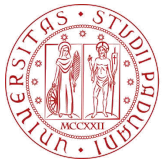

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Biomedical Science

Interuniversity Bachelor's degree programme
in Biology of Human and Environmental Health

Final dissertation

Investigating Endolysosomal Dysfunction in Two Ultra-Rare Hereditary Spastic Paraplegia Disorders via Immunofluorescence Confocal Microscopy

Supervisor
Professor Paola Galozzi

Candidate: ***J HUCKFELDT***
Student ID number: **2112421**

ACADEMIC YEAR 2025/2026

INDEX

ABSTRACT

Hereditary Spastic Paraplegias (HSP) represent a large, clinically and genetically heterogenous family of motor neurodegenerative disorders. They are characterized by corticospinal axonopathy which leads to progressive lower-limb spasticity and weakness. Current medical interventions only help to manage the physiological symptoms rather than address their molecular causes. Elucidating the underlying pathological mechanisms is crucial to the development of novel therapeutic treatments, and dysfunction of intracellular trafficking is emerging as an interesting target. Endo-lysosomal trafficking has been well established as being essential to cellular homeostasis and naturally its dysfunction and/or dysregulation has been implicated in the pathogenesis of several diseases, including various neurodegenerative disorders. Accordingly, several HSP-related genes have been found to be involved in the endo-lysosomal and autophagic pathways, suggesting a functional convergence. With a focus on two ultra-rare forms of HSP, KIDINS220-dependent SINO and ALS2-dependent IAHSP, this investigation aims at exploring potential abnormalities of the endo-lysosomal system in patient-derived fibroblasts, through the use of immunofluorescence confocal microscopy.

1. INTRODUCTION

1.1 Hereditary Spastic Paraplegia

Hereditary Spastic Paraplegia (HSP) constitutes a large family of clinically and genetically heterogenous motor neuron degenerative disorders. The predominant clinical features of HSP involve progressive lower-limb spasticity and weakness due to neuronal degeneration of the corticospinal tract. There are no current treatments to prevent, slow or reverse this condition; existing medical interventions only help to manage the symptoms (Bellofatto *et al.*, 2019).

Due to the phenotypical and genotypical diversity of HSP, several clinical and genetic subclasses have been defined. The clinical classification divides HSP into pure or complex; the pure form features neurological dysfunction that is limited to progressive lower-limb spastic weakness, sometimes involving urinary symptoms and impaired pallesthesia. Complicated HSP constitutes the presence of other accompanying neurological and/or non-neurological features such as ataxia, intellectual disability, epilepsy, and peripheral neuropathy. Genetic subclasses, on the other hand, are based on the inheritance pattern of HSP: Autosomal dominant; Autosomal recessive; X-linked inheritance; and Mitochondrial inheritance (Meyyazhagan *et al.,* 2022).

This thesis will be focused on examining two ultra-rare, autosomal and complex forms of HSP: Spastic paraplegia, Intellectual disability, Nystagmus, and Obesity (SINO) syndrome; and Infantile-onset Ascending Hereditary Spastic Paraplegia (IAHSP).

1.2 SINO syndrome

Spastic paraplegia, Intellectual disability, Nystagmus, and Obesity (SINO) syndrome is a rare autosomal dominant disorder, characterized by the combination of congenital spastic paraplegia, intellectual disability, ophthalmologic abnormalities (such as nystagmus, reduced visual acuity, and hyperopia), and obesity, as the SINO acronym indicates. Additionally, recent studies have supported the consideration of other clinical features, involving behavioural abnormalities and dysmorphic features such as ventriculomegaly and brachyplagiocephaly (Alstrup *et al.*, 2024).

SINO syndrome has been established as being dependent on mutations of the *KIDINS220* gene, which encodes for the protein Kinase D-interacting substrate of 220 kDa (KIDINS220), alternatively named Ankyrin repeat-rich membrane-spanning (ARMS) protein (Josifova *et al.*, 2016). KIDINS220 is a conserved transmembrane scaffold protein, whose sequence structure reveals various domains for protein-protein interactions, notably with major neurotrophins receptors like tropomyosin receptor kinases (Trks) and the p75 neurotrophins receptor (p75ntr). Its interactions with numerous receptors and its involvement in a variety of signalling pathways, often through the recruitment of adaptors and effectors, suggests it plays a functional role as a signalling platform, affecting neuronal development, differentiation and even synaptic function. An example of this includes the selective mediation of sustained MAPK/ERK activation through KIDINS220 recruitment of mediators (C3G and Rap1), which primarily occurs at late endosomes, promoting neuronal survival and differentiation (Neubrand *et al.*, 2012).

1.3 IAHSP

Infantile-onset Ascending Hereditary Spastic Paraplegia (IAHSP) is a rare autosomal recessive form of HSP that involves retrograde degeneration of the upper motor neurons of the pyramidal tracts. Onset typically occurs during the first two years of life and continues to progress to upper limbs and bulbar muscles within five to ten years. Tetraplegia, anarthria, dysphagia, and slow eye movements are frequently reported to develop in those following years, and yet survival beyond the third and fourth decades is not uncommon. The condition has been found to be related to mutations

in the *ALS2* gene, which has commonality with other distinct spastic disorders like Amyotrophic Lateral Sclerosis (ALS) and Primary Lateral Sclerosis (PLS) (Eymard-Pierre *et al.*, 2002).

The *ALS2* gene encodes for the ALSIN protein which acts as a guanine-nucleotide exchange factor (GEF) for the small GTPase Rab5. ALSIN is expressed primarily in the central nervous system, particularly in motor neurons of the cortex and spinal cord. ALSIN's GEF activity consists of binding and activating Rab5 in a superoxide dismutase 1 (SOD1)-dependent manner, triggering the formation of a protein complex with early endosome auto-antigen 1 (EEA1), which in turn promotes endosome vesicle fusion. ALSIN protein impairment has therefore been correlated with defective endosomal trafficking, which may consequentially contribute to disrupted intracellular signalling (Hadano *et al.*, 2007;Kim *et al.*, 2021).

1.4 The endosomal-lysosomal system

The endosomal-lysosomal system is a highly complex and dynamic network of tubulovesicular organelles, responsible for major intracellular trafficking pathways. It regulates and facilitates the delivery, localization, and processing of numerous kinds of molecules within the cell. It is strictly involved in nutrient uptake, cargo sorting, and degradation of cellular waste, delivered by both endocytic and autophagic routes. Accordingly, this system is crucial in maintaining cell homeostasis, establishing cell polarity, mediating cell signalling, and modulating synaptic transmission for proper neuronal function and survival (Li and DiFiglia, 2012; Hu *et al.*, 2015). Impaired function of this system has been increasingly associated with various neurodegenerative disorders, including a large number of HSP phenotypes (Herman *et al.*, 2023) (Blackstone, *et al.*, 2011).

Endosomal trafficking comprises the dynamic fusion and fission of cytosolic vesicles, which move through different maturation stages and compositions, and are responsible for the sorting of cellular content to a variety of destinations. Incoming extracellular material and membrane-associated elements, such as solutes and receptors, are internalized via endocytic vesicles, which then fuse into an early endosome (EE). The EE's slightly acidic intraluminal space facilitates the dissociation of any ligands from their receptors, allowing for their differential assortment and consequent delivery. Internalized cargo is typically routed to one of three destinations, according to their fate: cargo can be recycled back to the plasma membrane for reuse; retrogradely transported to the trans-Gogli network (TGN); or carried into late endosomes for lysosomal delivery and resultant degradation (Scott *et al.*, 2014; Hu *et al.*, 2015).

The recycling of receptors and membrane components is essential in maintaining plasma membrane integrity and homeostasis. It either occurs directly, via the fast recycling pathway which is mediated by Rab4, or through the indirect one. The indirect pathway, mediated by Rab11, is slower as cargo is first loaded into recycling endosomes (REs) before being delivered to the membrane. REs participate in supporting proper neuronal function through their redistribution of crucial adhesion molecules required for neuronal outgrowth and for their return of neurotransmitter transporters that enable synaptic transmission (Li and DiFiglia, 2012).

On the other hand, cargoes that are destined for degradation, for instance uncoupled ligands and solutes, are progressively accumulated in the EE as intralumenal vesicles. These eventually bud off from the EE as a multivesicular body (MVB), which then tends to migrate along a microtubule to the perinuclear region of the cell, progressively developing into a late endosome (LE). This maturation process is characterized by a change in association from EE-bound Rab5 to Rab7, accompanied by a progressive increase in intravacuolar acidity. Finally, LEs fuse with hydrolase-containing lysosomes forming a transitory hybrid vesicle, the endo-lysosome, which has a highly acidic intraluminal environment, optimal for proteolytic activity constituting content degradation. The endo-lysosome is eventually transformed back into a classical dense lysosome (Hu *et al.*, 2015; Behl *et al.,* 2022).

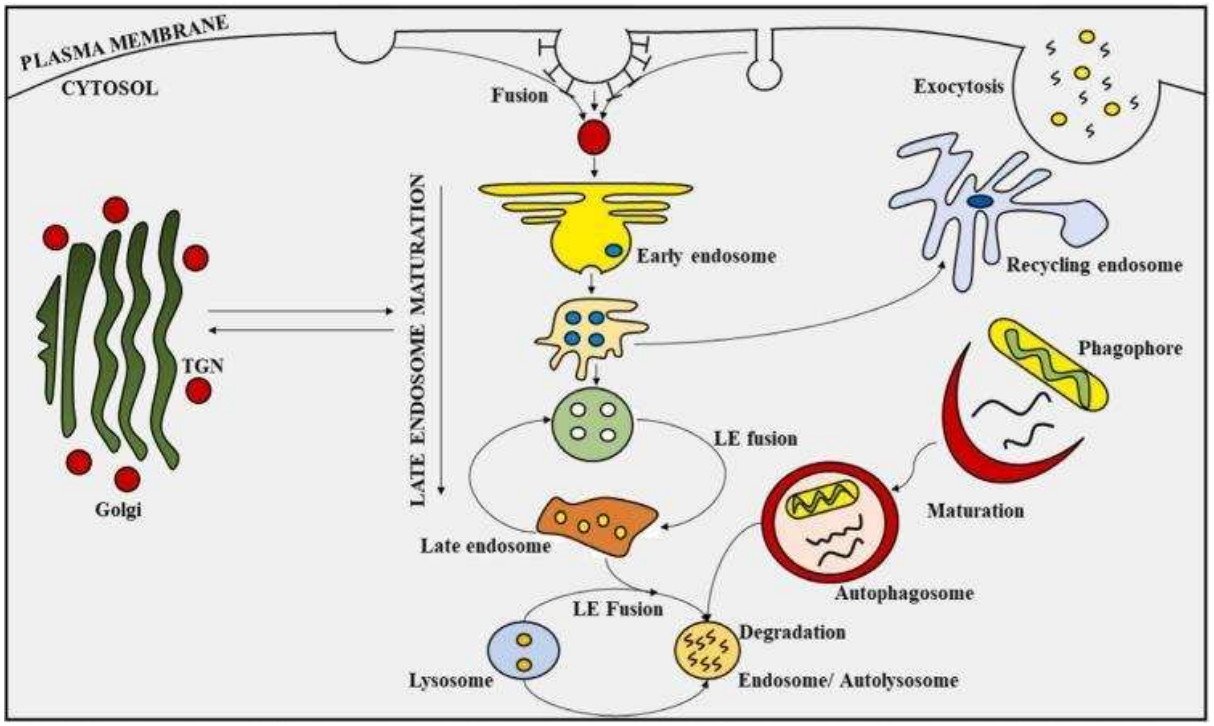

Figure 1. Overview of endolysosomal and autophagic trafficking.

Fig.1 Internalized cargo enters early endosomes, which mature into late endosomes (LEs) and fuse with lysosomes for degradation. Recycling endosomes return selected cargo to the plasma membrane, while trafficking from the trans-Golgi network (TGN) contributes to late endosome maturation. Autophagosomes formed from phagophores fuse with LEs or lysosomes to generate degradative endosome/autolysosome vesicles. *Adapted from: Behl et al. 2022*

Throughout this process, numerous different Rab proteins are affiliated, in association with distinct types of endosomes. These membrane-associated proteins are part of a large family of small GTP-ases, and they are the primary regulators of endo-lysosomal trafficking. They act as molecular switches, alternating from an inactive GDP-bound form to an active GTP-bound form that recruits various effector proteins in order to exert their regulatory function. These effectors include motor proteins for vesicle migration, as well as proteins involved in vesicle membrane tethering and fusion (Zhang, Jiang and Shi, 2022). Accordingly, Rabs have been described to crucially mediate synaptic function and neurotransmitter release, especially through their generation, mobilization and recycling of synaptic vesicles (Bellucci *et al.,* 2022).

1.5 Endo-lysosomal dysfunction

Considering the vital nature of the endo-lysosomal pathway in ensuring proper cell homeostasis, including its intrinsic connection to other crucial systems such as autophagy and phagocytosis, its disruption expectedly contributes to cellular dysfunction. Various studies have established this link in several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). These diseases often have the shared feature of presenting abnormal accumulations of misfolded proteins and dysfunctional organelles that then results in cellular toxicity, suggesting an impairment in intracellular trafficking and lysosomal waste clearance. Notably, accumulations of abnormal early endosomes were observed in postmortem brain tissue of AD patients and the synaptopathy present in PD has been associated with pathological alterations of Rab family protein activity (Bellucci *et al.,* 2022; Herman *et al.,* 2023).

In the context of HSP disorders, length-dependent maintenance and degeneration of corticospinal axons plausibly relies on the robustness of intracellular trafficking and organelle morphogenesis and distribution. Considering that these axons span very long distances in the spinal cord to facilitate rapid signal relay for voluntary movement, highly dynamic and complex mechanisms are required for the appropriate intracellular content organization and allocation. Accordingly, a considerably large set of proteins, identified to be associated with HSPs, are shown to be involved in membrane

trafficking. As an example, the Spastin protein, whose encoding gene *SPG4* was the first gene identified as a cause for autosomal dominant pure HSP, is highly implicated in membrane modelling and polarized endosomal trafficking. Moreover, as discussed for IAHSP, ALSIN dysfunction has shown to disrupt Rab5-dependent endosomal fusion and leads to the impairment of numerous intracellular signalling cascades essential for axonal morphology, synaptic plasticity and neuronal survival (Blackstone, *et al.*, 2011).

4. AIMS OF THE THESIS

The underlying pathological mechanisms which lead to the progressive corticospinal axonal degeneration in HSP are yet to be clearly defined, even less so in the two ultra-rare subtypes, SINO syndrome and IAHSP. When considering other motor neuron and neurodegenerative diseases -such as ALS and AD- disruption of the endo-lysosomal system, which governs intracellular trafficking and cellular homeostasis, is emerging as a significant contributor to their pathogenesis. Additionally, several HSP-related proteins are implicated in these trafficking pathways, with varying alterations impacting different aspects of the system, resulting in substantial neuronal defects. The aim of this study is to investigate potential irregularities of the endo-lysosomal pathway in SINO and IAHSP forms through the analysis of a set of selected proteins using immunofluorescence confocal microscopy of patient-derived dermal fibroblasts. These proteins are associated with different progressive stages of the endo-lysosomal pathway, allowing to distinguish the possible points of disruption. Early endosome antigen 1 (EEA1) is associated with early endosome formation, Rab5 with early endosome fusion, Rab7 with early-to-late endosome maturation.

By identifying these principal endo-lysosomal compartments affected in SINO syndrome and IAHSP, the objective of this paper is to provide an initial characterization of endocytic membrane trafficking alterations in rare HSP subtypes, laying the groundwork for further studies investigating the mechanisms underlying disease pathogenesis.

3. MATERIALS AND METHODS

3.1 Fibroblast cell culture

Fibroblast cells derived from patient and control dermal biopsies were provided by associated collaborators. As outlined in **Table 1**, a total of 6 HSP patients (3 SINO, 3 IAHSP), and 5 age and sex-matched healthy controls were enrolled in the Stella Maris Institute in Pisa, Italy. All participants signed an informed consent form before sample collection, according to the current regulations, under the responsibility of head neurologist, Dr. F.Santorelli.

	SINO (n=3)	IHASP (n=3)	Controls (n=5)	
Age/Sex	F3	F5	F3	
	M6	M6	F5	M11
	F12	F12	M6	F12

Table 1. *Ages and sexes of patients and controls at time of biopsy*

The obtained fibroblast cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% Fetal Bovine Serum (FBS), 2nM glutamine, 100 U/ml penicillin and 0.1mg/ml streptomycin. They were kept in a 5% CO2 humidified incubator at 37ºC. Once cells reached 80% confluence, medium was removed and Trypsin 0.5X was added and cells were left in the incubator for 5 min. Trypsin was stopped with DMEM medium once the cells had fully detached. Cells were then counted manually using a hemocytometer and seeded in 24-well plates containing 18mm round glass coverslips, at 15,000 cells/coverslip.

3.2 Immunocytochemistry/Immunofluorescence

Fibroblast cells on 12 mm round glass coverslips were washed with PBS 1X, fixed with paraformaldehyde (PFA) 4% for 15 min and washed three times for 5 min with PBS 1X. Cells were then permeabilized with PBS 1X-Triton X-100 0.5% for 15 min at room temperature (RT). The permeabilization solution was removed and the blocking solution of PBS 1X-Triton X-100 0.1% with Bovine Serum Albumin (BSA) 5% was added for 30 min at RT. After removing the blocking solution, cells were washed with PBS 1X and incubated with the primary antibodies for 1 h at RT while covered to limit evaporation. Two unconjugated primary antibodies deriving from different host species were administered once diluted in blocking solution, as indicated in **Table 2**. Following the incubation with the primary antibodies, three washes with PBS 1X-Triton X 0.01% were performed. Then, the cells were incubated with a solution containing both secondary antibodies, unconjugated and diluted in blocking solution according to **Table 2**, for 1 h at RT while covered in aluminium foil to minimize light exposure as well as evaporation. Phalloidin 647 actin marker, in

blocking solution at 1:200 dilution, was also added to the secondary antibody solution for incubation. Cells were washed three times with PBS 1X-Triton X 0.05% followed by a wash with PBS 1X and then kept in dd-milliQ water. Finally, coverslips were placed upside down on a 20µl droplet of Fluoromount mounting medium onto microscope slides.

	Antibody	Species	Dilution
Primary	Anti-EEA1	Rabbit	1:100
	Anti-RAB5	Rabbit	1:100
	Anti-RAB7	Mouse	1:100
Secondary	Alexa Fluor 488 anti-rabbit	Goat	1:500
	Alexa Fluor 568 anti-mouse	Goat	1:500

Table 2. *Details of primary and secondary antibodies with species and dilution.*

3.4 Confocal microscopy imaging and analysis

Immunofluorescence images of fixed cells were acquired at room temperature on a Leica SP5 confocal fluorescence microscope using a 40X oil immersion objective. Each coverslip had three fields imaged and serial confocal sections were captured from the top to the bottom of the cells. These were obtained as stacks in a 1024 x 1024 pixel format and at a speed of 400Hz. Images were converted to TIFF files to be processed using ImageJ as Z-projections. Regions of interest (ROI) were manually drawn around individual cells, from which the area, integrated density and mean grey values were measured for each separate channel. Background intensity was set per image as a manually-drawn ROI adjacent to each selected cell.

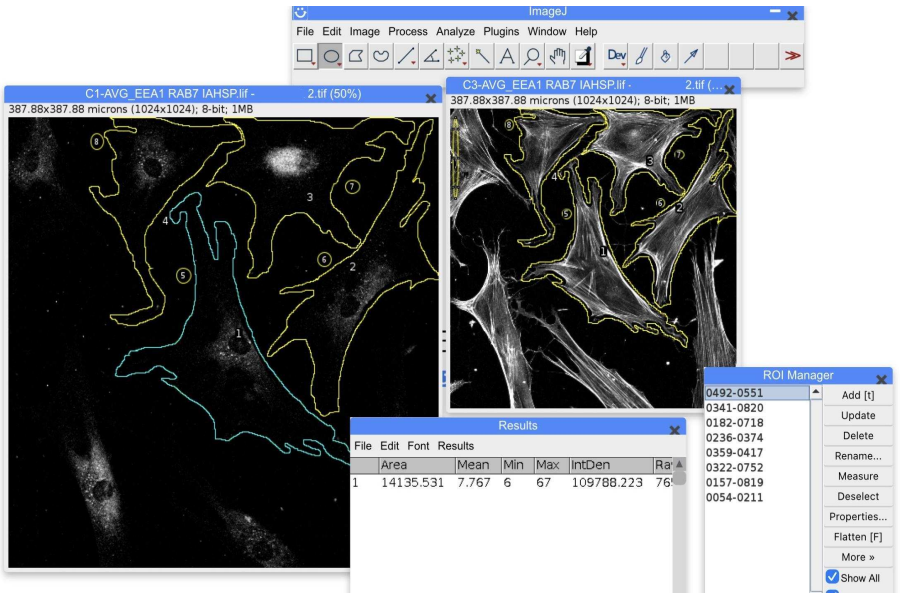

Figure 2. *Example of ROI selections and measurements -area, mean grey values, integrated density- taken from confocal images on ImageJ.*

Corrected total cell fluorescence (CTCF) was then calculated for each image using the following formula:

CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings)

All calculations were performed in Microsoft Excel (Office) to be then subjected to statistical analysis.

3.5 Statistical analysis

The statistical analysis of CTCF data was performed using GraphPad Prism 11 software (GraphPad Software, Inc). CTCF values of each patient were pooled into a single data set for IAHSP and SINO patients respectively, except for EEA1 marker analysis where IAHSP patients were treated as individual data sets. Data distribution was first tested with the Shapiro-Wilk normality test. Then data was analysed using the Kruskal-Wallis ANOVA test for non-parametric data, followed by Dunn's multiple comparisons test. All tests were set with an alpha level of 0.05% with 95% confidence intervals.

4. RESULTS

4.1 Rab5 intensities appear significantly reduced in IAHSP patients but not in SINO

To begin the assessment of altered endo-lysosomal dynamics, early endosome (EE) biogenesis and fusion was evaluated through the analysis of Rab5 fluorescence intensity. Rab5 is responsible for the formation and movement of new endocytic vesicles that derive from the plasma membrane and contain endocytosed material. When Rab5 is GDP-bound, it is in an inactive state, halting cargo transportation. Once activated by ALSIN GEF activity, Rab5 becomes GTP-bound and begins recruiting various effector proteins to the newly formed EE, facilitating the fusion of other incoming vesicles and mediating their assortment into the various branches of the endocytic pathway.

Fibroblasts from IAHSP, SINO and healthy controls were stained with an anti-Rab5 antibody, detected by Alexa-488-conjugated secondary antibodies (shown in red).

Strikingly, IAHSP patient fibroblasts show a significantly reduced fluorescence intensity of Rab5-positive structures compared to healthy controls (**Figure 3A**, middle row; **Figure 3B**).

Conversely, no stark differences of Rab5 staining were observed in SINO patient fibroblasts, being overall equivalent to control levels (**Figure 3A**, last row; **Figure 3B**). The prominent and specific diminution of Rab5 in IAHSP patients, but not in SINO, is coherent with the defective ALSIN mutation present in the disorder, considering its GEF activity is responsible for Rab5 activation on EE membranes.

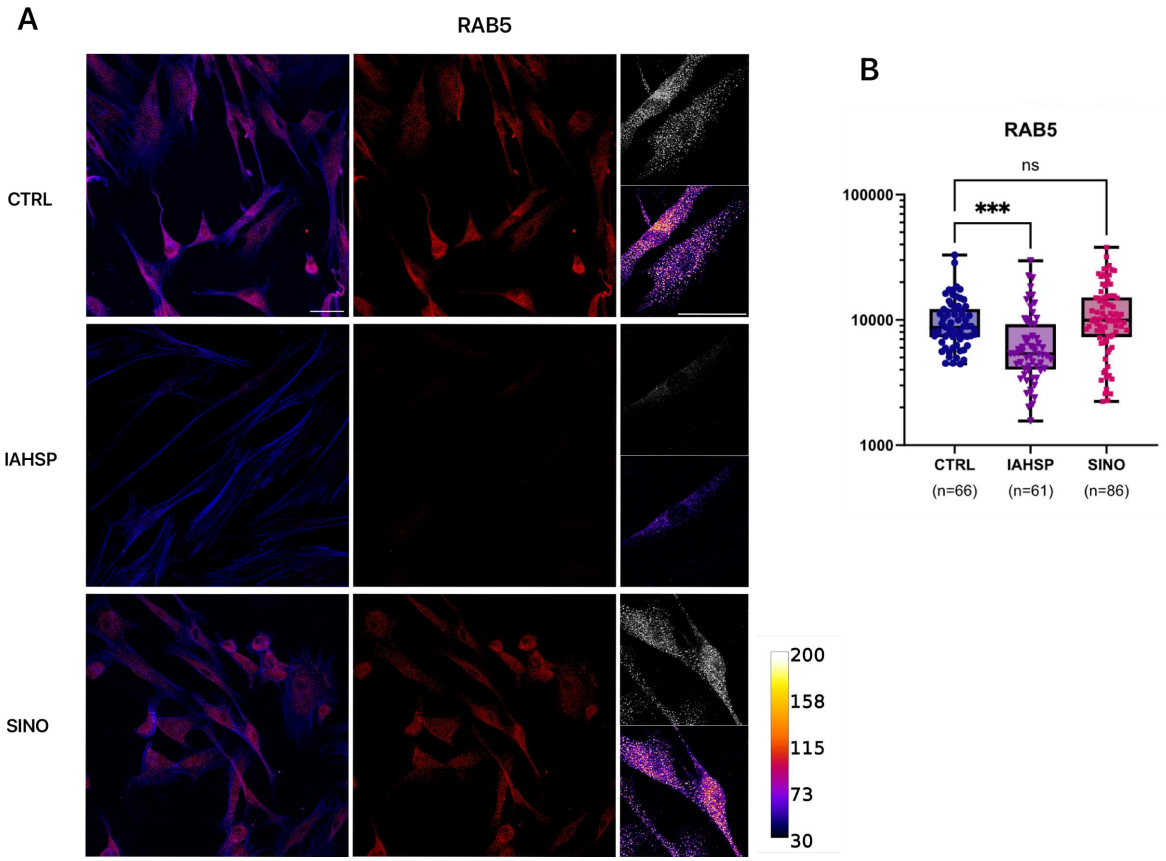

Figure 3. **IAHSP displays reduced Rab5 intensity whereas SINO is comparable to controls. (A)** Representative 3D image reconstructions of fibroblasts from healthy controls, IAHSP patients and SINO patients. Anti-Rab5 (RAB5) antibody was detected with Alexa-488-conjugated secondary antibodies (red channel). Actin structures were stained with Phalloidin-647 marker (blue channel), as seen in the composite images with RAB5 in the first column. The last column of the panel shows magnified areas in both greyscale and as a corresponding intensity map for better fluorescence intensity visualization. Scale bars: 50µm, indicated in the composite and intensity map control panels. Scales are maintained for all other panels. **(B)** The corrected total cell fluorescence (CTCF) intensity (arbitrary unit, a.u.) of RAB5-positive structures, set to a log10 scale ranging from 1,000-100,000. Single points indicate single cells and cell numbers for all groups are indicated by (n=x). Kruskal-Wallis test and Dunn's multiple comparison test for non-parametric data; not significant (ns), ***p<0.001.

4.2 Early endosome antigen 1-compartments differ across IAHSP patient fibroblasts

Considering the substantial alteration of Rab5 intensity that is specifically seen in IAHSP patients, it seemed appropriate to focus on assessing the downstream effector and specific marker of EE organelles, early endosome antigen 1 (EEA1), solely in IAHSP fibroblasts. EEA1 is a membrane protein that mediates tethering of incoming endocytic vesicles, allowing the fusion and development of an EE. Fibroblasts were stained with an antibody against EEA1 and detected with Alexa-488-conjugated secondary antibodies (shown in red).

Findings show that the three different IAHSP patients display varied fluorescence intensity signals, of which two patients, IAHSP_F and IAHSP_O, present significantly higher fluorescence intensities with respect to healthy controls (**Figure 4A**). Statistical analysis of the corrected total cell fluorescence (CTCF) intensities corroborates this range of EE intensity differences seen both across the patients, and when compared to healthy controls (**Figure 4B**). Visually, IAHSP patient EE vesicles also appear to be more diffused than the generally distinct puncta of control EEs, with IAHSP_H being the least dissimilar and IAHSP_O presenting the most apparent variation (**Figure 4A)**. Diffused EEA1 signal may be indicative of increased EE vesicle number and/or of fragmentation of EEA1-positive compartments distributed across the cytosol.

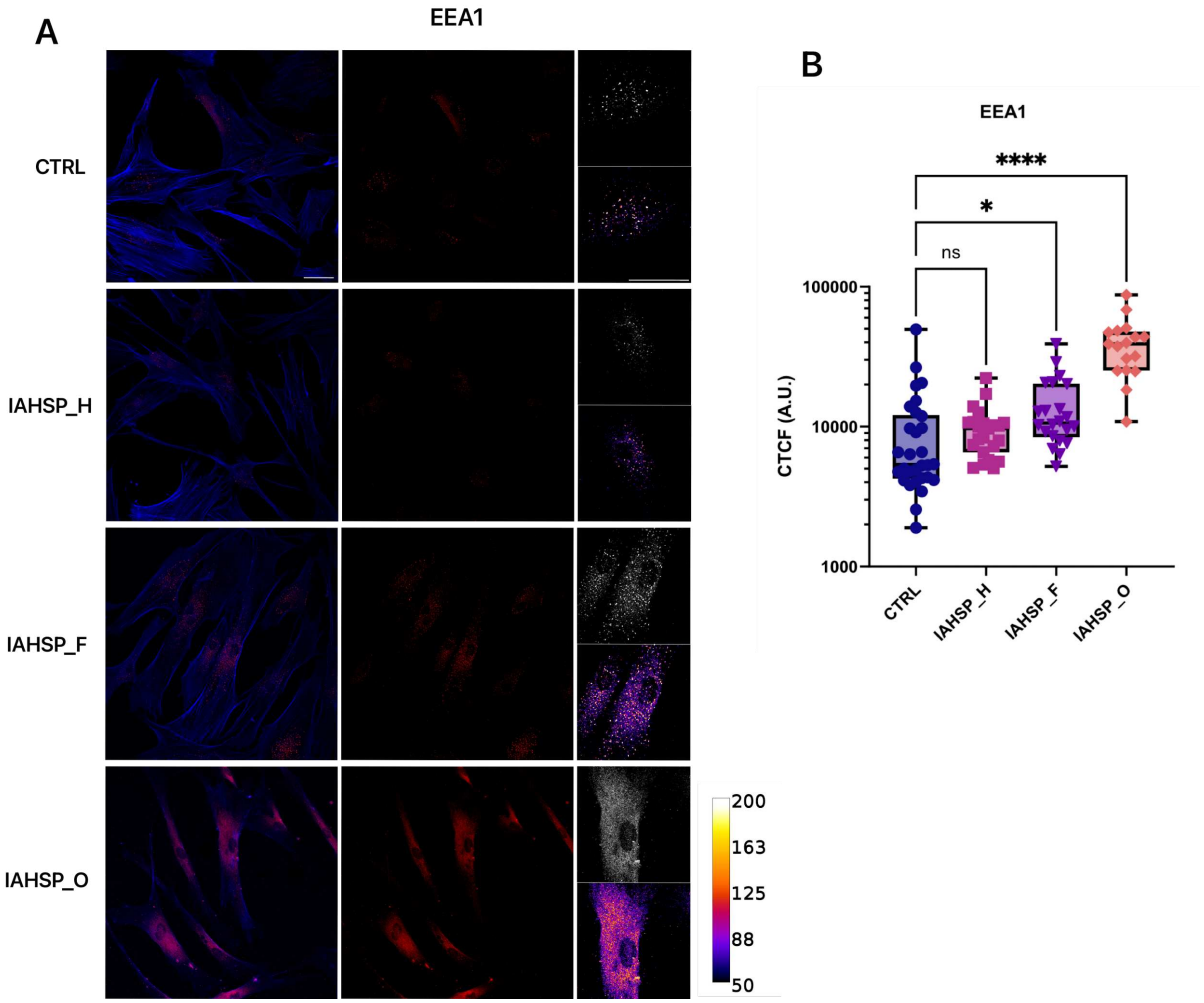

Figure 4. **Early endosome antigen 1-compartments differ across IAHSP patient fibroblasts.**
(A) Representative 3D image reconstructions of fibroblasts from 3 different IAHSP patients and healthy controls. Anti-Early endosome antigen 1 (EEA1) antibody was detected with Alexa-488-conjugated secondary antibodies (red channel). Actin structures were stained with Phalloidin-647 marker (blue channel), as seen in the composite images with EEA1 in the first column. The last column of the panel shows magnified areas in both greyscale and as a corresponding intensity map for better fluorescence intensity visualization. Scale bars: 50µm, indicated in the composite and intensity map control panels. Scales are maintained for all other panels. **(B)** The corrected total cell fluorescence (CTCF) intensity (arbitrary unit, a.u.) of EEA1-positive structures, set to a log10 scale ranging from 1,000-100,000. Single points indicate individual cells. Cell numbers: CTRL (n=30); IAHSP_H (n=23); IAHSP-F (n=22); IAHSP_O (n=23). Kruskal-Wallis test and Dunn's multiple comparison test for non-parametric data; not significant (ns), *p<0.05, ****p<0.0001.

4.3 Rab7 shows higher intensities in IAHSP cells compared to SINO and controls.

In order to follow subsequent EE maturation into late endosomes (LEs), which is characterized by Rab5-to-Rab7 conversion, Rab7 fluorescence intensity was analyzed. Rab7, similar to Rab5, is a small GTPase that, when GTP-bound and activated, regulates the trafficking of cargo from EEs into

mature LEs, characterized by a gradual augmentation of intravacuolar acidity levels. Rab7 is also involved in the fusion of lysosomes with LES and autophagosomes, forming endolysosomes and autolysosomes respectively.

Both IAHSP and SINO patient fibroblasts were stained with anti-Rab7 antibodies, detected by Alexa-568-conjugated secondary antibodies (shown in green). Interestingly, despite the observed decreased fluorescence intensity levels of Rab5, which is presumably associated with its impaired activation, IAHSP cells displayed high Rab7 intensity compared to both SINO and control cells, which are instead seen to be analogous (**Figure 5A**,**B**). Spatial distribution and size of Rab7-stained compartments appear to be consistent across all three groups.

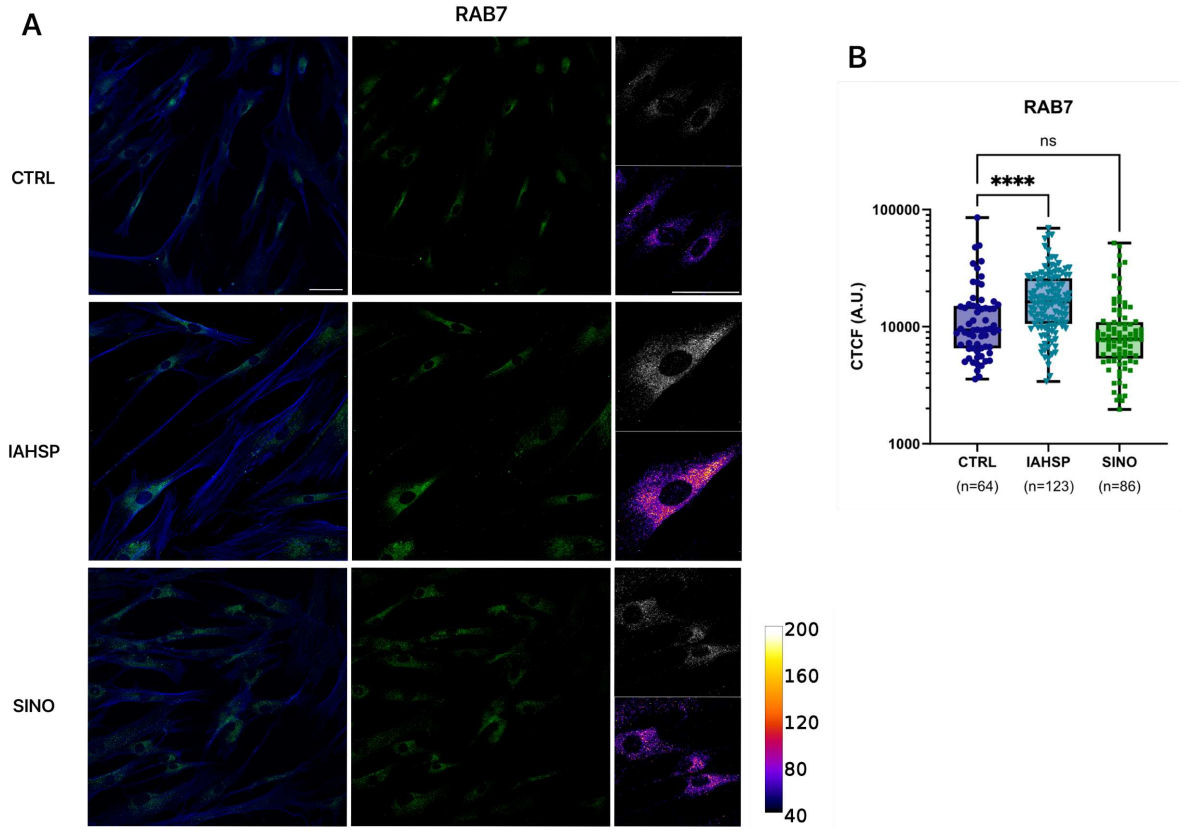

Figure 5. **IAHSP and SINO patient fibroblasts differ in Rab7 intensities. (A)** Representative 3D image reconstructions of fibroblasts from IAHSP patients, SINO patients and their respective healthy controls. Anti-Rab7 (RAB7) antibody was detected with Alexa-568-conjugated secondary antibodies (green channel). Actin structures were stained with Phalloidin-647 marker (blue channel), as seen in the composite images with RAB7 in the first column. The last column of the panel shows magnified areas in both greyscale and as a corresponding intensity map for better fluorescence intensity visualization. Scale bars: 50µm, indicated in the composite and intensity map control panels. Scales are maintained for all other panels. **(B)** The corrected total cell fluorescence (CTCF) intensity (arbitrary unit, a.u.) of RAB7-positive structures, set to a log10 scale ranging from 1,000-100,000. Single points indicate single cells and cell numbers for all groups are indicated by (n=x). Kruskal-Wallis test and Dunn's multiple comparison test for non-parametric data; not significant (ns), ***p<0.0001.

5. DISCUSSION AND FUTURE PERSPECTIVES

The endo-lysosomal system stands as a vitally dynamic pathway that is fundamental to the maintenance of cellular homeostasis, implicated in signalling mediation, intracellular transport and waste clearance of the cell. Consequences of its debilitation have been increasingly affiliated with numerous neurodegenerative diseases, associated with a disruption of signal transmission and accumulations of intracellular stressors. Therefore, an investigation into the presence of such alterations in two ultra-rare forms of HSP serves as a worthy route of exploration, aiming to contribute to the challenge of characterizing their complex pathogenic mechanisms.

In line with this rationale, results from the performed experiments indicate an alteration in endocytic trafficking that is particularly relevant in the HSP subtype IAHSP, rather than in SINO syndrome. This is consistent with the direct implication of IAHSP-specific *Als2* mutations, causing defective ALSIN GEF activation of Rab5, which is required for early endosomal biogenesis. Indeed, Rab5 intensities in IAHSP patient fibroblasts were markedly low with respect to both SINO patients and controls. However, whether this correlates with a decrease in Rab5 protein expression itself cannot be concluded solely through fluorescence microscopy imaging, and would require clarification through protein quantification via western blot analysis for example. Further analysis of Rab5 morphology, cellular distribution and EEA1 co-localization may also provide additional insights into this impairment.

Following the findings of a diminished Rab5 signal in IAHSP, analysis of this effect on early endosome antigen 1 (EEA1) intensity was performed in that specific IAHSP cohort. Two out of the three IAHSP patients (IAHSP_F/O) exhibited substantial increases in EEA1 fluorescence intensity. Interestingly, these same two patients have a similar mutation profile; one allele is truncated from a frameshift mutation, and the other allele features a missense point mutation affecting the same amino acid. Whereas in the IAHSP_H patient, whose fibroblasts display comparable EEA1 intensity with healthy controls, the *Als2* mutation consists of two distinct missense mutations at different points of the gene transcript, which corresponds to the milder clinical phenotype observed. The diffused EEA1 appearance that is seen amongst all IAHSP fibroblasts, in contrast to the relatively uniform and circular puncta of controls, may suggest a general augmentation of EEA1-associated vesicles and could arguably coincide with increased intensity values. However, diffused morphology could also indicate fragmentation, mislocalization and/or abnormal formation

of EEA1 compartments; this may manifest as a consequence of defective ALSIN activation of Rab5, directly disrupting its recruitment of EEA1 to EEs. Perhaps an increase in EEA1 fluorescence intensity in a diffused manner represents an abnormal and scattered generation of EEA1 endocytic compartments in response to impaired Rab5 activation. This aspect could be further elucidated through ultrastructure analysis of EEA1 vesicles using electron microscopy.

In the assessment of late endosome maturation, which is characterized by Rab5-to-Rab7 conversion, SINO fibroblast Rab7 fluorescence levels remained comparable to healthy controls, indicating a functional maturation process. In contrast, Rab7 fluorescence was found to be significantly increased in IAHSP cells. Despite Rab5-to-Rab7 conversion during functional LE maturation being generally associated with a decrease in Rab5-positive EEs, followed by the acquisition of Rab7, this interpretation cannot be directly assumed from the IAHSP data. For one, the marked divergence between Rab5 and Rab7 fluorescence intensities compared to control cells may reflect altered endosomal trafficking rather than effective endosomal maturation. Moreover, if Rab5-dependent EE dynamics are impaired in IAHSP cells, the subsequent transition toward Rab7-positive LEs would also be expected to be affected as a consequence. Therefore, the increased Rab7 signal may instead indicate an accumulation or altered turnover of Rab7-positive compartments.
Intriguingly, a 2014 study by *Girard* et al., expanded the role of Rab7 past LE maturation and endo-lysosomal fusion, revealing an additional functional role in the regulation of specific cargo sorting at EEs. Following these findings, perhaps a hypothesis could be formed around the compensatory development of Rab7 EE-cargo sorting, in order to make up for the loss of Rab5 activity present in IAHSP. Further experimentation and analysis is certainly required to support this idea, but it prompts one to reconsider the conventional categorization of the endo-lysosomal pathway as discrete steps, each with their own distinct mediators, and to instead embrace the system's dynamic complexity that is waiting to be fully understood.

Accounting for the lack of observable experimental alterations in endocytic trafficking apparent in SINO patient fibroblasts, continued investigation involving other aspects of the pathway, such as lysosomal biogenesis, the recycling endocytic pathway and autophagic routes, is strongly suggested. Experimental studies could be designed to specifically inquire about endo-lysosomal alterations affecting signalling cascades in which KIDINS220 is involved.

It is worth mentioning that, although this investigation is related to disorders of the corticospinal motor neurons, these studies are performed on patient dermal fibroblasts. Aside from being easily obtained through minimally-invasive dermal biopsies, providing a patient-specific study source, fibroblasts are also highly resilient and grow rapidly when cultured. They serve as a useful model to explore potential pathological mechanisms of a condition, before progressing to experiment replication and validation in neuronal cells. Any alterations of the endo-lysosomal pathway in IAHSP and SINO fibroblasts provide a valuable insight into the mechanisms of dysfunctional neurons, considering the universal essentiality of the pathway for all human cells. Furthermore, both ALSIN and KIDINS220 proteins are ubiquitous throughout the body; ALSIN is fundamental to early endosomal formation, and despite KIDINS200 preferential expression and role in neurons for neurotrophins signalling, it is also involved in other signalling pathways in some non-neuronal tissues (Neubrand *et al.*, 2012).

It should also be noted that due to the ultra-rare nature of IAHSP and SINO subtypes, patient cohort size is considerably restricted, affecting statistical robustness. This limitation highlights the importance of creating international collaborations to expand patient registries, involving natural history profiling, and introduction of adjustments to experimental designs and statistical measurements in order to improve reliability of results.

Overall, this investigation emphasizes the demand of further research into how endo-lysosomal impairment can contribute to the intricate pathogenesis of neurodegenerative disorders, especially for rare and complex forms like IAHSP and SINO syndrome. Such insights are not only of critical importance in promoting the development of effective and targeted treatments for these debilitating conditions, but they are also instrumental in evolving a greater understanding of one of the most fundamental systems of cellular physiology.

BIBLIOGRAPHY

1. Bellofatto M, De Michele G, Iovino A, Filla A, Santorelli FM. Management of Hereditary Spastic Paraplegia: A Systematic Review of the Literature. Front Neurol. 2019 Jan 22;10:3. doi:10.3389/fneur.2019.00003

2. Meyyazhagan A, Orlacchio A. Hereditary Spastic Paraplegia: An Update. Int J Mol Sci. 2022 Feb 1;23(3):1697. doi:10.3390/ijms23031697 PubMed PMID: 35163618; PubMed Central PMCID: PMC8835766.

3. Alstrup M, Cesca F, Krawczun-Rygmaczewska A, López-Menéndez C, Pose-Utrilla J, Castberg FC, et al. Refining the phenotype of SINO syndrome: A comprehensive cohort report of 14 novel cases. Genetics in Medicine. 2024 Nov 1;26(11):101219. doi:10.1016/j.gim.2024.101219

4. Josifova DJ, Monroe GR, Tessadori F, De Graaff E, Van Der Zwaag B, Mehta SG, et al. Heterozygous *KIDINS220/ARMS* nonsense variants cause spastic paraplegia, intellectual disability, nystagmus, and obesity. Hum Mol Genet. 2016 Jun 1;25(11):2158–67. doi:10.1093/hmg/ddw082

5. Neubrand VE, Cesca F, Benfenati F, Schiavo G. Kidins220/ARMS as a functional mediator of multiple receptor signalling pathways. J Cell Sci. 2012 Apr 15;125(8):1845–54. doi:10.1242/jcs.102764

6. Eymard-Pierre E, Lesca G, Dollet S, Santorelli FM, Di Capua M, Bertini E, et al. Infantile-Onset Ascending Hereditary Spastic Paralysis Is Associated with Mutations in the Alsin Gene. The American Journal of Human Genetics. 2002 Sep;71(3):518–27. doi:10.1086/342359

7. Hadano S, Kunita R, Otomo A, Suzuki-Utsunomiya K, Ikeda JE. Molecular and cellular function of ALS2/alsin: Implication of membrane dynamics in neuronal development and degeneration. Neurochemistry International. 2007 Jul;51(2–4):74–84. doi:10.1016/j.neuint.2007.04.010

8. Kim J, Kim S, Nahm M, Li TN, Lin HC, Kim YD, et al. ALS2 regulates endosomal trafficking, postsynaptic development, and neuronal survival. J Cell Biol. 2021 Mar 8;220(5):e202007112. doi:10.1083/jcb.202007112

9.

Herman M, Randall GW, Spiegel JL, Maldonado DJ, Simoes S. Endo-lysosomal dysfunction in neurodegenerative diseases: opinion on current progress and future direction in the use of exosomes as biomarkers. Philos Trans R Soc Lond B Biol Sci. 379(1899):20220387. doi:10.1098/rstb.2022.0387 PubMed PMID: 38368936; PubMed Central PMCID: PMC10874701.

10.

Blackstone C, O'Kane CJ, Reid E. Hereditary spastic paraplegias: membrane traffic and the motor pathway. Nat Rev Neurosci. 2011 Jan;12(1):31–42. doi:10.1038/nrn2946

11.

Scott CC, Vacca F, Gruenberg J. Endosome maturation, transport and functions. Seminars in Cell & Developmental Biology. 2014 Jul 1;Endosome dynamics & Tubulogenesis31:2–10. doi:10.1016/j.semcdb.2014.03.034

12.

Hu YB, Dammer EB, Ren RJ, Wang G. The endosomal-lysosomal system: from acidification and cargo sorting to neurodegeneration. Transl Neurodegener. 2015 Dec;4(1):18. doi:10.1186/s40035-015-0041-1

13.

Li X, DiFiglia M. The recycling endosome and its role in neurological disorders. Progress in Neurobiology. 2012 May;97(2):127–41. doi:10.1016/j.pneurobio.2011.10.002

14.

Behl T, Kaur D, Sehgal A, et al. Exploring the potential role of rab5 protein in endo-lysosomal impairment in Alzheimer's disease. *Biomed Pharmacother*. 2022;148:112773. doi:10.1016/j.biopha.2022.112773

15.

Zhang J, Jiang Z, Shi A. Rab GTPases: The principal players in crafting the regulatory landscape of endosomal trafficking. Computational and Structural Biotechnology Journal. 2022 Jan 1;20:4464–72. doi:10.1016/j.csbj.2022.08.016

16.

Bellucci A, Longhena F, Spillantini MG. The Role of Rab Proteins in Parkinson's Disease Synaptopathy. Biomedicines. 2022 Aug 10;10(8):1941. doi:10.3390/biomedicines10081941

17.

Girard E, Chmiest D, Fournier N, Johannes L, Paul J, Vedie B, et al. Rab7 Is Functionally Required for Selective Cargo Sorting at the Early Endosome. Traffic. 2014 Mar;15(3):309–26. doi:10.1111/tra.12143