associate with the PM and endosomal membranes (Lin et al., 2017a; Won et al., 2018).

1.3.1 Ras GTPase

Ras GTPases are present in four isoforms, H-Ras, N-Ras, K-Ras4A and K-Ras4B. These proteins have been extensively studied due to their role as oncogenes. Indeed Ras proteins transduce the signal from Growth Factor Tyrosine Kinase receptor into the activation of intracellular pathways associated with growth and proliferation of cells. These proteins are characterized by a conserved c-terminal sequence CAAX-box where “A” represents an aliphatic amino acid and “X” indicates the last c-terminal residue. Like Rabs, these proteins are synthesized in the cytosol as soluble proteins. After their synthesis, the proteins are prenylated by the addition of a farnesyl group to the cysteine of the CAAX box. This leads to their association with the ER membrane where the AAX sequence is cleaved and the farnesylated cysteine is methylated, transforming the c-terminal domain into a lipid domain that allows the association of the small GTPase to the membrane (Ahearn et al., 2011; Daniotti et al., 2017). Farnesylation is not sufficient for the stable association of the protein to the membrane. A second signal is required for the efficient membrane targeting of RAS. In the case of K-Ras4B, a polybasic lysine rich region works together with the farnesyl group to provide the necessary stability for the binding to the plasma membrane. H-Ras, N-Ras and K-Ras4A rely instead on palmitoylation for a stable association with the membrane (Ahearn et al., 2011; Lin et al., 2017a).

In particular H-Ras and N-Ras palmitoylation/depalmitoylatiion cycle modulates their intracellular localization. The DHHC9/GCP16 (Swarthout et al., 2005) dependent palmitoylation of these GTPases at the Golgi induces their association with the membrane and their vesicular trafficking to the PM, where they can perform their function (Daniotti et al., 2017). At the PM, the APT1- (Dekker et al., 2010; Kong et al., 2013; Pedro et al., 2017) and ABHD17- (Lin and Conibear, 2015) dependent depalmitoylation, induces the membrane release and the cytosolic diffusion of the protein that once at the Golgi, can initiate another round of trafficking. Ras proteins are kept soluble in the cytosol via interaction with farnesyl-protein binding proteins, GDI-like factors that mask the lipid tail of the Ras making the protein soluble (Chandra et al., 2011) (**Figure 1-8**).

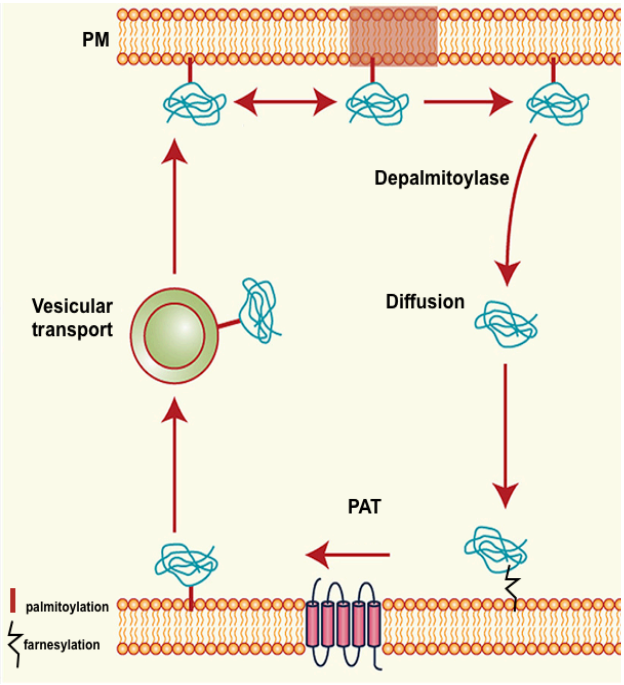

Figure 1-8 Schematic representation of Ras regulation via palmitoylation

At the Golgi, farnesylated Ras are palmitoylated by DHHC9/GCP6 Palmitoyl Transferase (PAT). the dual lipidation stabilize the association of the protein to the membrane allowing its vesicular transport toward the PM. Here the action of dapalmitoylases mediated the removal of the palmitate inducing the cytosolic diffusion of the protein via its binding to farnesyl-protein binding protein (not shown).

Adapted from Daniotti et al., Traffic, 2017

1.3.2 Rho GTPase

The family of Rho GTPases play a crucial role in regulating cell morphology and actin cytoskeleton rearrangement that influence cell motility and polarization, proliferation and differentiation (Hodge and Ridley, 2016; Olson, 2018). Like Ras, Rho GTPases are synthesized in the cytosol as soluble protein and post-translationally lipidated to allow the association with the membrane. Differently from Ras, Rho GTPase are geranylgeranylated by the by the Geranylgeranyltransferase type I (GGTase I) to the cysteine of the Rho CAAX box. The signal

driving the choice between geranylgeranylation and farnesylation relies in the CAAX box, indeed if the “X" residue is a leucine, like for the Rho GTPases, the geranylgeranyl group is added, while if the “X” residue is not a leucine, the protein is farnesylated (Resh, 2006).

Palmitoylation has been shown to have a role in modulating the function of some Rho GTPases. Rac1 palmitoylation for instance regulates the localization of the small GTPase in Detergent Resistant Microdomains (DRMs) at the PM. This leads to the activation of downstream effectors that results in the actin cytoskeleton rearrangement to promote cell migration (Navarro-Lerida et al., 2012). Palmitoylation has been also involved in the regulation of the activity of the brain-splice variant of CDC42 (bCDC42). In this case, the dual lipidation of prenylation and palmitoylation of a subpopulation of bCDC42, increases the association of this protein with the membrane while reducing the binding with the RhoGDIa. The increased association of prenyl-palmitoylated bCDC42 on the membrane increases the pool of small GTPase that can be activated, resulting in a potentiated downstream signal (Nishimura and Linder, 2013).

1.4 Thesis structure

In this thesis, I describe the results of my PhD project that was focused on the analysis of the role of post-translational modifications in modulating Rab7 functions. Such results are presented in the form of papers. The first one has been published in 2017, while the second is a manuscript in preparation and I plan to submit it by the end of December 2019.

The first work presented, “Rab7 palmitoylation is required for efficient endosome-to-TGN trafficking” has been published in the Journal of Cell Science in August 2017. In this work, we show that Rab7 is palmitoylated and that palmitoylation is required to recruit retromer to the endosome for efficient endosome-to-trans Golgi Network trafficking. Rab7 is a small GTPase regulating membrane trafficking at the late endosome, as such it coordinates late endosome-lysosome fusion, vesicle movement a positioning and endosome-to-TGN trafficking. Our work was driven by the goal of understanding how the same protein could coordinate so many pathways and which mechanisms were involved in modulating Rab7 functions.

Previously published data have involved palmitoylation in the regulation of endosome-to-TGN trafficking. McCormick et al., showed that palmitoylation has a role in controlling endosome-to-TGN trafficking by preventing the degradation of the cargo receptors sortilin ad CI-MPR. In this respect, palmitoylation and ubiquitination compete in modulating the stability of these proteins, with non-palmitoylatable sortilin being degraded in the lysosome and not recycled to the TGN (McCormick et al., 2008). Other work in yeast identified two palmitoyltransferases, Swf1 and Akr1, as involved in the trafficking of the vacuolar enzyme CPY. Indeed yeast strains where the genes coding for these two PAT were deleted, failed in localizing CPY to the vacuole and instead secreted the protease in the extracellular space (Bonangelino et al., 2002).

With this preliminary information and the fact that other small GTPase are palmitoylated, we hypothesized that palmitoylation could have a role in regulating retromer association with the membrane. We first asked if retromer or Rab7 were palmitoylated. To perform this analysis we optimized the Acyl-RAC (Resine Assisted Capture) protocol. This technique foresees the use of hydroxylamine (NH_2OH) to cleave palmitate chains from proteins, followed by the incubation with thiopropyl-beads that bind to the newly freed cysteine residues. The beads are then precipitated and proteins eluted with the reducing reagent Dithiothreitol (DTT). Samples obtained are run in acrylamide gel and subjected to Western Blot with the antibody against a protein of interest. After discovering that Rab7 was palmitoylated, and identifying the cysteines targeted by palmitoylation, used CRISPR/Cas9 to engineer a cell line knocked out for Rab7, and

used this cell to perform rescue experiments with wild-type and non-palmitoylatable Rab7 to clarify the role of palmitoylation in regulating retromer recruitment.

The second work “Role of phosphorylation in regulating Rab7 functions” is a manuscript in preparation.

While investigating the role of palmitoylation in modulating the Rab7/Retromer interaction, other work was published showing that Rab7 is phosphorylated on two residues, S72 and Y183 (Francavilla et al., 2016; Lin et al., 2017b; Shinde and Maddika, 2016). The model proposed at that time was that S72 phosphorylation was acting as an on/off switch to regulate Rab7 activity by modulating its membrane association, with S72 phosphorylation localization Rab7 to the cytosol. Subsequent studies showed that the phosphomimetic Rab7 S72 mutant failed to interact with the RabGGTase, therefore its cytosolic localization is likely due to the absence of prenylation rather than to a constitutive phosphorylated state (Heo et al., 2018). With respect to Y183 phosphorylation, it was shown that this PTM blocks the interaction with RILP (Lin et al., 2017b), however the consequences of this inhibition were not clear.

We hypothesized that different PTMs would regulated various Rab7 functions in different pathways. We decided to test our hypothesis by first testing if phosphorylation was affecting any Rab7/effector interactions. To do so, we used Bioluminescence Resonance Energy Transfer (BRET), a technique that allowed us to look at protein-protein interaction in live cells without altering the intracellular localization of the proteins in analysis. We also decided to use our previously generated Rab7-KO cells to probe the function of Rab7 phosphorylation.

In conclusion, with this project we were able to identify palmitoylation as a novel Rab7 PTM and to describe its role in modulating Rab7 activity. We also identified the existence of an interplay between S72 phosphorylation and palmitoylation in modulating Rab7/Retromer interaction and finally we suggest a novel role of Y183 phosphorylation as a molecular switch to favour ESCRT-II dependent cargo sorting into ILVs.

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3 RAB7 PALMITOYLATION IS REQUIRED FOR EFFICIENT ENDOSOME-TO-TGN TRAFFICKING

Authors:

Graziana Modica [1], Olga Skorobogata [1], Etienne Sauvageau [1], Adriano Vissa [2,3,4], Christopher M. Yip [3,4], Peter K. Kim [2,3], Hugo Wurtele [5] and Stephane Lefrancois [1,6]

1 Centre INRS-Institut Armand-Frappier, Institut National de la Recherche Scientifique, Laval, Québec, Canada H7V 1B7

2 Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada, M5G 1X8

3 Department of Biochemistry, University of Toronto, Toronto, Canada M5G 1X8

4 Institute of Biomaterials & Biomedical Engineering and Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 3E5

5 Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Montréal, Canada H1T 2M4 and Département de Médicine, Université de Montréal, Montréal, Québec, Canada H3C 3J7

6 Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada H3A 0C7

Journal of Cell Science

2017 Aug 1;130(15):2579-2590. Epub 2017 Jun 9.

doi: 10.1242/jcs.199729

Author contributions :

Conceptualization, S.L., G.M., H.W.; Design (Methodology), S.L., G.M., H.W., E.S., O.S., P.K.K.; Investigation, G.M., O.S., E.S., A.V.; Analysis, G.M., O.S., E.S., H.W., A.V., P.K.K., S.L. Writing – Original Draft, S.L. G.M.; Writing – Review & Editing, S.L., G.M., O.S., E.S., P.K.K., C.M.Y.; Funding Acquisition, S.L., H.W.; Supervision, S.L., H.W., C.M.Y., P.K.K.

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Summary Statement

Active Rab7 functions in various cellular pathways. We demonstrate that Rab7 is palmitoylated, a post-translational modification required for endosome-to-TGN trafficking, but not other functions of Rab7.

Abstract

Retromer is a multimeric protein complex that mediates endosome-to-TGN and endosome-to-PM trafficking of integral membrane proteins. Dysfunction of this complex has been linked to Alzheimer's and Parkinson's disease. The recruitment of retromer to endosomes is regulated by Rab7 to coordinate endosome-to-TGN trafficking of cargo-receptor complexes. We found that Rab7 is palmitoylated and that this modification is not required for membrane anchoring. Palmitoylated Rab7 co-localizes efficiently with and has a higher propensity to interact with retromer than non-palmitoylatable Rab7. Finally, rescue of Rab7 knock out cells with wild-type Rab7 restores efficient endosome-to-TGN trafficking, while rescue with non-palmitoylatable Rab7 does not. Interestingly, Rab7 palmitoylation does not appear to be required for the degradation of EGF receptor nor its interaction with RILP. Overall, our results indicate that Rab7 palmitoylation is required for the spatiotemporal recruitment of retromer and efficient endosome-to-TGN trafficking of the lysosomal sorting receptors.

3.1 Introduction

Retromer is an evolutionarily conserved multimeric complex first identified in the yeast S. cerevisiae as responsible for the retrieval of Vps10p receptor from the pre-vacuolar compartment to the trans-Golgi Network (TGN) (Seaman et al., 1997; Seaman et al., 1998). In yeast, retromer is a pentameric complex composed of a conserved trimer of Vacuolar Protein Sorting-26 (Vps26p), Vps29p and Vps35p, which is responsible for cargo recognition (referred to as cargo selective complex - CSC), and a dimer of sorting nexins (SNXs), Vps5p and Vps17p (Horazdovsky et al., 1997). Yeast strains deleted of each of the retromer subunits are unable to correctly traffic the lysosomal enzyme carboxypeptidase Y (CPY) to the vacuole and secrete it into the extracellular space (Bonangelino et al., 2002). In humans, the retromer CSC is conserved (Haft et al., 2000) and can associate with a varying combination of SNXs expanding the range of cargos that can be recognized and trafficked (Cullen, 2008; Gallon and Cullen, 2015). In human cells, retromer is responsible for the retrieval of cargo receptors from the endosome to the TGN (Arighi et al., 2004; Seaman, 2004) and in recycling transmembrane proteins from the endosomal compartment to the plasma membrane (Burd and Cullen, 2014; Feinstein et al., 2011; Steinberg et al., 2013; Temkin et al., 2011). Increasing evidence underlines how alteration in retromer expression or mutations in its subunits are associated with several neurodegenerative diseases including Alzheimer's and Parkinson's diseases (Follett et al., 2016a; Small and Petsko, 2015).

Rab7 and its yeast homologue Ypt7 are responsible for the recruitment and stabilization of the retromer CSC at the endosome (Balderhaar et al., 2010; Burd and Cullen, 2014; Rojas et al., 2008; Seaman et al., 2009). Deletion of Ypt7 in yeast is associated with CPY secretion (Bonangelino et al., 2002) and cargo-receptor accumulation at the endosome (Liu et al., 2012). In human cells, siRNA-mediated down-regulation of Rab7 expression is associated with displacement of the CSC from the endosomal membrane to the cytosol and with secretion of the lysosomal hydrolase cathepsin D (Rojas et al., 2008; Seaman et al., 2009).

In addition to its role in retromer recruitment, Rab7 also regulates endosome/lysosome fusion events via its interaction with Rab-interacting lysosomal protein (RILP) (Cantalupo et al., 2001). RILP in turn interacts with the Endosomal Sorting Complex Required for Transport (ESCRT) system (Progida et al., 2006) mediating the internalization of mono-ubiquitinated transmembrane proteins into multivesicular bodies (MVBs) leading to their degradation in lysosomes. Furthermore, Rab7 can regulate autophagy by modulating the interaction of

lysosomes with autophagosomes (McEwan et al., 2015). How cells regulate the function of Rab7 in these various pathways is not understood.

A RNAi screen designed to identify integral membrane proteins implicated in endosome-to-TGN trafficking found the palmitoyltransferase (PAT) DHHC5 (Breusegem and Seaman, 2014). PATs are transmembrane proteins characterized by a conserved catalytic domain containing Aspartic Acid, Histidine, Histidine, and Cysteine (DHHC) residues (Mitchell et al., 2006) that mediate palmitoylation, a reversible attachment of a palmitate chain onto a cysteine of a target protein. Palmitoylation can serve as a reversible membrane anchor, promote localization of proteins to detergent resistant membranes (DRMs) or modulate protein-protein interaction (Linder and Deschenes, 2007). Furthermore, two yeast PATs, Akr1 and Swf1, were identified in a screen for novel proteins implicated in CPY trafficking suggesting these enzymes play a critical role in lysosomal trafficking (Bonangelino et al., 2002).

Herein, we show that Rab7 is palmitoylated on two cysteine residues. The non-palmitoylatable Rab7 mutant, $Rab7_{C83,84S}$, localizes to membranes suggesting this modification is not required for membrane anchoring. Lack of Rab7 palmitoylation results in a reduced interaction with retromer leading to a less efficient endosome-to-TGN cargo-receptor trafficking, resulting in the missorting of the lysosomal protein cathepsin D. Interestingly, Rab7 palmitoylation is not required for the degradation of the epidermal growth factor receptor (EGFR) or for the Rab7/RILP interaction suggesting that this post-translational modification on Rab7 is required for its role in endosome-to-TGN trafficking.

3.2 Results

3.2.1 Rab7 is palmitoylated on cysteine residues 83 and 84

To determine the role of palmitoylation in modulating endosome-to-TGN trafficking, we first asked if any retromer subunits are palmitoylated as this post-translational modification could act as a reversible membrane anchor for retromer CSC. Due to the lack of an effective antibody for Vps29, we transfected HEK-293 cells with a FLAG-tagged form of Vps29 (Vps29-(FLAG)3) and performed Acyl-Resin Assisted Capture (Acyl-RAC) analysis, a well characterized method to test for protein palmitoylation (Forrester et al., 2011). None of the retromer CSC subunits were captured in our assay, while we recovered the well known palmitoylated protein TRAPPC3 (Kummel et al., 2006) suggesting none of the retromer CSC components are palmitoylated (Fig. 1A).

Rab7 is required to recruit retromer to endosomes (Rojas et al., 2008; Seaman et al., 2009) and was identified in a screen for palmitoylated neuronal proteins (Kang et al., 2008). We hypothesized that Rab7 palmitoylation could affect retromer endosomal recruitment by modulating the activity of this protein. We first tested endogenous Rab7 in HEK-293 cells for palmitoylation using Acyl-RAC and found that this small GTPase is palmitoylated (Fig. 3.1A) as it was captured in our assay when cells were treated with hydroxylamine (NH2OH), which cleaves palmitate groups from proteins exposing a free cysteine(s) to interact with the thiopropyl sepharose beads. Palmitoylation occurs on cysteine residues of proteins and we identified four evolutionarily conserved cysteine residues in Rab7 and Ypt7. The two C-terminal cysteines 205 and 207 for mammalian Rab7 and 206 and 208 for yeast Ypt7 are prenylated (Magee and Newman, 1992), a post-translational modification that is required for membrane binding, while no post-translational modifications on the other pairs of cysteine residues 83, 84 and 84, 85 for Rab7 and Ypt7, respectively, have been described.

To identify the residues targeted by this post-translational modification, we used myc-tagged Rab7 (myc-Rab7) and mutated the various cysteines using site-directed mutagenesis. HEK-293 cells were transfected with wild-type myc-Rab7 and the various mutants, and an Acyl-RAC assay was performed (Fig. 3.1B). We found that the single cysteine-to-serine mutation of residues 83 or 84 resulted in a decrease in palmitoylation compared to wild-type Rab7, while the double mutant, $Rab7_{C83,84S,}$ is not palmitoylated (Fig. 3.1B) as we did not recapture this mutant. Mutation of cysteine 143, not present in Ypt7, did not affect Rab7 palmitoylation (Fig. 3.1B). Mutation of the two C-terminal cysteines ($Rab7_{C205,207S}$) results in a reduction in the palmitoylation level of Rab7 (Fig. 3.1B). Prenylation of C-terminal cysteines in Rab GTPases serves as a membrane anchor (Chavrier et al., 1991), so the reduction in palmitoylation observed in the prenylation mutants may be due to the inability of the mutant protein to be membrane associated. Since all known PATs are integral membrane proteins, prenylation and membrane anchoring of Rab7 may be a prerequisite for palmitoylation.

3.2.2 Rab7 palmitoylation is not required for membrane anchoring

To determine how palmitoylation modulates the function of Rab7, we first analyzed the membrane distribution of wild-type and mutant proteins. We transfected HEK-293 cells with wild-type myc-Rab7 and the various mutants and performed a membrane separation experiment to determine the amount of Rab7 on membranes. Our separation was successful as the cytosolic protein α-tubulin and the integral membrane protein Lamp-2 were found in the

soluble and pellet fraction respectively (Fig. 3.1C). As expected, wild-type Rab7 is mostly membrane bound while Rab7$_{C205,207S}$ is localized to the soluble fraction (Fig. 3.1C). The membrane localization of the Rab7$_{C83S}$, Rab7$_{C84S}$ and the non-palmitoylatable mutant Rab7$_{C83,84S}$ is comparable to that of wild-type Rab7, suggesting that palmitoylation is not regulating the ability of the protein to bind membranes (Fig. 3.1C). Statistical analysis of 4 separate experiments found no significant changes in membrane distribution between myc-Rab7, myc-Rab7$_{C83S}$, myc-Rab7$_{C84S}$ and the non-palmitoylatable mutant (myc-Rab7$_{C83,84S}$) while the distribution of the prenylation (myc-Rab7$_{C205,207S}$) mutant was significantly different than the others as it was almost exclusively cytosolic (Fig. 3.1D).

Palmitoylation can serve as a membrane anchor, or it can serve to localize proteins to specific membrane compartments (Rocks et al., 2005). To test if the palmitoylation of Rab7 modulates its intracellular distribution, we compared the localization of wild-type myc-Rab7, myc-Rab7$_{C83,84S}$ and myc-Rab7$_{C205,207S}$ with the early endosomal marker Rab5 (RFP-Rab5, Fig. 3.2A - I) and the late endosomal marker RILP (Fig 3.2K - S). We found that myc-Rab7 and myc-Rab7$_{C83,84S}$ partially co-localized with RFP-Rab5 (Rwt = 0.2194, RC83,84S = 0.2554, Fig. 3.2J) and more strongly with RILP (Rwt = 0.4167, RC83,84S = 0.4832, Fig. 2T), while myc-Rab7$_{C205,207S}$ had a negative Pearson's correlation with both RFP-Rab5 (R205,207S = -0.02875, Fig. 3.2J) and RILP (R205,207S = -0.1222, Fig. 3.2T) suggesting that this mutant was not found in either early or late endosomes as expected. Overall, palmitoylation does not seem to be required for the localization of Rab7 to endosomal membranes.

3.2.3 Rab7 palmitoylation is required for the efficient recruitment of retromer CSC

To test the function of Rab7 palmitoylation, we engineered a Rab7 knockout HEK-293 cell line (Rab7-KO) using CRISPR/Cas9 (Fig. 3.3A). The absence of endogenous Rab7 allows us to study the function of Rab7 palmitoylation without the background of any residual expression of endogenous wild-type protein (Fig. 3.3A). To determine if Rab7 palmitoylation is required for the recruitment of retromer to endosomal membranes, we used a membrane separation experiment. Compared to HEK-293 or HEK-293 cells expressing myc-Rab7, retromer CSC is displaced to the soluble fraction (cytosol) in Rab7-KO cells (Fig. 3.3B) as shown by Western blot for both Vps26A and Vps35. This phenotype is rescued by the expression of wild-type myc-Rab7, but not myc-Rab7$_{C205,207S}$. The expression of myc-Rab7$_{C83,84S}$ did not efficiently restore Vps26A membrane localization (Fig. 3.3B).

To support our membrane separation assay, we transfected Rab7-KO cells with myc-Rab7, the non-palmitoylatable mutant myc- Rab7$_{C83,84S}$, and the prenylation defective mutant myc-Rab7$_{C205,207S}$ and examined the intracellular localization of the CSC subunit of retromer via Vps35 immunofluorescence (Fig. 3.4). In HEK-293 cells, Vps35 appears in punctate structures characteristic of endosomal membranes (Fig. 3.4A, arrowheads). In cells not expressing Rab7 (Rab7-KO cells), we observed an almost complete loss of Vps35 endosomal staining (Fig. 3.4B, stars) consistent with previously published results (Rojas et al., 2008; Seaman et al., 2009). Expression of myc-Rab7 in Rab7-KO cells rescues Vps35 localization to punctate structures (Fig. 3.4B, arrowheads). Quantification of the fluorescence intensity of Vps35 puncta was significantly increased in Rab7-KO cells expressing wild-type myc-Rab7 compared to non-transfected cells. Interestingly, the expression of myc-Rab7$_{C83,84S}$ in Rab7-KO cells (Fig. 4C, arrowheads) partially rescued the membrane localization of Vps35, but quantification shows that the increase is not significant compared to Rab7-KO cells. Finally, expression of myc-Rab7$_{C205,207S}$ fails to rescue Vps35 localization as expected since this mutant is not membrane bound (Fig. 3.4D).

3.2.4 Palmitoylation modulates the interaction between Rab7 and retromer

To better understand how Rab7 palmitoylation is required to recruit retromer, we tested how this post-translational modification affects the interaction between Rab7 and its effectors using bioluminescence resonance energy transfer (BRET), a method that detects molecular proximity in live cells. Wild-type Rab7 and Rab7$_{C83,84S}$ were fused at the N-terminus to the energy donor Renilla Luciferase II (RLucII-Rab7 and RLucII-Rab7$_{C83,84S}$), while Vps26A and RILP were fused at the C-terminus with the energy acceptor green fluorescence protein 10 (Vps26A-GFP10, RILP-GFP10). To ensure that tagging Rab7 with Renilla Luciferase II (RlucII-Rab7) did not affects its membrane localization and function, we performed rescue experiments using membrane separation and immunofluorescence microscopy to determine if RLucII-Rab7 could restore retromer CSC recruitment in Rab7-KO cells (Fig. 3.S1). Recruitment to membranes was not impaired by the RLucII tag as RLucII-Rab7 was membrane bound (Fig. 3.S1A). Furthermore, RLucII-Rab7 and myc-Rab7 both rescued retromer CSC membrane recruitment in Rab7-KO cells as shown by blotting for both Vps26A and Vps35 following a membrane separation assay (Fig. 3.S1A). To confirm these results, we expressed myc-Rab7 or RLucII-Rab7 in Rab7-KO cells, immunostained for Vps35 (Fig. 3.S1B) and quantified the fluorescence intensity of Vps35 puncta in Rab7-KO cells (Fig. S1C, black bar), Rab7-KO cells expressing myc-Rab7 (Fig. 3.S1C, white bar) and Rab7-KO cells expressing RLucII-Rab7 (Fig. 3.S1C, grey

bar). Both myc-Rab7 and RLucII-Rab7 were able to recruit retromer CSC to membranes as statistical analysis of the fluorescence intensity of Vps35 puncta showed a significant increase in both myc-Rab7 and RLucII-Rab7 expressing cells compared to untransfected cells (Fig. 3.S1C). The recruitment efficiency of myc-Rab7 and RLucII-Rab7 were comparable to one another as Vps35 intensity between these 2 conditions was not statistically different (Fig. 3.S1C).

To determine if palmitoylation was playing a role in modulating the Rab7/retromer interaction, HEK-293 cells were co-transfected with a constant amount of RLucII-Rab7 or RLucII-Rab7$_{C83,84S}$ and increasing concentrations of Vps26A-GFP10 or RILP-GFP10 to generate BRET titration curves (Kobayashi et al., 2009; Mercier et al., 2002). The BRET signal between RlucII-Rab7 and Vps26A-GFP10 rapidly increased with increasing amounts of expressed Vps26A-GFP10 until it reached saturation (Fig. 3.5A, black line), suggestive of a specific interaction (Mercier et al., 2002). In contrast, RLucII-Rab7$_{C83,84S}$ displayed lower BRET value with Vps26A-GFP10 that never fully reaches saturation (Fig. 3.5A, blue line). As controls, we tested the interaction of Rab1a (RLucII-Rab1a), which localizes to the Golgi apparatus, with retromer. As expected, we found no interaction as demonstrated by a linear curve (Fig. 3.5A, red line). Conversely, we tested the interaction between Rab7 and the μ1 (μ1-GFP10) subunit of the AP-1 clathrin adaptor. Once again we did not detect an interaction (Fig. 3.5A, green line) suggesting the BRET curves between Rab7 and retromer represent a specific interaction. BRET$_{50}$ is the value at which the concentration of the acceptor is required to obtain 50% of the maximal BRET signal (BRET$_{MAX}$) and is indicative of the propensity to interact between the donor and acceptor (Kobayashi et al., 2009; Mercier et al., 2002). We calculated BRET$_{50}$ for the interaction between wild-type Rab7 or Rab7$_{C83,84S}$ and Vps26A and found a 4 fold increase in the BRET$_{50}$ value for Rab7$_{C83,84S}$ binding to Vps26A (Fig. 3.5A, black bar) compared to wild-type Rab7 (Fig. 3.5A, grey bar) suggesting a much weaker interaction. Interestingly, palmitoylation of Rab7 did not appear to modulate the Rab7/RILP interaction, as we found no significant changes in the BRET titration curves between Rab7 and RILP (Fig. 5B, black line) or Rab7$_{C83,84S}$ and RILP (Fig. 3.5B, blue line) or the BRET$_{50}$ of these interactions (Fig. 3.5B, grey and black bars). As expected, Rab1a did not interact with RILP (Fig. 3.5B, red line). These data show that even if the palmitoylation deficient mutant is still membrane bound, its interaction with Vps26A is significantly less efficient than wild-type Rab7. However, it would appear that not all Rab7/effector interactions are modulated by palmitoylation, suggesting that this post-translational modification can mediate specific Rab7 functions.

To further explore how palmitoylation mediates the Rab7/retromer interaction, we performed a co-immunoprecipitation (co-IP) experiment by expressing wild-type myc-Rab7, the non-palmitoylatable mutant myc-Rab7$_{C83,84S}$ and (HA)3-Vps35 separately in HEK-293 cells. We then mixed lysates from each of the Rab7 expressing cells with lysate from cells expressing (HA)3-Vps35 and performed a co-IP using anti-HA antibody. If palmitoylation modulates the conformation of Rab7 to enable an interaction with retromer, myc-Rab7$_{C83,84S}$ would not bind (HA)3-Vps35 in this assay. On the other hand, if palmitoylation is required to localize Rab7 to facilitate an interaction with retromer, we would expect an interaction in this assay since the proteins are in a lysate and not membrane localized. We found an interaction between myc-Rab7 and (HA)3-Vps35 and between myc-Rab7$_{C83,84S}$ and (HA)3-Vps35 (Fig. 3.S2) suggesting that the role of palmitoylation is to localize Rab7 to a specific endosomal domain leading to an optimal interaction with retromer. As expected, (HA)3-Vps35 also interacted with another retromer subunit, Vps26A (Fig. 3.S2).

3.2.5 Retromer is not efficiently localized to Rab7$_{C83,84S}$ puncta.

To determine if palmitoylation is required to localize Rab7 to endosomal domains thereby modulating the Rab7/retromer interaction, we transfected U2OS cells with myc-Rab7 (Fig. 3.5C and D, green) or myc-Rab7$_{C83,84S}$ (Fig. 3.5E and F, green) to evaluate co-localization between these proteins and endogenous Vps26A (Fig. 3.5A - D, red) using structured illumination microscopy. Co-localization between myc-Rab7 or Rab7C83,84S and Vps26A was determined using Mander's coefficient. If the amount of Vps26A co-localizing with wild-type Rab7 or Rab7$_{C83,84S}$ puncta is determined, we observed reduced co-localization between Vps26A and Rab7$_{C83,84S}$(Fig. 3.5E, F and G, black bars) compared to myc-Rab7 (Fig. 3.5C, D and G, white bars). If the amount of Rab7 localized to Vps26A puncta is compared between wild-type Rab7 and Rab7$_{C83,84S}$ (Fig. 3.5G, white bars), we found a slight, but statistically significant decrease in the degree of co-localization observed. Indeed, Rab7 is required to recruit Vps26A to endosomes (Rojas et al., 2008), thus it is expected that Rab7 will be present in Vps26A-positive puncta on endosomes. However, together these data suggest that loss of palmitoylation does not completely prevent interaction between Rab7 and retromer, but due to reduced interaction ability, non-palmitoylatable Rab7 is not as efficient in recruiting retromer to endosomal membranes.

3.2.6 Rab7$_{C83,84S}$ is less efficient in coordinating retromer cargo-receptor retrieval.

Since Rab7 palmitoylation is required for an optimal interaction with retromer CSC, we hypothesized that the absence of palmitoylation would result in decreased retromer function. In humans and yeast, altered retromer function is associated with an impaired trafficking of lysosomal/vacuolar enzymes that results in their secretion into the extracellular space (Bonangelino et al., 2002; Follett et al., 2016b; Rojas et al., 2008). We asked if Rab7 palmitoylation is required for the correct trafficking of the lysosomal enzyme cathepsin D. To test the effect of Rab7 palmitoylation on cathepsin D trafficking, we compared the secretion of cathepsin D in HEK-293 cells, Rab7-KO cells and Rab7-KO cells transiently expressing wild-type myc-Rab7 and the various mutants (Fig. 3.6A). As expected, in HEK-293 cells, cathepsin D is correctly trafficked to the lysosome with almost no secretion of the protein into the media (Fig. 3.6A). Rab7-KO cells are not able to efficiently recruit retromer to endosomes resulting in inefficient trafficking of the lysosomal sorting receptors and the secretion of cathepsin D (Fig. 3.6A). The expression of myc-Rab7 in Rab7-KO cells rescues cathepsin D trafficking, therefore secretion is reduced compared to Rab7-KO cells (Fig. 3.6A). The expression of the prenylation mutant (Rab7$_{C205,207S}$) does not rescue Rab7-KO cells resulting in secretion of cathepsin D (Fig. 3.6A). Expression of myc-Rab7$_{C83,84S}$ does not efficiently rescue cathepsin D secretion, suggesting that efficient lysosomal localization of this protease is dependent on Rab7 palmitoylation (Fig. 3.6A). Functionally, palmitoylation of Rab7 plays a significant role in modulating efficient endosome-to-TGN trafficking of the lysosomal sorting receptors and the proper localization of lysosomal proteins.

3.2.7 Rab7 palmitoylation is not required for degradation of integral membrane proteins.

Although Rab7 is required for the spatiotemporal recruitment of retromer and therefore efficient endosome-to-TGN trafficking of CI-MPR and sortilin, it also regulates the degradation of integral membrane proteins such as Epidermal Growth Factor Receptor (EGFR) (Shinde and Maddika, 2016; Vanlandingham and Ceresa, 2009). Upon stimulation with epidermal growth factor (EGF), EGFR is internalized and degraded in lysosomes (Futter et al., 1996). We stimulated cells that had been previously incubated for 1 hour with cycloheximide with 100 ng/ml of EGF for either 15 or 120 minutes and compared the degradation of EGFR in HEK-293, Rab7-KO and Rab7-KO cells expressing wild-type myc-Rab7, myc-Rab7$_{C83,84S}$ or myc-Rab7$_{C205,207S.}$ Non-stimulated (NS) cells served as controls. As expected, HEK-293 cells and HEK-293 cells

overexpressing myc-Rab7 efficiently degrade EGFR following stimulation with EGF (Fig. 3.6B). However, compared to HEK-293 cells, Rab7-KO cells are not able to degrade EGFR as efficiently (Fig. 3.6B) while Rab7-KO cells expressing myc-Rab7 can. Rab7-KO cells expressing myc-Rab7$_{C83,84S}$ appear to degrade some EGFR but with slower degradation kinetics compared to HEK-293 cells while Rab7-KO cells expressing myc-Rab7$_{C205,207S}$ had a significant delay.

3.2.8 EGF is efficiently degraded in Rab7-KO cells expressing myc-Rab7$_{C83,84S}$.

Although EGFR degradation was mostly restored in Rab7-KO cells expressing myc-Rab7$_{C83,84S}$, the degradation assay determines the amount of EGFR remaining in all cells, not just in cells that have been rescued. To support our EGFR degradation data, we tested the degradation of EGF labeled with Alexa-488 (EGF-488) so we could evaluate degradation in rescued cells specifically. Cells were incubated with 300ng of EGF-488 for 30 minutes, washed and then chased for 0, 15 and 60 minutes. In HEK-293 cells, we found very little EGF-488 as it was efficiently degraded (Fig. 3.7A, F and K). Quantification of 20 cells found that, on average, HEK-293 cells contained 4.15, 4.35 and 3.2 EGF-488 puncta at 0, 15 and 60 minutes respectively (Fig. 3.7P, white bars). In Rab7-KO cells, we found a significant increase in the amount of EGF-488 puncta (Fig. 3.7B, G and L, white stars) and quantification found 10.65, 10.85 and 9.8 at 0, 15 and 60 minutes respectively (Fig. 3.7P, black bars). Expressing wild-type myc-Rab7 reduced the number of EGF-488 puncta in Rab7-KO cells (Fig. 3.7C, H and M) to levels comparable to HEK-293 cells (Fig. 3.7P, grey bars). Supporting our EGFR degradation data (Fig. 3.6B), expressing myc-Rab7$_{C83,84S}$ in Rab7-KO cells also decreased the number of EGF-488 puncta to levels comparable to HEK-293 cells (Fig. 3.7D, I and N). In fact, quantification of 20 cells found no statistical difference in the number of puncta (4.4, 5.65, 4.65 at 0, 15 and 60 minutes respectively) in Rab7-KO expressing myc-Rab7$_{C83,84S}$ compared to HEK-293 cells (Fig. 3.7P, stripped bars). Cells not expressing myc-Rab7$_{C83,84S}$ in the same field of view accumulated EGF-488 puncta (Fig. 3.7D and N, white stars). As expected, expression of myc-Rab7$_{C205,207S}$ in Rab7-KO cells (Fig. 3.7E, J and O) had no effect on the number of EGF-488 puncta (Fig. 3.7P, horizontal stripes) as these cells contained 8.7, 9.5 and 10.7 puncta at 0, 15 and 60 minutes respectively.

3.3 Discussion

Rabs are key regulators of the formation, trafficking, and fusion of transport vesicles at the endoplasmic reticulum (ER), Golgi apparatus and early and late endosomes (Hutagalung

and Novick, 2011). Rab7 functions in many pathways at endosomes including the recruitment of retromer (Rojas et al., 2008; Seaman et al., 2009), the recruitment of RILP (Cantalupo et al., 2001), and mediates the fusion of lysosomes with autophagosomes via the HOPS complex (McEwan et al., 2015). The molecular mechanisms that regulate and determine these various functions of Rab7 are not well understood. Herein, we demonstrate that Rab7 is palmitoylated, and that this post-translational modification plays a role in mediating the function of Rab7 in retromer recruitment and therefore in regulating endosome-to-TGN traffic.

3.3.1 Rab7 is palmitoylated on 2 cysteine residues

Rab7 and Ypt7 have 4 conserved cysteine residues. Two of these are the C-terminal cysteines that are prenylated, C205 and C207 in mammalian Rab7 and C206 and C208 in yeast Ypt7. As we and others have shown, these terminal residues are required for membrane anchoring. The other conserved cysteines are found between Switch I and Switch II, C83 and C84 in mammalian Rab7 and C84 and C85 in yeast Ypt7. We now demonstrate that these residues in Rab7 are palmitoylated. $Rab7_{C205,207S}$ is not palmitoylated and we suggest that membrane anchoring is a prerequisite for palmitoylation. The palmitoyltransferases (PATs), of which 24 have been found in humans and 7 in yeast, are all multispan integral membrane proteins (Linder and Deschenes, 2007). We propose that prenylation enables Rab7 to anchor into endosomal membranes. Subsequently, a subpopulation could be palmitoylated by one or more PAT(s). Further work will be required to identify the mechanism of Rab7 palmitoylation. The PAT DHHC5 is an excellent candidate as endosome-to-TGN trafficking was disrupted in cells depleted of DHHC5 by RNAi (Breusegem and Seaman, 2014). Furthermore, DHHC5 localizes to endosomes and could serve as a local mechanism for Rab7 palmitoylation.

3.3.2 Rab7 palmitoylation mediates its interaction with a subset of downstream effectors

Palmitoylation does not act as a membrane anchor for Rab7, but rather it modulates interactions. Using BRET, we found that $Rab7_{C83,84S}$ bound significantly less retromer than wild-type Rab7. However, RILP bound equally well to both wild-type Rab7 and $Rab7_{C83,84S}$. It will be interesting to determine how palmitoylation of Rab7 mediates interaction with other effectors such as HOPS, Rubicon and PLEKMH1. Based on our data, we find that palmitoylation modulates Rab7 function and its interaction with retromer by localizing the protein into specific endosomal sub-domains, and probably not by regulating its GTP bound status.

3.3.3 Rab7 post-translational modifications

It is becoming more apparent that the function of Rab7 is modified and regulated by post-translational modifications. We have demonstrated that Rab7 palmitoylation is required for the efficient endosome-to-TGN trafficking of the lysosomal sorting receptors. Recent studies have also shown how phosphorylation (Francavilla et al., 2016; Shinde and Maddika, 2016) and ubiquitination (Song et al., 2016) can regulate Rab7 function. Rab7 has at least 2 phosphorylation sites (serine 72 and tyrosine 183), which can act as a negative regulator or modulate function. Indeed, phosphorylation as serine 72 acts as an “off switch” for Rab7 as the phosphomimetic mutant was completely cytosolic (Shinde and Maddika, 2016), while phosphorylation at tyrosine 183 is required to mediate the degradation of the EGF receptor (Francavilla et al., 2016). Here we demonstrate that palmitoylation also regulates the function of Rab7. As far as we know, we are the first to confirm this post-translational modification on a mammalian Rab protein and the first to functionally characterize the role of palmitoylation on the function of a Rab. It will be interesting to confirm palmitoylation of other Rabs and how this modification would affect their function. Our work adds another regulatory mechanism to Rab7 biology. By combining phosphorylation, ubiquitination and palmitoylation, cells can modulate Rab7 function to various pathways and modulating these mechanisms could prove to be valuable targets in diseases where Rab7 is implicated.

3.4 Materials and Methods

Antibodies and reagents

Unless otherwise stated, all reagents were purchased from Fisher Scientific (Ottawa, ON). The following mouse monoclonal antibodies were used: anti-myc 9E10 (MMS-150P, Cedarlane Laboratories, Burlington, ON), anti-Vps35 (ab57632, Abcam, Cambridge, MA), anti-actin Ab5 (612657, BD Biosciences, Mississauga, ON), anti-α-tubulin (T9026, Sigma-Aldrich, Oakville, ON), anti-Lamp-2 (ab25631, Abcam), anti-Renilla luciferase (MAB4400, Fisher Scientific) and anti-HA (16B12, Cedarlane Laboratories). The following rabbit polyclonal antibody was used: anti-Vps26A (ab23892, Abcam), anti-TRAPPC3 (15555-1-AP, Cedarlane Laboratories), anti-RILP (ab128616, Abcam), anti-myc (2272, Cell Signaling Technology, Danvers, MA) and anti-FLAG (F7425, Sigma-Aldrich). The following rabbit monoclonal antibodies were used: anti-Rab7 (D95F2, Cell Signaling Technology), and anti-EGFR (ab52894, Abcam). All antibodies were used at a dilution of 1:1000 for Western Blotting (Wb) and immunofluorescence (IF) except anti-

myc, which was used at 1:500 for IF and Lamp-2, which was used at 1:500 for Wb.

Cloning and mutagenesis

All restriction enzymes used for cloning were purchased from New England Biolabs (Danvers, MA). Vps29-(FLAG)3 was generated by amplifying Vps29 cDNA from Vps29-YFP via PCR and cloned into BstbI/NotI of pEZ-M14 (GeneCopoeia, Rockville, MD). For myc-Rab7, the cDNA of Rab7 was amplified from RFP-Rab7 (a gift from Ari Helenius, plasmid # 14436, Addgene, Cambridge, MA) via PCR and cloned into the XbaI/NheI sites of pKMyc (a gift from Ian Macara, plasmid # 19400, Addgene). All the mutants described were generated via PCR mutagenesis using cloned PFU polymerase (Agilent Technologies, Santa Clara, CA). RLucII-Rab7 and RLucII-Rab7C83,84S were generated by amplifying Rab7 cDNA from myc-Rab7 and myc-Rab7C83,84S and cloned into the EcoRV/XhoI site of pcDNA3.1 Hygro (+) RLucII-GFP10-st2 plasmid. To obtain Vps26A-GFP10, Vps26A cDNA was amplified from Vps26A-YFP and cloned into the NheI/BamHI sites of pcDNA3.1 Hygro (+)-GFP10-RLucII. RLucII-Rab1a was generated by amplifying Rab1a cDNA from myc-Rab1a (a gift from Dr. Terry Hebert, McGill University) and cloned into the EcoRV/XhoI sites of pcDNA3.1 Hygro (+) RLucII-GFP10-st2 plasmid (pcDNA3.1 Hygro (+) RLucII-GFP10-st2 and pcDNA3.1 Hygro (+) GFP10-RLucII-st2 plasmids were generous gifts from Dr. Michel Bouvier (IRIC, Université de Montreal, Montreal, QC). RILP-GFP10 was generated by amplifying RILP cDNA from HA-RILP (a generous gift from Cecilia Bucci, University of Salento) and cloned into the NheI/KpnI sites of pcDNA3.1 Hygro (+)-GFP10-RLucII.

Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin (Thermo Fisher Scientific, Burlington, ON). All cells were originally obtained from ATCC (Manassas, VA) and periodically checked for contamination. All transfections, unless otherwise noted, were performed with polyethylenimine (PEI) (Fisher Scientific, Ottawa, ON). Briefly, solution A was prepared diluting plasmid into Optimem (Thermo Fisher Scientific). Solution B was prepared by diluting PEI (1µg/µl) in Optimen in a 1:3 ratio with the DNA to transfect. After 5 minutes incubation, the two solutions were mixed, vortexed for 3 second, incubated room temperature (RT) for 15 minutes and added to the cells.

Acyl-RAC to isolate palmitoylated proteins

The protocol to detect palmitoylated protein was adapted from a published protocol (Ren et al., 2013). Briefly, protein lysates were incubated overnight (O/N) at room temperature (RT) with 0.5% methyl methanethiosulfonate (MMTS) (Sigma-Aldrich) to block free cysteine residues. Proteins were then precipitated by adding 2 volumes of cold acetone and incubated at -20C° for 2 hours. After washing with cold acetone, the pellet was resuspended in binding buffer (100 mM HEPES, 1 mM EDTA, 1% SDS). Water-swollen thiopropyl sepharose 6B (GE Healthcare Life Sciences, Mississauga, ON) was added and samples were divided into two equal parts. One part was treated with hydroxylamine (Sigma-Aldrich) (NH2OH) pH7.5 to a final concentration of 0.2M to cleave palmitate residues off of proteins, the other part was treated with an equal amount of NaCl as control. After 3 hours incubation at RT, beads were washed 5 times with binding buffer and captured proteins were eluted with 75mM DTT.

Membrane separation assay

24 hours post-transfection, cells were harvested, snap frozen with liquid nitrogen and allowed to thaw at room temperature. Samples were resuspended in buffer 1 (0.1 M Mes-NaOH pH 6.5, 1 mM MgAc, 0.5 mM EGTA, 200 µM sodium orthovanadate, 0.2 M sucrose) and centrifuged at 10 000 g for 5 min at 4°C. The supernatant containing cytosolic proteins was collected (S, supernantant) and the pellet was resuspended in buffer 2 (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) and spun at 10 000 g for 5 min at 4 °C. The supernatant containing membrane proteins was collected (P, pellet fraction) for further analysis. Equal volumes of each fraction were loaded for Western blotting. The intensity of the bands on Western blots was determined using Fiji (Schindelin et al., 2012). Analysis of the distribution of proteins between the 2 fractions was performed by dividing the intensity of each fraction by the total in each condition and expressed as a percentage.

CRISPR/Cas9 Rab7-KO cell line

HEK-293 were transfected with an all-in-one CRISPR plasmid for Rab7 (Genecopoeia, plasmid # HTN218819). 72 hours post-transfection, cells were treated with 1mg/ml Geneticin (Thermo Fisher Scientific) for 1 week. Limiting dilution was performed to isolate single cells. Single

clones were allowed to grow for approximately two weeks prior to testing via immunoblotting to identify Rab7-KO clones.

Immunofluorescence

HEK-293 or U2OS cell immunofluorescence was performed as previously described (Dumaresq-Doiron et al., 2010). Briefly, cells were seeded on glass coverslips overnight and subsequently transiently transfected with indicated plasmids. 48 hours post-transfection, cells were washed once in PBS, fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature (RT) and washed twice with PBS. Cells were permeabilized with 0.1% Triton X-100, Bovine Serum Albumin (BSA) (Fisher Scientific) 1% in PBS for 5 minutes RT, washed once with PBS, and incubated for two hours with primary antibodies in a solution of 0.1% BSA in PBS. Cells were then washed 3 times in PBS and incubated 1 hour at RT with appropriate secondary antibodies conjugated to either AlexaFluor-594 (Thermo Fisher Scientific) or Alexa-Fluor-488 in a solution of 0.1% BSA in PBS. Cells were washed once with PBS and incubated with DAPI (Thermo Fisher Scientific) in PBS for 5 minutes at RT. Cells were then washed three times with PBS and mounted on glass slides with Fluoromount G (Fisher Scientific). Quantification of fluorescence intensity was performed using Fiji software by manually outlining either non-rescued or rescued Rab7-KO cells. Quantification of co-localization was performed using Fiji (Schindelin et al., 2012) Coloc2 by manually outlining the doubly labeled cells.

BRET2 Assay

BRET assay was performed as previously described (Gales et al., 2006; Kobayashi et al., 2009). Briefly, HEK-293 cells were seeded in 12 well plates (Fisher Scientific) and transfected with the indicated plasmids. 48 hours post-transfection, cells were washed once with PBS, detached with 5 mM EDTA in PBS and resuspended in 500 µl of PBS. Cell suspensions were distributed into 96 wells plate (Fisher Scientific), each samples was plated in triplicate. BRET signal was measured after the addition of the RLucII substrate DeepBlueC coelenterazine (Cedarlane Laboratories) to a final concentration of 5 µM. Readings were performed using the Infinite M1000Pro (Tecan, Chapel Hill, NC). The BRET signal was calculated as a ratio between GFP10 emission (500-535nm) over RLucII emission (370-450 nm). The BRET net signal was calculated as the difference between the total BRET signals and the one obtained from experiments where only the RLucII is expressed. The expression level of Vps26A-GFP10 was

measured by reading the emission of fluorescence at 510nm after exciting the fluorophore at 400 nm. The expression level of RLucII was measured by reading the emission at 370-450 nm after the addition of the substrate. BRET signal was plotted as function of GFP10 fluorescence over RLucII luminescence (GFP10/RLuc).

Structured Illumination Microscopy (SIM)

U2OS cells were seeded in 8-well LabTek #1.5 borosilicate chambered coverglass imaging dishes (Fisher Scientific) to 50% - 60% confluency. Wild-type myc-Rab7 and myc-Rab7C83,84S were transiently expressed for 36 hours in separate wells. Cells were then fixed with 2% paraformaldehyde (PFA) at 22° C (RT) for 20 minutes. Fixed cells were washed three times with 25 mM ammounium chloride (NH_4Cl) and 50 mM glycine to remove excess PFA and reduce autofluorescence and then three times with phospho- buffered saline (PBS). The cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS and blocked with 10% FBS (v/v) in PBS. Immunostaining was performed using anti-myc and anti-Vps26A antibodies. An anti-mouse Alexa 488 secondary antibody (Thermo Fisher Scientific) and an anti-rabbit Alexa 647 secondary antibodies (Thermo Fisher Scientific) were then used to indirectly label proteins with green and far-red fluorescence, respectively. After immunostaining, cells were washed three times with PBS prior to imaging. Imaging of samples was performed using structured illumination on the Zeiss Elyra PS.1 super-resolution inverted microscope. Samples were imaged at an effective magnification of 101x (63x objective + 1.6x optovar tube lens) on an oil immersion objective. Typically, 10 to 20 slices of 0.110 µm were captured for each field of view for an imaging volume of approximately 1.1 to 2.2 µm. A 647 nm (and subsequently 488 nm) laser line was directed into the microscope optical train via a multimode fiber coupler. The laser was passed through a diffraction grating, and a series of diffraction orders (-1, 0, +1) were projected onto the back focal plane of the objective. These wavefronts were collimated in the objective to create a three-dimensional sinusoidal illumination pattern on the sample. The diffraction grating was then rotated and translated throughout the acquisition to create patterned offset images containing higher spatial frequency information as compared to widefield imaging. Five lateral positions were acquired at each of three diffraction grating rotations (120°) for a total of 15 raw images per slice. SIM imaging with both the 488 nm and 647 nm laser was carried out at 50 ms exposures with laser power varying between 3-10%, and a gain level of 60-80. Raw SIM image stacks were processed in Zen software under the Structured Illumination toolbar. A series of parameters were set to generate an optical transfer function (OTF) used for 3D

reconstruction. The noise filter for Wiener de-convolution was set to a value of -6.0, and the maximum isotropy option was left unselected to recover all available frequency information at exactly the 120° rotation angles. Super resolution frequency weighting was set 1.0. Negative values arising as an artifact of the Wiener filter were clipped to zero using the Baseline Cut option. Sectioning filters used to remove the 0-frequencies from the 0th order and non-shifted 1st order (+1, -1) were set to 100 and 83, respectively. Processed SIM images were then aligned via an affine transformation matrix of pre-defined values obtained using 100 nm multicolor Tetraspeck fluorescent microspheres (Thermo Fisher Scientific). Dual-channel aligned images were analyzed using the co-localization function in Zen. Regions of interest were drawn in the cytoplasm (omitting the nucleus) of 10-20 cells for cells expressing WT and mutant Rab7. The average Manders co-localization coefficients over the imaging volume were calculated for both channels. The average Manders co-localization coefficients and standard deviations were calculated across the sample size for wild-type and all mutants.

Cathepsin D Secretion assay

A cathepsin D secretion assay was performed as previously described (Follett et al., 2016b). Briefly, HEK-293 and Rab7-KO cells were transfected with the indicated plasmids. 48 hours post-transfection, cells were incubated in serum-free media (OptiMem, Thermo Fisher Scientific) supplemented with 100 µg/ml of cycloheximide and chased for 4.5 hours. The media was collected, precipitated with trichloroacetic acid and the pellet was resuspended in Laemmli sample buffer. Cells were collected, lysed in TNE (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail) for 30 minutes on ice as previously described (Dumaresq-Doiron et al., 2013). The precipitated proteins collected from the media and the cell lysate were analyzed by Western blotting.

EGFR degradation assay

HEK-293 or Rab7-KO cells were transfected or not with myc-Rab7 and the various Rab7 mutants. 48 hours post-transfection, the serum-starved cells were treated with 50 µg/ml cycloheximide for 1 h to prevent de novo synthesis EGFR during EGF stimulation. Cells were subsequently stimulated with 100 ng/ml EGF for the indicated times, harvested and lysed in TNE as described above. Following Western blotting, the levels of EGFR were determined by

Western blotting and analyzed using Fiji software. The amount of EGFR remaining at each time point was calculated relative to the non treated.

EGF-488 pulse-chase

Cells were seeded on coverslips. 24 hours later, cells were transfected with wild-type myc-Rab7 or mutant constructs. 24 hours post-transfection, cells were serum-starved in Opti-Mem for one hour followed by a 30-minute pulse of Alexa Fluor 488 EGF complex (ThermoFisher) at a concentration of 300 ng/ml. Cells were then washed with PBS and fixed in 4% paraformaldehyde at the following chase time points: 0, 15 or 60 minutes. Cells were immunostained with anti-myc primary and Alexa Fluor 594 secondary antibodies. Cells were imaged using Zeiss LSM 780 confocal microscope. The number of puncta per cell was counted manually (20 cells per condition for each time point).

Statistics

Statistical analysis was performed using GraphPad Prism Version 7 (GraphPad Software Inc., La Jolla, CA) and described in the corresponding figure legend.

Author Contributions

Conceptualization, S.L., G.M., H.W.; Design (Methodology), S.L., G.M., H.W., E.S., O.S., P.K.K.; Investigation, G.M., O.S., E.S., A.V.; Analysis, G.M., O.S., E.S., H.W., A.V., P.K.K., S.L. Writing – Original Draft, S.L. G.M.; Writing – Review & Editing, S.L., G.M., O.S., E.S., P.K.K., C.M.Y.; Funding Acquisition, S.L., H.W.; Supervision, S.L., H.W., C.M.Y., P.K.K.

Acknowledgments

We would like to thanks Dr. Peter J. McCormick (University of Surrey) for critical reading of the manuscript and for helpful discussions.

Competing Interests

The authors declare no conflicts of interest.

Funding

This work was supported by Canadian Institutes of Health Research operating grants to SL (MOP-10754) and HW (MOP-123438) and Alzheimer Society of Canada to SL (Grant 14-27). SL and HW are supported by salary awards from Fonds de recherche du Quebec - Santé. OS is supported by a post-doctoral fellowship from the Natural Sciences and Engineering Research Council. The authors have no conflicts of interest to declare.

3.5 Figures

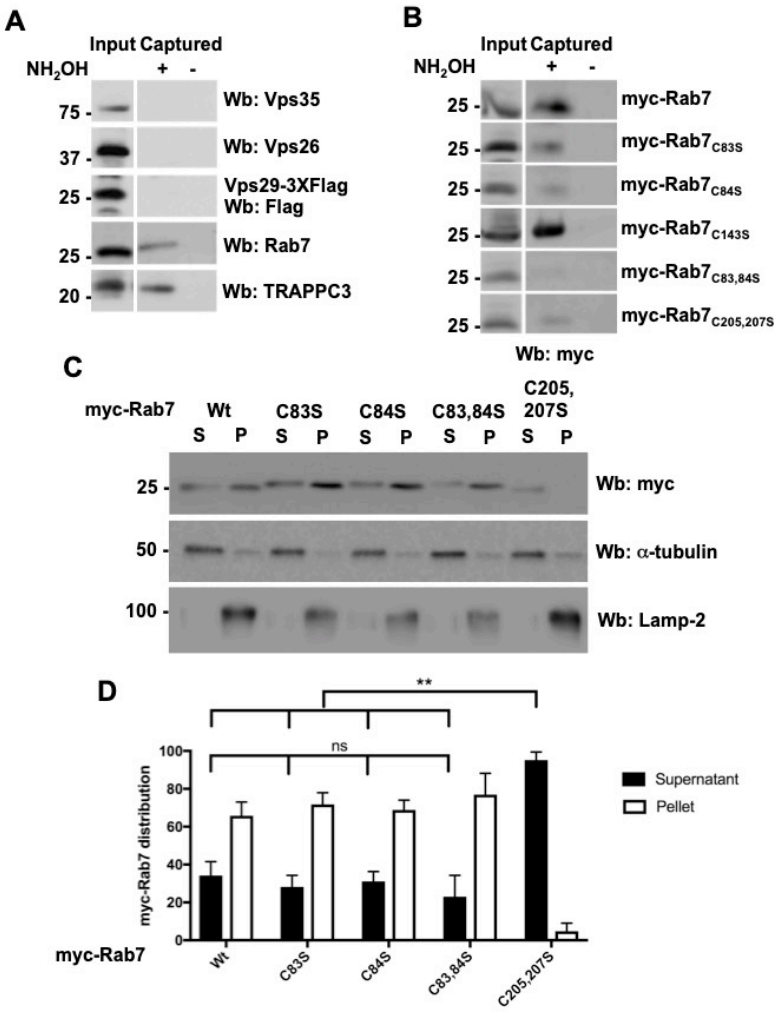

Figure 3-1 Rab7 is palmitoylated but this modification is not required for membrane binding.

(A) HEK-293 cells were transfected with Vps29-(FLAG)3 and cell lysate was obtained 24 hours post-transfection. 30µl of whole-cell lysate was kept as loading control (Input) and the rest was subjected to the Acyl-RAC protocol. Eluted samples (Pulldown) and input were loaded onto a 12% acrylamide gel and subjected to Western Blot (Wb) with anti-Vps35, anti-Vps26A, anti-FLAG, anti-Rab7 and anti-TRAPPC3. NH2OH: Hydroxylamine. (B) HEK-293 cells were transfected with myc-Rab7, myc-Rab7C83S, myc-Rab7C84S, myc-Rab7C83,84S and myc-Rab7C205,207S. 24 hours post-transfection, cell lysates were subjected to Acyl-RAC analysis and Wb with anti-myc antibody was performed. NH2OH: Hydroxylamine. (C) HEK-293 cells were transfected with myc-Rab7 and the indicated mutants. 24 hours post-transfection, membrane separation assays were performed and samples were analyzed via Western blot (Wb) with anti-myc, anti-Lamp-2 (a membrane marker) and anti-α-tubulin (a cytosolic marker) antibodies. (D) Quantification of membrane distribution from 4 separate membrane separation experiments as performed in A. Data is represented as mean ± SD. ns, not significant; ** $P < 0.01$; One-way ANOVA followed by Tukey's post-hoc test.

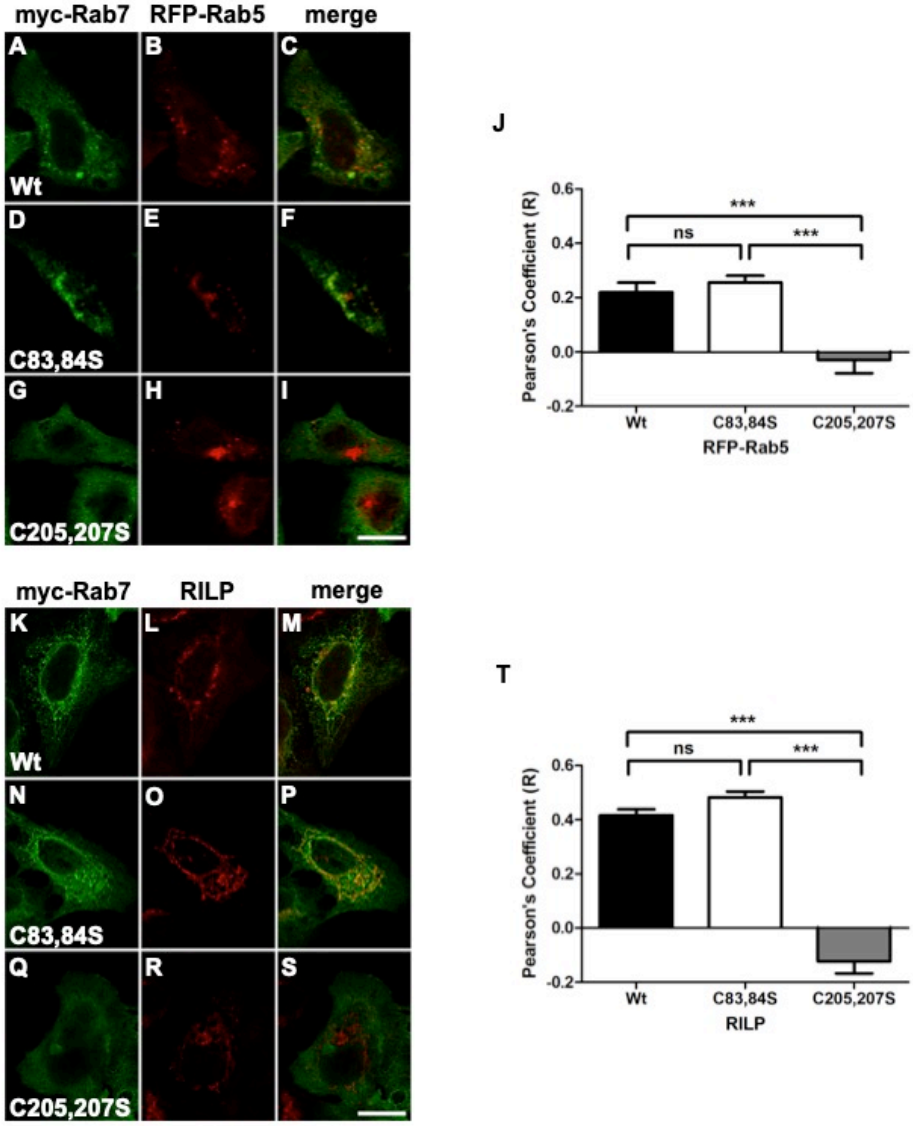

Figure 3-2 Palmitoylation is not required to localize Rab7 to late endosome.

(A - I) U2OS cells co-expressing myc-Rab7 (A and C), myc-Rab7C83,84S (D and F) or myc-Rab7C205,207S (G and I) and RFP-Rab5 were immunostained with anti-myc antibody. Representative images are shown. Scale bar = 10 μm. (J) Pearson's correlation coefficient of myc-Rab7 (black bar), myc-Rab7C83,84S (white bar) or myc-Rab7C205,207S (grey bar) with RFP-Rab5 was calculated from 16, 13 and 16 cells respectively. One way ANOVA with Tukey's post hoc test. *** $P < 0.001$, ns, not significant. (K - S) U2OS cells expressing myc-Rab7 (K and M), myc-Rab7C83,84S (N and P) or myc-Rab7C205,207S (Q and S) were immunostained with anti-myc and anti-RILP antibodies. Representative images are shown. Scale bar = 10 μm. (T) Pearson's correlation coefficient of myc-Rab7 (black bar), myc-Rab7C83,84S (white bar) or myc-Rab7C205,207S (grey bar) with RILP was calculated from 27, 19 and 23 cells respectively. One-way ANOVA with Tukey's post hoc test. *** $P < 0.001$, ns, not significant.

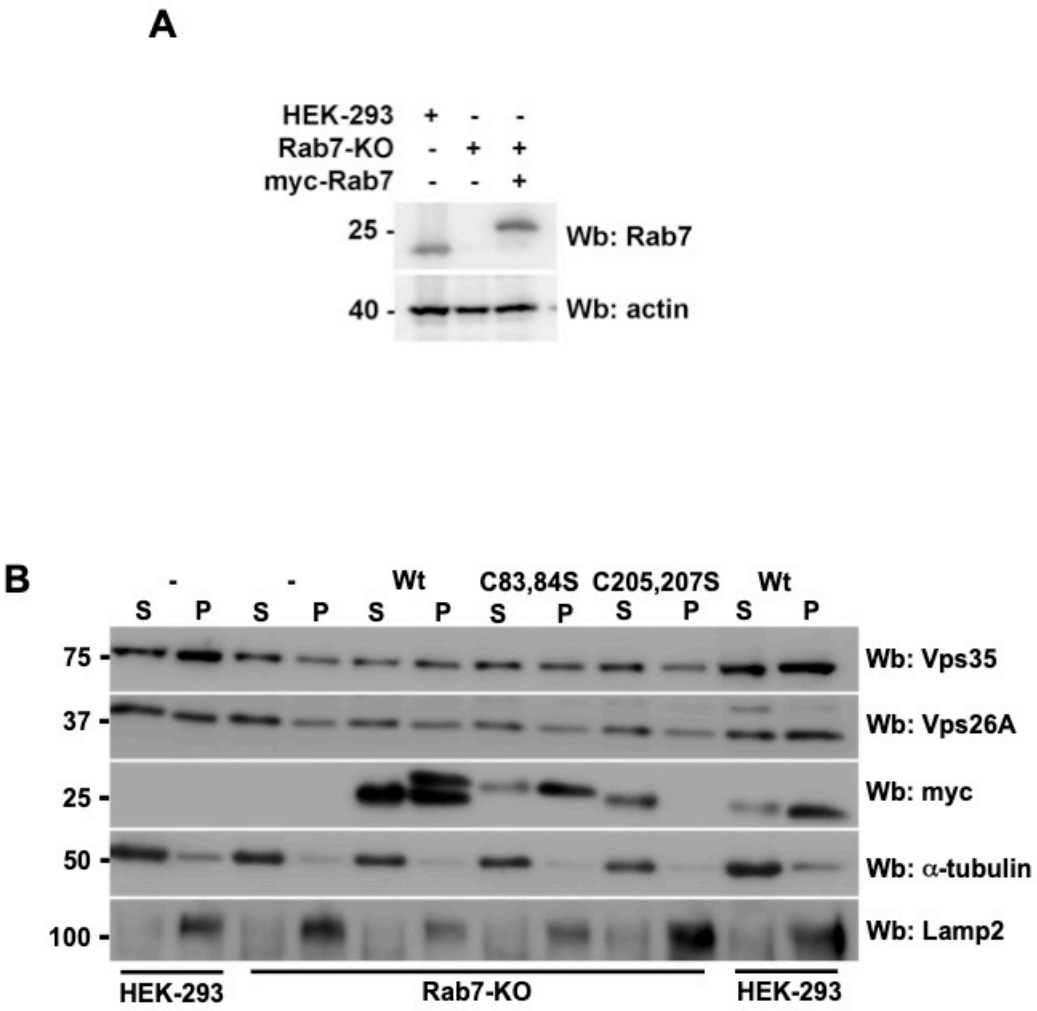

Figure 3-3 Rab7 palmitoylation is required for retromer recruitment.

(A) Whole-cell lysates of HEK-293, Rab7-KO cells (generated using CRISPR/Cas9), or Rab7-KO cells transiently transfected with myc-Rab7 were analyzed via Western blot (Wb) with anti-Rab7 antibody. Anti-actin staining was used as a loading control. (B) HEK-293 or Rab7-KO cells were transfected with myc-Rab7 and indicated mutants. 48 hours post-transfection, a membrane separation assay was performed and samples were subjected to Western blot (Wb) with anti-myc, anti-Vps26A, anti-Vps35, anti-α-tubulin (a cytosolic marker) and anti-Lamp2 antibody (a membrane marker).

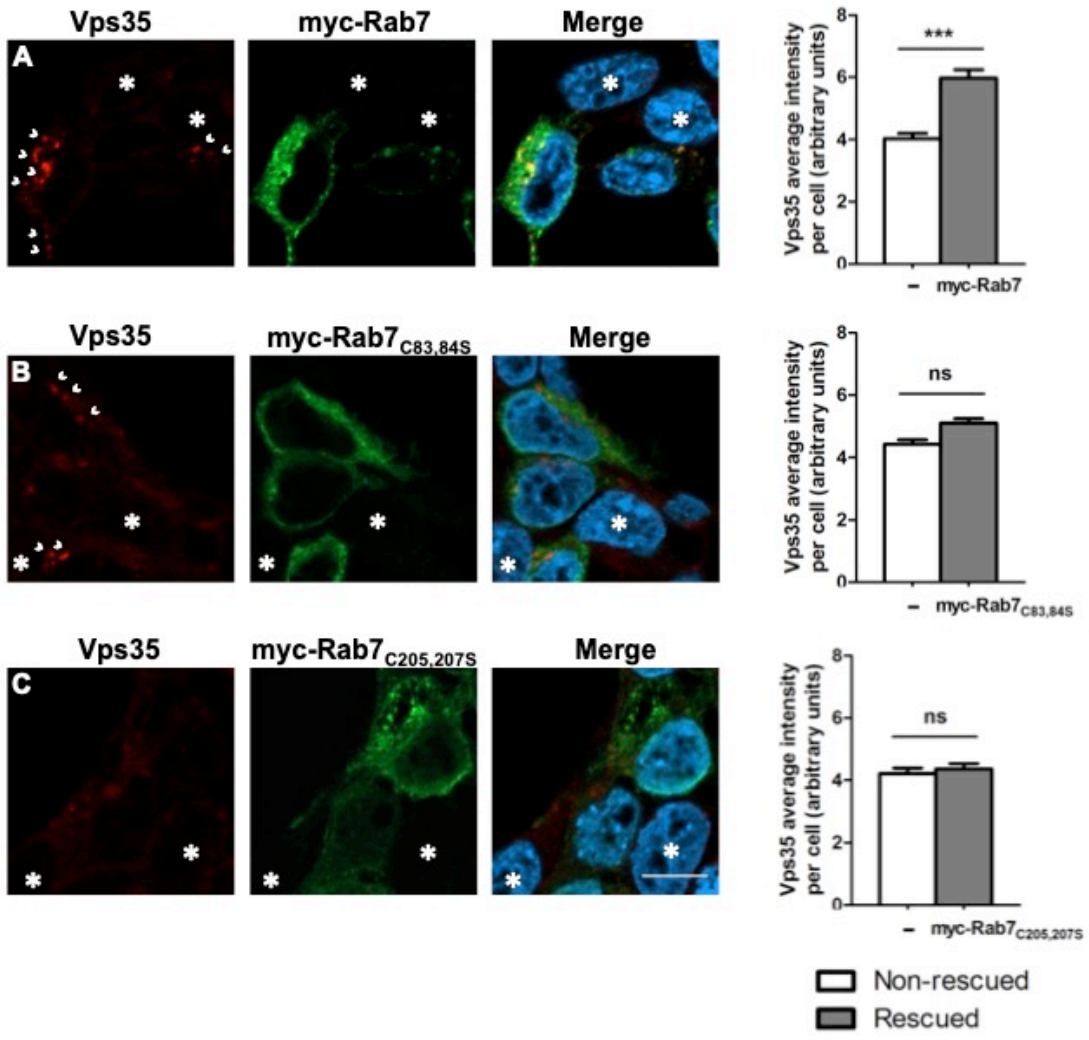

Figure 3-4 Rab7$_{C83,84S}$ is not efficient in recruiting retromer to endosomes.

(A) HEK-293 cells were fixed with 4% PFA and immunostained with anti-Vps35 antibody (red, arrowheads). Graph shows the average Vps35 puncta intensity in HEK-293 cells (black bar). Data represented as mean ± SEM. (B) Rab7-KO cells were transfected with myc-Rab7. 24 hours post-transfection, cells were fixed with 4% PFA and immunostained with anti-myc (green) and anti-Vps35 antibodies (red, arrowheads). Fluorescence intensity of Vps35 puncta from 55 transfected and 59 non-transfected cells (stars) was determined. Graph shows the average Vps35 puncta intensity in Rab7-KO cells (white bar) and Rab7-KO cells expressing myc-Rab7 (grey bar). Data represented as mean ± SEM. *** $P < 0.0001$ (C) Same as in B, but cells were transfected with myc-Rab7$_{C83,84S}$. Fluorescence intensity of Vps35 puncta from 57 transfected and 57 non-transfected cells (stars) was determined. Graph shows the average intensity of Vps35 puncta in Rab7-KO cells (white bar) and Rab7-KO cells expressing myc- Rab7$_{C83,84S}$ (grey bar). Data represented as mean ± SEM. ns, not significant (D) Same as in B, but cells were transfected with myc-Rab7$_{C205,207S}$. Fluorescence intensity of Vps35 puncta from 58 transfected and 57 non-transfected cells (star) was determined. Graph shows the average Vps35 puncta intensity in Rab7-KO cells (white bar) and Rab7-KO cells expressing myc- Rab7$_{C205,207S}$ (grey bar). Data represented as mean ± SEM. Ns, not significant. Scale bar = 10 µm.

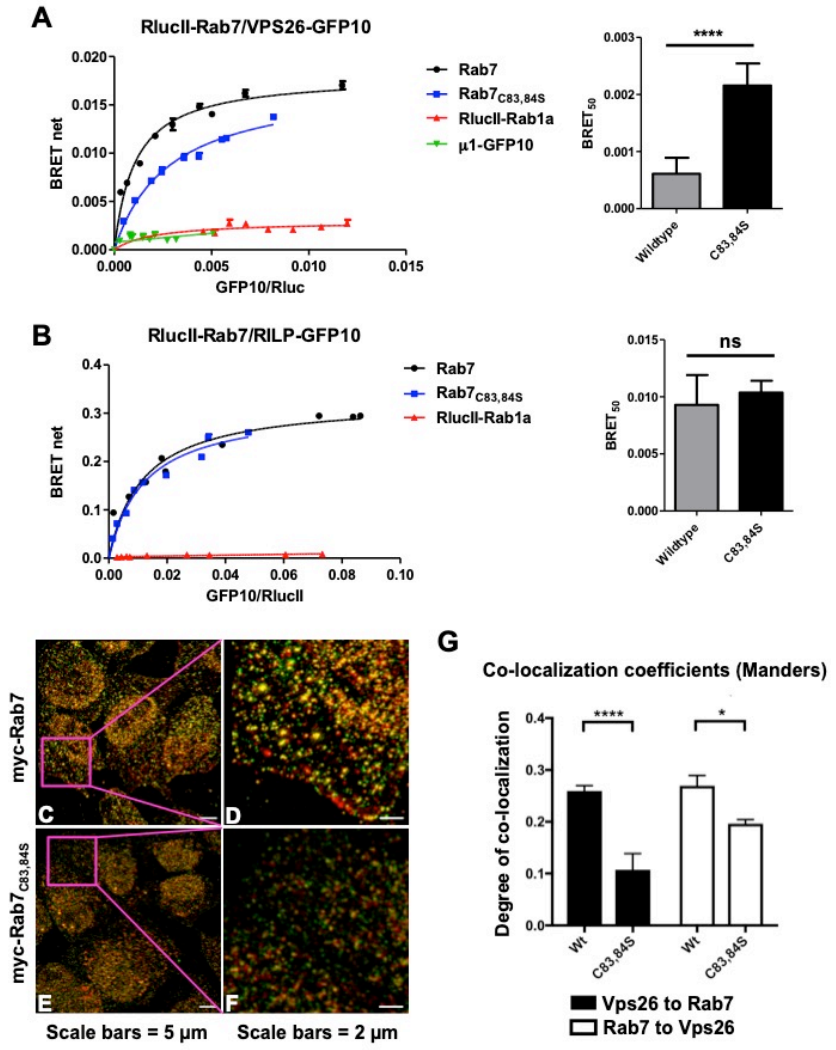

Figure 3-5 Palmitoylation modulates the interaction between Rab7 and retromer

HEK-293 cells were transfected with a constant amount of RLucII-Rab7, RLucII-Rab7$_{C83,84S}$ or RLucII-Rab1a and increasing amounts of Vps26A-GFP10 or µ1-GFP10. 48 hours post-transfection BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RLucII luminescence. BRET$_{50}$: concentration of the acceptor required to obtain 50% of BRET$_{MAX}$. The average of the BRET$_{50}$ from 3 separate experiments is shown. Data is represented as mean ± SD. **** P < 0.0001; two-tailed paired t-test. (B) HEK-293 cells were transfected with a constant amount RLucII-Rab7, RLucII-Rab7$_{C83,84S}$ or RLucII-Rab1a increasing amounts of RILP-GFP10. BRET$_{50}$: concentration of the acceptor required to obtain 50% of BRET$_{MAX}$. The average of the BRET$_{50}$ from 3 separate experiments is shown. Data is represented as mean ± SD. ns, not significant; two-tailed paired t-test. (C - F) U2OS cells were transiently transfected with either myc-Rab7 (C and D) or Rab7$_{C83,84S}$ (E and F) and immunostained with anti-myc (green, C - F) and anti-Vps26A (red, C - F) antibodies and analyzed via SIM. Images on the right are a magnified area. (G) 10 to 20 cells per condition were analyzed and the average Mander's co-localization coefficient was calculated. Data is represented as mean ± SEM. **** P < 0.0001; * P < 0.05, two-tailed unpaired t-test

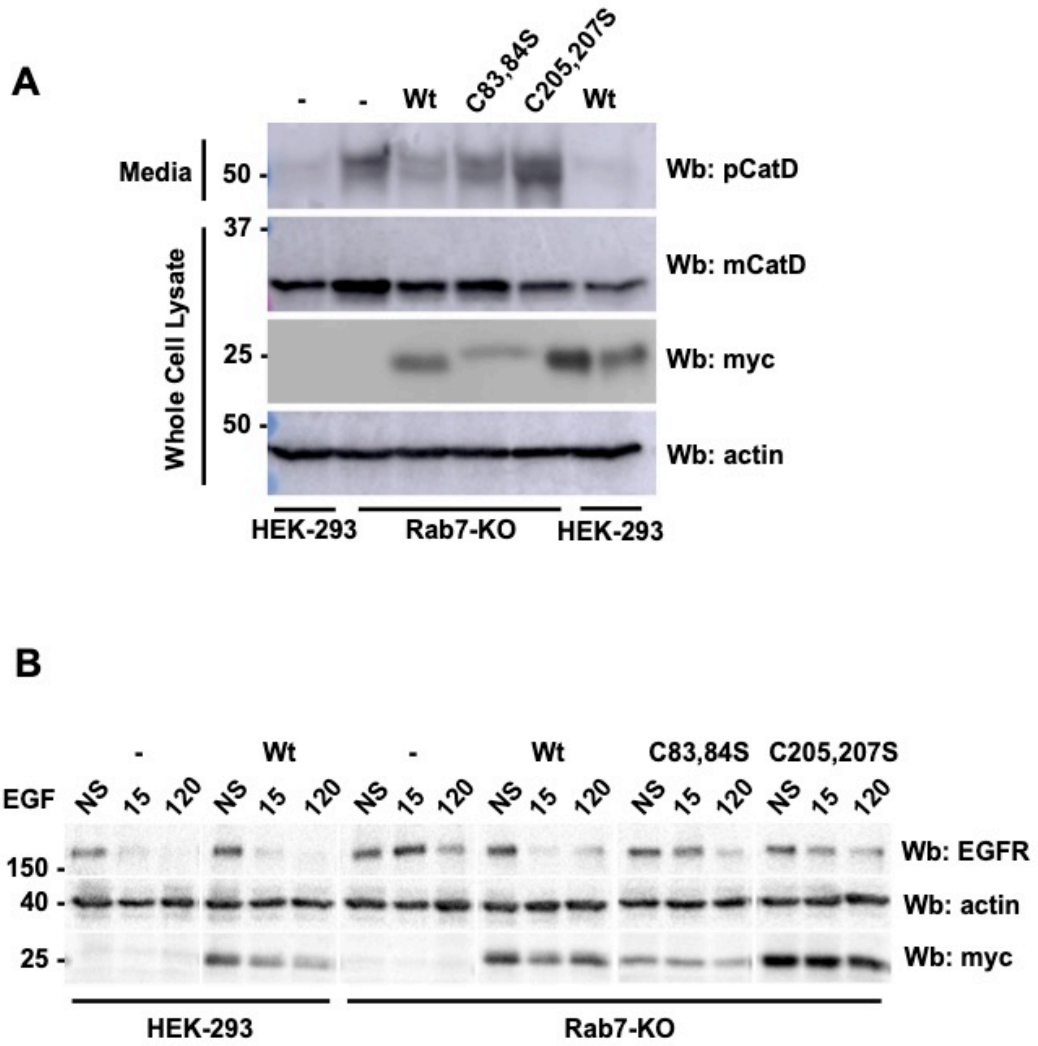

Figure 3-6 $Rab7_{C83,84S}$ is not efficient in regulating endosome-to-TGN cargo-receptor retrieval.

(A) HEK-293 or Rab7-KO cells were transfected with either wild-type myc-Rab7 or the indicated mutants. 48 hours post-transfection, the media was changed to Opti-MEM supplemented with cycloheximide and collected after 4.5 hours. Proteins were precipitated and resuspended in Laemmli buffer (Conditioned media). Whole cell lysate and conditioned media were run on a 12% polyacrylamide gel and Western blotted (Wb) with anti-cathepsin D, anti-myc and anti-actin antibodies. (B) HEK-293 or Rab7-KO cells were transfected as indicated. After a 1 hour treatment with 50 µg/ml of cycloheximide to prevent de novo protein synthesis, cells were treated with 100 ng/ml of EGF for either 15 or 120 minutes. Non-stimulated (NS) cells served as control. After EGF stimulation, the cells were lysed and whole cell lysate was run on a 12% polyacrylamide gel, transferred to nitrocellulose membrane and Western blotting (Wb) was performed using anti-EGFR, anti-actin and anti-myc antibodies.

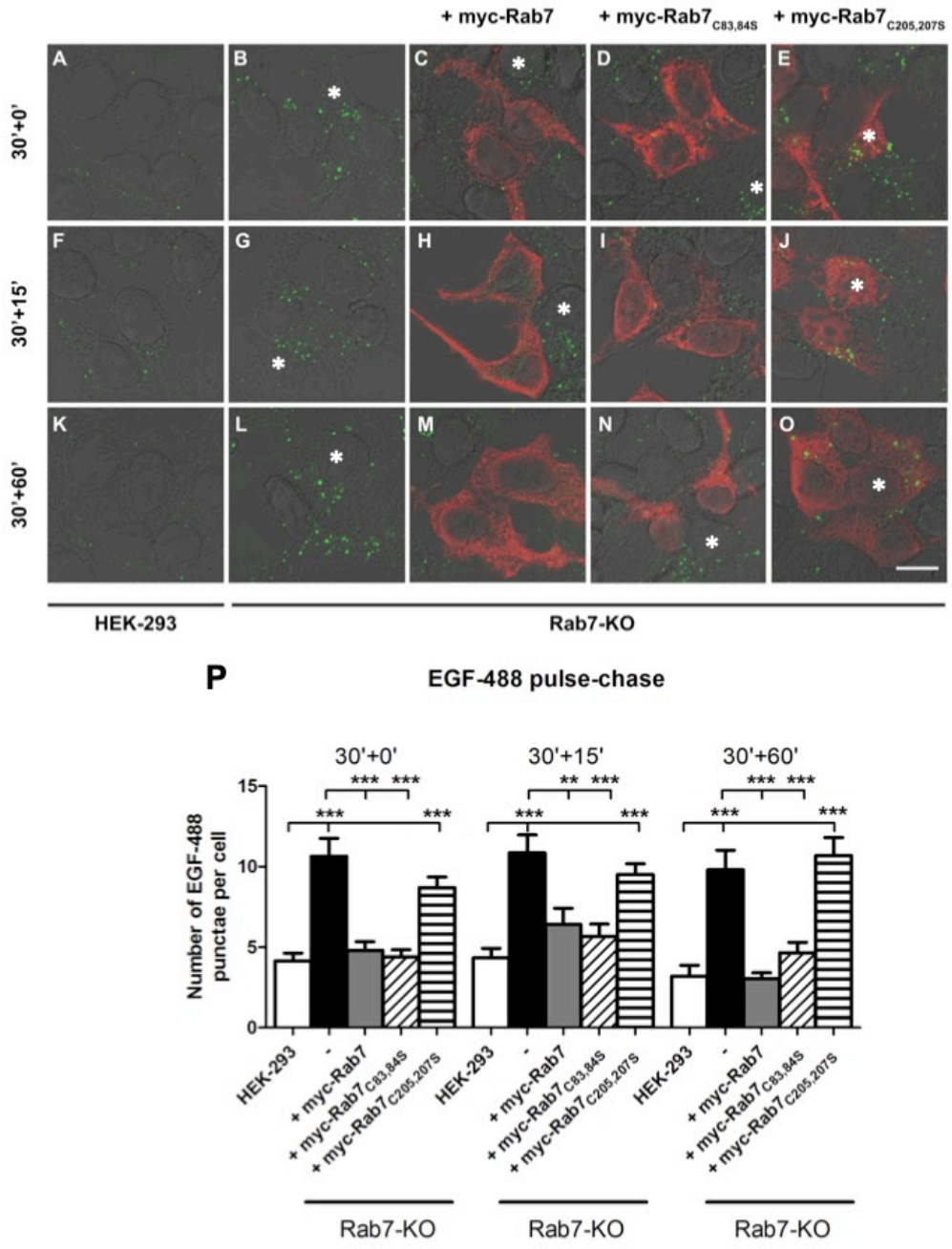

Figure 3-7 Rab7 palmitoylation is not required for EGF degradation

(A - O) HEK-293 (A, F and K), Rab7-KO cells (B, G and L), Rab7-KO cells transiently expressing myc-Rab7 (C, H and M), myc-Rab7$_{C83,84S}$ (D, I and N) or myc-Rab7$_{C205,207S}$ (E, J and O) were incubated with 300 ng of Alexa-488 tagged EGF and chased for the indicated times. Stars highlight cells accumulating EGF-488 that are not expressing Rab7 or one of the Rab7 mutants. Representative images are shown. (P) Quantification of 20 cells per condition. Data is represented as mean ± SD. *** P < 0.0001, ** P < 0.01, One-way ANOVA with Tukey's post-hoc test

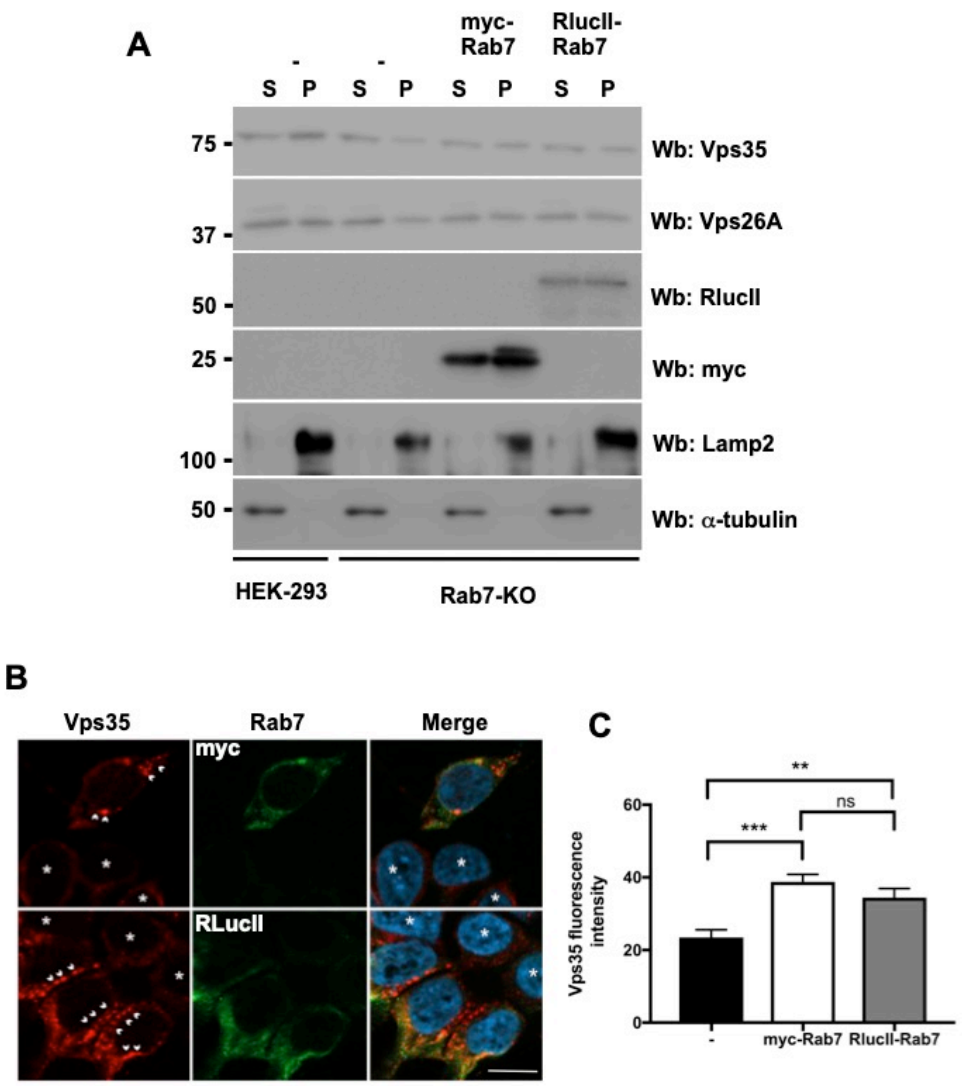

Figure 3-(S) 1 RLucII-Rab7 is properly membrane recruited and functional

HEK-293 or Rab7-KO cells were transfected with myc-Rab7 or RLucII-Rab7. 48 hours post-transfection, a membrane separation assay was performed and samples were subjected to Western blot (Wb) with anti-Vps35, anti-Vps26A, anti-RLucII, anti-myc, anti-Lamp2 antibody (a membrane marker) and anti-α-tubulin (a cytosolic marker). (B) HEK-293 or Rab7-KO cells were transfected with myc-Rab7 or RLucII-Rab7. 48 hours post-transfection, the cells were fixed in 4% paraformaldehyde and immunofluorescence staining was performed with anti-myc (green) or anti-RLucII (green) and anti-Vps26A (red) antibodies. Scale bar = 10 μm. (C) Fluorescence intensity of Vps35 staining was measured in Rab7-KO cells (black bar), Rab7-KO cells expressing myc-Rab7 (white bar) or RLucII-Rab7 (grey bar). Data is represented as mean Vps35 intensity ± SEM from 31, 14, and 16 cells per condition. *** $P < 0.001$, ** $P < 0.01$, ns, not significant, One-way ANOVA with Tukey's post-hoc test.

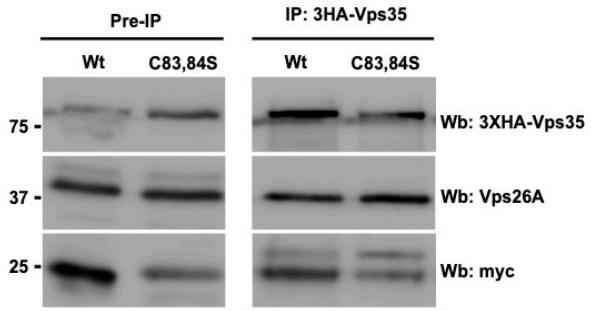

Figure 3-(S) 2 Palmitoylation does not affect the Rab7/retromer interaction *per se*.

HEK-293 were transfected with myc-Rab7, myc-Rab7$_{C83,84S}$ or (HA)3-Vps35. 24 hours after transfections, equal amounts of cell lysates from myc-Rab7 or myc-Rab7$_{C83,84S}$ transfected cells were mixed with lysate from HEK-293 expressing (HA)3-Vps35, followed by immunoprecipitation with anti-HA antibody. Eluted samples were loaded onto a 12% polyacrylamide gel and subjected to Western blot (Wb) analysis with anti-myc, anti-Vps26A and anti-HA antibodies

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Preface to the next paper

With my first paper, we showed that palmitoylation modulates the ability of Rab7 to recruit retromer on the endosomal membrane. While this paper was in preparation, the work of Francavilla et al., Lin et al., and Shinde & Maddika were published describing the role of phosphorylation in modulating Rab7 functions. However the conclusions drawn from the above mentioned works were not clear in defyining the role of these modifications in regulating Rab7 function.

This prompted us to ask how phosphorylation regulates Rab7 functions and in particular if this PTM was also playing a role in controlling Rab7/retromer interaction.

To answer these questions we decided to generate Rab7 phospho-mimetic and phospho-null mutants and to analyze via BRET their interactions with Rab7 effectors. We also decided to use our previously generated Rab7-KO cell line to perform functional studies to understand how these modifications affected Rab7 downstream pathways.

5 THE ROLE OF PHOSPHORYLATION IN MODULATING RAB7 FUNCTION

Authors

Graziana Modica [1], Olga Skorobogata [1], and Stephane Lefrancois [1, 2]

[1] Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique, Laval, Québec, Canada H7V 1B7

[2] Department of Anatomy and Cell Biology, McGill University, Montreal, Canada H3A 0C7 and Centre d'Excellence en Recherche sur les Maladies Orphelines - Fondation Courtois (CERMO-FC), Université du Québec à Montréal (UQAM), Montréal, Canada H2X 3Y7

Correspondence to: Stephane Lefrancois, PhD stephane.lefrancois@iaf.inrs.ca

Manuscript in preparation

Author contribution

Conceptualization, S.L., G.M. Design (Methodology), S.L., G.M., Investigation G.M., O.S., Writing - Original Draft, S.L., G.M: Writing - Review & Editing S.L., G.M., Funding Acquisition, S.L.

Abstract

The small GTPAse Rab7 has a key role in regulating membrane trafficking at late endosomes. By interacting with several downstream effectors, this small GTPase controls late endosome mobility, orchestrates fusion events between late endosomes and lysosomes, and participates in the formation of and regulates the fusion between autophagosomes and lysosomes. At late endosomes, Rab7 is also responsible for the spatiotemporal recruitment of retromer, which is necessary for the endosome-to-TGN trafficking of cargo-receptors such as sortilin and CI-MPR. Recently, several post-translational modifications have been shown to modulate Rab7 functions, but the mechanisms behind this regulation are not completely understood. In this work, we investigated the role of phosphorylation in regulating Rab7 functions. We show that phosphorylation of Rab7 at serine 72 is important to modulate its interaction with retromer as the non-phosphorylatable $Rab7_{S72A}$ mutant is not able to interact with and recruit retromer to late endosomes. We have previously shown that Rab7 palmitoylation is also required for efficient retromer recruitment. Interestingly palmitoylation of $Rab7_{S72A}$ is reduced compared to the wild-type protein, suggesting an interplay between serine 72 phosphorylation and palmitoylation in tightly regulating the Rab7/retromer interaction. We also show that Rab7 phosphorylation on Y183 is required for the degradation of EGF as the non-phosphorylatable $Rab7_{Y183F}$ does not rescue EGF degradation in Rab7-KO HEK293 cells. Overall, our data supports a role for post-translational modifications in regulating various Rab7 pathways.

5.1 Introduction

Late endosomes (LE) are a highly dynamic compartment where the fate of proteins arriving from various cellular compartments is decided. The small GTPase Rab7A (from now on Rab7) is the principal regulator of trafficking events at late endosomes. Indeed by engaging different effectors, Rab7 can coordinate several pathways. The movement and positioning of late endosomes is dependent on the interaction between Rab7 and RILP with the oxysterol-binding protein–related protein (ORP)1L [1] and by the ability of RILP to recruit the $p150^{Glued}$ subunit of the dynein motor complex for the minus end-directed transport on microtubules [2, 3]. The Rab7/RILP complex has also been shown to be required for the efficient fusion between late endosomes and lysosome by recruiting the homotypic fusion and vacuole protein sorting (HOPS) complex, the tethering complex required for the fusion at the late endosome [4]. For efficient fusion to occur, a second Rab7 effector, Pleckstrin homology domain containing protein family member 1 (PLEKHM1) [5] is recruited. The latter interacts with HOPS, and contributes to the stability of the tethering complex on vesicle membranes [6]. Finally, Rab7 can interact and recruit retromer, which is required for endosome-to-trans Golgi network (TGN) cargo retrieval [7, 8]. An increasing amount of data highlights the role of post-translational modifications (PTMs) as a mechanism that regulates multiple Rab7 functions [9]. Rab7 is irreversibly prenylated right after its translation on two c-terminal cysteines (C205, C207), and this modification is required for its proper membrane localization. Indeed, the $Rab7_{C205,207S}$ mutant fails to bind to endosomal membranes and appears mostly cytosolic [10]. In addition to prenylation, other modifications such as ubiquitination [11, 12], phosphorylation [13, 14] and palmitoylation [10] have been shown to play a role in regulating Rab7 function.

We have previously shown that Rab7 can be palmitoylated on cysteines 83 and 84 and that this reversible modification is required for efficient retromer recruitment and function [10]. Retromer is an evolutionary conserved complex composed of a trimer of vacuolar sorting proteins (Vps) 26, Vps35 and Vps29, that is responsible for the endosome-to-TGN retrieval of the lysosomal cargo receptors sortilin and Cation-Independent Mannose-6-phosphate receptors (CI-MPR) bind binding to their cytosolic tails [15-17]. At the TGN, these receptors recognize and bind lysosomal resident proteins such as cathepsin D and prosaposin (cargos), and mediate their trafficking to the endosome via clathrin coated vesicles [18]. Once at the endosome, the acidic pH induces the release of cargo that is eventually trafficked to the lysosome [19-21]. The now empty receptor is recognized and bound by retromer, and trafficked back to the TGN for a subsequent round of trafficking [16]. Impaired retromer function results in the accelerated

lysosomal degradation of CI-MPR and sortilin and dysfunction of lysosomes [15, 22]. Rab7 is required for retromer recruitment at the late endosome, and down regulation or knock out of this small GTPase results in a significant displacement of retromer from the membrane to the cytosol [7, 8, 10]. Palmitoylation modulates the ability of Rab7 to efficiently bind retromer. Indeed in Rab7-KO HEK293 cells rescued with non-palmitoylatable Rab7 ($Rab7_{C83,83S}$), retromer is not efficiently recruited to late endosomal membranes [10].

Rab7 can be phosphorylated on at least two sites, serine 72 (S72) and tyrosine 183 (Y183). Leucine rich repeat kinase 1 (LRRK1) has been shown to phosphorylate Rab7 on S72, leading to an increased interaction with the Rab7 effector, Rab7 Interacting Lysosomal Protein (RILP) [23]. Tank Binding Kinase 1 (TBK1) also phosphorylates Rab7 on S72, and this PTM is necessary for mitophagy [24]. Finally, Y183 phosphorylation is mediated by Src kinase and inhibits the ability of Rab7 to interact with RILP [25].

In this work, we show that the non-phosphorylatable Rab7 ($Rab7_{S72A}$) mutant is not able to interact with retromer, and fails to rescue retromer membrane recruitment when expressed in Rab7-KO HEK293 cells. This phenotype recapitulates the behaviour of non-palmitoylatable Rab7 mutants, and indeed we show that $Rab7_{S72A}$ is not efficiently palmitoylated. Furthermore, we show that tyrosine phosphorylation of Rab7 is required for EGF degradation, and that this process is independent of the interaction between Rab7 and RILP.

5.2 Results

5.2.1 $Rab7_{S72A}$ does not efficiently bind retromer

To investigate the role of phosphorylation in modulating the ability of Rab7 to interact with retromer, we first generated Rab7 mutants mimicking constitutively phosphorylated forms (phopshomimetic, $Rab7_{Y183E}$) of tyrosine 183 or non-phosphorylatable versions (phospho-null, $Rab7_{S72A}$ and $Rab7_{Y183F}$) of both serine 72 and tyrosine 183. Recently, it was shown that the phosphomimetic $Rab7_{S72E}$ does not interact with the Rab geranylgeranyl transferase (RabGGTase), the enzyme responsible for Rab7 prenylation. Hence, the protein could not properly localize to endosomal membranes because of the missing prenylated tail [24]. Therefore since we cannot establish if the effects observed would be related to the lack of this

lipid modification, or to the phosphomimetic mutation, we decided not to include this mutant in our analysis.

To test the ability of the phosphomutants to interact with retromer, we used Bioluminescence Resonance Energy Transfer (BRET). Renilla Luciferase II was fused at the N-terminus of Rab7 and the different mutants to generate RlucII-Rab7 constructs. We have previously shown that the addition of RlucII at the N-terminus of Rab7 does not alter the ability of the protein to bind membranes and to properly rescue retromer in Rab7-KO HEK293 cells [10]. We then fused GFP10 at the C-terminus of the Rab7 effector RILP (RILP-GFP10) and the Vps26A subunit of retromer (Vps26A-GFP10). We previously showed that Vps26A-GFP10 is integrated into the retromer trimer, and these effectors efficiently bind RlucII-Rab7 but not RlucII-Rab1a, suggesting specificity and functionality [10, 22]. To analyze the role of phosphorylation in modulating the Rab7/Vps26A interaction, we transfected HEK293 cells with constant amounts of RlucII-Rab7, RlucII-Rab7$_{S72A}$ (serine phospho-null), RlucII-Rab7$_{Y183E}$ (tyrosine phosphomimetic) and RlucII-Rab7$_{Y183F}$ (tyrosine phospho-null) and increasing amounts of RILP-GFP10 or Vps26A-GFP10 to generate BRET titration curves (**Figure 5.1A and C**). By plotting the BRET$_{NET}$ values as a function of the ratio between the fluorescence (the GFP10 emission) and the luminescence (the RlucII emission), we generated BRET titration curves and calculated the BRET$_{50}$ value from these curves. This value describes the propensity of a protein pair to interact, and the lower the value the stronger the interaction (**Figure 5.1B and D**). Previously published co-Immunoprecipitation (co-IP) data showed that phosphorylation on tyrosine Y183 inhibits the interaction between Rab7 and RILP [25]. We found that the BRET$_{50}$ of the phosphomimetic mutant Rab7$_{Y183E}$ is more than two fold higher than the value obtained with wild-type Rab7 (BRET$_{50}$ respectively of 0.02904±0.0046 and 0.01288±0.0060). We did not observe changes to the Rab7/RILP interaction with RlucII-Rab7$_{S72A}$ or RlucII-Rab7$_{Y183F}$ (**Figure 5.1A and B**). Since we demonstrated that we can recapitulate with BRET what was observed with co-IP [25], we tested the ability of the Rab7 phosphomutants to bind the retromer. The mutations at tyrosine 183 had no impact on the interaction (**Figure 5.1C and D**). Interestingly RlucII-Rab7$_{S72A}$ shows a two-fold increase in BRET$_{50}$ compared to wild-type Rab7 (BRET$_{50}$ respectively of 0.0061±0.0011and 0.0026±0.000512), suggesting that phosphorylation of S72 is required for the interaction with retromer (**Figure 5.1C and D**).

To determine whether the altered interaction of Rab7$_{S72A}$ with Vps26A is due to a change in the localization of the mutant protein, we co-transfected U2OS cells with the late endosome lysosomal protein Cerulean-Lamp1 with myc-tagged Rab7 (myc-Rab7) or the different

phosphomutants and performed co-localization analysis (**Figure 5.2**). Wild-type Rab7, $Rab7_{S72A}$, $Rab7_{Y183E}$ and $Rab7_{Y183F}$ all co-localize to the same extent with Cerulean-Lamp1 (**Figure 5.2A - D, F**). The Rab7 prenylation mutant, $Rab7_{C205,207S}$, appears diffused in the cytosol, and the mutant does not co-localize with Cerulean-Lamp1 as expected, since this mutant lacks the lipid anchor to bind the membrane (**Figure 5.2E and F**). As $Rab7_{S72A}$ shows no difference in co-localizing with Cerulean-Lamp1 (**Figure 5.2B and F**) compared to wild-type Rab7, this suggests the reduced binding to the retromer subunit Vps26A is not due to an altered localization of the serine phospho-null mutant.

5.2.2 Phosphorylation on S72 is required for retromer recruitment

We next asked if S72 phosphorylation is required to efficiently recruit retromer to endosomes. To answer this question, we performed rescue experiments in our previously generated Rab7-KO HEK293 cell line [10]. We transfected wild-type and Rab7-KO HEK293 cells with wild-type myc-Rab7 or the different phosphomutants and determined the intensity of Vps26A using image analysis. As Rab7 is required for retromer recruitment [7, 8], the absence of Rab7 results in the dissociation of Vps26A from the membrane causing a significant decrease of Vps26A puncta in Rab7-KO cells compared to the parental HEK293 cells (**Figure 5.3A and B**). The expression of myc-Rab7 (**Figure 5.3C**), myc-$Rab7_{Y183E}$ (**Figure 5.3E**) and myc-$Rab7_{Y183F}$ (**Figure 3F**) rescued Vps26A intensity to the same extent (**Figure 5.3H**) as wild-type Rab7. The expression of myc-$Rab7_{C205,207S}$ did not rescue Vps26A intensity (**Figure 5.3G and H**) as expected. Interestingly the phospho-null mutant, myc-$Rab7_{S72A}$, failed to rescue Vps26A intensity (**Figure 5.3D and H**), which is consistent with the lack of interaction we observed in BRET. These results suggest that Rab7 S72 phosphorylation is required for proper retromer recruitment to endosomes.

5.2.3 S72 phosphorylation modulates Rab7 palmitoylation level.

We have previously shown that Rab7 palmitoylation is required for optimal retromer recruitment and function at endosomes [10]. Given that $Rab7_{S72A}$ is not able to bind and recruit retromer, we wondered if this phenotype could be due to an impaired palmitoylation of $Rab7_{S72A}$. We therefore performed Acyl-Resin Assisted Capture (RAC) analysis and found that $Rab7_{S72A}$ palmitoylation is reduced by almost 40% compared to wild-type Rab7 (**Figure 5.4A and B**). Since non-phosphorylatable $Rab7_{S72A}$ is less palmitoylated, we wondered if increased S72 phosphorylation could result in higher Rab7 palmitoylation. TBK1 was recently shown to

phosphorylate Rab7 on serine 72 [24]. We first confirmed the kinase activity of TBK1 on Rab7 by co-transfecting TBK1 and myc-Rab7 in HEK293 cells and and used a phos-tag acrylamide gel strategy to determine Rab7 phosphorylation (**Figure 5.S1A**). We found significant phosphorylation of myc-Rab7 as shown by the shift in mobility in the phos-tag gel when expressing TBK1-myc (**Figure 5.S1A, lane 2, top arrowhead**), which was blocked when cells were also treated with the TBK1 inhibitor MRT67307 (**Figure 5.S1A, lane 3**). We then transfected HEK293 cells with increasing amounts of TBK1 and looked at Rab7 palmitoylation. Our Acyl-RAC results show that over expression of TBK1 does not lead to a significant increase in Rab7 palmitoylation (**Figure 5.4C and D**). Moreover we found that the distribution of Rab7 (**Figure 5.4E and F**), or of the retromer subunits Vps26A (**Figure 5.4E and G**) or Vps35 (**Figure 5.4E and H**) does not change upon TBK1 - dependent Rab7 phosphorylation.

Although we found no changes in Rab7 palmitoylation when we over expressed TBK1, we wondered if inhibiting the function of TBK1 could have an affect. We decided to investigate the impact on Rab7 palmitoylation if the activity of TBK1 is pharmacologically inhibited. Using this approach, we looked at endogenous Rab7 palmitoylation in HEK293 cells treated with the TBK1 inhibitor MRT67307 (**Figure 5.5A**). We found that the drug treatment leads to a decrease in Rab7 palmitoylation level by almost 25% compared to DMSO treated cells (**Figure 5.5B**). To test if this reduction in Rab7 palmitoylation was affecting retromer membrane recruitment, we decided to look at retromer distribution upon DMSO or MRT67307 treatment (**Figure 5.5D**). We found that the treatment with TBK1 inhibitor did not cause a significant displacement of Vps26A (**Figure 5.5D**) or of the Rab7 effectors RILP (**Figure 5.5E**) from the membrane. This data suggests that TBK1-dependent Rab7 phosphorylation does not have a crucial role in regulating retromer activity in basal conditions.

5.2.4 Rab7 Y183 phosphorylation is required for EGF degradation.

The phosphorylation of Rab7 on tyrosine 183 blocks the Rab7/RILP interaction (**Figure 5.1A and B)** [25]). How this modification affects the degradative pathways in cells it is still not understood. The expression of the phosphomimetic $Rab7_{Y183D}$ or $Rab7_{Y183E}$ in HeLa cells has been associated with delayed EGFR degradation [14, 25]. In other work, it was shown that the expression of the phospho-null $Rab7_{Y183F}$ blocks EGFR degradation [13]. The studies above were performed by expressing the mutant proteins in the presence of endogenous Rab7. Therefore, we decided to determine the effect of Rab7 phosphorylation on the degradation of EGF in Rab7-

KO HEK293 cells. HEK293, Rab7-KO or Rab7-KO rescued with myc-Rab7 or the phosphomutants were stimulated with Alexa-488 tagged EGF for 30 min. After a 1 hour chase, the residual amount of EGF-488 was calculated (**Figure 5.6A**). We found that wild-type HEK293 cells degraded a significant amount of EGF-488 with only 0.7500±0.9105 puncta per cell remaining (**Figure 5.6A and B**), while Rab7-KO cells did not, as 10.65±3.249 puncta remained per cell. Rab7-KO cells rescued with myc-Rab7$_{C205,207S}$ also did not degrade EGF-488 as 9.105±4.396 puncta remained (**Figure 5.6A and B**). Rab7-KO cells rescued with myc-Rab7 or myc-Rab7$_{S72A}$ did degrade EGF-488 at levels comparable to wild-type HEK293 were observed (1.778±2.074 and 2.778±2.365 puncta respectively), suggesting that phosphorylation on S72 is not required in this pathway. Interestingly, we found that phosphomimetic myc-Rab7$_{Y183E}$ rescued Rab7-KO cells, while phospho-null myc-Rab7$_{Y183F}$ failed to do so (2.550±2.585 and 9.550±4.751 puncta remaining respectively). These data are consistent with the results published showing that Rab7 phosphorylation is required for EGF degradation [13].

Following EGF stimulation, Rab7 is rapidly tyrosine phosphorylated and this modification is important in the sorting of EGFR [13]. The Rab7 effector RILP has been shown to play a key role in the degradation of EGF and EGFR [26]. RILP interacts with Vps22 and Vps36, subunits of the Endosomal Sorting Complex Required for Transport (ESCRT)-II complex [27, 28]. At endosomes, the ESCRT complex is responsible for the sorting of ubiquitinated cargos and for the biogenesis of the intraluminal vesicles (ILVs) [29]. RILP interacts with and stabilizes the Vps22 subunit on the membrane. Given the importance of RILP for the correct sorting of EGFR into ILVs and hence its degradation, we wondered if the rapid Rab7 Y183 phosphorylation induced by EGF stimulation acts as a molecular switch to release RILP and favour the interaction of the latter with ESCRT-II for the efficient sorting of EGFR. To test this hypothesis, we generated GFP10-Vps22, GFP10-Vps36 and RILP-RlucII constructs and used BRET to look at the interaction between RILP and the two ESCRT-II subunits in HEK293 cells or in HEK293 cells expressing HA-Src, the EGFR activated kinases responsible of Rab7 Y183 phosphorylation [25]. We confirmed the role of Src in phosphorylating Rab7 using co-IP and anti-phosphotyrosine antibodies as expression of HA-Src or the constitutively active form of Src (Src$_{Y530F}$) resulted in a significant increase in Rab7 phosphorylation (**Figure 5.S1B**). We next tested the effect of expressing Src on the Rab7/RILP interaction using BRET. When HA-Src is expressed, we found an increase in the BRET$_{50}$ between Rab7 and RILP suggesting a weaker interaction (**Figure 5.6C and D**). Our BRET data shows that, consistent with previously published co-IP data [27] and GST-pulldown results [28], RILP interacts with both Vps22 (**Figure 5.6E and F**) and Vps36 (**Figure 5.6G and H**). Interestingly, upon HA-Src

expression, we found that the $BRET_{50}$ value for both the RILP/Vps22 (**Figure 5.6F**) and RILP/Vps36 interactions decrease (**Figure 5.6H**), suggesting that the expression of Src favours a RILP/ESCRT interaction over the RILP/Rab7 interaction. Although further analysis is required, these findings suggest that Rab7 phosphorylation could serve as a molecular switch to release RILP and favour the efficient sorting and degradation of internalized EGFR.

5.2.5 RILP interaction with the fusion machinery at the LE is Rab7 dependent.

The efficient fusion between late endosomes and lysosomes requires the engagement of several Rab7 effectors. The interaction between Rab7 and RILP is required for the efficient fusion between late endosomes and lysosome by recruiting the homotypic fusion and vacuole protein sorting (HOPS) complex, the tethering complex required for fusion at late endosomes [4]. RILP is able to interact with several HOPS subunits, including Vps41 and Vps39. Furthermore, Rab7 recruits a second effector, PLEKHM1, which interacts with both Vps39 and RILP, which is required to stabilize HOPS onto the membrane [6]. These interactions are also controlled by another Rab7 effector, the oxysterol binding protein related protein 1L (ORP1L). The latter is a cholesterols sensor that prevents the ability of Rab7/RILP to interact with HOPS and PLEKHM1 in low cholesterol condition [4, 6]. We showed that EGF is degraded when the Rab7/RILP interaction is blocked suggesting that Rab7-dependent RILP engagement may not be necessary for late endosome-lysosome fusion. To better understand the contribution of RILP in this process, we decided to analyze the ability of RILP to interact with some of the partners involved in this process in the context of Rab7-KO. Since the membrane recruitment of retromer is dependent on Rab7, we first asked if this was the case for RILP and HOPS as well. We performed a membrane separation assay in wild-type and Rab7-KO HEK293 cells and looked at the distribution of RILP and Vps41 (**Figure 5.S2A**). We found that the membrane association of both proteins is Rab7 independent. Indeed, in the context of Rab7-KO, both HOPS (**Figure 5.S2B**) and RILP (**Figure 5.S2C**) are still localized onto membranes. We then decided to investigate the role of Rab7 in modulating the RILP\HOPS, RILP\PLEKHM1 and RILP/ORP1L interactions. Wild-type and Rab7-KO HEK293 cells were transfected with RILP-RlucII and Vps39-GFP10, PLEKHM1-GFP10 or ORP1L-GFP10 and BRET analysis was performed (**Figure 5.7**). While all these pairs are able to interact in HEK293 cells (**Figure 5.7A, C, E, black curves**), we were not able to detect any interaction in Rab7-KO HEK293 cells (**Figure 5.7A, C, E, red lines**). To clarify the role of Rab7 phosphorylation in modulating the ability of RILP to interact with the above mentioned partners, we transfected RILP-RlucII and

PLEKHM1-GFP10 or RILP-RlucII and ORP1L-GFP10 in Rab7-KO cells, or in Rab7-KO cells rescued with myc-Rab7 or the phosphomutants. We found that myc-Rab7$_{Y183E}$ fails to rescue both the RILP/PLEKHM1 (**Figure 5.8A and B**) and RILP\ORP1L interactions (**Figure 5.8C and D**). This data are not surprising given the inability of Rab7$_{Y183E}$ to interact with RILP.

5.2.6 Rab7 Y183 phopshorylation regulates Rab7/ORP1L interaction

Our data suggest that Y183 phosphorylation could act as a molecular switch to release RILP from Rab7, and favour its interaction with ESCRT-II complex for the efficient sorting of EGFR. We wondered if, beyond modulating the interaction with RILP, Y183 phosphorylation is involved in regulating the interaction between Rab7 and other effectors. For this purpose we used BRET to test the interaction between RlucII-Rab7 or its phosphomutants and PLEKHM1-GFP10, GFP10-FYCO1 and ORP1L-GFP10. We did not find any significant changes in the Rab7/PLEKHM1 or the Rab7/FYCO1 interactions (**Figure 5.9A - D**). Interestingly, we found that the BRET$_{50}$ of the phospho-null RlucII-Rab7$_{Y183F}$ is statistically significantly smaller that the one calculated for RlucII-Rab7 (BRET$_{50}$ respectively 0.0043 ±0.0005 and 0.0095±0.0013). These data, together with the observation that Rab7$_{Y183F}$ fails in rescuing EGF degradation, suggest a possible contribution of ORP1L in this pathway.

5.3 Discussion

Rab7 activity at late endosomes is crucial for several pathways, including late endosome-lysosome and autophagosome-lysosome fusion [5][4], late endosome movement and positioning [2, 3, 30, 31], and late endosome-to-TGN protein retrieval [7, 8]. The ability of Rab7 to coordinate all these aspects of late endosome function is due to the capacity of this small GTPase to interact with different effectors. In this respect, PTMs are able to modulate Rab7 and favour interactions with one specific effector over another according to cellular needs. In particular, our previous work has shown how Rab7 palmitoylation is required to interact with and recruit retromer to endosomes [10]. Here we characterize a further layer of regulation, where the interplay between Rab7 serine 72 phosphorylation and cysteine 83 and 84 palmitoylation is required for the small GTPase to efficiently recruit retromer. Moreover we clarify the role of Rab7 Y183 phosphorylation in modulation EGF degradation. In this respect, the phosphorylation of Y183 acts as a molecular switch to weaken the Rab7 interaction with RILP, and favours the interaction of the latter with ESCRT-II. This is necessary for the efficient sorting of EGFR into ILVs and its lysosomal degradation.

5.3.1 Serine 72 phosphorylation is required for efficient Rab7 palmitoylation and retromer interaction and recruitment

The modulation of protein activity via the combination of several PTMs has been shown previously for several proteins, including members of Ras GTPase superfamily [32, 33]. Phosphorylation and palmitoylation are two major reversible post-translational modifications used by cells to modulate the activity of proteins according to cellular needs. These PTMs can work in synergy or can have opposite effects in the regulation of a protein [34-38]. As for Rab7, we found cooperation between serine phosphorylation and palmitoylation in modulating the ability of this small GTPase to interact with and recruit retromer. Our data suggest that phosphorylation on serine 72 is required to achieve optimal palmitoylation levels, as the non-phosphorylatable $Rab7_{S72A}$ is substantially less palmitoylated compared to wild-type Rab7. We speculate that serine phosphorylation could facilitate the interaction of Rab7 with the palmitoylation machinery, possibly by modifying the conformation of the protein itself to favour the interaction with a yet unidentified palmitoyltransferase responsible for the addition of the palmitate chain. Conversely, phosphorylation at S72 could block the interaction of Rab7 with a thioesterase, a group of enzymes that remove the palmitate group.

5.3.2 TBK1-mediated Rab7 phosphorylation does not affect retromer function.

TBK1 is related to the family of IKK kinases ($I\text{-}_{\kappa}B$ Kinase), and was first identified for its role in promoting the translocation of transcription factors during innate immune responses [39-41]. In this context, TBK1 also has a role in activating autophagy via the phosphorylation of the autophagic adaptor optineurin (OPTN), a process required for the lysosomal degradation of pathogens [42]. More recently the TBK1-OPTN axis has been described as crucial in coordinating the turnover of damaged mitochondria via mitophagy, and hence in maintaining cellular homeostasis [43-45]. During mitophagy, TBK1 and Rab7 are independently recruited to the Mitochondrial Outer Membrane (MOM). There, TBK1 can phosphorylate Rab7 on serine 72 and this PTM is required for the recruitment of ATG9+ membranes for the formation of the autophagosome [24]. However, the mechanisms behind this process have not been fully described. We found that the pharmacological inhibition of TBK1 results in a small reduction in Rab7 palmitoylation, that does not substantially affect the ability of retromer to localize to membranes, suggesting that although TBK1 phosphorylates Rab7 in the context of mitophagy activation, its activity has no effect on the function of retromer. Further work is required to identify and elucidate other kinases in modulating the role of Rab7 phosphorylation in retromer

recruitment and function. However, in this work, we have investigated the effect of TBK1 inhibition in steady-state conditions. We cannot exclude that TBK1 could modulate Rab7/Retromer activity during stress conditions, such as starvation, rapamycin-induced autophagy or mitophagy. Indeed, during mitophagy, TBK1-dependent Rab7 phosphorylation is required for the maturation of the mitophagosome. Moreover, cells expressing $Rab7_{S72A}$ or TBK1-KO fail to mobilize ATG9+ membrane during mitophagy [24]. Given that ATG9+ membrane recruitment is retromer dependent [46], we cannot exclude the possibility that TBK1 phosphorylation of the fraction of Rab7 localized on the mitochondrial membrane is required to recruit retromer to mitochondria and in turn ATG9+ membrane for mitophagosome maturation.

5.3.3 Rab7 Y183 phosphorylation regulate EGF degradation

The role of Rab7 Y183 phosphorylation in the degradation of endocytic cargo has been previously shown by several groups. However the conclusions drawn about the role of this PTM in regulating EGFR degradation are not completely clear. Some work suggests that the over-expression of Rab7 phosphomimetic mutants Y183D or Y183E blocks EGFR degradation in HeLa cells [14, 25], while other work shows that the expression of the phospho-null mutant Y183F impairs EGFR degradation [13]. Although we cannot exhaustively explain the reasons behind these opposite conclusions, in all these studies, the experiments were performed in the presence of endogenous Rab7, which could possibly have biased the results. In this work, we tested the ability of Rab7 phosphorylation mutants to degrade EGF in a Rab7-KO background, in order to eliminate any possible interference from the endogenous protein. In this setting, we found that the expression of $Rab7_{Y183F}$ in Rab7-KO cells fails to degrade EGF, while both $Rab7_{S72A}$ and $Rab7_{Y183E}$ show the same ability to rescue EGF degradation as wild-type Rab7. This suggests that Rab7 phosphorylation is required for the efficient lysosomal degradation of EGFR following EGF stimulation.

Rab7 is rapidly phosphorylated by the EGFR activated kinase Src following EGF stimulation [13, 25]. This phosphorylation substantially reduces the ability of Rab7 to engage RILP [25]. We show that the reduced ability of Rab7 to interact with RILP corresponds to an increased interaction between RILP and the Vps22 and Vps36 subunits of the ESCRT-II complex. This interaction is required for the efficient sorting of ubiquitinated receptor into ILVs, a necessary step for their degradation [29]. We suggest that upon EGFR activation, Rab7 Y183 phosphorylation act a molecular switch to release RILP and favour the interaction of the latter with ESCRT-II for the efficient sorting of the ubiquitinated proteins directed towards lysosomal

degradation.

5.3.4 Rab7/RILP interaction is not required for late endosome-lysosome fusion and EGF degradation

Published work suggests that to efficiently accomplish fusion, several Rab7 effectors must be recruited onto late endosomes. The Rab7/RILP interaction is required for the RILP-dependent recruitment of the late endosome-lysosome tethering complex HOPS [4]. To stabilize the association of this complex on the membrane, the Rab7/PLEKHM1 interaction is also required [6]. Our data shows that the Rab7/RILP interaction is dispensable to achieve degradation, indeed the expression of phosphomimetic $Rab7_{Y183E}$ rescues EGF degradation in Rab7-KO cells. Using BRET, we show that the ability of RILP to be engaged in the complex of proteins required for fusion is strictly dependent on Rab7. Indeed, in Rab7-KO cells, RILP looses the ability to interact with the Vps39 subunit of HOPS and with PLEKHM1. We confirmed that RILP is still membrane bound in Rab7-KO cells, suggesting that the absence of interaction between RILP and HOPS and RILP and PLEKHM1 is not due to the impaired recruitment onto membranes, but it is due to the missing interaction with Rab7 to possibly re-localize RILP to specific late endosome subdomain. Moreover, we show that this effect is strictly dependent on the phosphorylation status of Rab7, since the $Rab7_{Y183E}$ mutant does not rescue the interaction between RILP\HOPS and RILP\PLEKHM1.

5.3.5 Rab7 phosphorylation and late endosome positioning.

Late endosome movement is coordinated by the interaction between Rab7 and different effectors, depending on the direction of the traffic. The Rab7\FYCO1 interaction is required to recruit kinesin-1 for the anterograde movement on microtubules [47]. Retrograde traffic is mediated by the interaction between Rab7/RILP/ORP1L with the $p150^{Glued}$ subunit of dynein [2, 3]. Interestingly ORP1L also coordinates late endosome positioning by interacting with VAMP-associated ER protein (VAP)-A. This interaction blocks the ability of RILP to interact with $p150^{Glued}$, and keeps the late endosome locked at late endosome-ER contact sites [31]. Interestingly we show that the RILP\ORP1L interaction is Rab7 dependent and is blocked by Rab7 phosphorylation on tyrosine 183. The inability of phosphorylated Rab7 to bind RILP could result in the impairment of anterograde transport of late endosome, which would remain localized to the periphery of the cell. Further work is required to confirm this hypothesis. Interestingly, we found a stronger interaction between $Rab7_{Y183F}$ and ORP1L compared with wild-type Rab7. We are currently investigating the possibility that this increased interaction could

translate into a stronger ORP1L/VAP-A interaction. This could lead to the immobilization of late endosomes at the late endosome-ER contact site. The impaired mobility of the late endosome when Rab7 phosphorylation is blocked could potentially explain why $Rab7_{Y183F}$ mutants fail in degrading EGFR.

Overall our data uncovered new layers of regulation of Rab7 mediated by phosphorylation. We show that S72 phosphorylation and palmitoylation work together to regulate Rab7/retromer recruitment at the endosome. We show also that TBK1 does not affect the ability of Rab7 to recruit retromer in steady state condition, suggesting that other kinases are involved in regulating Rab7/retromer recruitment.

We also clarify the role of Y183 phosphorylation in regulating EGF degradation, we show indeed that Rab7 phosphorylation while blocking Rab7/RILP interaction, favours the RILP/ESCRT-II interaction to efficiently degrade EGFR. We propose preliminary data pointing to an important role of Y183 in modulating Rab7-dependent late endosome movement and positioning, disclosing further implications on the role of Rab7 PTMs in coordinating late endosome functions.

5.4 Materials and Methods

Reagents, Cloning and mutagenesis

Unless otherwise stated, all reagents used in this study were bought from Fisher Scientific (Ottawa, ON). The antibodies used in this study are listed on Supplementary Table 1.

The plasmids used in this study are listed on Supplementary Table 2. All the restriction enzymes use in this study were purchased from New England Biolabs (Danvers, MA). All the mutants described in this work were generated via PCR mutagenesis using cloned PFU polymerase (Agilent Technologies, Santa Clara, CA).

Cell culture

All cell lines used in this study were originally obtained from ATCC (Manassas, VA) and regularly screened for contamination. HEK293T, U2OS or HeLa were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin (Thermo Fisher Scientific, Burlington, ON). Rab7-KO cell lines were generated using CRISPR/Cas9 approach as previously described [9]. Transfections were performed with

polyethylenimine (PEI) (Fisher Scientific, Ottawa, ON) as previously described [10].

Membrane Separation Assay

Cell pellets were snap frozen in liquid nitrogen and thawed at room temperature (RT). Samples were then resuspended in buffer 1 (0.1 M Mes-NaOH pH 6.5, 1 mM MgAc, 0.5 mM EGTA, 200 µM sodium orthovanadate, 0.2 M sucrose) and centrifuged 5' at 10000g at 4°C. Supernatant (fraction indicated as S in the text) containing cytosolic proteins was collected and the remaining pellet was resuspended in buffer 2 (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) and centrifuged 5' at 10000g at 4°C to isolate the supernatant containing membrane proteins (fraction indicated as P in the text). Isolated fractions were analyzed via Western Blot as described in Modica et al., 2017.

Immunofluorescence

HEK-293, U2OS immunofluorescence was performed as previously described [48]. HeLa cell immunofluorescence was performed by seeding the cells on coverslip overnight. The following day cells were transfected or treated as indicate on the figure. 24 hrs or 48 hrs after treatment or transfection, coverslips were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature (RT). PFA was removed by washing the samples three times with PBS for 5 minutes. Cells were blocked with 0.1% Saponin (SIGMA), BSA 1% in PBS for 1hr at RT followed by incubation with primary Antibody diluted in blocking solution for 2hrs at RT. Coverslips were washed three times for 5 minutes in PBS and incubated 1 hour at RT with secondary antibodies conjugated to AlexaFluor-594 Alexa-Fluor-488 in a solution of 0.1% Saponin, BSA 1% in PBS. After one wash of 5 minutes in PBS, cells were incubated with DAPI, washed three times for 5 minutes in PBS and mounted glass slides with Fluoromount G.

Acyl-RAC

The isolation of palmitoylated protein was adapted from Modica et al., 2017. Briefly cells were lysate in TNE (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail) supplemented with 50mM N-Ethylmaleimide (NEM) and incubated 30 minutes in rotating wheel at 4°C. Samples were centrifuged 10 minutes at 10000g at 4°C and the collected supernatants were incubated 2 hrs at RT on a rotating wheel. Samples were then precipitated over night with two volumes of cold Acetone at -20°C to remove NEM. After washing with cold acetone, the pellet was resuspended in binding buffer (100 mM HEPES, 1 mM EDTA, 1% SDS) with 250 mM hydroxylamine (Sigma-Aldrich) (NH_2OH) pH7.5 to cleave

palmitate residues off of proteins. Control samples were resuspended in binding buffer containing 250mM NaCl. When the pellet was completely resuspended, Water-swollen thiopropyl sepharose 6B beads (GE Healthcare Life Sciences, Mississauga, ON) were added and samples were incubated 2 hrs at RT on rotating wheel. Beads were then washed 4 times with binding buffer and captured proteins were eluted with 100mM DTT.

BRET^2 Assay

HEK293 were seeded on 12 well plates overnight followed by transfection with the indicated constructs. 48 hrs after transfections, cells were washed with PBS, detached with PBS 5mM EDTA and resuspended in 500uL PBS. Samples were then plated in triplicated (90uL/well) on a 96 well plates (VWR Canada, Mississauga, ON). Total fluorescence was measured with infinite M1000 Pro plate reader (Tecan Group Ltd., Mannedorf, Switzerland), with the excitation set at 400nm and the emission at 510nm. The Renilla Luciferase substrate coelenterazine 400a (GoldBio, Saint Louis, MO) was added to each well to a final concentration of 5uM and BRET signal was read after 2 minutes incubation at RT. BRET value is calculated as a ratio between the GFP10 emission (500-535nm) over RlucII emission (370-450 nm). To calculate the BRETnet, the BRET obtained by cells expressing only RLucII was subtracted from the BRET value registered from the cells expressing both GFP10 and RLucII. To generate saturation curves, the BRETnet values were plotted as a function of the ratio between the GFP10 signal (Fluorescence) over the RLucII signal (Luminescence).

Phos-Tag

The phos-tag gel was prepared as follows. Stacking gel 4% Acrylamide/bis-acrylamide (bio basic), 125mM Trsic/HCl pH 6.8, 0.1 % (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.01% tetramethylenediamine. Resolving gel: 10% Acrylamide/bis-acrylamide (BIoBasic), 375mM Tris/HCl pH 8.8, 0.1% (w/v) SDS, 75uM Phos Binding Reagent Acrylamide acrylamide (APExBIO), 150uM MnCl2, 0.05% (w/v) ammonium persulfate, 0.01% tetramethylenediamine. Samples to analyze via phos-tag were precipitated with 2 volumes of cold-acetone o.n. at -20C and resuspended in EDTA free lysine buffer (100mM Tris, 150mM NaCl, 1% Triton-X100). After resuspension samples were mixed with Laemmli sample buffer, loaded and electrophoresis was performed. Before the transfer onto nitrocellulose membranes (Amersham Protran Premium 0.45um NC; GE Healthcare), gel was washed twice with transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) supplemented with 0.05% (w/v) SDS and 10mM EDTA followed by 2 washes in transfer buffer 0.05% (w/v) SDS. Transfer was performed at 350mA, 60 minutes on

ice.

Image analysis and statistics

Image analysis was performed using Fiji [49] and the coloc2 plugins for the co-localization analysis. Statistical analysis was performed using GraphPad Prism Version 8.2.1 (GraphPad Software, San Diego, California USA, www.graphpad.com) and described in the corresponding figure legend.

Author contribution

Conceptualization, S.L., G.M. Design (Methodology), S.L., G.M., Investigation G.M., O.S., Writing - Original Draft, S.L., G.M: Writing - Review & Editing S.L., G.M., Funding Acquisition, S.L.

Acknowledgments

We would like to thank Michel Bouvier (IRIC and University de Montreal), Paul Odgren (University of Massachusetts Medical School, Addgene #73592), and Dr. John H. Brumell, Sickkids Hospital, Toronto) for providing plasmids.

Competing Interests

The authors declare no conflict of interests

Funding

This work was supported by Joint Programme in Neurodegenerative Diseases Grant (Neuronode to SL), the Canadian Institutes for Health Research (ENG-387575 to SL), the Canadian Foundation for Innovation (35258 to SL). G.M. is supported by a PhD scholarship from Fonds de Recherche du Quebec - Santé. O.S. was supported by a post-doctoral fellowship from Fonds de Recherche du Quebec - Santé.

5.5 Figures

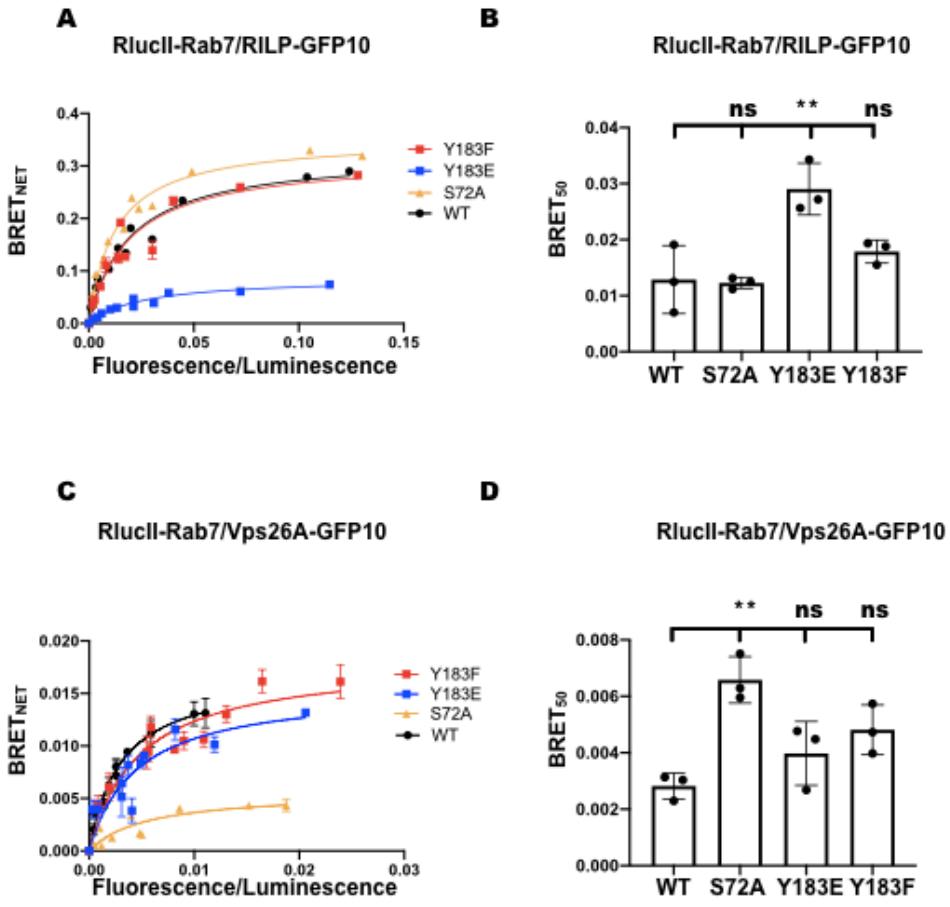

Figure 5-1 Rab7 S72 phosphorylation regulates Rab7/Retromer interaction.

(A) HEK293 cells were transfected with a constant amount of RlucII-Rab7, and the indicated phosphomimetic or phospho-null serine and tyrosine mutants, and increasing amounts of RILP-GFP10. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (B) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. ,** P< 0.01; One-way Anova with Tukey's post-hoc test. (C) HEK293 cells were transfected with a constant amount of RlucII-Rab7, and the indicated phosphomimetic or phospho-null serine and tyrosine mutants, and increasing amounts of Vps26A-GFP10. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (D) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. ,** P< 0.01; One-way Anova with Tukey's post-hoc test.

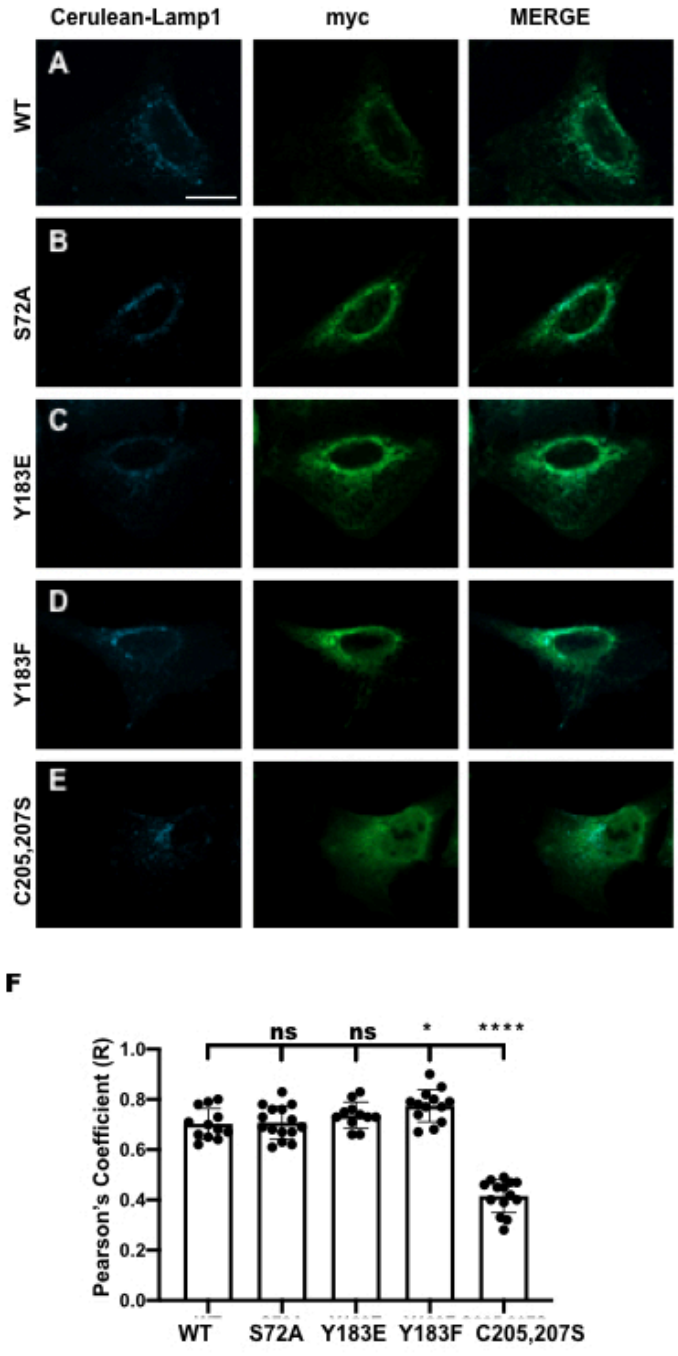

Figure 5-2 Phosphorylation does not alter Rab7 localization

(A -E) U2OS were co-transfected with cerulean-Lamp1 and the indicated myc-Rab7 constructs . 24 hrs after transfection, cells were fixed with PFA 4% and immunostained with anti-myc antibody (green). Representative images are shown, scale bar: 10μm. (F) Pearson's correlation coefficient from n ≥ 12 cells per condition. Data are represented as mean ± SD. , n.s. not significant, * $P < 0.05$, **** $P < 0.0001$; One-way Anova with Tukey's post-hoc test.

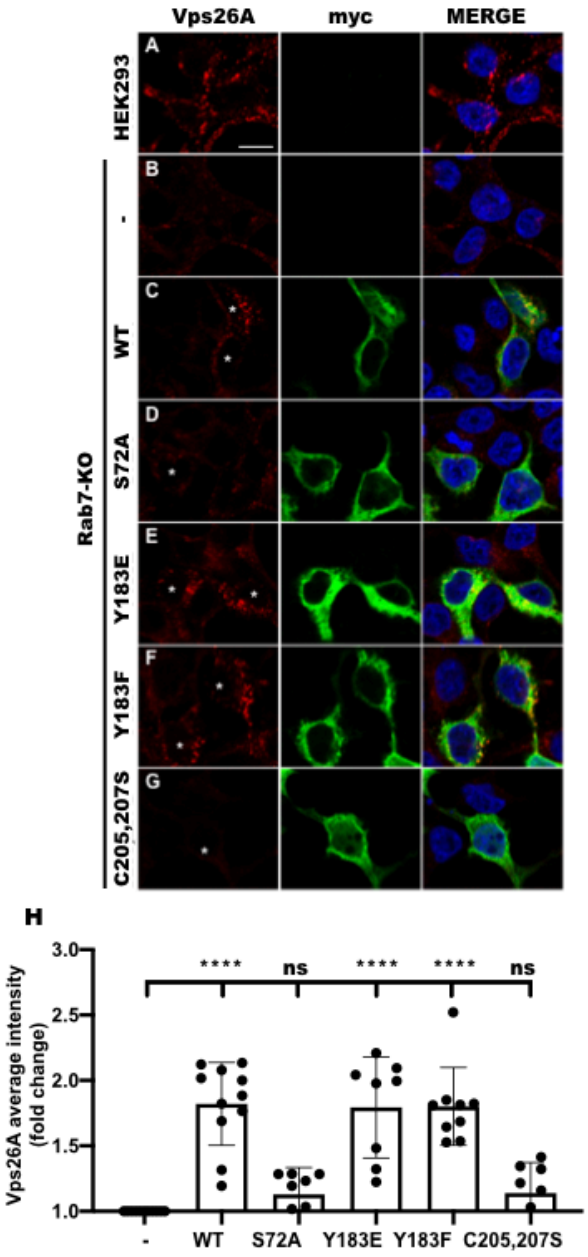

Figure 5-3 Rab7$_{S72A}$ does not rescue retromer distribution in Rab7-KO cells

A) Wild-type or (B) Rab7-KO HEK293 cells were fixed with PFA4% and immunostained with anti-Vps26A antibody (red) and counter stained with DAPI (blue). (C - G) Rab7-KO HEK293 cells were transfected with the indicated myc-Rab7 constructs. 48 hrs after transfections, cells were fixed with PFA4% and immunostained with anti-myc antibodies (green), anti-Vps26A antibodies (red) and counter stained with DAPI (blue). Representative images are shown, scale bar:10µm. (H) Quantification of Vps26A average intensity from n ≥ 10 cells per condition, values are reported as fold increase compared to Rab7-KO. Data are represented as mean ± SD. not significant, **** P < 0.0001; One-way Anova with Tukey's post-hoc test.

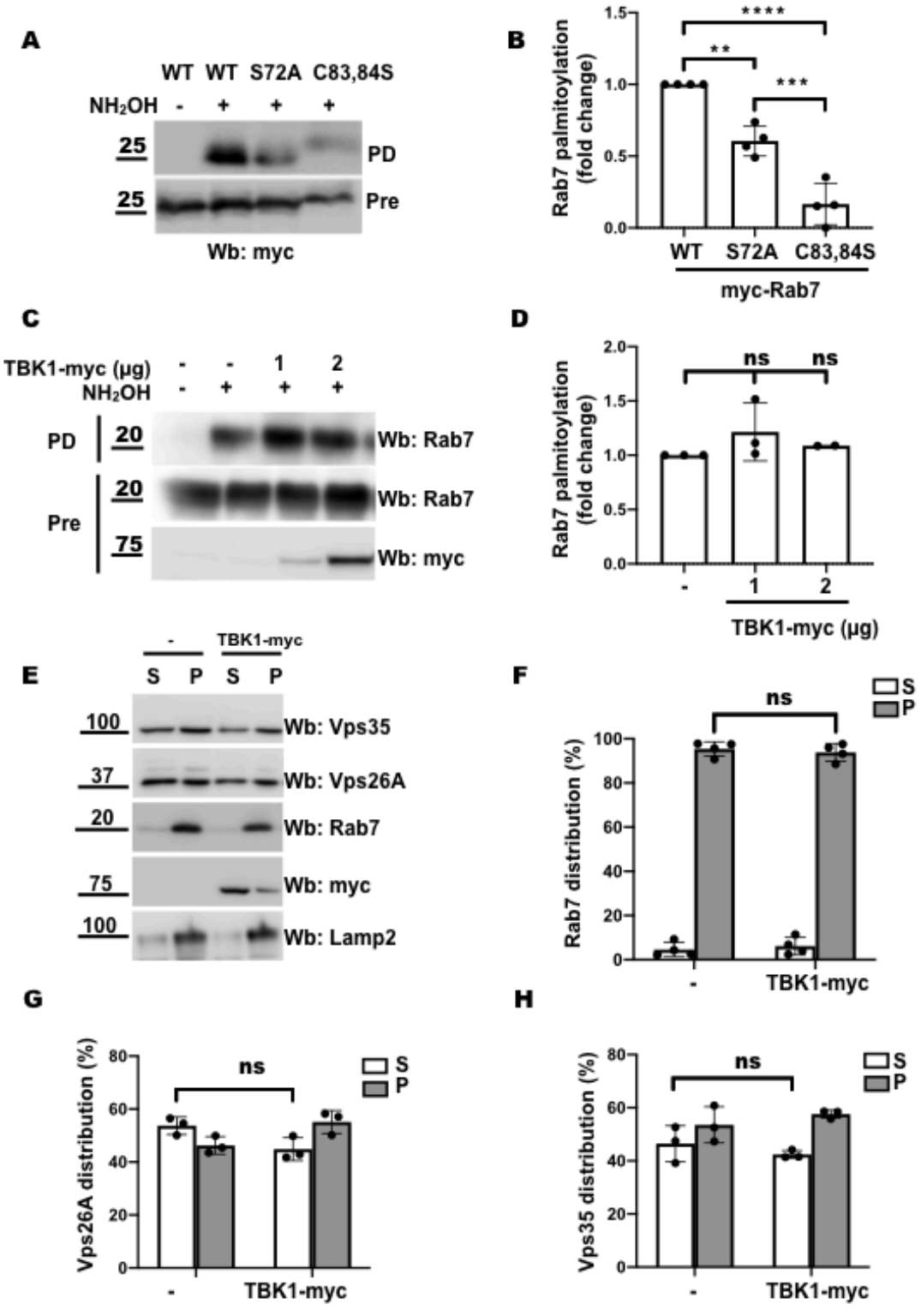
A
WT WT S72A C83,84S
NH2OH - + + +
25
PD
25
Pre
Wb: myc
B
Rab7 palmitoylation (fold change)
1.5
1.0
0.5
0.0
****
**
***
WT S72A C83,84S
myc-Rab7
C
TBK1-myc (μg) - - 1 2
NH2OH - + + +
PD
20
Wb: Rab7
Pre
20
Wb: Rab7
75
Wb: myc
D
Rab7 palmitoylation (fold change)
2.0
1.5
1.0
0.5
0.0
ns ns
- 1 2
TBK1-myc (μg)
E
- TBK1-myc
S P S P
100
Wb: Vps35
37
Wb: Vps26A
20
Wb: Rab7
75
Wb: myc
100
Wb: Lamp2
F
S
P
ns
Rab7 distribution (%)
100
80
60
40
20
0
- TBK1-myc
G
Vps26A distribution (%)
80
60
40
20
0
ns
S
P
- TBK1-myc
H
Vps35 distribution (%)
80
60
40
20
0
ns
S
P
- TBK1-myc

Figure 5-4 S72 phosphorylation modulates Rab7 palmitoylation

(A) HEK293 cells were transfected with myc-Rab7, myc-Rab7$_{S72A}$ or myc-Rab7$_{C83,84S}$. 24hrs after transfection, cell lysates were collected and Acyl-RAC was performed to determine the palmitoylation level. Samples were analyzed via Wb. NH_2OH: Hydroxylamine (B) Quantification of 4 independent experiments, values are represented as fold change compared to wild-type myc-Rab7. Data are represented as mean ± SD. *** P<0.001, **** P < 0.0001; One-way Anova with Tukey's post-hoc test. (C) HEK293 cells were transfected or not with increasing concentrations of TBK1-myc. 48 hours post transfection, an Acyl-RAC experiment was performed. (D) Quantification of 3 independent experiments, values are represented as fold change compared to non-transfected cells. Data are represented as mean ± SD. *** P<0.001, **** P < 0.0001; One-way Anova with Tukey's post-hoc test. (E) HEK293 cells were transfected or not with TBK1-myc. 48 hours post-transfection, the cells were collected for a membrane isolation assay. Samples were subjected to Western blot (Wb) with anti-Vps26A, anti-Vps35, anti-Rab7A antibody, anti-Lamp2 antibody (a marker for the membrane fraction), and anti-myc antibody (to determine the expression of TBK1-myc). S, supernatant, P, pellet. (F) Quantification of 4 separate membrane isolation assay experiments for Rab7 distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test. (G) Quantification of 4 separate membrane isolation assay experiments for Vps26A distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test. (H) Quantification of 4 separate membrane isolation assay experiments for Vps35 distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test.

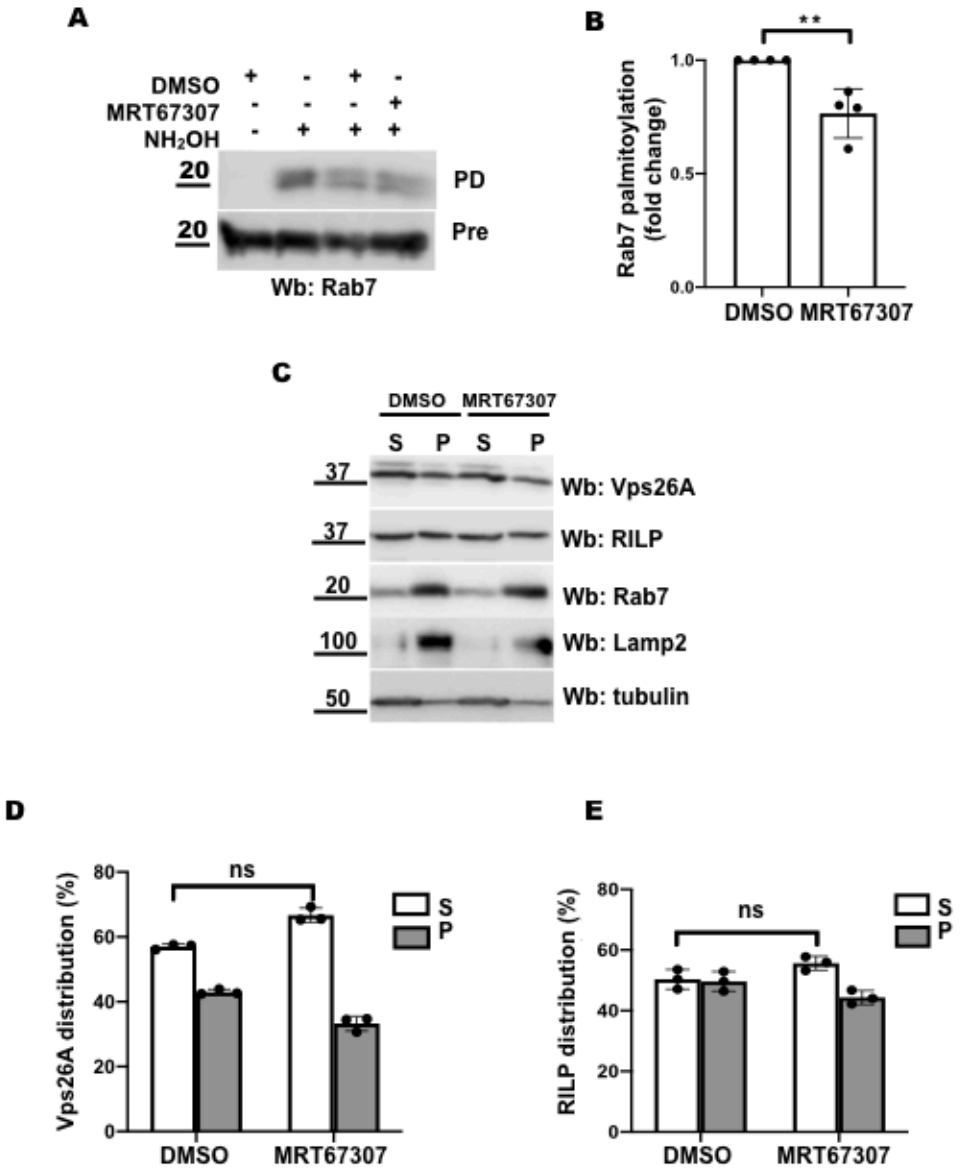

Figure 5-5 Inhibiting TBK1 function has no effect on retromer of RILP

(A) HEK293 cells were treated overnight with DMSO or 10 μM MRT67307. Collected lysates were subjected to Acyl-RAC and eluted samples analyzed via Wb. (B) Quantification of 4 independent Acyl-RAC experiments. Values are represented as fold change compared to DMSO treated cells. Data are represented as mean ± SD. ** P< 0.01, two tailed unpaired t test. (C) HEK293 cells were treated overnight with DMSO or 10μM MRT67307 and subjected to a membrane separation assay. Samples were subjected to Western blotting (Wb) with anti-Vps26A antibody, anti-Rab7A antibody, anti-RILP antibody, anti-Lamp2 antibody (a marker for the membrane fraction), and anti-tubulin antibody (a marker for the cytosolic fraction). S, supernatant, P, pellet. (D) Quantification of 3 separate membrane isolation assay experiments for Vps26A distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test. (E) Quantification of 4 separate membrane isolation assay experiments for RILP distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test.

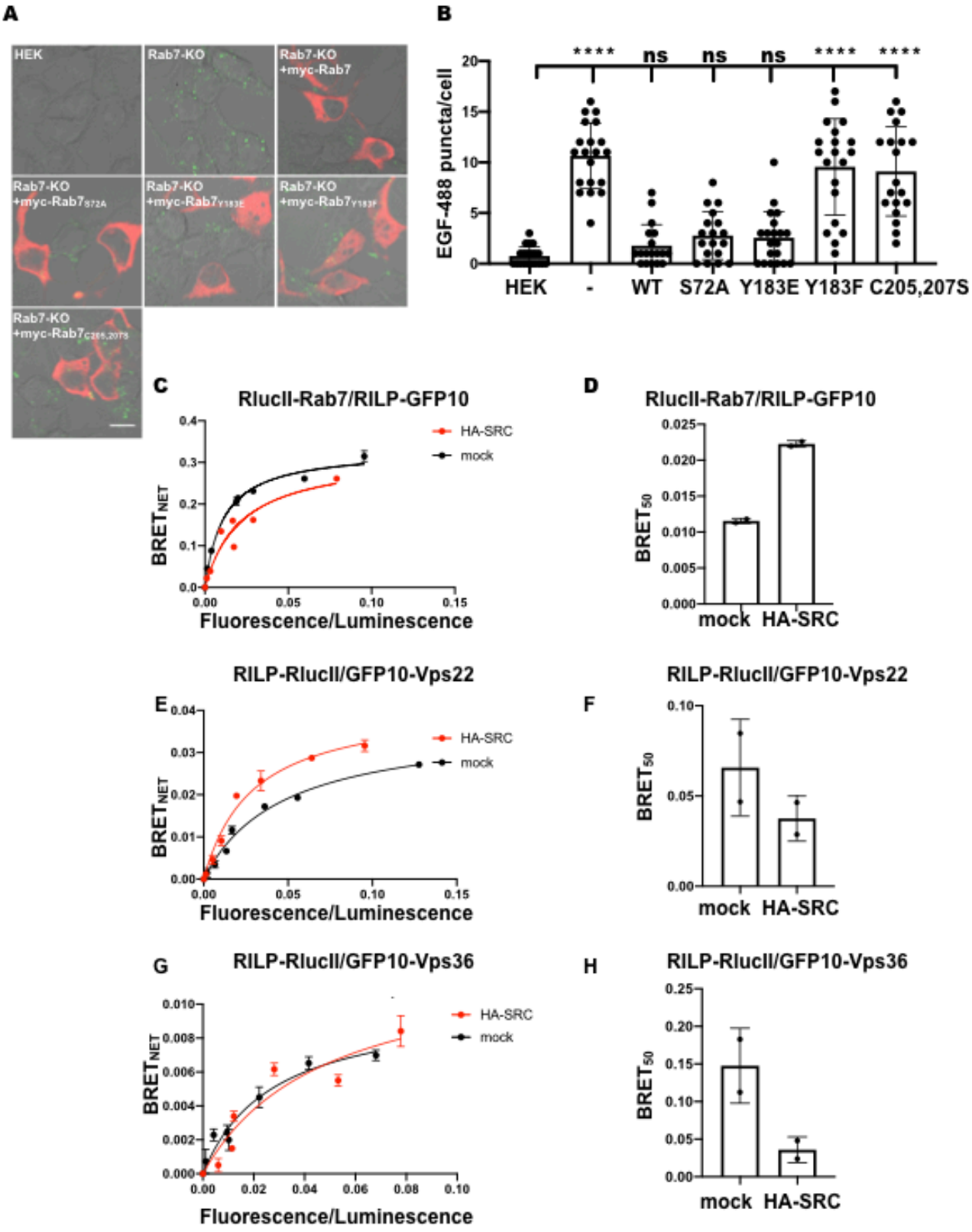
A
HEK
Rab7-KO
Rab7-KO +myc-Rab7
Rab7-KO +myc-Rab7S72A
Rab7-KO +myc-Rab7Y183E
Rab7-KO +myc-Rab7Y183F
Rab7-KO +myc-Rab7C205,207S
B
EGF-488 puncta/cell
**** ns ns ns **** ****
HEK - WT S72A Y183E Y183F C205,207S
C
RlucII-Rab7/RILP-GFP10
HA-SRC
mock
BRETNET
Fluorescence/Luminescence
D
RlucII-Rab7/RILP-GFP10
BRET50
mock HA-SRC
E
RILP-RlucII/GFP10-Vps22
HA-SRC
mock
BRETNET
Fluorescence/Luminescence
F
RILP-RlucII/GFP10-Vps22
BRET50
mock HA-SRC
G
RILP-RlucII/GFP10-Vps36
HA-SRC
mock
BRETNET
Fluorescence/Luminescence
H
RILP-RlucII/GFP10-Vps36
BRET50
mock HA-SRC

Figure 5-6 Rab7 Y183 phosphorylation regulates EGF degradation

(A) Wild-type, Rab7-KO and Rab7-KO HEK293 cells transiently expressing myc-Rab7, myc-Rab7$_{S72A}$, myc-Rab7$_{Y183E}$, myc-Rab7$_{Y183F}$ or myc-Rab7$_{C205,207S}$ were pulsed for 30 minutes with 300 ng/ml EGF conjugated with 488 Alexa flour (green) and chased for 1 hour before being fixed with 4% PFA and immunostained with anti-myc antibody (red). Representative images are shown, scale bar: 10µm. (B) Quantification of EGF puncta per cell, n ≥ 18 cells per condition. Data are represented as mean ± SD. , n.s. not significant, **** P < 0.0001; One-way Anova with Tukey's post-hoc test. (C) HEK293 cells were transfected with a constant amount of RlucII-Rab7 and increasing amounts of RILP-GFP10 with or without a constant amount of HA-Src. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (D) The average of the BRET$_{50}$ from 2 separate experiments is shown. Data are represented as mean ± SD. Preliminary data, no statistical analysis performed. (E, G) HEK293 cells were transfected with a constant amount of RILP-RlucII and increasing amounts of GFP10-Vps22 or GFP10-Vps36 with or without constant amount of HA-Src. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (F, H) The average of the BRET$_{50}$ from 2 separate experiments is shown. Data are represented as mean ± SD. Preliminary data, no statistical analysis performed

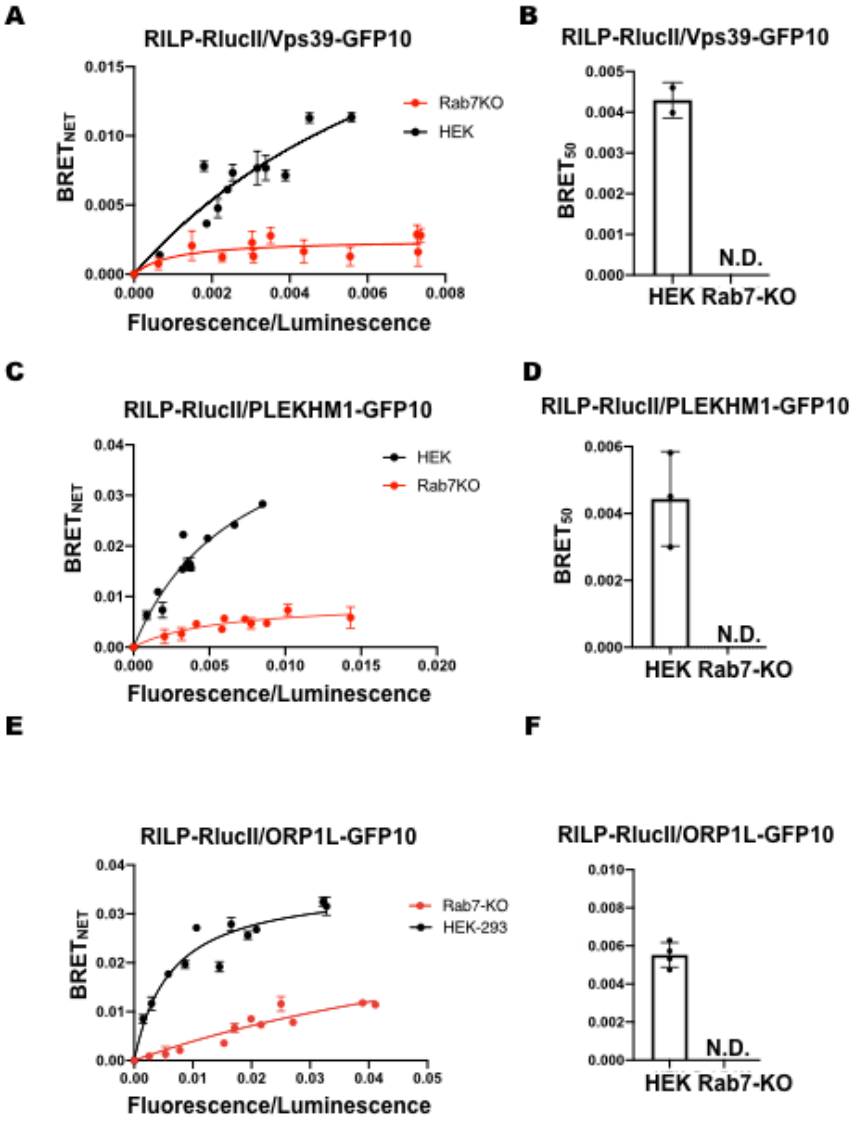

Figure 5-7 RILP interaction with the fusion machinery at the LE is Rab7-dependent

(A) Wild-type or Rab7-KO HEK293 cells were transfected with a constant amount of RILP-RlucII and increasing amounts of Vps39-GFP10. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (B) The average of the $BRET_{50}$ from 2 separate experiments is shown. N.D., not determined. Data are represented as mean ± SD. Preliminary data, no statistical analysis performed. (C) Same experiment as in A, but with PLEKHM1-GFP10. (D) The average of the $BRET_{50}$ from 2 separate experiments is shown. N.D., not determined. Data are represented as mean ± SD. Preliminary data, no statistical analysis performed. (E) Same experiment as in A, but with ORP1L-GFP10. (E) The average of the $BRET_{50}$ from 2 separate experiments is shown. N.D., not determined. Data are represented as mean ± SD. Preliminary data, no statistical analysis performed.

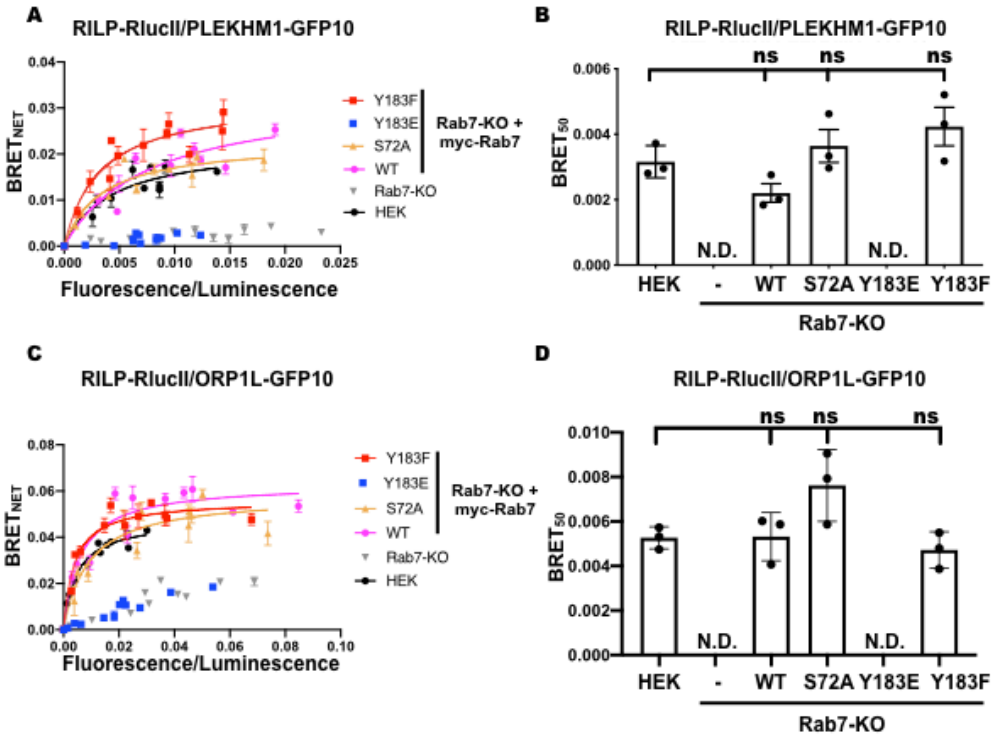

Figure 5-8 Rab7 Y183 phosphorylation blocks RILP/PLEKHM1 and RILP/ORP1L interaction

(A) Wild-type or Rab7-KO HEK293 cells were transfected with a constant amount of RILP-RlucII and increasing amounts of PLEKHM1-GFP10, with or without a constant amount of the indicated myc-Rab7 constructs. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (B) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. n.s, not significant; One-way Anova with Tukey's post-hoc test. (C) HEK293 or Rab7-KO cells were transfected with a constant amount of RILP-RlucII and increasing amounts of ORP1L-GFP10, with or without a constant amount of the indicated myc-Rab7 constructs. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (D) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. n.s, not significant; One-way Anova with Tukey's post-hoc test.

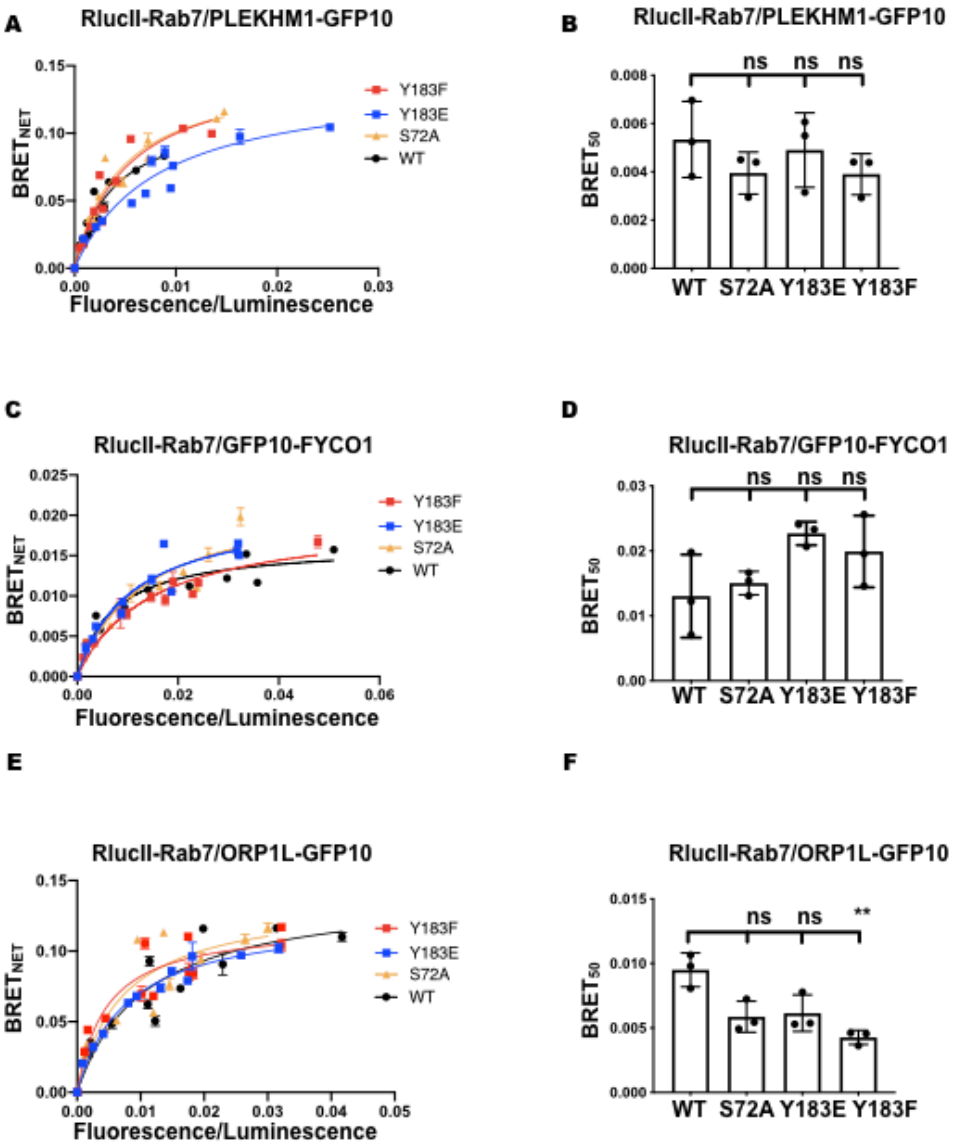

Figure 5-9 Rab7 Y183 phosphorylation regulates ORP1L interaction

(A) HEK293 cells were transfected with a constant amount of RlucII-Rab7, or the indicated phosphomimetic or phospho-null serine and tyrosine mutants, and increasing amounts of PLEKHM1-GFP10. 48 hours post-transfection, BRET analysis was performed. BRET signals are plotted as a function of the ratio between the GFP10 fluorescence over RlucII luminescence. (B) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. , n.s, not significant; One-way Anova with Tukey's post-hoc test. (C) Same experiment as in A, but with GFP10-FYCO1. (D) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. , n.s, not significant; One-way Anova with Tukey's post-hoc test. (E) Same experiment as in A, but with ORP1L-GFP10. (E) The average of the $BRET_{50}$ from 3 separate experiments is shown. Data are represented as mean ± SD. , n.s, not significant, ** P< 0.01; One-way Anova with Tukey's post-hoc test.

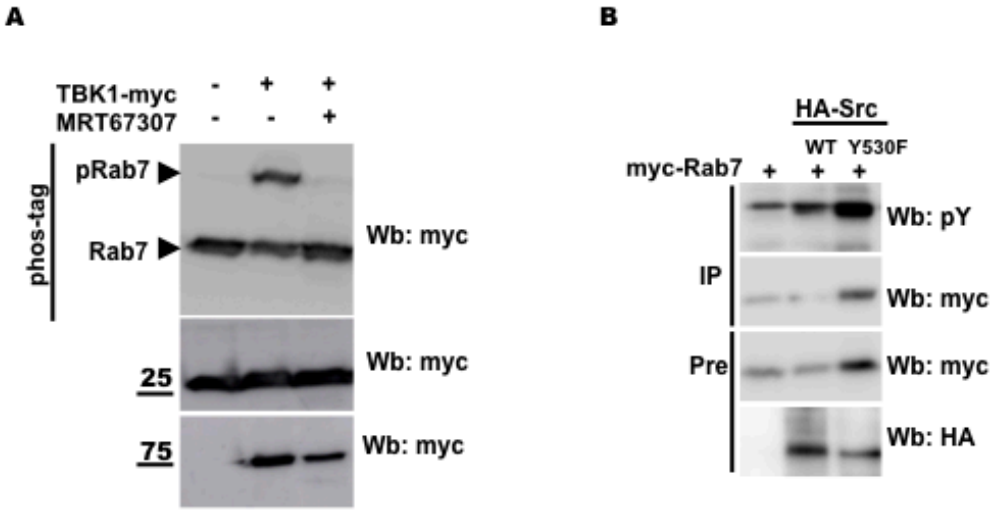

Figure 5-(S) 1

(A) HEK293 were transfected with myc-Rab7 and TBK1-myc as indicated. 24 hrs after transfection, cells were treated 1hr with 10μM MRT67307 as indicated. Lysates were collected and run on a phos-tag acrylamide gel to separate phosphorylated Rab7 fraction (top arrowhead) from the non-phopshorylated pool (bottom arrowhead). Samples were also run on a regular acrylamide gel to serve as loading control. Western Blotting (Wb) was performed, staining with anti-myc antibody in shown. (B) HEK293 were transfected with myc-Rab7 and the indicated HA-Src constructs. 24 hrs after transfection, cell lysates were immunoprecipitated with anti-myc antibody. Samples were subjected to Wb with anti-phospho tyrosine (pY), anti-myc and anti HA antibody.

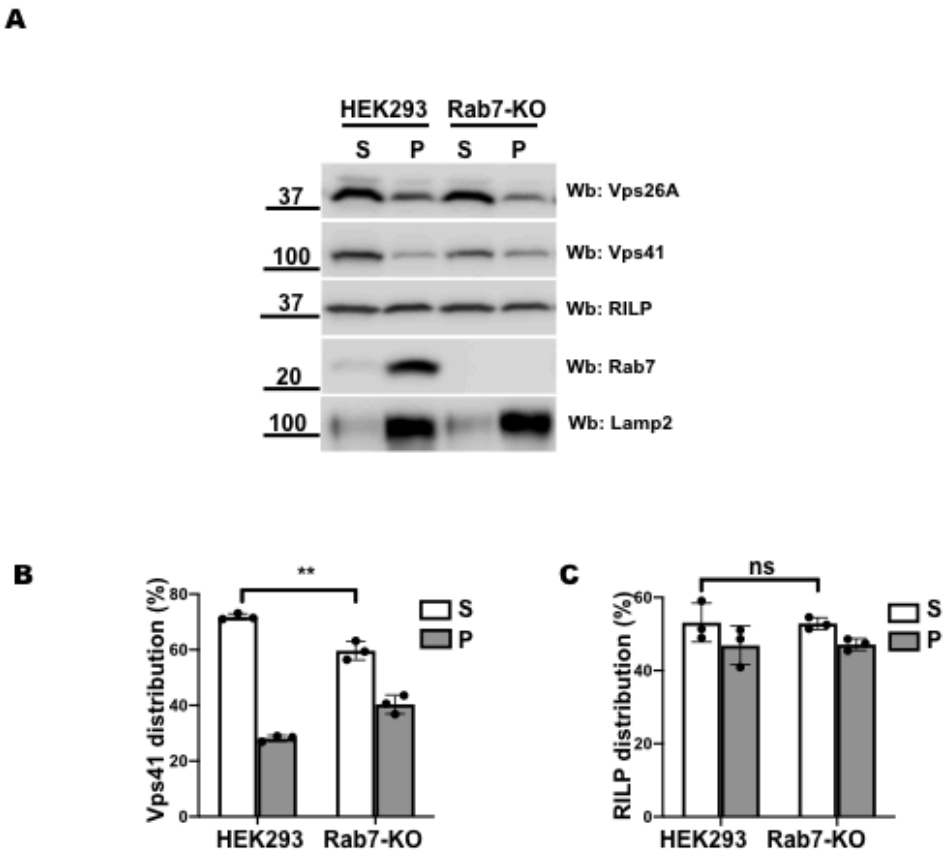

Figure 5-(S) 2

(A) Wild-type or Rab7-KO HEK293 cells were subjected to a membrane separation assay. Samples were subjected to Western blotting (Wb) with anti-Vps26A, anti-Vps41, anti-RILP, anti-Rab7A and anti-Lamp2 (a marker for the membrane fraction) antibodies. S, supernatant, P, pellet. (B) Quantification of 3 separate membrane isolation assay experiments for Vps41 distribution. Data is represented as mean ± SD. ** P< 0.01; two tailed unpaired t test. (E) Quantification of 3 separate membrane isolation assay experiments for RILP distribution. Data is represented as mean ± SD. ns, not significant; two tailed unpaired t test.

Designation	Source or reference	Additional information
myc- or RLucII-Rab7	Modica et al, 2017	
myc- or RlucII-Rab7$_{C83,84S}$, myc-Rab7$_{C205,207S}$	Modica et al, 2017	
myc- or RlucII-Rab7 phospho-mutants	This study	The mutants were engineered using site-directed mutagenesis from myc-Rab7 and RlucII-Rab7 plasmids
Vps26A-GFP10	Modica at al., 2017	
RILP-GFP10	Modica et al, 2017	
cerulean-Lamp1	This study	Addgene
TBK1-myc-DKK	This study	OriGene, Rockville, MD USA
GFP10-Vps22	This Study	This plasmid was generated by inserting Vps22 cDNA from Vps22-myc-DDK (OriGene, Rockville, MD USA) into the KpnI/ Xba sites of pcDNA3.1 Hygro (+) GFP10-RLucII-st2
GFP10-Vps36	This Study	This plasmid was generated by inserting Vps36 cDNA from Vps36-myc-DDK (OriGene, Rockville, MD USA) into the KpnI/ Xba sites of pcDNA3.1 Hygro (+) GFP10-RLucII-st2
Vps39-GFP10	This Study	This plasmid was generated by by inserting Vps39 cDNA from myc-Vps39, a generous gift from Dr. Steve Caplan (University of Nebraska)
PLEKHM1-GFP10	Yasa et al., 2019	
ORP1L-GFP10	This Study	This plasmid was generated by inserting ORP1L cDNA from ORP1L-myc-DDK (OriGene, Rockville, MD USA) into the Nhe/ KpnI sites of pcDNA3.1Hygro(+)RlucII-GFP10-st2
GFP10-FYCO1	Yasa et al., 2019	
RILP-RlucII	This Study	This plasmid was generated by amplifying RILP cDNA from RILP-GFP10
HA-SRC, HA-SRC$_{Y530F}$	Fessart et al., 2005	HA-SRC and HA-SRC$_{Y530F}$ plasmids were a generous gift from Dr. Laporte (McGill University).

Table 5-(S) 1 List of expression plasmids used in this study

		Dilution
Anti-mouse horseradish peroxidase-conjugated IgG	(7076S) New England Biolabs	1:5000
anti-rabbit horseradish peroxidase-conjugated IgG	(7074S) New England Biolabs	1:5000
anti-myc mouse monoclonal	(9E10) Thermo-Fisher	1:1000 WB/1:500 IF
anti-Rab7A rabbit monoclonal	(D95F2) Cell Signalling Technology	1:1000
anti-α-tubulin mouse monoclonal	(T9026) Sigma- Aldrich	1:1000
anti-Lamp-1 mouse monoclonal	(ab25631) Abcam	1:500
anti-Vps26A rabbit polyclonal	(ab23892) Abcam	1:1000 WB/1:500 IF
anti-Vps35 mouse monoclonal	(ab57632) Abcam	1:1000
anti-RILP rabbit polyclonal	(ab128616) Abcam	1:1000
anti-FLAG rabbit polyclonal	(F7425) Sigma-Aldrich	1:1000
anti-CD63 mouse monoclonal	(556019) BD Biosciences	1:500
anti-sortilin monoclonal mouse	BD Biosciences	1:1000
anti-Vps41 rabbit monoclonal	(ab246360) Abcam	1:1000
anti-pY	(ab10321) Abcam	1:1000

Table 5-(S) 2 List of antibodies used in this study

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7 DISCUSSION AND CONCLUSION

The small GTPase Rab7 has key roles in controlling membrane trafficking events at the Late Endosomes. Rab7 is indeed involved in the regulation of movement and positioning of late endosomes (Johansson et al., 2005; Johansson et al., 2007; Jordens et al., 2001), it coordinates fusion events between late endosomes and lysosomes (McEwan et al., 2015; van der Kant et al., 2013), and its activity is crucial to coordinate trafficking from late endosomes to the trans Golgi network (TGN) (Rojas et al., 2008; Seaman et al., 2009). The regulation of each of these pathways requires the engagement of a specific group of proteins by Rab7, known as effectors, to activate the steps required for these processes to happen. The regulatory mechanisms required to precisely modulate these various function-specific effector interactions are poorly understood. In this respect, post-translational modifications (PTMs) are emerging as a powerful tool to finely tune by favouring or temporarily restricting the ability of Rab7 to interact with effectors. However, how these modifications modulate Rab7 functions is not completely understood. With my PhD project, I contributed to this field by elucidating some of the functional consequences that PTMs have on the function of this small GTPase.

Rab7 is synthesized as a cytosolic protein that is rapidly prenylated on its two terminal cysteines (Joberty et al., 1993). Prenylation consist in the attachment of lipidic tail that allows the protein to associate to the membrane where it can regulate downstream functions. Like all small GTPases, Rab7 cycles between active and inactive states according to its binding to GTP or GDP respectively. In its active GTP-bound state, Rab7 can engage its effectors to activate downstream pathways. The action of GTPase activating proteins (GAPs) triggers the hydrolysis of GTP into GDP, terminating Rab7 activity (Barr and Lambright, 2010). In its inactive GDP-bound state, Rab7 is extracted from the membrane by a guanine-nucleotide dissociation inhibitor (GDI) (Soldati et al., 1993) that by masking the prenylated tail keeps the Rab7soluble in the cytosol (Muller and Goody, 2018). Here, the action of Guanine Exchange Factors (GEF) induce the release of Rab7 from GDI, enable the exchange of GDP for GTP and possibly contributes to locate Rab7 on the membrane where its function is required (Burton et al., 1994).

Once on the membrane, other regulatory mechanisms contribute in deciding which effectors need to be engaged. Recently published data have identified post-translational modification (PTM) as crucial regulators of Rab7 activity (Modica and Lefrancois, 2017). Indeed, beyond prenylation, Rab7 can be phosphorylated (Francavilla et al., 2016; Lin et al., 2017; Shinde and Maddika, 2016), ubiquitinated (Sapmaz et al., 2019; Song et al., 2016) and with my

work, I showed that Rab7 can also be palmitoylated (Modica et al., 2017).

7.1 Rab7 palmitoylation, retromer regulation and neurodegenerative diseases

With my work I showed that Rab7 is palmitoylated and that this PTM regulates the ability of Rab7 to interact with and recruit retromer to endosomes.

Retromer is a trimeric complex of Vacuolar protein Sorting (Vps)-35, Vps26 and Vps29, that coordinates the retrieval pathway from the endosome to the TGN. This route is extremely important for the recycling of cargo receptors such as sortilin and CI-MPR. At the Golgi, these receptors recognize and bind lysosomal resident proteins (cargos) and coordinate their trafficking to the endosome. Here, the acidic pH induces the release of cargo from the receptors; while the cargo is further trafficked to the lysosome, the receptor is recognized and bound by the retromer and retrieved back to the TGN where it is now available for another round of trafficking. The function of this pathway is extremely important to maintain optimal lysosomal functions. Indeed impaired retromer activity affects the recycling of the cargo receptors such that they accumulate at endosomes and are subsequently sorted for lysosomal degradation. Reduced amount of cargo receptor in turns results in impaired trafficking of lysosomal resident proteins, that are released in the extracellular space rather than reaching the lysosome. The reduction in the amount of lysosomal proteins affects the degradative capacity of lysosomes and causes the accumulation of undegraded materials in the cell. This is the underlying mechanism of several neurodegenerative diseases.

Parkinson's disease (PD) is characterized by the accumulation in neurons of α-synuclein aggregates, known as Lewy bodies. The mutation D620N of the retromer subunit Vps35 has been associated to familial cases of PD (Vilarino-Guell et al., 2011; Zimprich et al., 2011), and post-mortem analysis of brains of affected individuals have shown reduced levels of Vps35 (Zhao et al., 2018), pointing out the importance of retromer in PD. Alterations of several retromer dependent pathways have been linked to the pathogenesis of this neurodegenerative disease. Mutation of, or reduced expression of Vps35 in models of PD results in the rapid degradation of CI-MPR, a cargo-receptor responsible for the trafficking from the TGN to the endosome of lysosomal hydrolases, among which cathepsin D, is the main enzyme responsible for the degradation of α-synuclein (Cullen et al., 2009; Sevlever et al., 2008). Reduced levels of CI-MPR results in a less efficient trafficking of cathepsin D to the lysosome, that finally leads to the accumulation of non-degraded α-synuclein and the formation of Lewy bodies (Cui et al.,

2018; Miura et al., 2014). The retromer dependent endosome-to-Golgi protein retrieval is also important for the stability of the lysosomal integral membrane protein, Lamp2 (Tang et al., 2015). This protein is required for chaperone-mediated autophagy, a process also important for the degradation of α-synuclein (Vogiatzi et al., 2008). Increased degradation of Lamp2 due to impaired retromer function causes accumulation and aggregation of α-synuclein (Tang et al., 2015). The D620N mutation on Vps35 affects also the ability of retromer to traffic ATG9+ membranes, which is necessary for the formation and maturation of autophagosomes. Given that autophagy is the main route for the elimination of α-synuclein aggregates, defective autophagy translates in the accumulation of Lewy bodies (Zavodszky et al., 2014). Vps35 mutations are also associated to defect in the SNX27/retromer mediated PM receptor recycling. In the context of PD, Vps35 D620N mutation causes the aberrant trafficking of AMPAR and dopamine D1 receptor (Cui et al., 2018). This retromer pathway is independent of Rab7.

Alzheimer's disease (AD) is characterized by the extracellular accumulation of Aβ-peptide in amyloid plaques and the intracellular deposit of tau protein in neurofibrillary tangles (Scheltens et al., 2016). Aβ is the product of the proteolytic processing of Amyloid precursor protein (APP), a type I transmembrane protein expressed in several tissues with no clear function. APP is cleaved by specific proteases via two possible processing pathways, amyloidogenic and non-amyloidogenic. In the amyloidogenic pathway, APP is cleaved resulting in the production of pathogenic extracellular Aβ (Haass et al., 2012; Willnow and Andersen, 2013). The two pathways compete for APP cleavage, and the regulation of intracellular trafficking of the protein is extremely important to prevent the production of Aβ. APP is synthesized in the ER where it is post-translationally modified and trafficked via the secretory pathway to the plasma membrane (PM) where it enters the non-amyloidogenic pathway. However, only a small amount of APP reaches the PM, while the majority of the protein accumulates in the trans-Golgi network (TGN) (Haass et al., 2012; Scheltens et al., 2016). Here APP is bound by cargo-receptors of the Vps10-domain family, trafficked to the endosome and retrieved back to the TGN. APP accumulation in endosomes results in the amyloidogenic cleavage of APP by β-amyloid APP-cleaving enzyme 1 (BACE1) and γ-secretase (Willnow and Andersen, 2013; Willnow et al., 2008). The efficient retrieval of APP from the endosome by retromer plays a key role in reducing APP endosome dwelling time and prevents Aβ production (Li et al., 2016; Reitz, 2012; Small and Petsko, 2015). Indeed gene expression profiles of the brains from AD affected patients reveals a reduction in the expression of the retromer subunits Vps35 and Vps26, highlighting the importance of this complex in AD (Small et al., 2005).

Neuronal Ceroid Lipofuscinoses (NCL) represent a group of inherited neurodegenerative diseases characterized at the cellular level by the accumulation of auto-fluorescent material, lipofuscin. Clinical manifestations include progressive decline of visual and motor functions, intellectual impairment, epileptic seizures and shorten life span (Mole and Cotman, 2015; Radke et al., 2015). Among the genes involved in the pathogenesis of this disease, CLN3 and CLN5 have been shown to be involved in the regulation of retromer function. CLN5 is a soluble lysosomal protein (Jules et al., 2017) whose function is still not clear. Depletion of CLN5 causes the displacement of the retromer from the membrane that functionally results in the degradation of the cargo receptor sortilin and CI-MPR (Mamo et al., 2012). Moreover, mutations causing the ER retention of CLN5 result in decreased lysosomal trafficking of cathepsin D, supporting the role of CLN5 in regulating retromer function. We have recently shown that the endolysosomal membrane protein CLN3 is involved in the regulation of retromer function. CLN3 has been shown to interact with Rab7 (Uusi-Rauva et al., 2012; Yasa et al., 2019) and we have recently showed an interaction between CLN3/retromer and CLN3/sortilin. Interestingly, depletion of CLN3 weakens the Rab7/retromer and retromer/sortilin interaction. The decrease in these interaction functionally results in the degradation, rather then the recycling of, the cargo receptors sortilin and CI-MPR (Yasa et al., 2019).

Given its crucial role in the maintenance of a healthy nervous system, retromer and the complex of proteins that contribute to its regulation could represent an important therapeutic target. Pharmacological chaperones that stabilize retromer against thermal denaturation have been shown to increase retromer level in hippocampal neurons and to lower the production of Aβ fragments by reducing the residence time of APP in endosome (Mecozzi et al., 2014). This preliminary study shows the therapeutic potential of the modulation of retromer function in the context of neurodegeneration. It is therefore critical to understand the several layers of retromer function regulation to expand the pool of therapeutical targets. In this respect, our discovery that Rab7 palmitoylation has a role in modulating the ability of the small GTPase to interact with retromer, could have an important significance from a therapeutic point of view. Our work suggests that Rab7 palmitoylation is specifically involved in the regulation of retromer function, while it does not significantly affect the ability of Rab7 to control the lysosomal degradation pathway. Indeed although the non-palmitoylatable Rab7 mutant, $Rab7_{C83,84S}$, fails in rescuing retromer recruitment to endosomes, it is able to rescue the degradation of EGF/EGFR complex in a way comparable to wild-type Rab7. Interestingly, supporting the inability of this mutant to

efficiently engage retromer, we observe a slight delay in the degradation efficiency of cells rescued with $Rab7_{C83,84S}$ after 15 minute chase, a delay that disappear after 2 hour chase. This delay likely reflects the reduced degradative capacity of lysosomes, a consequence of retromer function defect. Indeed Rab7-KO cells rescued with $Rab7_{C83,84S}$ secrete the lysosomal enzyme cathepsin D into the extracellular space instead of trafficking the hydrolase to the lysosome. I can hypothesize that the modulation of Rab7 palmitoylation level could translate in the regulation of retromer association on the membrane. In this respect, controlling the activity of the palmitoyltransferase(s) or thioesterase(s) involved in the addition and removal of the palmitate chain on Rab7 respectively, could finally translate in the regulation of retromer recruitment and function at the endosome. Unfortunately the identity of the palmitoylation machinery in regulating the Rab7 palmitoylation/depalmitoylation cycle is currently unknown.

Interestingly, in my second work, I show that phosphorylation of Rab7 on serine 72 is important to regulate the ability of the small GTPase to interact with and recruit retromer at the late endosome membrane. Indeed, the expression of the non-phosphorylatable $Rab7_{S72A}$ mutant in Rab7-KO cells phenocopies what we observed with the non-palmitoylatable mutant $Rab7_{C83,84S}$. Moreover we show that $Rab7_{S72A}$ is less palmitoylated than wild-type Rab7, consistent with the inability of this mutant to recruit retromer. Since we found a correlation between palmitoylation and phosphorylation, we hypothesized that by increasing Rab7 serine 72 phosphorylation level, we could in turn increase the palmitoylation of the small GTPase. Unfortunately, we could not rely on the use of the phoshomimetic $Rab7_{S72E}$ for these experiments. This mutant is supposed to mimic a constitutively phosphorylated serine 72 on Rab7. However, a recently published study shows that the substitution of the serine with glutamic acid strongly impairs the interaction of Rab7 with RabGGTase, hence $Rab7_{S72E}$ is not efficiently prenylated (Heo et al., 2018). Indeed this mutant is mainly localized in the cytosol and mimics the behaviour of the prenylation mutant $Rab7_{C205,207S}$. We therefore decided to increase Rab7 phosphorylation level by over-expressing the recently identified Rab7 kinase TBK1. This enzyme phosphorylates mitochondria-associated Rab7 during mitophagy and this modification is required for the efficient lysosomal degradation of damaged mitochondria (Heo et al., 2018). In our hands, the over expression of TBK1 was not associated with increased Rab7 palmitoylation levels and did not substantially change the amount of retromer associated with the membrane. Interestingly we found that by treating the cells with TBK1 inhibitor MRT67307, Rab7 palmitoylation levels were reduced, although not enough to have a significant effect of retromer membrane recruitment. In the context of mitophagy, the knock out of TBK1 or the expression in a Rab7-KO background of $Rab7_{S72A}$ resulted in impaired mitophagy and reduced recruitment of ATG9+ membranes to the

mitophagosome, a process that is mediated by retromer (Jimenez-Orgaz et al., 2018). My data suggest that TBK1-mediated Rab7 phosphorylation is required for retromer recruitment exclusively during mitophagy, and has no major role in regulating the overall retromer membrane distribution. Given that the expression of $Rab7_{S72A}$ has an important effect on retromer membrane recruitment, it is likely that beyond TBK1, other kinases participate in phopshorylating Rab7. In this respect a recent published work showed that the dominant active mutant of the leucine-repeat reach kinase 1 (LRRK1) $LRRK1_{Y944F}$ phosphorylates Rab7 on S72 (Hanafusa et al., 2019). According to these data the LRRK1 dependent phosphorylation increases Rab7/RILP interactions without affecting the one with FYCO1. LRRK1-dependent phosphorylation would therefore be required for Rab7/RILP interaction and vesicles movement (Hanafusa et al., 2019). Our findings are in contrast with these results. Indeed our BRET analysis shows no difference between Rab7/RILP and Rab7S72A/RILP interaction, suggesting that phosphorylation of S72 is not required to engage RILP nor ORP1L, the other player regulating retrograde trafficking. A possible explanation for this inconsistency could be the use of the dominant active form of LRRK1, that could have reduced substrate specificity. Further work is required to confirm the role of LRRK1 as Rab7 kinase, and in this context, it would be interesting to evaluate if the expression of LRRK1 WT induces as well cause the increase in Rab7 S72 phosphorylation.

A recently published proximity-labeling screen using APEX-2, identified WNK1, Raf1 and SIK3 as Rab7-interacting kinases (Del Olmo et al., 2019). It would be interesting to test if these kinases contribute to Rab7 S72 phosphorylation and if this is the case, how this phosphorylation affects Rab7 palmitoylation and retromer functions. These would contribute to a further enlargement of the pool of therapeutic targets for the treatment of neurodegenerative diseases such as AD and PD.

Palmitoylation is a PTM common among several players involved in endosome-to-TGN trafficking. Indeed, beyond Rab7, sortilin and CI-MPR are palmitoylated and this PTM is required for their trafficking to the TGN (McCormick et al., 2008). Unpublished data from our lab show that CLN3 involved in the regulation of retromer/Rab7 interaction, is palmitoylated as well. Since one of the functions of palmitoylation is to localize protein in specific membrane subdomain, is it possible that in the context of endosome-to-TGN trafficking, palmitoylation serves as a mechanism to bring all the involved players in close proximity in specific endosomal domain. Supporting this hypothesis, my previous work showed that non-palmitoylatable Rab7 does not co-localize with retromer using SIM microscopy, even though both are on the

membrane on late endosomes (Modica et al., 2017). In addition, previous work from our lab showed that non-palmitoylatable sortilin was not in the same membrane fraction as retromer, although wild-type sortilin was (McCormick et al., 2008). The formation of an endosome-to-TGN trafficking platform by palmitoylation would assure the efficient spatiotemporal endosomal recruitment and function of retromer, and efficient retrograde trafficking.

7.2 Rab7 Y183 phosphorylation

Rab7 and its effector RILP play a key role in the degradation of Epidermal Growth Factor receptor (EGFR). Activation of this receptor at the plasma membrane (PM) leads to its dimerization and initiation of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways to promote cell growth, differentiation, motility and proliferation (Bakker et al., 2017; Ceresa and Peterson, 2014). Given the plethora of effects associated with the stimulation of EGFR, the tight spatiotemporal regulation of the active state of this receptor is crucial to prevent oncogenic transformation. In this respect, the rapid endocytic internalization of the receptor is the first step to limit its signaling. Under low ligand stimulation, EGFR is preferentially internalized via clathrin-dependent endocytosis (CME) and trafficked in the endosomal compartment, where it can either be recycled back to the PM, or sorted for lysosomal degradation. It is important to note that at the endosome, EGFR is still able to activate signaling cascades. In this respect, the spatial compartmentalization of EGFR influences cellular outcomes of certain signaling pathways (Bakker et al., 2017). Under high ligand stimulation (EGF> 20ng/ml) (Sigismund et al., 2013; Sigismund et al., 2005), EGFR is rapidly internalized in a clathrin independent way and trafficked to the endosome for degradation. Indeed high ligand concentration induces the ubiquitination of EGFR that once at the endosome, is recognized by the ESCRT complex and sorted into the intra luminal vesicles (ILVs) en route to the lysosome. ESCRT-dependent sorting of EGFR into ILVs causes the removal of the intracellular tail of the receptor from the cytosol, actually down-regulating downstream signaling (Eden et al., 2009).

Rab7 has a multifaceted role in regulating the fate of EGFR that involves the regulation of the trafficking of MVBs vesicle toward the perinuclear region to meet and fuse with lysosomes for the degradation of internalized EGFR and the recruitment of the fusion machinery to organize the late endosome-lysosomal fusion. The RILP/Rab7 interaction is considered crucial to accomplish these functions. Indeed, the combined action of RILP and ORP1L in a tripartite

complex with Rab7 leads to the recruitment of the dynein motor complex to link the vesicles to microtubules to approach perinuclear lysosomes (Johansson et al., 2005; Johansson et al., 2007; Jordens et al., 2001). Here RILP/Rab7/ORP1L together with PLEKHM1 can recruit and stabilize the late endosome-lysosomal tethering complex to the membrane and accomplish fusion (McEwan et al., 2015; Wijdeven et al., 2016). Surprisingly, we found that the recruitment of RILP in this context is dispensable, as the tyrosine phosphomimetic $Rab7_{Y183E}$ mutant, that is not able to engage RILP, is still capable of coordinating the trafficking and fusion events for the degradation of EGFR. However, RILP is required to direct EGFR sorting into ILVs and degradation in lysosomes. Down regulation of the Rab7 effector induces the accumulation of EGFR in early endosomal compartments. This effect is likely due to the ability of RILP to recruit and stabilize the Vps22 and Vps36 subunits of ESCRT-II complex on the membrane, for the efficient sorting of ubiquitinated cargo into the degradative compartment. Rab7 Y183 phosphorylation is dependent upon EGF stimulation (Francavilla et al., 2016; Lin et al., 2017), and it is mediated by the kinase Src (Lin et al., 2017). We show that over expression Src leads to a decreased RILP/Rab7 interaction and corresponds to a stronger RILP/ESCRT-II interaction. The level of Rab7 Y183 phosphorylation rapidly increases after EGFR stimulation (Francavilla et al., 2016; Lin et al., 2017; Sapmaz et al., 2019) and returns to basal level after 40 to 90 minutes (Francavilla et al., 2016; Sapmaz et al., 2019). This might suggest that during the early phase of EGF/EGFR internalization, RILP is required to work with ESCRT-II to ensure faster sorting into ILVs to quickly inactivate downstream signaling pathways by EGFR. Our preliminary data concerning the interaction between RILP and ESCRT-II were performed in Src over expressing cells. In this setting Rab7 is constantly over phosphorylated, therefore we are not able to take into account the reversible nature of this modification and how this cycle affects this pathways. Furthermore, in this context, it would also be interesting to look at how different concentration of EGF affect EGFR internalization and degradation rate. In our work, we only tested high EGF concentrations. In this condition the internalization of EGF/EGFR is also mediated by the fast clathrin-independent endocytosis that has the main object to degrade overactive EGFR. We have no data about the mechanisms that take place under low-EGF stimulation. Further work will help to clarify these points.

Although the Y183F mutant blocks the degradation of EGF, at a molecular level, it does not show any major alteration in the ability to interact with RILP or PLEKHM1 nor affect RILP/PLEKHM1 interaction when expressed in Rab7-KO cells. Although we do not have direct evidence at the moment about the ability of this mutant to recruit HOPS on the membrane, the observation that RILP and PLEKHM1 can both be recruited by Rab7, suggests that the absence

of phosphorylation of Y183 does not affect the recruitment of the fusion machinery on the membrane. Furthermore, our data revealed a stronger interaction between $Rab7_{Y183F}$ and ORP1L. This protein regulates late endosome/ER contact sites by interacting with the ER protein VAP-A. This interaction is cholesterol dependent, in particular low cholesterol levels favour ORP1L/VAP-A interaction and the dissociation of $p150^{GLued}$ from RILP, maintaining late endosome/ER contact site (Wijdeven et al., 2016). On the other hand, high cholesterol levels cause the release of ORP1L from VAP-A while restoring the RILP/$p150^{Glued}$ interaction and favouring late endosome movement. The increased $Rab7_{Y183F}$/ORP1L interaction that we observed could have consequences in the regulation or ORP1L\VAP-A interaction, possibly by reducing the ability of ORP1L to release the ER protein. This would result in turn in the impairment of RILP/p150Glued interaction with the consequent block of late endosome movement. This could finally result in an impaired late endosome-lysosomal fusion that could justify the accumulation of EGF that we observe in Rab7-KO cells rescued with $Rab7_{Y183F}$. It would also be interesting to evaluate the contribution of FYCO1/kinesin-1 plus-end directed trafficking in this process. Interestingly, plus-end directed trafficking of late endosomes is dependent upon the bind of the ER protein protrudin to Rab7, allowing the transfer of the motor protein kinesin-1 from protruding to FYCO1 (Raiborg et al., 2015). Therefore it would be interesting to evaluate the role of Y183 phosphorylation in regulating the Rab7/protudin interaction. It also important to note that the mechanisms regulating movement and positioning of late endosomes, play a key role in the context of autophagosome-lysosome fusion. In the respect, it would be interestingly to analyze how Y183 phosphorylation affects the clearance of autophagosomes.

Membrane trafficking is critical for the maintenance of neuronal physiology. Indeed, these cells are highly polarized and rely on endosomal activity to maintain their architecture (Neefjes et al., 2017). In this context, Rab7 is involved in several aspects of neuronal trafficking. The interaction between Rab7/FYCO1/Protrudin has shown to promote neurite outgrowth by mediating late endosome trafficking towards the periphery in a kinesin-1 dependent manner (Raiborg et al., 2015). Rab7 is also crucial for retrograde trafficking of vesicles from the axon towards the soma, a pathway that is vital for the communication between the axon and the soma. The tripartite complex Rab7/RILP/ORP1L, via the recruitment of $p150^{Glued}$, is primarily responsible in accomplishing this task. Mutations in Rab7 have been linked to the inherited neuropathy, Charcot-Marie-Tooth type 2B (CMT2B) (Verhoeven et al., 2003). Interestingly all the identified Rab7 mutations cause an over activation of the small GTPase (McCray et al., 2010; Spinosa et al., 2008) that functionally translates in increased binding with RILP (Spinosa

et al., 2008) and in faster axonal transport (Zhang et al., 2013). This leads to the faster internalization, transport and degradation of Nerve Grows Factor (NGF) receptor TrkA upon NGF stimulation. This process finally results in a premature termination of NGF signalling leading to the degeneration and atrophy of the axon (Neefjes et al., 2017; Neefjes and van der Kant, 2014; Zhang et al., 2013). There is no information on how PTMs could affect axonal trafficking. In this context, it would be of particular interest to look at the impact of Y183 phosphorylation in the regulation of axonal retrograde trafficking.

7.3 Conclusion

With my work, I contributed to the identification and characterization of new layers of regulation of Rab7 functions. Cells use post-translational modification as a way to regulate the function of proteins. Transient PTM such as phosphorylation, ubiquitination and palmitoylation represent a clever tool to customize protein functions according to cellular needs. Indeed, through the combination of phosphorylation, ubiquitination and palmitoylation, cells can regulate Rab7 activity in the context of several different trafficking pathways. In particular, my work has experimentally shown that Rab7 is palmitoylated and we also revealed the importance of this modification for the spatiotemporal recruitment of retromer at the endosome. Moreover we show that an interplay between serine phosphorylation and palmitoylation exist, and is involved in the regulation of retromer function.

Despite the fact that Rab7 phosphorylation on Y183 had already been shown its functional significance on Rab7 was not clear. With my work, I elucidated some of the functional consequences that Y183 phosphorylation has on Rab7 function. I found that Y183 phosphorylation is required for EGF degradation and we provide evidence that this process is independent of the Rab7/RILP interaction. Furthermore our preliminary observations suggest a possible involvement in late endosome/ER contact sites in the control of Rab7-depended vesicle trafficking and movement.

Although future work is required to exhaustively understand how these PTMs are regulated and the enzymes that are involved, this work suggests new possibilities for the design of new therapeutic approach aimed to stabilize or improve retromer function, whose optimal functions are required to prevent the pathogenesis of neurodegenerative disorders like AD or PD.

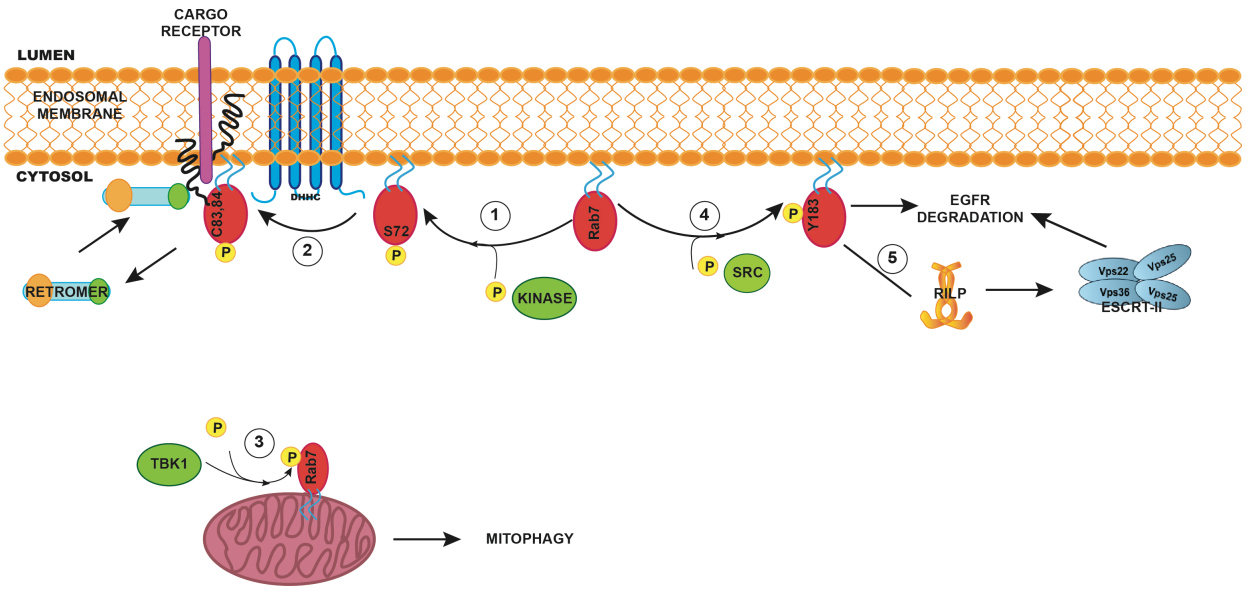

Figure 7-1 Schematic representation of Rab7 PTMs analyzed in this work

(1) At the endosome phosphorylation on S72 favours Rab7 palmitoylation (2) and the efficient recruitment of retromer on the endosomal membrane. (3) During mitophagy, TBK1-mediated Rab7 S72 phosphorylation is required for efficient mitophagosome maturation and degradation. (4) Rab7 phosphorylation on Y183 mediated by Src is required for Epidermal Growth Factor Receptor (EGFR) degradation. (5) Y183 phosphorylation inhibits the interaction between Rab7 and RILP while favouring the interaction of the latter with ESCRT-II for proper EGFR sorting and degradation.

Adapted from Modica & Lefrancois, Small GTPase 2017

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