UNIVERSITY OF NAPLES FEDERICO II

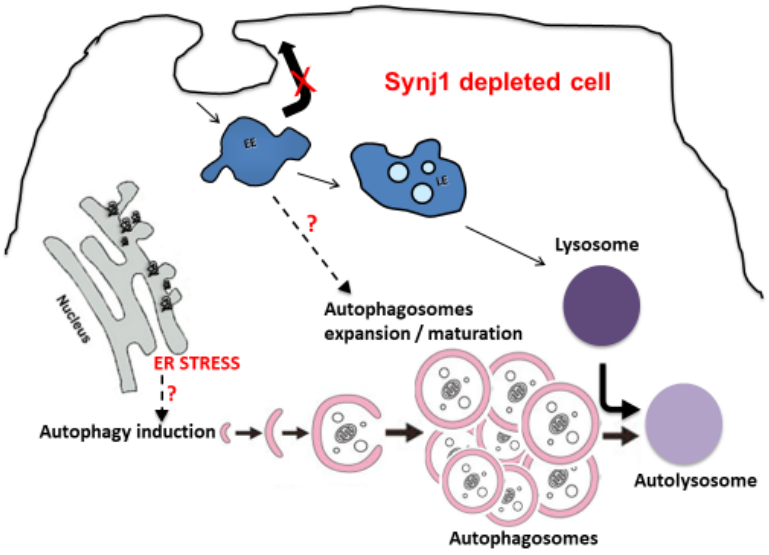
DOCTORATE IN

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXII CYCLE

Lucrezia Zerillo

The role of Synj1 in membrane trafficking and its impact in PARK20 pathogenesis.



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The role of Synj1 in membrane trafficking and its impact in PARK20 pathogenesis.

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List of Abbreviations used

AD: Alzheimer's disease
ALR: autophagic lysosome reformation
AMPK kinases: adenosine monophosphate-activated protein kinase
AP2: adaptor protein complex 2
ATF6: activating transcription factor 6
ATG: autophagy-related proteins
CCPs: clathrin-coated pits
CCV: clathrin‐coated vesicles
CMA: chaperone-mediated autophagy
DS: Down's syndrome
EE: early endosome
EEA1: early endosome antigen 1
Eps15: epidermal growth factor receptor pathway substrate
ER: endoplasmic reticulum
ERGIC: endoplasmic reticulum-Golgi intermediate compartment
ERGIC-53: ER-Golgi intermediate compartment 53kDa protein
FBS: Fetal Bovine Serum
GAPs: GTPase-activating proteins
GEF: guanine nucleotide exchange factor
HGNC: HUGO Gene Nomenclature Committee
IRE1: inositolrequiring enzyme1
LE: late endosomes
mTOR: mammalian target of rapamycin
PD: Parkinson disease
PERK: PRKR-like ER kinase
PI: phosphoinositide
PRD: proline-rich domain
PtdIns: phosphatidylinositol
Sac1: suppressor of actin1 domain
SQSTM1: sequestosome 1
Synj1: Synaptojanin-1
TGN: Trans-Golgi Network

TSC2: Tuberous sclerosis complex 2
UPR: Unfolded Protein Response
XBP1: X-box binding protein

Abstract

Homeostasis of eukaryotic cells is largely dependent on the dynamic compartmentalization of the endomembrane system. Proper membrane trafficking, which connects different organelles, is essential to maintain the proper composition of cellular compartments as well as to ensure their homeostasis and function.

With respect to other cell types, nervous system is more sensitive to alterations of membrane trafficking. In the last years, several genes responsible for hereditary forms of Parkinson's disease are implicated in regulating membrane trafficking. We have recently highlighted that the alteration of homeostasis and functions of early endosomal compartments is associated with the early-onset parkinsonism (PARK20) caused by R258Q mutation in the phosphoinositide (PI) phosphatase Synaptojanin 1 (Synj1), emphasizing the role of endosomal trafficking in the pathogenesis of Parkinson's disease.

Thanks to two consecutive phosphatase domains, Synj1 dephosphorylates various substrates and for this peculiarity it might play multiple roles; hence defects of Synj1 activity might compromise different intracellular pathways.

Main goal of my PhD project was to elucidate the role of Synj1 in membrane trafficking and its impact on PARK20 pathogenesis. Specifically, first aim was to study the role of Synj1 in the secretory pathway since the PI(4)P, which is enriched in the Golgi membranes, is one of Synj1 substrate. Here, we show that the exit machinery from the endoplasmic reticulum (ER) and the ER-to-Golgi trafficking is markedly compromised in PARK20 fibroblasts. As a consequence, they accumulate large amounts of cargo proteins within the ER, leading to the induction of ER stress and, in turn, activation of PERK/eIF2α/ATF4/CHOP pathway of the Unfolded Protein Response (UPR). All together these findings suggest that dysfunction of early secretory pathway might contribute to the pathogenesis of the disease.

Second, we studied the role of Synj1 in the autophagy pathway starting from the observation that lysosome structure is altered, despite trafficking toward lysosomes is unaffected, and therefore hypothesizing that these alterations could be due to changes in this process. We observed higher levels of the autophagic markers in Synj1 deficient cells and in PARK20 fibroblasts as well as the increase of the number of autophagosomes. Moreover, autophagic flux results perturbed and the clearance of autophagy substrates was drastically reduced in Synj1-depleted cells. All these data indicate a role of Synj1 in the autophagy pathway from one side, and highlight a potential role of autophagy dysregulation in PARK20 pathogenesis from the other one.

Overall, our findings pointed out multiple roles of Synj1 in different cellular pathways. In addition, they highlight that beside the role of endosomal system, defects of early secretory pathway and a dysregulation of autophagy could contribute to PARK20 pathogenesis.

1. BACKGROUND

Membrane trafficking

The eukaryotic cells are composed of membrane compartments called organelles and their homeostasis is largely dependent on the dynamic compartmentalization of this endomembrane system. Intracellular compartments are designed so that they can exchange materials and undergo dramatic morphological changes in order to meet the demands of metabolism, growth, and environment.

Each compartment has distinct membrane composition and unique function. Membrane trafficking, which connects different organelles, is essential to maintain a proper composition of various compartments by their continuous sorting, as well as to ensure their homeostasis and function.

Two pathways mainly achieve this: the biosynthetic secretion and the endocytic route (Figure 1.1). The anterograde flow is counterbalanced by retrograde transport, which is essential for the maintenance of organelle homeostasis and re-use of components of the trafficking machineries. The out-going and in-coming pathways communicate through bidirectional transport between the Golgi complex and endosomes.

Thus, the molecular machinery regulating membrane trafficking has a key role in the maintenance of organelle functionality and cell viability. Thus, alteration of membrane trafficking might lead to alterations of different intracellular pathways inducing a modification of cell fate in dependence of the cell context. Hence, it is not surprising that alterations in membrane trafficking can result in different pathologies (e.g. cancer, neurological disorder) (De Matteis 2011 and Schreij 2016).

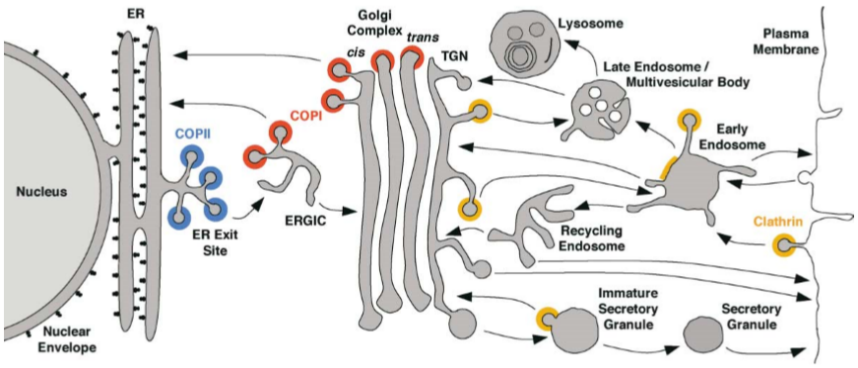

Figure 1.1 Intracellular Transport Pathways.
Scheme of exocytic and endocytic routes. The different vesicle coats (COPII in blue; COPI in red; and clathrin in yellow) involved in these pathways are shown. The arrows indicate the directions of the different trafficking steps. (from Bonifacino 2004)

Secretory pathway

The secretory pathway synthesizes, modifies, and transports about one third of all cellular proteins. It is responsible for proper lipid and protein sorting and transport from the site of synthesis to their correct final destination from one side, and it also allows cell-to-cell communication through the secretion of various molecules from the other side. To avoid dysfunction of this system, these activities must be coordinated with the rest of the cell and with environmental needs (Pigino 2012).

Along the secretory pathway, endoplasmic reticulum (ER) is the site of lipid and proteins synthesis and where proteins acquire proper folding. ER quality control is an essential requirement for the exit from this organelle. Via the sequential action of several sorting signals and multiple sorting events proteins reach the final destination: Golgi complex, endo-lysosomal system, plasma membrane or the outside of the cell. Secretion can occur either in a constitutive or a regulated fashion. The constitutive secretory pathway operates in all eukaryotic cells. Many soluble proteins are

continually secreted from the cells by this pathway, which also supplies the plasma membrane with newly synthesized membrane lipids and proteins. Instead, the regulated pathway operates only in specialized cells.

A series of basic steps allows the transfer of cargo from the lumen and/or the membrane of a donor compartment to a target/acceptor compartment. First, specific cargo is selected for packaging in the donor organelle and this process is concurrent with the formation of a specific protein coat on the cytosolic surface of the donor membrane, which is also crucial for membrane curvature and vesicle formation. Once formed, vesicle buds off from the donor organelle and rapidly shed their protein coats in an active process that involves either an ATPase/chaperone or a small GTPase. After uncoating, vesicles are actively translocated across the cytoplasm, usually through mechanisms mediated by microtubule-based molecular motors. Transport vesicles eventually reach, recognize and tether to the appropriate target organelle. Finally, unloading of the transport vesicle cargo to the target membrane occurs by membrane fusion (Pigino 2012). In all the intracellular trafficking, phosphoinositides, Rab GTPase, tethering factors, v-SNARE and t-SNARE complexes ensure the specificity and efficiency of cargo selection, vesicle targeting, and fusion (Bonifacino 2004).

In the specific for the secretory pathway, the newly synthesized proteins are transported from the endoplasmic reticulum to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) through COPII coated vesicles (Figure 1.1). These vesicles are generated from specialized regions of the ER membranes ‘ER exit sites’, which contain the specific protein (Sec16) involved in the recruitment of COPII components. From the ERGIC the cargo is transported to the Golgi complex, where, the cargo enters the cis-Golgi network and proceed to the trans-Golgi network. The vesicles that are implicated in the Golgi network transport are COPI-coated vesicles (Figure 1.1). These types of coated vesicles are also involved in the return of cargo to the ER from the Golgi

complex. In the trans-Golgi, the different cargoes are packaged in different vesicles and sorted to their final destinations (De Matteis 2011; Pigino 2012). Interestingly, also lysosomes can secrete their contents outside of the cell through the fusing with the plasma membrane (lysosomal "exocytosis"). This process is very active in particular cell types, such as in the osteoclasts, hematopoietic lineage, and melanocytes, but any cell type can perform this function (Settembre 2015).

Endocytic pathway

Cells ingest fluid, molecules and particles by endocytosis through invagination of localized regions of the plasma membrane and pinch off to form endocytic vesicles.

In most cells, endocytosis internalizes a large fraction of the plasma membrane every hour. Importantly, plasma membrane maintains the proper size thanks to the continuous arrival of new components (proteins and lipids) through the exocytic pathway.

The endocytic pathway is responsible for the uptake of essential nutrients, for the control of the plasma membrane composition, and cell signaling (De Matteis 2011). Internalization of molecules can occur through the classical, clathrin-mediated pathway for which many molecular components are known and/or via non-classical clathrin-independent routes. In the clathrin-mediated endocytosis, there is the formation of morphologically distinct clathrin-coated vesicles (CCVs) on the cytoplasmic side of the plasma membrane and, this pathway mainly mediates internalization of plasma membrane receptors such as epidermal growth factor receptors, transferrin, and low-density lipoproteins. The clathrin-independent endocytosis consists of the several internalization pathways, which use an alternative protein coat or do not require a protein coat for the formation of endocytic vesicles (Vassilieva and Nusrat 2008).

Early endosomes (EEs) are the initial destination for all internalized material (Figure 1.1) (Jovic 2010). EEs are very

dynamic compartments undergoing frequently to fusion with each another (Naslavisky 2018; Scott 2014; Jovic 2010). The functional attributes of the EEs are related to the association of specific proteins to the cytosolic surface of EEs membrane. One of these key components is Rab5 that, such as other Rab proteins, with phosphoinositides, tethering factors, v-SNARE and t-SNARE complexes helps to manifest the identity of the organelles (see in Membrane trafficking regulators) (Christoforidis 1999; Zerial and McBride 2001; Behnia and Munro 2005). From EEs the cargo molecules can have different destinations: can be trafficked to the late endosomes (LE) and lysosome for degradation or can be returned to the plasma membrane by various routes (Figure 1.1). In fact, some receptors are recycled to the plasma membrane directly from the EEs through a rapid recycling pathway, whereas other receptors are transported to more specialized recycling endosomes which are mainly clustered in the perinuclear-localized endocytic recycling compartment adjacent to the microtubule-organizing center (Naslavsky 2018). EEs also communicate with the Trans-Golgi Network (TGN) through bidirectional vesicle exchange (Figure 1.1). This pathway depends on specific factors (such as the retromer complex, Rab7, Rab9) and is involved in the delivery of lysosomal components and removal of endosomal components during endosome maturation (Bonifacino and Hurley 2008; Pfeffer 2010). The Rab proteins are the main trafficking progression regulators, for example, in the progression between EEs to LEs is necessary the generation of Rab7 domain on EEs (Rink 2005). Such as EEs, also LEs are a heterogeneous group of organelles, but respect to EEs, LEs have a lower pH (Griffiths 1989). In bases of their lower pH and other characteristics, LEs are considered more closely related to lysosomes. In fact, like lysosomes, they can sense nutrients change through the mTOR pathway (Flinn 2010). The last destination of extracellular material that is direct to the degradation is the lysosome (Figure 1.1). Lysosomes have an acidic lumen that contains several types of hydrolases devoted to the degradation

of specific substrates. In the single-bilayer lipid membrane of the lysosome, there are proteins involved in the acidification, in the transport of substances into and out, and the fusion of the lysosome with other cellular structures. Moreover, also intracellular components intended to degradation are transported to the lysosome by autophagy. Thus, lysosomal principal function is cellular clearance, but they can also mediate a range of biological processes, such as plasma membrane repair, cell homeostasis, energy metabolism, and the immune response (Settembre 2015).

Membrane trafficking regulators

The specificity of membrane tethering and fusion is crucial to preserve organelle identity and the key mechanism of this process is the reversible and specific recruitment of cytosolic proteins or protein complexes to appropriate membranes. This mechanism is finely regulated in the space and time.

The main actors of this process are the Rab GTPases proteins and the phosphoinositides, which act as a platform for the recruitment of effector proteins to membranes. Both these components are membrane-associated and are implicated in different steps of membrane trafficking such as cargo selection, vesicle budding and transport, membrane tethering and fusion. Their function depends on their specific localizations to specific membrane compartments or specific membrane microdomains (Figure 1.2 and table 1) (Jean 2012).

Phosphoinositides (PIs) are phosphorylated derivatives of phosphatidylinositol (PtdIns). Although they comprise less than 10% of total cellular lipids, PIs are essential components of cellular membranes and play key roles in many fundamental biological process (Di Paolo and De Camilli 2006; Mayinger 2012). The precursor of phosphoinositides, phopshatidilinositol, is synthesized primarily in the endoplasmic reticulum and is then delivered to other membranes either by vesicular transport or via cytosolic PtdIns transfer proteins (Di Paolo and De Camilli 2006;

Cockcroft and Raghu 2018). Reversible phosphorylation of the myo-inositol head group at position 3, 4 and 5 gives rise to seven PI isoforms identified in eukaryotes cells. The conversion between these isoforms is controlled by specific phosphoinositide kinases and phosphatases that regulate the equilibrium among these lipids. Each of the seven PIs has a unique subcellular distribution (Figure 1.2) (Di Paolo and De Camilli 2006).

Proteins can selectively recruited to specific organelles through recognition of specific PI. So far, 11 PI-binding modules have been found in different proteins. The interaction of PI-binding modules with unique PIs with variable affinity generates a PIs code (Kutateladze 2010). This interaction depends on electrostatic interactions with the negative charges of the phosphate(s) of the inositol ring and, in some cases, adjacent hydrophobic aminoacids can potentiate the interaction (Lemmon 2003).

As aforementioned, other crucial regulators of membrane trafficking are the Rabs, which are small Ras-like GTPases and account to more than 70 members in humans (Wandinger-Ness and Zerial 2014). Their localization and activity depend on the switch between GTP- and GDP-bound conformations (Figure 1.2). Importantly, for their role in the traffic regulation is crucial their cycle between the cytosol and the specific membrane (respective transport compartment) because this cycle gives rise to the temporal and spatial regulation to membrane transport (Hutalung 2011).

Importantly, there is a coordinated regulation between specific RABs and phosphoinositides. In fact, the RABs can mediate the recruitment of phosphoinositide enzymes. For example, when RAB5 is activated on endocytic vesicles can recruit a phosphoinositide 5-phosphatase and a phosphoinositide 4-phosphatase that are implicated in the conversion of PI (3,4,5)P3 to PI(3,4)P2 and finally to PI(3)P. Furthermore, is true also the contrary: the PIs can mediate the recruitment of RABs regulators. One example is what occurs in the yeast

secretion, where, the PI (4)P regulates the Rab cascade at the Golgi (Jean 2012).

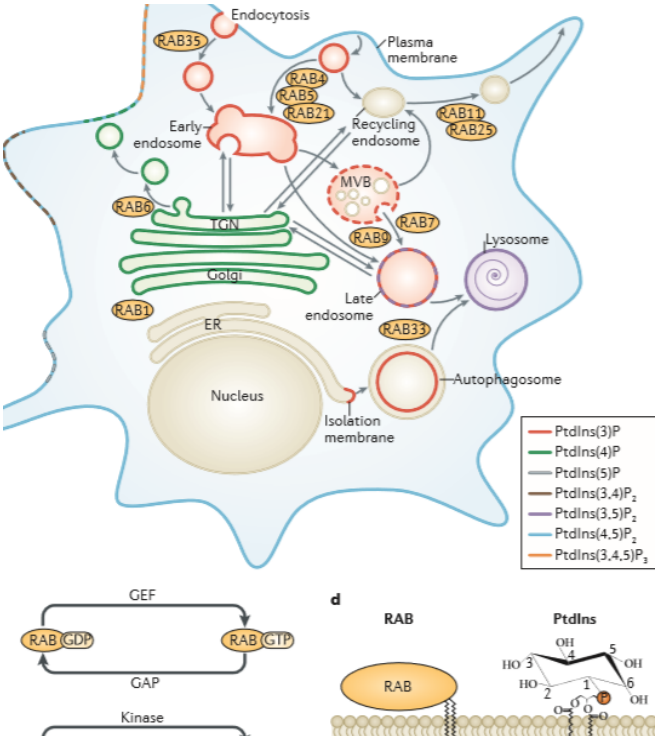

Figure 1.2 The coordination between the principal regulators of membrane trafficking (RABs and phosphoinositides). The image shows the know localization of a subset of RABs and phosphoinositides. Lower left there is a scheme of the RAB GTPase cyclic activity that is under the control of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Lower right there is a scheme of phosphoinositide cyclic regulation, which is controlled by phosphoinositide kinases and phosphatases (the different phosphorylation state of PtdIns is denoted as XP). (From Jean 2012)

Thus, PIs and RABs codes have a crucial role in cells. To support this, several human genetic diseases have been found to be associated to mutations of RABs or alterations of PI

levels in the last decade (Nicot and Laporte 2008; Waugh 2015; Kiral 2018).

Table 1. Subcellular localization of phosphoinositides and RABs.

Cellular localization	RABs	Phosphoinositides	
		Major	Minor
Plasma membrane	RAB5, RAB21, RAB35	$PtdIns(4,5)P_2$	$PtdIns(3,4,5)P_3$*,‡ $PtdIns(3,4)P_2$*,‡ $PtdIns(5)P$ $PtdIns(3)P$
Early endosomes	RAB4, RAB5, RAB10, RAB14,RAB15, RAB17, RAB21,RAB22, RAB23, RAB35	$PtdIns(3)P$	$PtdIns(3,4,5)P_3$ $PtdIns(3,4)P_2$ $PtdIns(4,5)P_2$
Late endosomes, multivesicular body, lysosomes	RAB7, RAB9	$PtdIns(3,5)P_2$	$PtdIns(3,4)P_2$*
Recycling endosomes	RAB11, RAB13, RAB17, RAB25, RAB35, RAB40		$PtdIns(3,4,5)P_3$ $PtdIns (4,5)P_2$
Autophagic compartments			
• Autophagosome precursors			$PtdIns(4,5)P_2$
• Phagophore assembly site	RAB24, RAB33,	$PtdIns(3)P$	
• Autophagosome	RAB7, RAB27, RAB33	$PtdIns(3)P$	
Endoplasmic reticulum	RAB1, RAB2, RAB24		$PtdIns(3)P$ $PtdIns(5)P$ $PtdIns(4,5)P_2$ $PtdIns(3,4)P_2$* $PtdIns(3,4,5)P_3$*
Golgi	RAB1, RAB24, RAB33, RAB34, RAB36, RAB39	$PtdIns(4)P$	$PtdIns(3)P$ $PtdIns(5)P$ $PtdIns(3,4,5)P_3$*
Trans-Golgi network	RAB6, RAB8, RAB10, RAB13, RAB14, RAB30, RAB31, RAB40	$PtdIns(4)P$	
Secretory vesicles	RAB3, RAB8, RAB26, RAB27, RAB37	$PtdIns(4)P$	
Nucleus			$PtdIns(4,5)P_2$ $PtdIns(5)P$
Cytokinetic apparatus			
• Cleavage furrow	RAB11, RAB21	$PtdIns(4,5)P_2$	
• Intercellular bridge	RAB11, RAB35		$PtdIns(3)P$

(from Jean 2012)

Membrane trafficking and neurodegenerative diseases

As mentioned before membrane trafficking linking different organelles is essential to maintain the proper composition of various compartments and to the transport of various molecules to appropriate compartments.

Neuronal cells have highly polarized intracellular compartmentalization that is critical for neuronal function. To achieve this, ubiquitous and specialized membrane trafficking machineries are required. The specialized membrane trafficking regulates protein and organelle compositions in dendrites and axons, synaptic transmission, and the correct distribution of countless cell surface receptors (Hanus 2016; Kiral 2018).

With respect to the other cell types, the functions and viability of neurons is strictly dependent on the proper membrane trafficking. It is not surprising that alterations in membrane trafficking are implicated in various neurodegenerative disorders such as Parkinson disease (PD; see box 1), amyotrophic lateral sclerosis, and hereditary spastic paraplegias (Schreij 2016).

Interestingly, there are 24 Rab proteins specifically enriched in the central nervous system and some mutations in Rab genes or genes encoding Rab-associated proteins were reported related to some neurodegenerative diseases (Kiral 2018). Moreover, also alterations in the PIs metabolism have been found to be associated to neurodegenerative disease or neurological disorders (Volpicelli-Daley and De Camilli 2007; Di Paolo and De Camilli 2006).

BOX 1: Parkison disease

Parkinson disease (PD) has been described for the first time by James Parkinson in 1817 and later refined by Jean-Martin Charcot (Goetz 2011). The principal features of disease are the dopamine deficiency, Lewy bodies (abnormal intracytoplasmic aggregates enriched of α-synuclein protein) and Lewy neurites (Thomas and Beal 2007). The aetiology of PD is still enigmatic today. Majority of PD cases are sporadic with unknown aetiology, possibly a complicated interplay of genetics and environmental factors concur to the pathogenesis of the disease (Calabresi and Tambasco 2017). Indeed, epidemiological studies showed that there are a lot of environmental and genetics risk factors involved in PD. Age is the principal risk factor: the disease incidence increases with age, showing a peak after 80 years of age (Kalia and Lang 2015). Another risk factor is the gender with the male-to-female ratio being approximately 3:2 (De Lau and Breteler 2006). Moreover, the exposure to environmental factors (e.g. pesticides, toxins), head injury and the use of β-blocker drugs are associated with an increase of risk of development the disease. On the contrary, the exposure to tobacco smoking, coffee drinking, alcohol consumption and the use of non-steroidal anti-inflammatory drug seem associated with a decrease of development of disease (Kalia and Lang 2015). Such as environmental risk of factors also genetic risk of factors can be divided into two groups: enhancers and reducers of risk. Furthermore, it seems that there exists a complex interplay between the various factors (Kalia and Lang 2015). However, not all PD have this complicate scenario, indeed, around 15% of PD patients have the hereditary form of disease. Respect to the sporadic forms, the familial form is more severe, starts at an earlier stage of life (<50 years) and have a quick progression (Klein and Westenberger 2012). At least 23 loci have been identified and designated as PD-causing genes by the HUGO Gene Nomenclature Committee (HGNC) table 2 (Deng 2018; Kalinderi 2016).

Alteration of membrane trafficking in PD

Recent developments in PD genetics highlighted that dysregulation of membrane trafficking might play a crucial role in the pathogenesis of this disease. Several genes causative for familial PD (in red in the table 2) are involved in the regulation of the membrane dynamics (Perret 2015; Vidyadhara 2019).

Interestingly, different steps of the membrane trafficking process have been reported to be altered in familial PD. In particular, many mutations of genes involved in the endosomal pathway have been identified in PD.

PARK19 is caused by autosomal recessive mutations of auxilin, a co-chaperone belonging to the DNAJC family highly expressed in the brain, a subclass of heat shock proteins (HSP40). Auxilin is involved in the clathrin uncoating during the vesicles recycling. Specifically, auxilin recruits ATP-activated Hsc70 to CCVs, which in turn, disassembles clathrin from vesicles (Lemmon 2001; Gorenberg and Chandra 2017). The brain of homozygous auxilin knockout (KO) mice shows an increased number of CCVs (Yim 2010). Furthermore, some of the mutations associated with PARK19 lead to a aberrant splicing and decrease of auxilin mRNA (Vidyadhara 2019).

Moreover, alterations of endosomal pathway is associated to PARK20 and PARK21 (Malik 2019). PARK20 is caused by mutations in SYNJ1 (see in detail in the next paragraph). PARK21 is caused by a mutation in the receptor-mediated endocytosis 8 or DNAJC13 that is implicated in the regulation of retromer and clathrin uncoating dynamics on endosomes (Gorenberg and Chandra 2017). In vitro studies showed that the mutant form leads to endolysosomal cargo trafficking deficits (Norris 2017).

Table 2 Hereditary forms of PD

Locus (OMIM)	Location	Full Gene Name Approved by HGNC
PARK1 **(168601)**	4q22.1	synuclein alpha
PARK2 **(600116)**	6q26	parkin RBR E3 ubiquitin protein ligase
PARK3 **(602404)**	2p13	Parkinson disease 3
PARK4 **(605543)**	4q22.1	synuclein alpha
PARK5 **(613643)**	4p13	ubiquitin C-terminal hydrolase L1
PARK6 **(605909)**	1p36	PTEN induced putative kinase 1
PARK7 **(606324)**	1p36.23	parkinsonism associated deglycase
PARK8 **(607060)**	12q12	leucine rich repeat kinase 2
PARK9 **(606693)**	1p36.13	ATPase 13A2
PARK10 **(606852)**	1p32	Parkinson disease 10
PARK11 **(607688)**	2q37.1	GRB10 interacting GYF protein 2
PARK12 **(300557)**	Xq21-q25	Parkinson disease 12
PARK13 **(610297)**	2p13.1	HtrA serine peptidase 2

Locus (OMIM)	Location	Full Gene Name Approved by HGNC
***PARK14* (612593)**	22q13.1	phospholipase A2 group VI
***PARK15* (260300)**	22q12.3	F-box protein 7
***PARK16* (613164)**	1q32	Parkinson disease 16
***PARK17* (614203)**	16q11.2	VPS35, retromer complex component
***PARK18* (614251)**	3q27.1	eukaryotic translation initiation factor 4 gamma 1
***PARK19* (615528)**	1p31.3	DnaJ heat shock protein family (Hsp40) member C6
***PARK20* (615530)**	21q22.1	synaptojanin 1
***PARK21* (616361)**	20p13	transmembrane protein 230
***PARK22* (616710)**	7p11.2	coiled-coil-helix-coiled-coil-helix domain containing 2
***PARK23* (616840)**	15q22.2	vacuolar protein sorting 13 homolog C

The table shows the 23 loci associated with hereditary PD that are identified and designated by the HUGO Gene Nomenclature Committee (HGNC). In red are highlighted the genes that could have a role in membrane trafficking (modified from Denga 2018).

Moreover, the perturbation of retromer activity is also implicated in PARK21 and PARK19 (Malik 2019). Alterations of retromer complex seem to be causative of other familial PDs such as PARK17 that is caused by a single allelic mutation in VPS35 (Vilarino and Guell 2011). VPS35 is a core component of the retromer complex and, it is involved in the retrograde trafficking and sorting of protein cargos from endosomes to the TGN, facilitating their recycle and reuse (Seaman 1998; Haft 2000). The molecular mechanisms by which VPS35 mutations induce PD are not yet known, but several studies demonstrate that silencing and overexpression VPS35 leads to damage for cellular health (Williams 2017). For example, the loss of function mutations of VPS35 in vitro induces enlargement of late endosomes and aggregation of α−synuclein oligomers (Follett 2016). Several studies demonstrate that alterations in the retromer complex functions could have a role also in the PARK2, PARK8, and PARK16 pathogenesis. In fact, Parkin (an E3 ubiquitin ligase causative of PARK2) can interact with VPS35 and mediate its ubiquitination (Martinez 2017; Linhart 2014). It has hypothesized that LRRK2 and RAB7L1 (causative of PARK8 and PARK16, respectively) can form a functional network with the retromer complex regulating the endolysosomal and the endosome-Golgi traffic (Tang 2017).
Another step of membrane trafficking frequently altered in PD is at the level of LEs, as shown by the fact that mutations in LRRK2 and the LE associated RAB7L1 are common risk factors for the development of sporadic PD. As mentioned before, the multidomain protein with kinase activity LRRK2 is also causative of a genetic form of PD (PARK8) (Paisan-Ruiz 2004). Fibroblasts derived from PARK8 patients show altered late endosomal trafficking related to the reduction of Rab7 activity (Gomez-Suaga 2014). Studies showing that LRRK2 colocalizes with the late endosomal marker Rab7 and with the lysosomal marker LAMP2 in α-synuclein positive brainstem LBs corroborate its role in regulating late endocytic compartments (Higashi 2009).

Interestingly, parkin mutants can regulate the levels and activity of Rab7 promoting its ubiquitination (Song 2016).
The lysosomal dysfunctions also are involved in PD. The homozygous and heterozygous missense mutations in the transmembrane endo/lysosomal-associated P5 type transport ATPase (ATP13A2) cause PARK9 (Di Fonzo 2007; Ning 2008; Santoro 2011). This protein is involved in the transports of inorganic cations. The ATP13A2 mutations affect localization of the pump or cause the impairment its ATPase activity (Podhajska 2012). Moreover, fibroblasts derived from PARK9 patients show altered lysosomal acidification that disrupts autophagosome clearance (Dehay 2010).
Moreover, the last gene discovered to be implicated in familial PD (VPS13C) is involved in endolysosomal system (Vidyadhara 2019). Moreover, its functions are also related to mitochondrial membrane potential and mitophagy (Lesage 2016). In addition, VPS13C seems to also have a role in the formation of membrane contact sites and lipid transfer between the ER and mitochondria (Gao 2018).
Thus, all these studies indicate a strict correlation between membrane trafficking alteration and PD.
Importantly, α-synuclein, the protein enriched in the Lewy bodies and Lewy neurites (Thomas and Beal 2007) that plays a central role in the pathogenesis of both sporadic and familial PD (PARK1/4) also have a role in membrane trafficking. Several studies indicate that α-Synuclein could have a role in the regulation of synaptic vesicle trafficking and subsequent neurotransmitter release. In fact, α-Synuclein promoted SNARE-complex assembly through the binds to Synaptobrevin, a protein involved in the formation of the SNARE complexes (Burre 2010). Furthermore, α-Synuclein seems to have also a dose-dependent effect on the dilation of the exocytotic fusion pore (Logan 2017; Huang 2018).
Thus, it might be interesting to investigate the role of membrane trafficking in the pathogenesis of idiopathic PD and other PD genetic forms.

Synj1

Synaptojanin 1(Synj1) is a polyphosphoinositide phosphatase, belonging to the family of Sac domain containing proteins. It was discovered in 1994 as 145 kDa nerve terminal protein interacting with growth factor receptor-bound protein 2 (Grb2) (Mcpherson 1994).

The SYNJ1 gene is located on chromosome 21q22.11 (Cremona 2000) and spans 99.29 kb of genomic DNA. This gene contains two open reading frames separated by an in-frame stop codon (Ramjaun and McPherson 1996). Two Synj1 isoforms of 170 and 145 kDa (isoform a, NP_003886.3, 1612 amino acids, and isoform b, NP_982271.2, 1350aminoacids) are generated. There are two additional isoforms of SYNJ1 gene, but their function is unknown (Drouet 2014).

The isoforms a and b are ubiquitously expressed, but the 145kDa isoform is found in very high concentrations in the brain, specifically in the presynaptic nerve terminals (Ramjaun and McPherson 1996).

Both the isoforms (a and b) have identical functional domains (Figure 1.3):

1) at the N-terminus the Sac domain, homologous to the yeast Sac1p, dephosphorylates predominantly PI monophosphates present in cell membranes, including those of the Golgi apparatus and endosomes.
2) the central 5'-phosphatase domain dephosphorylates phosphatidylinositol bis- or trisphosphates localized in plasma membranes to activate downstream pathways (Perera 2006; Mcpherson 1996; Cremona 1999; Guo 1999).
3) at the C-terminus, PRD domain comprises about 250 amino acid and contains at least five potential SH3 domain-binding consensus sequences (Mcpherson1996).

The 170 kDa isoform harbors an additional smaller PRD with at least three additional SH3 binding sites (Ramjaun and McPherson 1996).

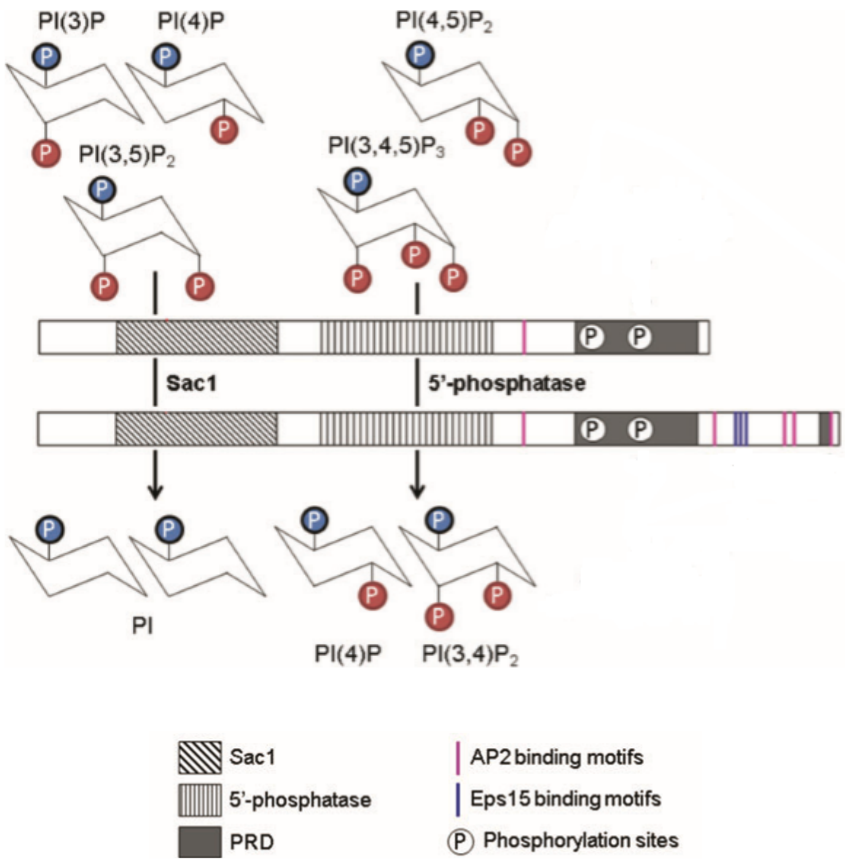

Figure 1.3 The structure and functions of the two major Synj1 isoforms.
Scheme of Synj1 isoform a (1612 aminoacids/170 kDa) and isoform b (1350 aminoacids/145 kDa) and their enzymatic activity.
Sac1 domain hydrolyzes phosphatidylinositol mono- and bisphosphate (PI(3)P, PI(4)P, and PI (3,5)P2).
5-phosphatase domain hydrolyzes phosphatidylinositol bis- and trisphosphate (PI(4,5)P2 and PI(3,4,5)P3).
PRD domain allows interactions with partner proteins.
(from Drouet 2014)

Besides Grb2, the C-terminal region common to both SYNJ1 isoforms interacts with the SH3 domains of a variety of proteins implicated in endocytosis, subcellular targeting, and

signalling such as endophilin, amphiphysin, syndapin/pacsin, intersectin, and many others (Heuvel 1997; Itoh 2005).
The additional C-terminal tail of 170 kDa isoform contains binding sites for clathrin and AP2 via three types of binding motifs (WxxF, FxDxF, and DxF), and for Eps15 through asparagine-proline-phenylalanine (NPF) domain (Haffner 1997; Praefke 2004).
It is important to note that Synj1 is subject to post-translational modifications, mainly phosphorylations, which modulate the Synj1 phosphatase activity and its interaction with partner proteins. For istance, a study performed on Synj1 purified from rat brain showed that Cdk5 can phosphorylate Synj1 at the Ser1144 (S1202 in human) and this phosphorylation inhibits Synj1 5-phosphatase activity (Lee 2004).

Phosphoinositides regulated by Synj1
As aforementioned, thanks to its different functional domains, Synj1 can act on different phosphoinositides, the principals are PI(4,5)P2, PI(3)P and PI(4)P Figure 1.3. PI(4,5)P2 is enriched at the plasma membrane, but a small amount of PI(4,5)P2 was also reported at the Golgi and the nuclear envelope (Watt 2002). It is involved in the invagination of the clathrin-coated pits (CCPs), in particular, it is implicated in the assembly of plasmalemmal CCPs. In fact, PI(4,5)P2 can binds the endocytic clathrin adaptors such as AP-2, AP180/CALM, epsin and many other endocytic factors (Wenk and De Camilli 2004; Owen 2004). Genetic inactivation of SYNJ1 causes accumulation of CCVs related to the increase of PI(4,5)P2 (Cremona 1999). Consistently, overexpression of the membrane-targeted 5-phosphatase domain of Synj1 causes depletion of PI(4,5)P2 and mislocalization of AP-2 and clathrin, resulting in the impairs of receptor-mediated uptake of transferrin or epidermal growth factor (Haucke 2005). Furthermore, PI(4,5)P2 is also implicated in the fusion of secretory granules with the plasmalemma and in micropinocytosis (De Matteis 2004).

PI(4)P is a precursor of PI(4,5)P2 and is localized in the plasma membrane and at the Golgi complex where it is involved in the control of Golgi trafficking and morphology (De Matteis 2007; D'Angelo 2008). PI4P is a master regulator of protein and lipid trafficking, the deficiency of PI(4)P gives rise to the disfunction and structure alteration of the Golgi complex (Di Paolo and De Camilli 2006).
Another substrate of Synj1 is PI(3)P. It is enriched in the cytosolic face of the limiting membrane of early endosomes and at the intraluminal vesicles contained within multivesicular bodies (MVBs) (Di Paolo and De Camilli 2006; Gillooly 2000). PI(3)P is generated by the lipid kinase PI(3)P kinase or also through the sequential dephosphorylation of PI(3,4,5)P3 by inositol phosphatases specifically for the 5 and 4 positions (including SYNJ1 for position 5) (Corvera 1999). PI(3)P can control the function of endosomes thanks to its effectors, such as early endosome antigen 1 (EEA1) and Hrs which contain the P(3) P-binding modules (Lemmon 2003; Birkeland 2003). Interestingly, EEs membranes, in addition to PI(3)P, also contain the other substrates of SYNJ1: PI (4,5) and some PI(4)P (Yoshida 2017). Furthermore, PI(3)P can regulate various steps of autophagy processes by recruiting specific protein effectors to target membranes. During autophagosome formation, it is produced thanks to the action of the VPS34 kinase complex. In this step, PI(3)P presence is necessary for the recruitment of specific proteins involved in the autophagosome formation. Moreover, the remotion of PI(3)P by a myotubularin-related PI phosphatase is implicated in autophagosome maturation and autolysosome biogenesis (Bento 2016).
Interestingly, PI(4)P and PI(4,5)P2 were also shown to play an important role during prolonged starvation in the autophagic lysosome reformation (ALR). ALR is the process of novo biogenesis of lysosomes from existing autophagolysosomes and occurs through tubulation and fission of autophagolysosomes membranes. Some studies indicated that during ALR there is a conversion of PI(4)P to PI (4,5) P2 by

PIP5K1B and subsequent recruitment of clathrin to the autolysosomes (via its adapters) (Yu 2010). Moreover, PIP5K1A (distinct isoform of PIP5K1B) control the fission of tubules reforming from autolysosomes (Rong 2012).

Synj1 functions

Thanks to its different functional domains and overall to the capacity to interact with different proteins, Synj1 has been implicated in different cellular process both in nerve terminals where it has been first discovered and in other cell types (Drouet and Lesage 2014).

First, Synj1 is implicated in synaptic vesicle recycling because it has been found localized in close proximity to clathrin- and dynamin coated endocytic intermediates in nerve terminals (McPherson 1994, David 1996; Haffner 1997). A lot of studies showed that Synj1 has a key role in nerve terminals. Synj1-deficient mice showed neurologic defects (weakness, ataxia, spontaneous epileptic seizures, and poor motor coordination) and died shortly after birth. In the neurons of these mice it was found an increase of phosphatidylinositol (4,5)-bisphosphate (PI (4,5) P2) levels and an accumulation of clathrin-coated vesicles in the cytomatrix-rich area surrounding the synaptic vesicle cluster in nerve endings (Cremona 1999). Moreover, Synj1 was reported localized in proximity to clathrin- and dynamin coated endocytic intermediates in nerve terminals (McPherson 1994; David 1996).

The loss of Synj1 function also leads to synaptic transmission alteration in other species (Drosophila, Zebrafish, and C. Elegans) (Harris 2000, Schuske 2003; Verstreken 2003; Van Epps 2004).

In addition, another role has been identified for Synj1 post-synaptically: it is involved in the internalization of AMPA receptors in postsynaptic neurons (Gong and De Camilli 2008), suggesting that Synj1 could participate in the signal transmission through postsynaptic reorganization.

Like in neurons, in podocytes Synj1 participates in endocytosis with its interacting partners dynamin and

endophilin by acting on phosphoinositides and actin filaments (Soda 2012). Recently, Synj1 has been reported as a potential regulator of allogenic T cell responses (Sun 2013).

Role of Synj1 in membrane trafficking

The involvement of Synj1 in regulating membrane trafficking is discovered firstly in Zebrafish. In photoreceptor neurons of Zebrafish the loss of Synj1 gives rise to enlarged acidic vesicles, abnormal late endosomes and, an increase in autophagosomes, thus alterations in all endolysosomal pathway (Holzhausen 2009; George 2014; George 2016). This role has recently confirmed in mammalian cells.

Studies performed in my laboratory have demonstrated that Synj1 plays a crucial role in the homeostasis and functions of early endosomes (Fasano 2018). They have shown that the loss of Synj1 causes the dysfunction of the early endosomal compartments in neural and in non-neural human cells. Interestingly, on the contrary of what was reported for the photoreceptor neurons of Zebrafish our data show that the loss of Synj1 in human cells does not affect late endosomes. Moreover, although the internalisation of different ligands, such as Tf or EGF, is unaffected in Synj1-depleted cells, the loss of Synj1 impairs the recycling of the TfR to the plasma membrane (Fasano 2018). In contrast, it does not alter the trafficking of EGFR to the lysosomes, indicating that the recycling pathway is specifically affected by the lack of Synj1.

Disease associated to genetic alterations of Synj1

Phosphoinositide balance in the cell is fundamental, and their disequilibrium leads to neurological symptoms. In fact, alteration of expression levels and activity of Synj1 are involved in several disorders (Figure 1.4). Synj1 mutations were implicated in intractable epilepsy with tau pathology (Dyment 2015), and early-onset refractory seizures with progressive neurological decline (Hardies 2016). Epileptic encephalopathy is a heterogeneous group of severe childhood onset epilepsies characterized by refractory seizures,

neurodevelopmental impairment, and poor prognosis. EIEE53 is autosomal recessive form of epileptic encephalopathy caused by mutations in the SYNJ1 gene. This disorder was found in 3 unrelated families and the mutations were in a homozygous or compound heterozygous state. Specifically, the aminoacid substitution p.Tyr888Cys affects the dual phosphatase activity of Synj1, while three premature stop variants (homozygote p.Trp843* and compound heterozygote p.Gln647Argfs*6/p.Ser1122Thrfs*3) produce a nonsense-mRNA decay and almost complete loss of protein expression (Hardies 2016).

SYNJ1 triplication observed in Down's syndrome (DS) might contribute to the complex pathogenesis of this disease. DS is caused by a trisomy of chromosome 21 and is the most common genetic cause of mental retardation. With the use of linkage analysis was definite the sub-regions of chromosome 21 responsible for mental retardation and Synj1 is present in this region. It was reported the Synj1 overexpression and an alteration of PI(4,5)P2 metabolism in the brain of Ts65Dn mice, commonly used as animal model of DS. Interestingly, these defects are abolished in Ts65Dn mice lowering Synj1 expression at levels comparable to diploid karyotype. Furthermore, the transgenic mice overexpressing Synj1 from BAC constructs exhibit the same defects and show deficits in the performance of the Morris water maze task (Voronov 2008; Drouet and Lesage 2014). Moreover, many studies have also reported the alteration of Synj1 levels in Alzheimer's disease (AD) (Drouet and Lesage 2014). It appears that there exists a direct correlation between Synj1 overexpression and amyloid-beta oligomerization, suggesting a potential role of this protein in the development of AD. Further studies will be needed to elucidate this aspect. Interestingly, recent data have shown that Synj1 reduction could be a possible therapeutic strategy to counteract AD pathology: Synj1 reduction was shown to be protective in a mouse model expressing the Swedish mutant of amyloid precursor protein and in an

Aapolipoprotein E4 knock-in mouse model (McIntire 2012; Zhu 2015).

PARK20

Synj1 mutations are also causative of an inherited form of Parkinson's disease. In 2013, through the use of homozygosity mapping followed by exome sequencing two teams independently identified the same homozygous missense mutation (R258Q) in the SYNJ1 gene in sibs of Iranian (Krebs 2013), and an Italian consanguineous family (Quadri 2013) with autosomal recessive early-onset Parkinson disease-20 (PARK20). Three years later was identified another homozygous mutation in Synj1 associated with PARK20 that is a transversion (c.1376C-G, NM_203446.2) in exon 11 of the SYNJ1 gene and it results in a substitution of a highly conserved residue in the Sac1 domain (R459P) (Kirola 2016). Recently, it was reported a frameshift mutation (p.S552Ffs$*$5) in heterozygous state with the benign p.T1236M missense variant in one late-onset PD (Bouhouche 2017).

As mentioned before, Synj1 has a crucial role in the endolysosomal system in different cell types. In particular, previous data of my laboratory demonstrate that endomal alterations are involved in PARK20 pathogenesis (Fasano 2018). In fact, fibroblast-derived from PAR20 patients display alterations in early endosomes and recycling trafficking (Fasano 2018). Moreover, also mice carryng Synj1 R259Q Sac mutation have synaptic endocytosis defects, including accumulating of CCVs (Cao 2017). Interestingly, in neurites of human PARK20 induced pluripotent stem cell-derived neurons some alterations in synaptic autophagosome formation were observed (Vanhauwaert 2017). What is the role of Synj1 in autophagy and whether this role has an impact on the pathogenesis of PAR20 remains to be determined.

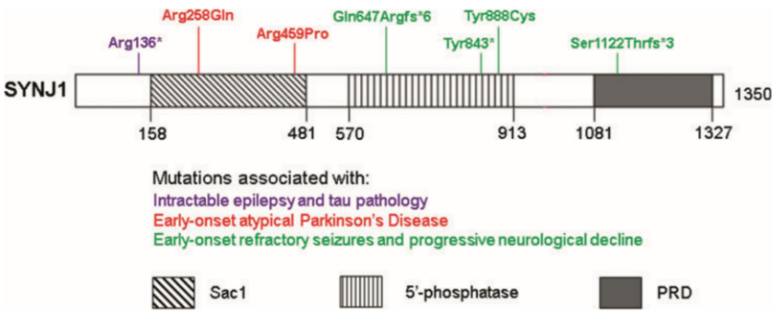

Figure 1.4. Genetic alterations of Synj1 associated to disease. Scheme of the mutations founded in Synj1 associated with pathologies. The Synj1 isoform in the image is the shorter 145 kDa because it is the most abundant in the brain. (from Drouet 2014)

Autophagy

Autophagy is a highly conserved lysosomal degradation pathway and it is a vital process for all organisms. Indeed, through the degradation of cytoplasmic organelles, proteins and macromolecules, and the recycling of the breakdown products, autophagy plays important roles in cell survival (Papandreou 2017).

There are different types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Kaur and Denath 2015). Microautophagy consists of the direct uptake of particulate or soluble cellular constituents through the direct engulfment action of the endosomal or lysosomal membrane. Thus, after this direct engulfment, the substrates are degraded by lysosomal proteases (Li 2012). CMA is a type of selective autophagy, which consists in the targeting of cytosolic proteins for degradation and their direct delivery to the lysosome (Kaushik and Cuervo 2012).

Macroautophagy (hereafter called autophagy) involves dedicated double-membrane vesicles, called autophagosomes and proceed through different steps (Kliosnky 2016; Galluzzi 2017).

Autophagosome flux

As shown in Figure 1.5A autophagy is a multistep process and every step of this process is strictly controlled by different proteins. The main proteins involved in autophagy are the autophagy-related proteins (ATG) that are more than 30 and, some of them assemble into multisubunit functional complexes after their activation (Nakatogawa 2009).

The first step of autophagy is autophagosome biogenesis or formation that can be divided into three stages: nucleation, expansion, and closure (Soreng 2018).

Autophagosome nucleation

The autophagosome nucleation consists of the initial nucleation of a 'crescent-shaped' phagophore, which occurs in specific and multiple sites such as the ER, Golgi, endosomes, mitochondria, mitochondria-associated membranes and the plasma membrane (Papandreou and Tavernarakis 2017).

At molecular level, autophagy initiation starts from the activation of the ULK complex. It consists of the kinases ULK1 or ULK2, ATG13, FIP200 (FAK family kinase-interacting protein of 200kDa), and ATG101 (Chan 2009; Figure 1.5B).

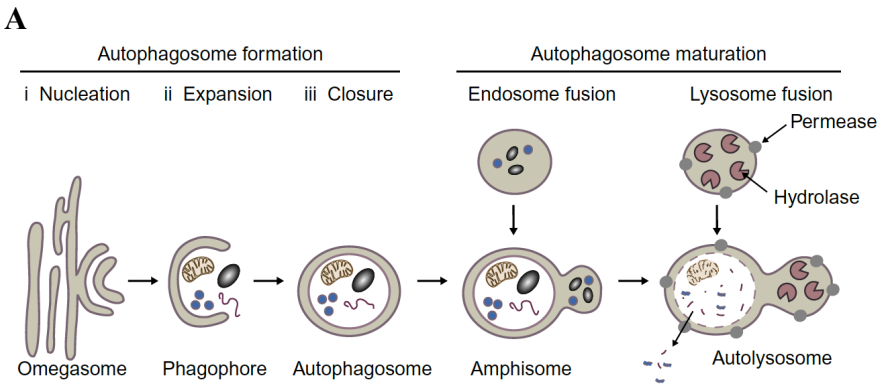

Figure 1.5 Autophagy pathway. (A) Overview of the macroautophagy pathway. (i). The phagophore membrane expands and elongates to sequester cytoplasmic components into a double membrane structure (ii) which upon closure becomes an autophagosome (iii). Maturation of the autophagosome involves its fusion with endosomes to create an amphisome, which further fuses with lysosomes to generate an autolysosome. The sequestered cargo is degraded by lysosomal hydrolases in the acidic environment of the autolysosome, and the degradation products are released back into the cytoplasm through permeases in the autolysosomal membrane to be reused by the cell as energy source or building blocks for synthesis of new molecules. **B** Nucleation and expansion of the phagophore membrane in mammalian cells. In the black rectangle: Th eubiquitin-like proteins ATG12 and LC3/GABARAP (here

shown as LC3) and their conjugation systems operate sequentially and are important during autophagosome formation. (From Soreng 2018)

The activity of the ULK complex is crucial for the recruitment of the class III PI3K complex to the phagophore. The class III PI3K complex is composed of PIK3C3 (also known as the VPS34), its adaptor protein p150 (PIK3R4), ATG14L (also called Barkor) and BECLIN1 and, their function is the production of phosphatidylinositol 3-phosphate [PI(3)P] at the sites of phagophore initiation (Soreng 2018). PI(3)P is recognized by PI(3)P autophagic effectors that are double FYVE-containing proteins such as DFCP1 (an omegasomes marker) and the WIPI family proteins (Soreng 2018; Papandreou and Tavernarakis 2017). Another important protein involved in the first step of autophagy is ATG9, a multispanning membrane protein. ATG9 is implicated in providing lipid bilayers required for the formation of the autophagosome cycling between the site of the phagophore formation and cytosolic pools (Figure 1.5 B) (Reggiori 2012).

Autophagosome expansion

After nucleation, phagophore undergo to expansion, growth and membrane remodelling through several membrane sources and the sequestration of cytoplasmic cargo. The actors involved in this step are two ubiquitin-like proteins: ATG12 and LC3 and their conjugation machinery. ATG12 is conjugated to ATG5 by ATG7 and ATG10 (E1-like and, E2-like enzyme) binds ATG16L1. This complex acts as an E3 ligase mediating lipidation of LC3. As shown in Figure 1.5 B, LC3 exists such as pro-protein that is cleaved in the LC3-I through the action of the cysteine protease ATG4. Subsequently, LC3-I became lipited and inserted in the highly curved phagophorerim (LC3-II) through the action of ATG7, ATG3 and, as mentioned before, the ATG12-5-16L1 complex. LC3-II in the inner autophagosomal membrane remains bound to autophagosome and is degraded in the autolysosome, on the

contrary, LC3-II present in the outside membrane can become deconjugated through the ATG4 action (Soreng 2018).

Autophagosome closure

The last stage of autophagosome formation is the phagophore closure to form an autophagosome (Soreng 2018) (Figure 1.5 A). The clearance of PI3P is essential in this step. In fact, the dephosphorylation of PI3P by PI3 phosphatases reduces the interaction of PI3P-binding proteins and, the change in lipid composition gives rise (at least in part) to the remotion of the majority of the membrane-bound Atg proteins before phagophore closure (Soreng 2018). After closure, completed autophagosome acquires STX17, a SNARE protein. Importantly, STX17 is required for autophagosomal fusion with the endosome/lysosomes and, it is specifically present on completed autophagosomes but not on isolated membranes (Itakura 2012).

Autophagosome maturation

After autophagosome biogenesis there is the autophagosome maturation. In this step the autophagosome is transported through microtubules and actin to the endolysosomal system where the autophagosome can fuse with endocytic lesions and lysosomes to generate the autolysosome (Monastyrska 2009). In some cells, such as neurons, autophagosomes need to be transported for long distance. In fact, in thise types of cells, autophagosomes trafficking results more sensible to disregulations (Papandreou and Tavernarakis 2017; Cheng 2015). Thus, during autophagosome maturation, autophagosomes form hybrid vesicles (amphisomes) through fusion with different types of endocytic lesions (Berg 1998; Liou 1997). Moreover, early endosomes in this step seems to have an important role, as supported by the fact that inhibition of early endosomes functions give rise to an accumulation of autophagosomes (Razi 2009; Rusten 2009).

The next step of autophagy after maturation is the fusion of the autophagosome with the lysosome, in which are involved the

SNARE complexes (autophagosomal Stx17, synaptosomal-associated protein 29 (SNAP29) and endosomal/lysosomal membrane VAMP8), the homotypic fusion and protein sorting (HOPS)-tethering complex and Rab7 (Itakura 2012; Jiang 2014). In the lysosomes, terminal point of this tortuous multiprocess, there is the degradation of autophagosomal contents in the resultant metabolites that are transported into the cytosol.

Regulation of autophagy

Autophagy can be regulated by different signaling mechanisms in mammalian cells (Mehrpour 2010). The two main autophagy regulators are the mammalian target of rapamycin (mTOR) and the adenosine monophosphate-activated protein kinase (AMPK) Figure 1.5 B. mTOR negatively regulates the initiation step of autophagy (autophagosome formation) through inhibitory phosphorylation of ATG13 and ULK1 (Tamargo-Gomez and Marino 2018). On the contrary, AMPK is a positive regulator of autophagy through direct phosphorylation of ULK1 that increase the recruitment of autophagy-relevant proteins (ATG proteins) to the membrane domains involved in the autophagosome formation. Thus, mTOR and AMPK play antagonistic roles in autophagy. Moreover, one of the principal mechanisms in which AMPK induces the autophagy is the inhibition of mTORC1 through the specific phosphorylations/activation of mainly inhibitor of the mTOR Complex 1 (mTORC1): TSC2 (Tuberous sclerosis complex 2) (Tamargo-Gómez and Mariño 2018). mTOR and AMPK activity are subjected to multiple positive and negative regulations originating from extracellular growth and stress stimuli. It has been reported that in the presence of insulin and other growth factors, mTORC1 is activated by phosphatidylinositol 3-OH kinase (PI3K) and its downstream effector, Akt.

Akt can activate mTORC1 through direct phosphorylation/inactivation of TSC2, on four different sites.

Furthermore, TSC2 can be directly phosphorylated and inactivated also by other kinases such as ERK2, RSK, and the p38-activated kinase MK2, that are induced by growth factors (Bhaskar and Hay 2007). AMPK is mainly regulated by intracellular levels of ATP and AMP; it is in an active form when there are concomitantly low ATP levels and high AMP levels. The binding of AMP promotes stimulatory phosphorylation of AMPK by the AMP kinase (Manning and Toker 2017).

Alterations of autophagy in PD

Since the fact that neurons are post-mitotic cells, they can't continuously divide. Thus, they don't have the advantage to dilute potential toxic aggregates with the cellular division. One of the principal functions of autophagy is to clear out aggregated proteins, so it is not surprising that malfunctioning in this process could be involved in neurodegeneration's development (Tooze 2008; Karabiyik 2017). In support of the role of autophagy in neurodegeneration there are several studies (Karabiyik 2017). For example, the ATG5 and ATG7 loss in the mice leads to the accumulation of neuronal inclusion body giving rise to a phenotype simillar to those observed in neurodegenerative diseases (Hara 2006; Komatsu 2006). In particular, autophagic vacuoles number is increased in the dopaminergic neurons of substantia nigra of PD (Toulorge 2016) and, also autophagic markers were found altered in the postmortem PD brain respect to the controls as show in table 3. LC3-II, the principal marker used for analisis of autophagy, and Beclin-1, involved in the formation and in the maturation of autophagosomes, were reported increased in the SN of PD patients respect to control SN samples (Dehay 2010; Rohn and Catlin 2011; Miki 2016). On the contrary, lysosomal-associated membrane protein 2 A (LAMP2A) and heat shock cognate 70 (hsc70) protein levels were reported reduced in the SN and amygdala of PD brains respect to the control brain samples (Alvarez-Erviti 2010). Interestingly, some of the PD-associated proteins could have a role in

autophagy (Li 2015). The α-synuclein is degraded through ubiquitin-proteasome system (UPS), the CMA and the autophagy, but when it is mutated or overexpressed blocks its degradation (Ebrahimi-Fakhari 2011; Webb 2003; Cuervo 2004). Moreover, α-synuclein can regulate autophagosomes formation. In fact, studies performed from Rubinsztein et al. in mammalian cells and in transgenic mice show that overexpression of alpha-synuclein causes a reduction of autophagosome synthesis that is probably related to the disruption of the normal localization and mobilization of Atg9 to LC3-positive vesicle (Winslow 2011). Furthermore, also the protein causative of the most common autosomal dominant form of PD (LRRK2) has a role in autophagy (Li 2015). The loss of LRRK2 in mice causes an altered level of autophagy marker (LC3-II and p62) and, accumulation of lipofuscin granules (Tong 2010). The mutations in LRRK2 associated with PARK8 cause alterations in autophagy that can be positive or negative in the dependence of different mutations and cellular systems (Li 2015). The other proteins causative for young-onset autosomal recessive PD that could have a role in autophagy are PINK1 and Parkin. Both the proteins are involved in the autophagic degradation of mitochondria (mitophagy) and, when they are in the PD associated mutated forms are not able to perform these actions giving rise to accumulation of defective mitochondria (Karabiyik 2017). Hence, all these findings support a role of autophagy in the pathogenesis of Parkinson's disease.

Table 3 Lysosome and autophagy alteration in post-mortem PD brain

Protein	References	Structure	Observation in PD
Beclin 1	Murphy *et al.* (2014)	Cg Cx	↓ levels
Beclin 1	Miki *et al.* (2015)	SN	↑ levels
Beclin 1 (caspase-cleaved)	Rohn and Catlin (2011)	SN	↑ levels
Beclin1 (phosphorylated)	Miki *et al.* (2015)	SN	= levels
Cathepsin A	Murphy *et al.* (2014)	Cg Cx	↑ levels
Cathepsin B	Mantle *et al.* (1995)	Fr Cx	= levels
Cathepsin D	Murphy *et al.* (2014)	Cg Cx	↑ levels
Cathepsin D	Chu *et al.* (2009)	SNc	↓ levels in neurons
Cathepsin D	Mantle *et al.* (1995)	Fr Cx	= levels
Cathepsin H	Mantle *et al.* (1995)	Fr Cx	= levels
Cathepsin L	Mantle *et al.* (1995)	Fr Cx	= levels
Dipeptidyl aminopeptidase I	Mantle *et al.* (1996)	Fr Cx	= activity
Dipeptidyl aminopeptidase II	Mantle *et al.* (1996)	Fr Cx	↓ activity
Glucocerebrosidase	Murphy *et al.* (2014)	Cg Cx	↓ levels
Glucocerebrosidase	Gegg *et al.* (2012)	Crb	↓ levels and activity
Glucocerebrosidase	Gegg *et al.* (2012)	SN	↓ levels and activity
Glucocerebrosidase	Gegg *et al.* (2012)	Ag, Fr Cx, Pu	= levels
Hsc70	Alvarez-Erviti *et al.* (2010) and Mandel *et al.* (2009)	Ag, SN	↓ levels
HSP73	Chu *et al.* (2009)	SN	↓ levels in neurons
LAMP1	Dehay *et al.* (2010)	SN	↓ levels
LAMP1	Chu *et al.* (2009)	SN	↓ levels in neurons
LAMP2	Alvarez-Erviti *et al.* (2010) and Murphy *et al.* (2014)	Ag, Cg Cx, SN	↓ levels
LC3II	Dehay *et al.* (2010)	SN	↑ levels
LIMP-2	Rothaug *et al.* (2014)	SN	↑ levels
LMX1B	Laguna *et al.* (2015)	SN	↓ levels in TH neurons
MEF2D	She *et al.* (2011)	Str	↑ levels
P-type ATPase (ATP13A2)	Dehay *et al.* (2012)	SN	↓ levels
P-type ATPase (ATP13A2)	Ramonet *et al.* (2012)	Cx, Str	↑ levels
TFEB	Decressac *et al.* (2013)	SN	↓ nuclear levels in TH neurons
ULK1 (UNC-51-like kinase 1)	Miki *et al.* (2015)	SN	= levels
ULK2 (UNC-51-like kinase 2)	Miki *et al.* (2015)	SN	= levels
VPS34	Miki *et al.* (2015)	SN	= levels

Am, amygdala; Ca, caudate nucleus; Cb, cerebellum; Cb Cx, cerebral cortex; Cg Cx, cingulate cortex; Fr Cx, frontal cortex; SN, substantia nigra; Pu, putamen; Str, striatum (from Toulorge 2016)

2. Aims of the study

Proper membrane trafficking, which connects different organelles, is essential to maintain the proper composition of cellular compartments, by their continuous sorting, as well as to ensure their homeostasis and function. This is crucial for the homeostasis of eukaryotic cells and their vitality. Thus, membrane trafficking has to be finely regulated and it is not surprising that defects of the molecular machinery regulating membrane trafficking might lead alterations of intracellular pathways resulting in the development of diseases.

With respect to other cell types, neuronal cells are more sensitive to alterations of the membrane trafficking as highlighted by the increasing number of human neurodegenerative diseases and neurological disorders associated to the dysfunction of components of the trafficking machinery.

Interestingly, several genes responsible for hereditary forms of Parkinson disease have been reported to be implicated in distinct steps of the endolysosomal pathway.

We have recently highlighted the defective cellular pathways in the autosomal recessive early-onset parkinsonism (PARK20) caused by the mutation in the phosphoinositide (PI) phosphatase Synaptojanin 1 (Synj1). Specifically, we showed that Synj1 plays a crucial role in regulating the homeostasis and functions of early endosomal compartments in different cell types. Importantly, alterations of these compartments and trafficking defects occur in PARK20 fibroblasts, strengthening the link between endosomal trafficking and Parkinson's disease.

With respect to other inositol phosphatases, Synj1 has two consecutive phosphatase domains, Sac1 and 5'-phosphatase, through which it can act on diverse substrates: it mainly dephosphorylates PI bi- or triphosphates localized in plasma membrane, and PI monophosphates, PI(3)P and PI(4)P enriched in the membranes of endosomes and Golgi apparatus, respectively. Moreover, PI(3)P is also crucial for the biogenesis of autophagosomes. Hence, for this peculiarity

Synj1 might be responsible of different functions, and on the other hand defects of Synj1 activity might compromise different intracellular pathways.
Main goal of my PhD project was to elucidate the role of Synj1 in membrane trafficking and neurodegenaration and its impact on PARK20 pathogenesis.
Specifically, two main aims:

1) To study the role of Synj1 in the secretory pathway.
For this purpose, we analyzed whether i) Synj1 could control the homeostasis and functions of early secretory compartments and regulate the vesicular trafficking among them; and ii) whether the pathological mutation might induce ER stress, with consequent activation of the Unfolded Protein Response (UPR) pathway.

2) To study the role of Synj1 in the autophagy pathway.
For this purpose, we deeply monitored the autophagy pathway in Synj1 deficient cells and in PARK20 fibroblasts by analyzing the levels of autophagic markers, the formation of autophagosomes, the autophagy flux and the clearance of autophagy substrates.

3. MATERIALS AND METHODS

All materials and methods concerning the Project 1 are in the published article.

Reagent and antibodies

• Bafilomycin A_1 (B1793) has been purchased from Sigma-Aldrich (Saint Louis, MO, USA).
• Polyclonal anti-LC3 rabbit antibody (Novus) used for western blotting at a concentration of 1: 1000;
• Polyclonal anti-p62/SQSTM1 rabbit antibody (Progen) used for western blotting at a concentration of 1: 1000;
• Polyclonal anti α-synuclein rabbit antibody (Santa Cruz) used for western blotting 1: 1000;
• Polyclonal anti-AKT rabbit antibody (Cell Signaling) used for western blotting at a concentration of 1: 1000;
• Polyclonal anti-pAKT (Ser473) rabbit antibody (Cell Signaling) used for western blotting at a concentration of 1: 1000;
• Monoclonal anti-S6 rabbit antibody (Cell Signaling) used for western blotting at a concentration of 1: 1000;
• Polyclonal anti-pS6 (Ser240/244) rabbit antibody (Cell Signaling) used for western blotting at a concentration of 1: 1000;
• Polyclonal anti-pATG13 (Ser318) rabbit antibody (Abnova) used for western blotting at a concentration of 1: 1000;
• Monoclonal anti-α-Tubulin mouse antibody (Abcam) used for western blotting as a normalizer at the concentration 1: 2000;
• Monoclonal anti-GAPDH mouse antibody (Santa Cruz) used for western blotting as a normalizer at the concentration 1: 1000;
• Secondary Horseradish Peroxidase-linked anti-rabbit and anti-mouse antibodies were from GE Healthcare and used for western blotting at concentration of 1: 2000;
• Polyclonal anti-LC3 rabbit antibody (Abcam) used for immunofluorescence at 1: 100 concentration;

•Polyclonal anti-p62 guinea pig antibody (Progen) used for immunofluorescence at 1: 100 concentration;
• Polyclonal anti α-synuclein rabbit antibody (Santa Cruz) used for immunofluorescence at a concentration of 1: 100;
• Alexa Fluor 488 anti-rabbit used for immunofluorescence at concentration 1: 200
• Alexa Fluor 488 anti-guinea pig used for immunofluorescence at concentration 1: 200
• Secondary anti-rabbit antibody Jackson 546 tetramethylrodamine used for immunofluorescence at concentration 1: 100;
• X-Treme Gene 9 DNA Transfection Reagent (Roche);
• Lipofectamine® RNAiMax Transfection Reagent (Thermo Fisher Scientific);
• FUGENE® HD Transfection Reagent (Promega).

Cell cultures

HeLa cells were cultured in RPMI-1640 (EuroClone) with 10% FBS (Fetal Bovine Serum) and 2 mM L-glutamine. Moreover, the medium was supplemented with puromycin (0.6 μg/ml) to maintain the selection of pools and clones previously obtained in our laboratory (Fasano, 2018).
HeLa cells stably expressing GFP-mRFP-LC3 were a kind gift of D. Rubinsztein (Cambridge Institute for Medical Research).
Patients and control fibroblasts were derived directly from the skin punch biopsies and cultured in Dulbecco's Modified Eagle's Medium supplemented with 20% FBS and penicillin/streptomycin.
All cells were cultured at 37°C in a humidified atmosphere containing 95% air and 5% CO2. The culture medium was changed 2-3 times a week to provide the nutrients necessary for cell survival and growth. The cells were observed regularly under a phase-contrast microscope and, reaching a state of confluence of about 80%, were detached from the plate through the use of 0.3% trypsin containing 0.1% glucose, 2 mM EGTA in PBS pH 7.3 (KCl 13.7 mM, KH2PO4 1.47 mM, NaCl 137 mM, Na2HPO4 7H2O 8.06 mM) at 37°C. When

necessary, cells were counted with Neubauer chamber and seed in new cell culture dishes of different sizes or on other supports based on the type of experiment. All operations were carried out in conditions of absolute sterility using sterile instruments and working underneath a laminar flow hood.

Plasmids and transfection

The GFP-LC3 and GFP-p62 plasmids were provided from Dr. M. Renna (DMMBM, University of Naples).

Cells were transfected using different transfecting agents: Lipofectamine, Fugene or X-TremeGene XP9 and for each the manufcaturer's instructions were followed. In the different systems the DNA/reagent ratio is different: 1:2.5 for Lipofectamine, 1:6 in the X-Treme Gene XP9 and 1:4 in the Fugene. Moreover, while Lipofectamine and Fugene were removed after 5 hours avoiding toxicicty, X-Treme Gene XP9 reagent is kept over the whole transfection period.

Cells were analyzed 48 or 72 hours after transfection in dependence on the experiment.

Western blot analysis

After being cultured for 1 or 3 days and after the different treatments, cells were washed three times with ice-cold phosphate-buffered saline and lysed with JS lysis buffer (Hepes pH 7.5 50mM, NaCl 150mM, glycerol 1%, Triton X-100 1%, MgCl2 1.5mM, EGTA 5mM) or NP40 (Tris HCl pH 7,5 20mM, NaCl 120mM, NP40 2%) with 15 µg/ml of protease inhibitor (Antipain, Pepstatin, and Leupeptin all from Sigma). Protein concentration was measured by the Lowry or Bradford procedure. Protein extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes. Membranes were incubated with specific primary antibodies and revealed with HRP-conjugated secondary antibodies. For the detection ECL reagents (GE Healthcare or Pierce) was used according to the provided protocol.

Fold change values were calculated with a densitometric analysis by using the ImageJ software, each sample intensity value was normalized respect to its proper control sample that therefore have been arbitrary assigned as 1. The threshold for statistical significance (p-value) was set at 0.05.

Fluorescence microscopy

For immunofluorescence analysis cells were grown on coverslips, washed with PBS, fixed with 4% paraformaldehyde for 20 minutes and quenched with 50 mM NH4Cl for 15 minutes. Cells were permeabilized with 0,2% Triton/PBS for a time ranging from 5 to 10 minutes in dependence on the specific antigen and then incubated for 40 minutes in a blocking solution containing 0.5%BSA and 1-2% serum. Primary antibodies were diluted in the same blocking solution, incubated for a time-dependent on the specific antibody and detected with Alexa Fluor 488 or Alexa Fluor 546 conjugated secondary antibodies.

For Lysotracker staining, cells were fixed after the incubation for 1 h with Lysotracker (1:1000) in complete medium.

The images were acquired using a Zeiss LSM 510 META confocal scanning microscope equipped with a planapo 63X oil-immersion (NA 1.4) objective lens. The same settings (laser power, detector gain) were used.

The quantitative analysis was carried out using Zeiss LSM 510 or ImageJ software.

Bafilomycin treatment

Cells, grown on dishes for 3 days, were incubated with bafilomycin in complete medium at two different concentrations: 400 nM for 4 hours or 100 nM for 6h as preciously described (Klionsky, 2016; Conte, 2017)

Statistical Analysis

Data are expressed as mean ±SD. The data were statistically validated by Student's T-test.

4. RESULTS

Project 1: Role of Synj1 in the secretory pathway and its impact in PARK20 pathogenesis.

The Manuscript related to the first part of my project has been published on the journal "Frontiers in Neuroscience" and is appended at the end of this section, see at page 77 (**7.List of publications)**.

Aims

• To analyze whether Synj1 could control the homeostasis and functions of early secretory compartments and the vesicular trafficking among them.

• To analyze whether the pathological mutation might induce ER stress, with consequent activation of the Unfolded Protein Response (UPR) pathway.

Specific Background

The ER is an organelle involved in the translocation of the secretory proteins, in promoting their folding and in their quality control, which is crucial for the consequent exit of cargoes from the ER, to reach their final destination traversing the Golgi complex where the diverse cargoes can be modified and properly sorted.

Alterations of the ER functions lead to the accumulation of unfolded or misfolded proteins within the ER lumen with consequent induction of ER stress. The adaptive response that counteracts the ER stress is the Unfolded Protein Response (UPR), a group of signaling pathways that activate a potent transcriptional program aimed to restore ER proteostasis within the secretory pathway and ensuring cell survival. It is worth noting that with respect to other cells neurons, which lack the dilution effect produced by cell amplification, are completely dependent on UPR for counteracting misfolding proteins and in turn for cell survival (Remondelli and Renna 2017).

The UPR relies on the activation of PRKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Schroder and Kaufman 2005). These proteins are ER transmembrane proteins that in normal conditions are inactive thanks to the physical interaction with the chaperone BIP/GRP78 (binding immunoglobulin protein/78-kDa glucose regulated protein). During ER stress, BIP/GRP78 preferentially binds the unfolded polypeptides permitting the activation of PERK, IRE1, and ATF6. Once active, they play different actions:

- PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) attenuating protein translation. However, some proteins are preferentially translated and among them ATF4. ATF4 regulates the expression of genes involved in regulating protein folding, aminoacid metabolism, redox control, and ERGIC-53 (ER-Golgi intermediate compartment 53kDa protein) that is involved in the ER-to-Golgi transport of secretory proteins.
- IRE1α, which has a kinase and an endoribonuclease activity, drives the mRNA splicing of a 26-nucleotide-length fragment from X-box binding protein1 (XBP1) (Calfon 2002), permitting the translation of XBP1, that, in turn enhances the expression of UPR-dependent genes, including those for ER folding (calnexin, calreticulin), ERAD factors, autophagy components (e.g., ATG5, ATG12, Beclin) and redox metabolism.
- ATF6 is transported from the ER to the Golgi where it undergoes to proteolytic cleavage by S1P/S2P. The ATF6 active fragment is ultimately translocated into the nucleus where it activates the transcription of several genes such as chaperones (BIP, GRP94), XBP1, but also cell death activator CHOP.

These 3 stress sensors and their branches can act together or independently and are crucial to maintain protein homeostasis (Remondelli and Renna 2017). However, during chronic stress they can also trigger the cell death (Urra 2013).

Main results

Here we show that PARK20 fibroblasts display:

• Abnormal structure and distribution of Golgi membranes;

• Reduction of COPII vesicle formations and impairment of ER-to-Golgi transport with consequent accumulation of proteins within the ER;

• Activation of the PERK/eIF2a/ATF4 branch of the unfolded protein response.

Moreover, the persistent activation of PERK pathway induces oxidative stress and mitochondrial dysfunction. Interestingly, the treatment with specific PERK inhibitor (GSK2606414) restored the mitochondrial morphology and ROS levels.

Project 2: Role of Synj1 in the autophagy and its impact in PARK20 pathogenesis

Cellular model

To analyze the role of loss of Synj1 function on autophagy, we decided to use as cellular model the human HeLa cell line for several reasons: i) as aforementioned Synj1 is ubiquitously expressed; ii) previous studies in our laboratory have demonstatrated that the silencing of Synj1 affects the homeostasis and functions of early endosomes in different cell type, among them HeLa cells (Fasano 2018); iii) such cell line is widely used to monitor autophagic process.

In particular, we used stably silenced HeLa cells for Synj1 previously obtained in our laboratory by transfecting specific short hairpin RNA (Sh-1 and Sh-2; Fasano 2018). As control, we used a pool of cells interfered with a short hairpin RNA against a random GFP sequence. After selection with puromycin and collection of different pools and clones, the efficiency of knockdown was assessed by western blotting. As shown in Figure 4.1, pools and clones display a range of silencing from 30 to 80% and those with a reduction of about 40-45% were used for further experiments.

We also used PARK20 fibroblasts to evaluate the role of autophagy in the pathogenesis of the disease.

The loss of Synj1 alters the structure of lysosomes

As aforementioned, the Synj1 loss affects the homeostasis of early endosomal compartments, which result enlarged and sometimes mislocalized in the paranuclear region as well as the dynamic of the main markers of this compartments (such as EEA1, rab5) is altered (Fasano 2018). While upon Synj1 depletion LEs result unaffected, a moderate alteration of the structure of lysosomal compartments was observed (Fasano 2018). Here we confirmed our previous observation.

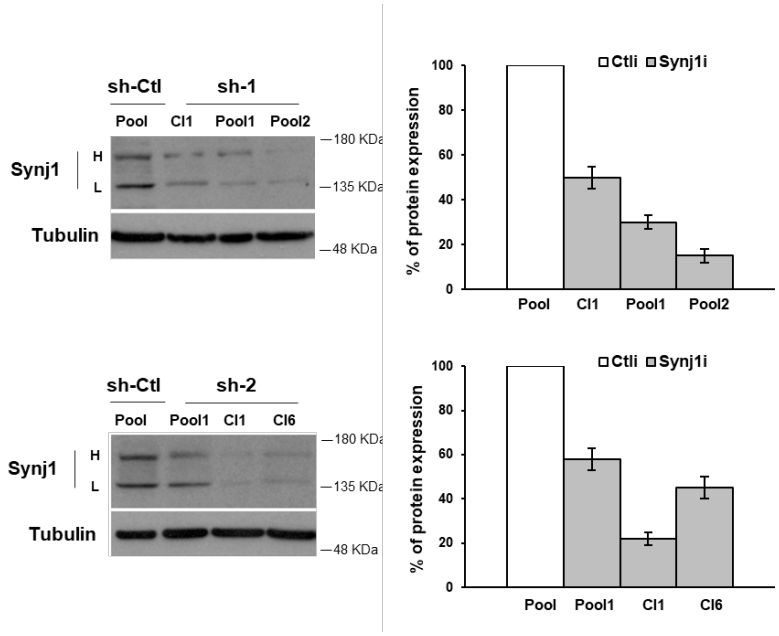

Figure 4.1 The expression of Synj1 was stably interfered in HeLa cells by using short hairpin RNAs. Representative immunoblotting of HeLa cells stably transfected with scrambled (sh-ctl) or specific anti-Synj1 (sh-1 and sh-2; see "Methods section" for more details) shRNA. Tubulin was used as a loading control. H and L correspond to 170 and 145 kDa isoforms, respectively. Densitometric analysis of 3 different experiments is shown. Results are expressed as mean value ± SD of the Synj1-interfered pools and clones compared with scrambled interfered cells (set to 100%). (From Fasano 2018).

We labeled lysosomes with the specific dye Lysotracker and we found that in Synj1 silenced (Synj1i) cells the lysosomes were numerically higher and showed an increase in the intensity of signal respect to control cells (Figure 4.2), indicating an expansion of this compartment. In addition, we obtained comparable results for Lamp-1 (Lysosomal-associated membrane protein 1) by immunofluoresce assays (Figure 4.2).

Interestingly, while western blot analyses showed higher levels of the EE markers EEA1 and Rab5 in Synj1i cells suggesting an alteration of the dynamics of these

compartments, the levels of the lysosomal proteins Lamp-1 and cathepsin D were comparable between the silenced and control cells (Figure 4.3).
Altogether these findings indicate that Synj1 knockdown affects the lysosomal compartments, but likely through a different mechanism. The lysosomes alteration could be a consequence of the altered trafficking from early endosomes.

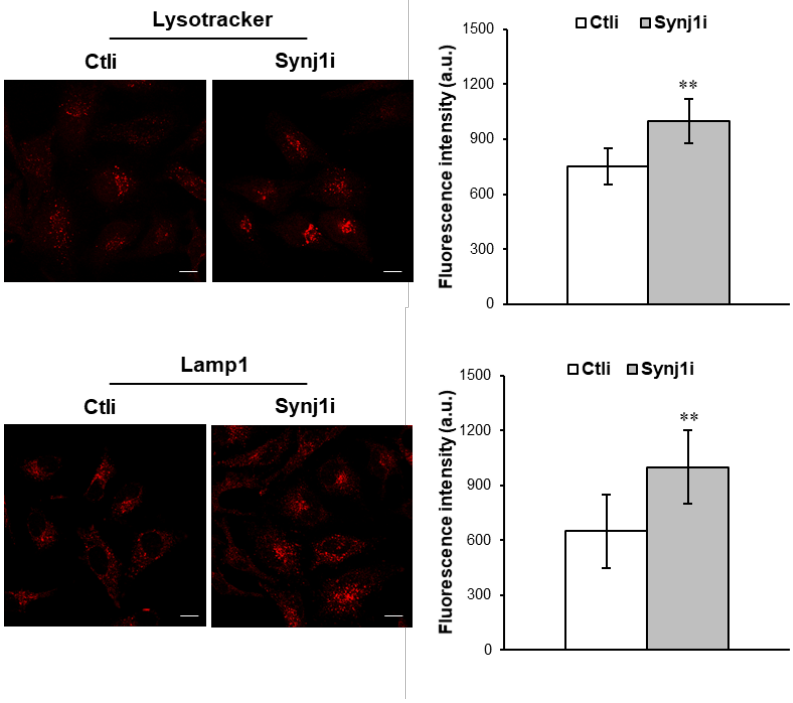

Figure 4.2. The loss of Synj1 affects the lysosomal compartments. Cells were labeled by using Lysotracker dye, which was added to living cells for 1h at 37°C or using a specific antibody against Lamp1 after fixation and permeabilization with Triton X-100 (see Methods). Representative single confocal sections of control (Ctli) or Synj1 (Synj1i) interfered HeLa cells are shown. Images were acquired by confocal microscopy using the same settings (laser power, detector gain). Scale bar 10 µm. Error bars, means ±SD. N>30 ** $p<0.01$, Student's t-test.

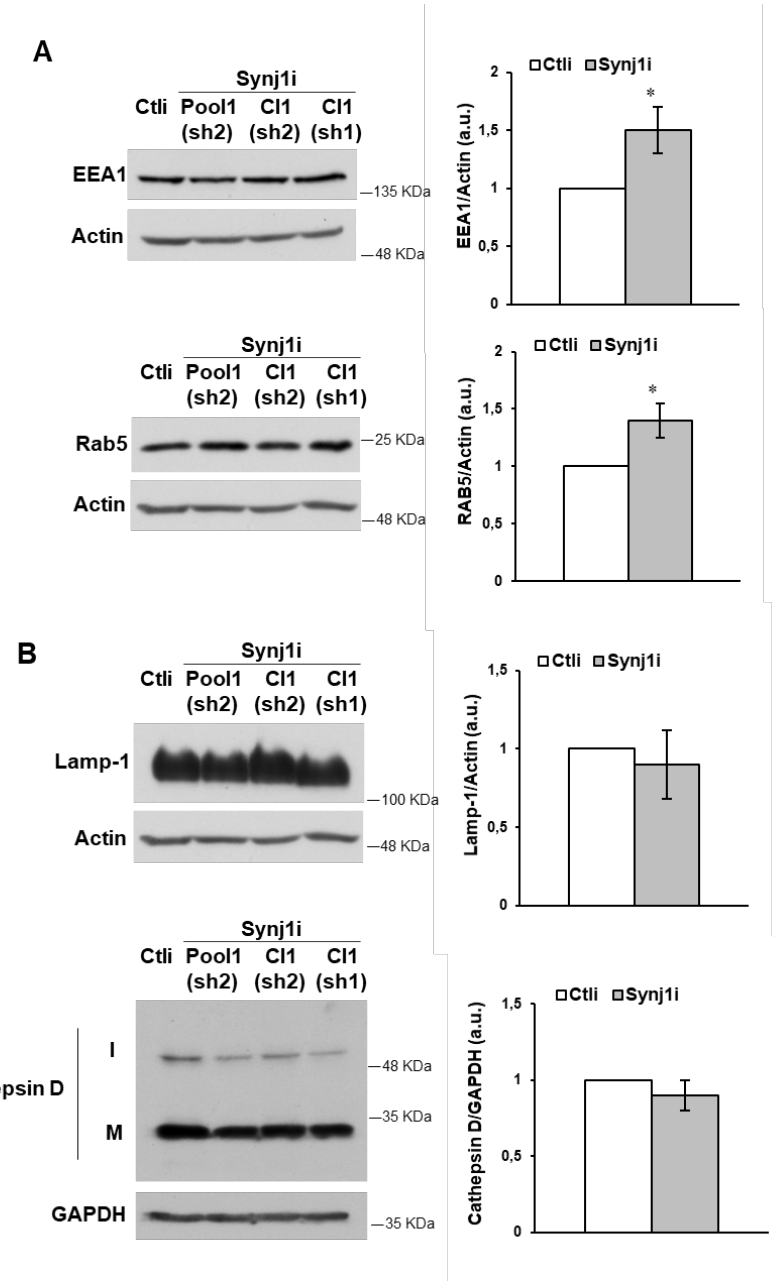

Figure 4.3 Expression levels of endolysosomal markers in Synj1i HeLa cells.

From Figure 4.3
Representative immunoblotting of eraly endosomal markers, EEA1 and Rab5 (**A**), and lysosomal markers, Cathepsin D and Lamp-1 **(B)** in Ctli and Synj1i HeLa cells. Actin or GAPDH was used as a loading control. M and I correspond to the mature (33 kDa) and immature (52 kDa) forms of Cathepsin D, respectively. Densitometric analysis of 3 different experiments is shown. Error bars, means ±SD. * $p < 0.05$; Student's t-test. (From Fasano 2018).

However, this hypothesis is not supported by the fact the trafficking of EGF receptor toward lysosomes is not altered in Synj1-silenced cells (Fasano 2018). Other plausible hypothesis is that the alteration of lysosomes could be related to changes in the autophagic pathway, whose activity is critical in many neurodegenerative diseases (Tooze 2008; Karabiyik 2017).

Levels of autophagic markers and the number of autophagosomes are increased in Synj1 silenced cells

To test the hypothesis that changes in the autophagic pathway may be responsible to the alteration of lysosomes, we monitored the autophagy pathway in control interfered (ctli) and Synj1-silenced (Synj1i) cells. To this aim, by western blot analysis we evaluated the levels of the lipidated form of LC3 (LC3II), which is recruited on autophagosome membranes upon autophagy activation, and those of p62 (also called sequestosome 1; SQSTM1), an ubiquitin binding protein that targets polyubiquitinated cargoes to autophagosome degradation (Klionsky 2016). HeLa cells were grown in full medium for 3 days to determine the basal levels of autophagy. As shown in Figure 4.4A the levels of LC3II were increased in Synj1-dpeleted cells. Moreover, about twofold ratio of LC3II-LC3I isoforms was observed in Synj1i with respect to Ctli cells, indicating an increase of LC3I-LC3II conversion (Figure 4.4A).

Furthermore, Synj1i pools and clones showed a variable increase of p62/SQSTM1 that correlates with the degree of

knockdown (Figure 4.4B). The highest levels were detected in the most silenced clone (cl1, sh2).

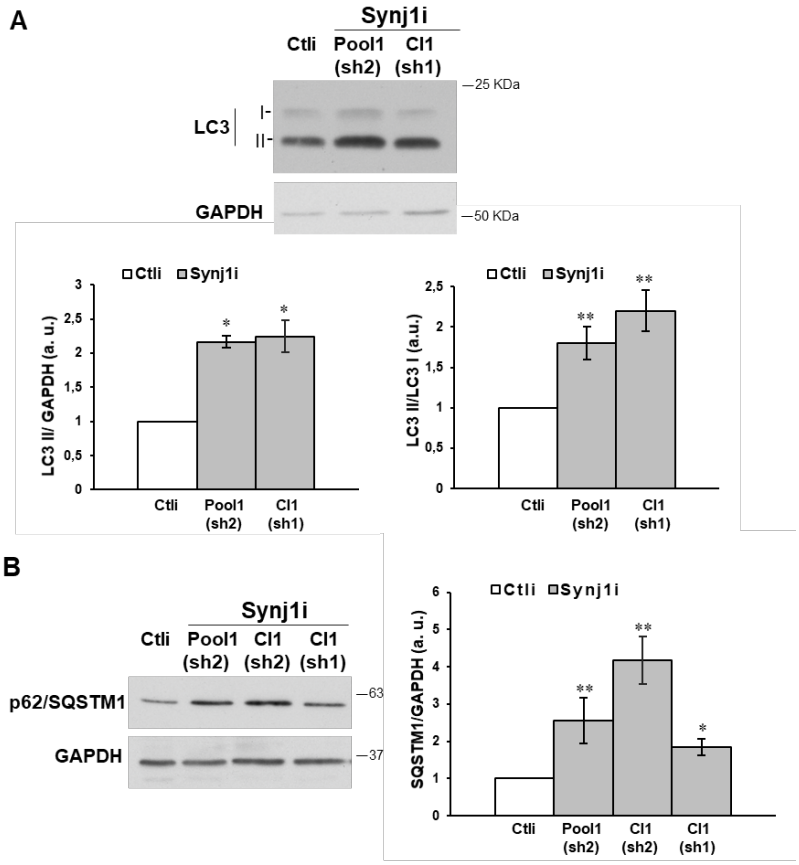

Figure 4.4. Levels of autophagic markers are increased in Synj1 knockdown cells. Representative immunoblotting of LC3 **(A)** and p62/SQSTM1 **(B)** in Ctli and Synj1i HeLa cells. GAPDH was used as loading control. I and II correspond to cytosolic (17 kDa) and lipidated (14 kDa) forms of LC3, respectively. Densitometric analysis of 4 different experiments is shown. Error bars, means ±SD. *p <0.05, ** p <0.01; Student's t-test.

To further corroborate the biochemical data, we assessed the distribution of the same autophagy markers by confocal

microscopy since the autophagosome number is one of the main mechanistic feature through which the amplitude of autophagic activity is regulated. In agreement with biochemical data, we observed a strong increase of LC3 and p62/SQSTM1 positive dots (or puncta) in Synj1 silenced cells, indicating their recruitment in autophagosomes (Figure 4.5).

All these data indicate an increase of autophagy pathway in Synj1 cells. However, it is worth noting that many of LC3 and p62/SQSTM1 resulted brighter and larger in Synj1 silenced cells with respect to control cells (Figure 4.5, see also magnifications), indicating the expansion of these compartments. To clarify this observation, we used an alternative approach taking the advantage of the chimeric proteins GFP-LC3 and GFP-p62/SQSTM1 in which the Green Fluorescent Protein (GFP) is fused to LC3 or p62/SQSTM1, respectively. Accordingly to immunofluorescence data, the number of GFP-LC3 and GFP-p62 puncta is significantly increased in Synj1-depleted cells with respect to control cells (Figure 4.6). Moreover, the GFP positive puncta results larger in Synj1 silenced cells as clearly evident in 3D reconstructions (Figure 4.6), further supporting an enlargement of autophagosomal compartment.

Altogether these findings clearly indicate the activation of autophagy pathway in Synj1 cells. However, the accumulation of p62/SQSTM1 and the expansion of autophagosomes suggest that this pathway is dysregulated rather than upregulated.

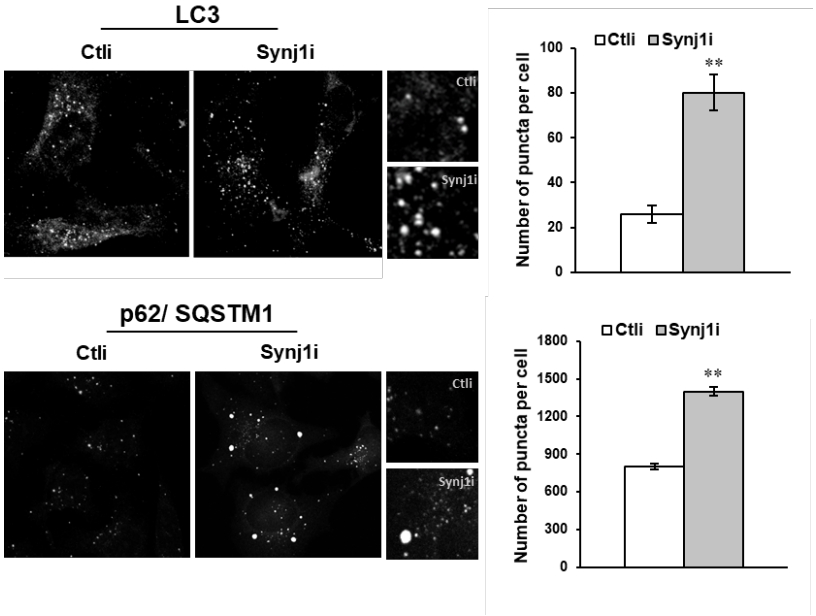

Figure 4.5. The number of autophagosomes is increased in Synj1 deficient cells. Ctli and Synj1i HeLa cells were labeled with specific antibodies against LC3 or p62/SQSTM1. Images were acquired from the top to the bottom of cells using confocal microscopy and representative 3D reconstructions are shown. Higher magnification pictures are shown in the insets. The number of LC3 or p62/SQSTM1 dots per cell was counted and the mean of 3 independent experiments is shown. N>30. Error bars, means ±SD; n ≥ 80 cells. ****** $p<0.01$, Student's t-test.

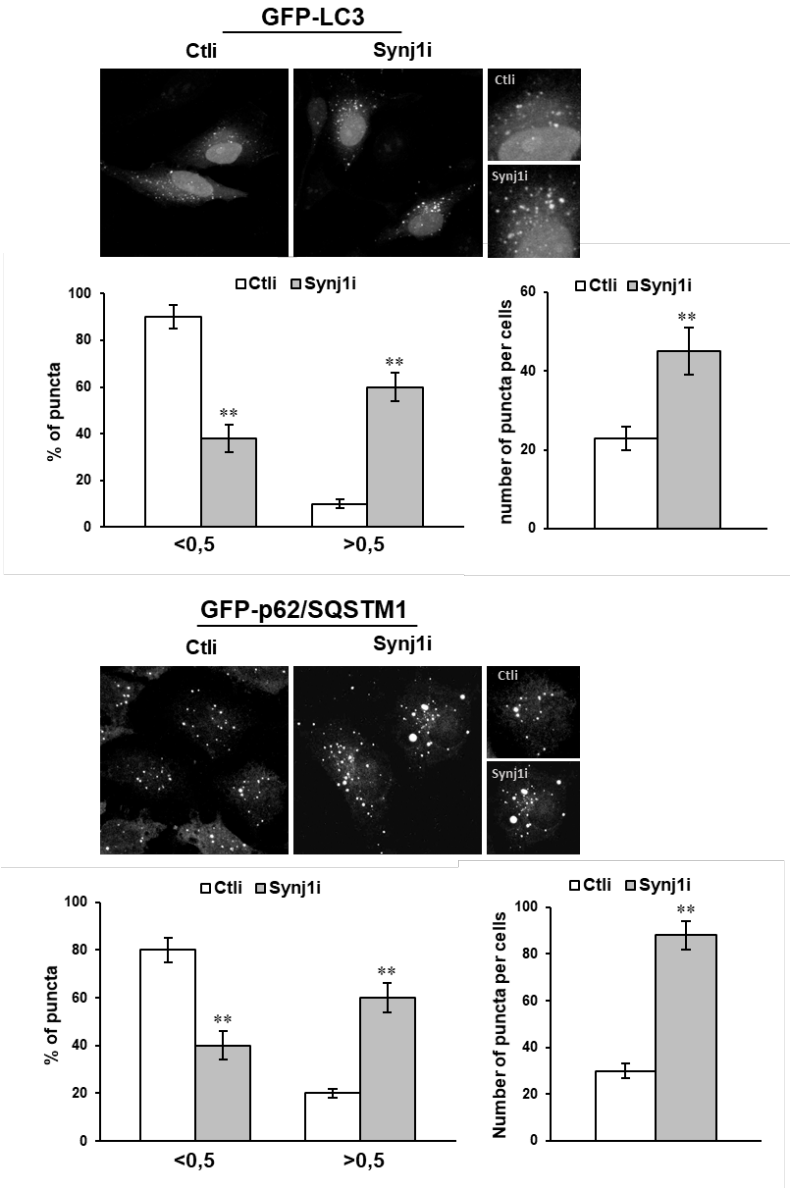

Figure 4.6. Autophagosomes are altered in number and in size in Synj1 deficient cells. Ctli and Synj1i HeLa cells were transiently transfected with cDNA encoding GFP-LC3 or GFP-p62/SQSTM1. The number of dots and the percentage of smaller (<0.5 μm) and larger (>0.5

μm) dots per cell are shown in the graphs. N>30. Error bars, means ±SD; n ≥ 50 cells. ** p<0.01, Student's t-test.

Autophagic flux is perturbed in Synj1-silenced cells.

To understand how the Synj1 loss impacts on autophagy pathway, we monitored the autophagic flux using two different approaches, a biochemical and an imaging approach (Figure 4.7 and 4.8).

In the biochemical approach, we evaluated the levels of LC3II and p62/SQSTM1 after incubation with Bafilomycin A_1, an inhibitor of the vacuolar-type H^+-ATPase, which prevents the lysosomal degradation by perturbing the lysosome acidification. Specifically, control and Synj1-silenced HeLa cells were treated with two different concentrations of Bafilomycin A_1 (100 nM or 400 nM for 6 and 4 hours, respectively). The autophagic flux was measured by the ratio between the amount of autophagic markers in treated and untreated cells.

At both concentrations, the levels of LC3II and p62/SQSTM1 were higher in presence of Bafylomicin A_1 than in basal condition both in Ctli and in Synj1i cells (Figure 4.7A); however the ratio between treated and untreated cells is lower in the silenced cells (Figure 4.7A), indicating that autophagic flux is delayed upon Synj1 loss.

In the imaging approach, we used a GFP-mRFP-tandem tagged LC3 (Vicinanza 2015; Klionsky 2016), which allows discriminating between autophgosomes (GFP and mRFP positive) and autolysosomes that result positive only for mRFP because the GFP signal is quenched in the acidic lumen of lysosomes. In particular, HeLa cells stably expressing GFP-mRFP-LC3 were transiently transfected, for 48 or 72 hours, with two specific stealth RNAs against Synj1 or with a control Stealth RNA (Figure 4.8).

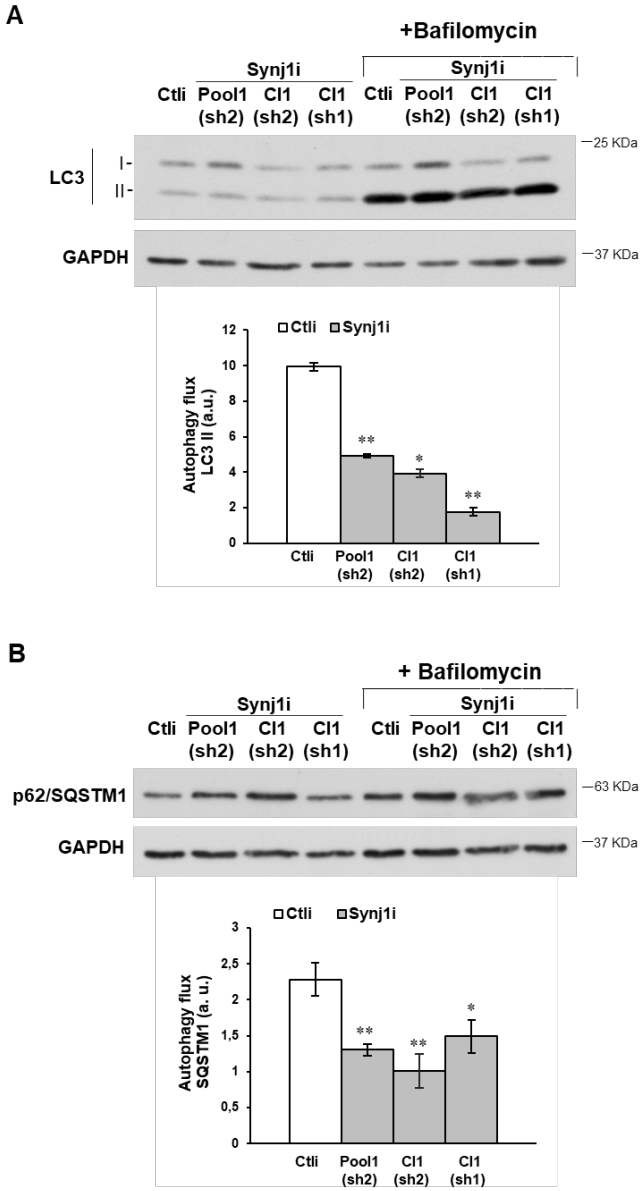

Figure 4.7. Autophagic flux is perturbed in Synj1-silenced cells. Representative immunoblot of LC3 **(A)** and p62/SQSTM1 **(B)** of Ctli and Synj1i HeLa cells untreated or treated with bafilomycin A_1 (400 nM) for 4 h The autophagic flux was calculated as the ratio between treated and

untreated of the same sample.. Error bars, means of 3 different experiments ±SD. *p <0.05, ** p <0.01; Student's t-test

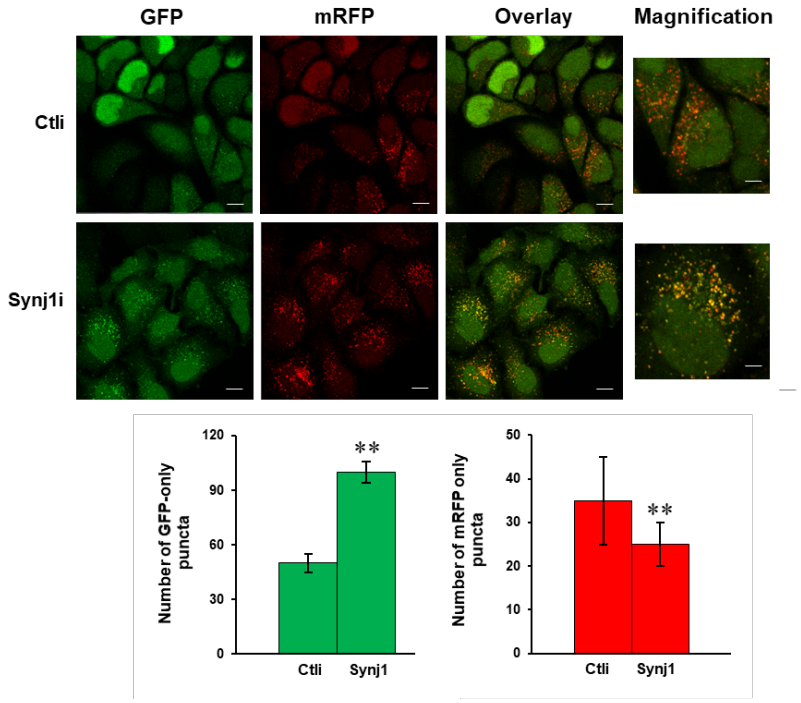

Figure 4.8 Analysis of autophagic flux in GFP-mRFP-LC3 expressing HeLa cells. Representative images of HeLa cells stably expressing GFP-mRFP-LC3 probe transfected with control or specific stealth RNAs against. Scale bar of single confocal sections and higher magnification inset is 10 µm and 5 µm, respectively. The number of GFP- or mRFP-positive puncta per cell is shown. N>30. Error bars, means ±SD; n ≥ 50 cells. ** p<0.01, Student's t-test.

In agreement with the results obtained by immunofluorescence experiments and with GFP-LC3 probe, we observed that the number of GFP-positive puncta was increased in silenced cells with respect to control-interfered cells. On the contrary, the number of red-only LC3 puncta was higher in control than in

silenced cells (Figure 4.8). Thus, the number of autophagosomes is increased in Synj1-silenced cells, but such increase is not paralleled by an equal increase in their maturation.
Overall, biochemical and imaging data indicate that autophagic flux is not inhibited, but partially delayed in Synj1-depeleted cells.

The loss of Synj1 reduces the clearance of autophagic substrates

Lysosomes are at the end of autophagic road: autophagosomes fuse with lysosomes and their content is degraded inside autolysosomes. Lysosomal function is crucial for cellular homeostasis; indeed dysfunction of the lysosome has been implicated in several human diseases, including neurodegenerative diseases (Shen and Mizushima 2014; Settembre 2015).
In order to understand which step of autophagy is dysregulated in Synj1-deficient cells, we evaluated the lysosomal clearance of autophagosomes in Synj1i and Ctli cells. To this aim, we used an imaging and a biochemcal approach, both based on the expression of proteins that are prone to form aggregates usually degraded by lysosomes.
In particular, in the imaging approach Ctli and Synj1 HeLa cells were transiently transfected with an expressing vector encoding the first exon of human Huntingtin (Htt) containing a polyglutamine repeat of 103Q fused to GFP (GFP-HttQ103). We measured the percentage of GFP-positive cells containing Htt aggregates (Figure 4.9A). No such inclusions were observed in cells expressing GFP alone. As shown in Figure 4.9A, a remarkable accumulation of GFP-Htt aggregates was observed in Synj1 silenced cells: about 50-55% of the Synj1i cells resulted GFP-positive compared to about 20-25% of the control cells.
In the biochemical approach, we monitored the lysosomal clearance of the mutant form of α-synuclein (A53T), which

has the tendency to form aggregates. After transient transfection of the cDNA encoding GFP-synuclein in Ctli and Synj1i HeLa cells, the levels of GFP-synuclein were analyzed

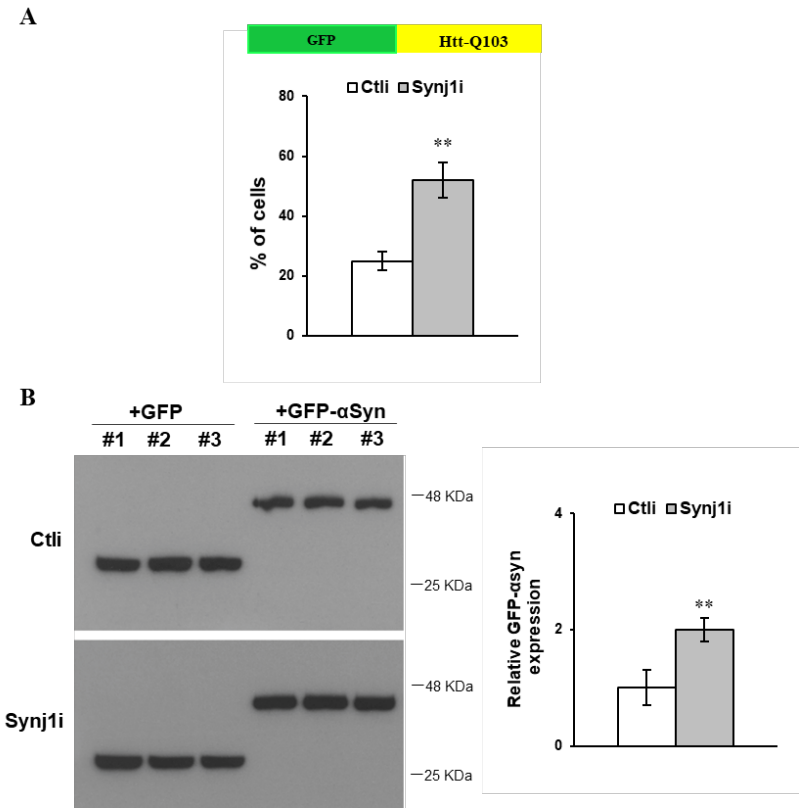

Figure 4.9. The clearance of autophagy substrates is reduced in Synj1 depleted cells. (A) Ctli and Synj1i HeLa cells were transiently transfected with cDNA encoding polyQ expanded form of Huntingtin (Htt-Q103) and fixed after 48h. The percentage of cells containing fluorescent dots is shown in the graph. N>30. Error bars, means ±SD; n ≥ 50 cells. **(B)** Ctli and Synj1i HeLa cells were transiently transfected with cDNA encoding GFP fused to the α-Synuclein (α-Syn) A53T mutant or only GFP as control, lysed 48h after transfection. Samples were revealed by immunoblotting using a specific GFP-antibody. Error bars, means ±SD.
** $p<0.01$, Student's t-test.

by western blotting using a specific anti-GFP antibody (Figure 4.9B); as control for transfection efficiency, cells were transfected with a cDNA encoding only GFP. In agreement with imaging assay, Synj1i cells showed higher levels of GFP-synuclein with respect to control cells (Figure 4.9B), indicating that upon Synj1 loss GFP-synuclein is less degraded and accumulates within cells.

Overall, these findings indicate that the clearance of autophagic substrates is reduced in the Synj1 silenced cells.

Because α-synuclein is a typical intrinsically disordered protein with natural tendency to aggregate and lysosomal degradation is crucial to prevent the accumulation of these aggregates over time (Uversky 2002; Webb 2003; Rodriguez 2018) we decided to analyze the aggregation state of the endogenous α-synuclein. To this aim, protein extracts of Ctli and Synj1i HeLa cells were separated by gradient SDS polyacrylamide gel electrophoresis (6-15% acrylamide) for better resoloution of a broader range of protein size. As shown in Figure 4.10A, α-synuclein is mostly in its monomeric form (14 kDa) and in less amount in its oligomeric isoforms. In particular, dimeric and trimeric forms were observed both in control and in Synj1 silenced cells with variable amount, whereas the tetramer is present almost exclusively in Synj1i cells. It is plausible that this isoform is part of a big aggregate that is partially degraded after boiling. It will be interesting validate this hypothesis by using native gels. Interestingly, by densitometric analysis of all isoforms the α-synuclein levels resulted statistically significant increased in Synj1 silenced cells with respect to control cells (Figure 4.10A), indicating accumulation of the protein upon Synj1 loss. In agreement with these data, by immunofluorescence assays we found that a portion of the α-synuclein appears as large and brighter fluorescent dots in silenced cells for Synj1 (Figure 4.10B), and the percentage of cells containing these structures was drastically higher in Synj1i than in Ctl1i cells (~60 vs 10%;

Figure 4.10B), further corroborating α-synuclein accumulation.

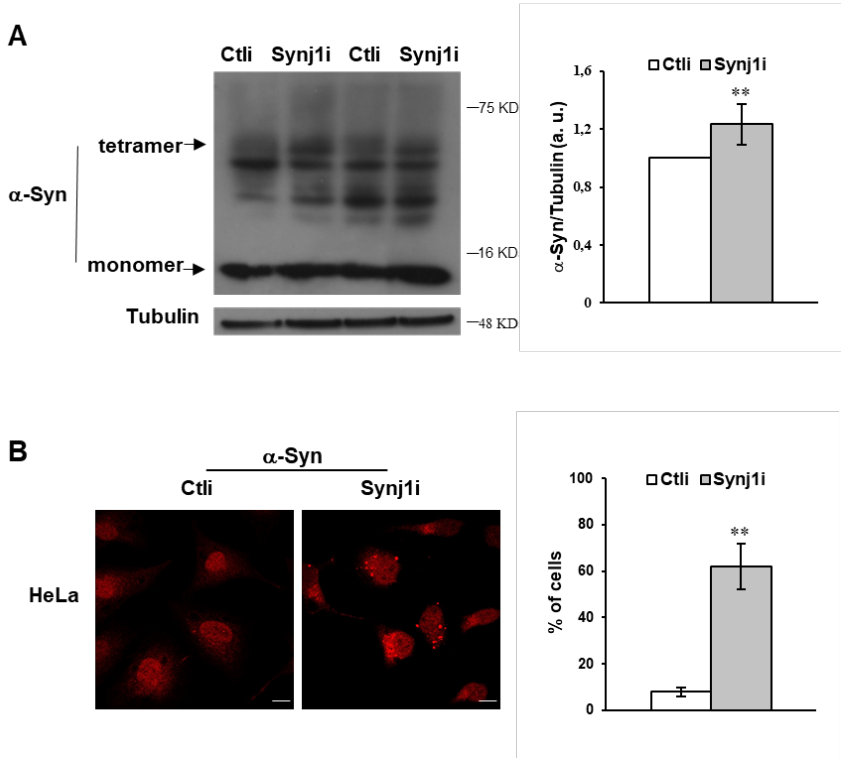

Figure 4.10. Synj1 knockdown cells display α-Synuclein aggregates. **(A)** Representative immunoblotting of α-synuclein in Ctli and Synj1i HeLa cells. After lysis in JS buffer, samples were were separated by gradient SDS-polyacrylamide gel electrophoresis (6-15% acrylamide). Tubulin was used as a loading control. Densitometric analysis of 3 different experiments is shown. Error bars, means ±SD.
(B) Representative confocal images of immunofluorescence assay using anti-α-Synuclein antibody carried out in Ctli and Synj1i HeLa cells. Scale bars, 10 μm. The percentage of cells containing fluorescent dots is shown in the graph. N>30. Error bars, means ±SD; n ≥ 30 cells.
** $p<0.01$, Student's t-test

Interestingly, Akt resulted hyperphosphorylated at Ser473 in Synj1 silenced cells with respect to control cells, while the total levels are comparable (Figure 4.11), thus indicating the activation of the survival pathway in knockdown cells (Brunet 1999; Vadlakonda 2013). This pathway might be induced as response to the lysosomal stress caused by protein accumulation within this organelle. Further studies will be necessary to validate this hypothesis. It will be interesting to understand if the loss of Synj1 may lead to the activation of the specific transcriptional program regulating lysosomal biogenesis and function, so called CLEAR (Sardiello 2009), for instance the master regulator TFEB.

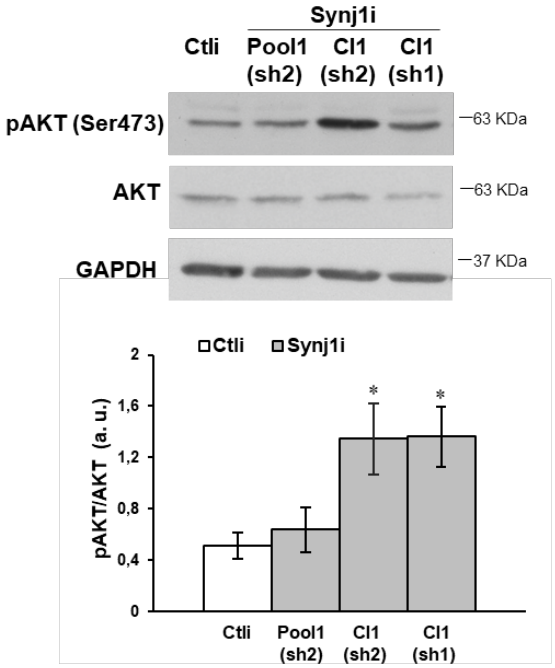

Figure 4.11. AKT is hyperphospshorylated in Synj1 knockdown cells. Representative immunoblotting of AKT and pAKT in Ctli and Synj1i HeLa cells. GAPDH was used as loading control. Densitometric

analysis of 4 different experiments is shown. Error bars, means ±SD. *p <0.05; Student's t-test.

Levels of autophagic markers and the number of autophagosomes are increased in PARK20 fibroblasts

The data obtained in HeLa cells clearly indicate that the Synj1 loss leads to a dysregulation of autophagic pathway. To understand the role of autophagy in PARK20 pathogenesis, we analyzed the abundance of LC3 and p62/SQSTM1 on extracts of healthy fibroblasts (wt/wt) and PARK20 fibroblasts (R258Q/R258Q) derived from skin biopsies (Figure 4.12A). Similarly to the data obtained in HeLa cells, we observed higher levels of the lipidated form of LC3 and p62/SQSTM1 in PARK20 fibroblasts with respect to healthy fibroblasts (Figure 4.12A).

Furthermore, immunofluorescence analysis also revealed that the number of p62/SQSTM-postive dots is drastically increased in PARK20 fibroblasts (Figure 4.10B). The phenotype of healthy heterozygous p.R258Q carriers is comparable to healthy individuals (Figure 4.12B). In agreement with the well-established role of autophagy in Parkinson's disease (Tooze and Schiavo 2008; Karabiyik 2017), we also observed a statistically significant increase of p62/SQSTM1 positive dots in idiopathic fibroblasts (PD) compared to both control and heterozygous fibroblasts, albeit lower than of PARK20 cells (Figure 4.12B).

All these findings indicate that the autophagic pathway could contribute to the PARK20 pathogenesis. The difference observed between idiopathic and PARK20 fibroblasts might imply a different involvement of autophagy in the pathogenesis of these diseases. On the basis of data obtained in knockdown HeLa cells, it is likely that the dysregulation of autophagy pathway has a potential role in PARK20 pathogenesis.

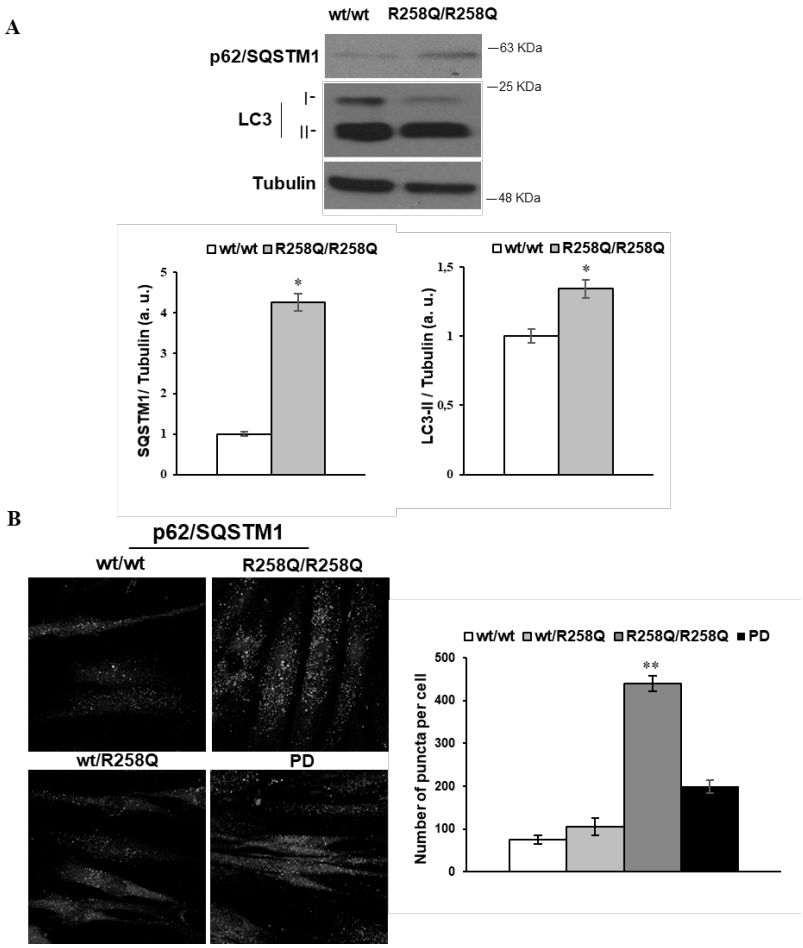

Figure 4.12. PARK20 fibroblasts display high levels of autophagic markers and increased number of autophagosomes. (A) Representative immunoblotting of LC3 and p62/SQSTM1 from healthy control (wt/wt) and homozygous PARK20 (R258Q/R258Q) patients. Tubulin was used as a loading control. Densitometric analyses of 2 different experiments is shown. Error bars, means ±SD. **(B)** Immunolabelling of p62/SQSTM1 in healthy control, homozygous PARK20, heterozygous carrier (wt/R258Q) and idiopathic PD (PD) fibroblasts. Representative 3D reconstructions are shown. The number of puncta per cell is shown in the graph. Error bars, means ±SD; n ≥ 30 cells.
* p <0.05; ** p <0.01 T-student test.

5. DISCUSSION

Defects of Synj1 loss: role in the autophagy

Previous studies in our laboratory have shown that Synj1 plays a crucial role in the homeostasis and functions of early endosomes in different cell types, including neuronal cells (Fasano 2018). Indeed, the Synj1 knockdown causes the enlargement of the early endosomal compartment, alters the dynamic of endosomal proteins, such as EEA1 and Rab5, and impairs the recycling trafficking towards the plasma membrane (Fasano 2018).

Beyond a dysfunction of early endosomes we have also observed the alteration of lysosome structure (Fasano 2018). As we confirmed here (Figure 4.2), lysosomes result enlarged, despite any substantial difference in the level of two lysosomal markers, LAMP-1 and Cathepsin-D. Because trafficking toward lysosomes is unaffected upon Synj1 loss (Fasano 2018), we hypothesized that the alteration of lysosomes could be due to changes in the autophagic pathway, whose activity is critical in many neurodegenerative diseases.

Through biochemical and imaging approaches, we found an increase in the levels of two main autophagic markers, LC3-II and p62/SQSTM1, and in the number of autophagosomes in Synj1 knockdown cells (Figure 4.4), indicating an upregulation of the autophagic pathway in Synj1 knockdown cells. Similarly, an enlargement of the lysosomes and an increase of the autophagosomes have been observed in the photoreceptor cones of zebrafish lacking Synj1 (George 2016), supporting a role of Synj1 in the autophagy in different species. In addition, a role of Synj1 in regulating autophagy has been proposed in Drosophila (Vanhauwaert 2017).

Interestingly, microscopy quantitative analysis of LC3 and p62/SQSTM1 revealed that the autophagosomes appear as fluorescent dots very bright, large and accumulated in the perinuclear region, suggesting dysfunction of the autophagic pathway (Figure 4.5). This is supported by the observation that the ratio between the amount of LC3 and p62/SQSTM1 in

basal conditions and upon bafilomycin treatment is strongly reduced in Synj1 knockdown cells (Figure 4.7). Consistently, the increase of autophagosomes with respect to autolysosomes, as shown by using GFP-mRFP-LC3 reporter, indicates that autophagic flux is delayed. Thus, it is likely that the maturation of autophagosomes is altered in Synj1 knockdown cells. From one side, PI3P levels could be critical for autophagosome maturation and fusion with lysosomes properly recruiting the molecular machinery involved in this step (Bento 2016). From the other side, the Synj1 activity might be critical providing early endosomal membranes for autophagy pathway. Several studies have shown that the sequential, stepwise maturation of autophagosomes through the fusion with the early endosomes, late endosomes and lysosomal compartments is fundamental for autophagy pathway (Razi 2009). Further studies will be necessary to distinguish between these two hypotheses.

Of note, the clearance of various autophagic substrates is significantly reduced in Synj1 knockdown cells (Figure 4.9 and 4.10), suggesting an even more complex scenario. Different steps of autophagy pathway could be compromised upon Synj1 loss. The engulfment of lysosomes with undegraded substrates may cause the alteration of their structure. The observation of AKT hyperphosphorylation supports the activation of autophagy stress. It will be interesting to evaluate whether the loss of Synj1 may lead to the activation of the specific transcriptional program regulating lysosomal biogenesis and function, so called CLEAR, for instance the master regulator TFEB (Sardiello 2009). In addition, it will be important to understand the molecular mechanisms underlying the reduced degradative capacity of lysosomes.

It has been reported that Synj1 reduces the extracellular and intracellular levels of amyloid peptide in mouse neuroblastoma cells N2a stably expressing the APP "Swedish" mutant (causative of familial form of Alzheimer's disease) and in a mouse model of Alzheimer (Zhu 2013; McIntire 2012).

Although the mechanism is unclear, Zhu and colleagues proposed that the reduction of Synj1 induces the amyloid peptide clearance. The discrepancy between these and our data could be due to the different physiopathological context. It is plausible that in the Alzheimer's pathological context, where different cell pathways could be compromised, the reduction of Synj1 levels may play a protective role. It would be interesting to understand if the amyloid peptide can affect endolysosomal compartments and/or endosomal traffic.

Overall, our data indicate that the Synj1 knockdown leads to a dysfunction of autophagy pathway. However, they have also highlighted a more complex scenario, which will need further investigations. We are currently analyzing the levels and phosphorylation status of ULK1 and ATG13, other key proteins in the regulation of autophagic flux.

How can Synj1 loss lead to the autophagy dysfunction? It is well demonstrated that phosphoinositides are essential components of cell membranes and important regulators of membrane trafficking thanks to their selective localization and the consequent ability to mediate the recruitment of effector proteins at specific cellular compartments (Di Palo and De Camilli 2006). As previously described, the phosphoinositide levels must be finely regulated and this occurs through the spatiotemporal control of the kinases and phosphatases that generate them (Di Palo and De Camilli 2006). In particular, PI(3)P, one of the Synj1 substrates, has a crucial role in the autophagic process (Dall'Armi 2013). The production of PI3P mediated by phosphatidylinositol III kinase (also known as VPS34) is fundamental for the phagophore expansion. Furthermore, recent studies have highlighted that regions of endosomal membranes PI(3)P-enriched form "contact sites" with the ER membranes promoting the formation of the autophagosome (Nascimbeni 2017). A model has been proposed in which the endoplasmic reticulum areas in contact with the VPS34-positive endosomes are enriched with PI(3)P assuming a particular Ω-shaped structure (called homegasome) and these domains would have an important role in the

autophagosome formation (Nascimbeni 2017). It is plausible that the PI(3)P imbalance, as a consequence of the loss of Synj1 activity might induce the autophagic pathway alteration.

Autophagy and PARK20

Our biochemical and immunofluorescence studies revealed an increase in LC3 and p62/SQSTM1 and autophagosome levels in PARK20 fibroblasts compared to healthy fibroblasts (Figure 4.12), indicating activation of the autophagic pathway in PARK20. Fibroblasts of healthy heterozygous individuals have negligible defects and are comparable to control fibroblasts, suggesting a potential role of autophagy in the pathogenesis of PARK20.

Based on the data obtained in HeLa cells, it is likely to hypothesize that the dysfunction of the autophagic process could contribute to the onset of the disease.

In the last years, many studies highlighted a critical role of autophagy pathway in the pathogenesis of various neurodegenerative disorders (Tooze 2008; Karabiyik 2017; Fleming 2017). However, how perturbation of autophagic processes contributes to these diseases is still elusive. If the dysfunction autophagic pathway is a consequence or cause of the accumulation of toxic protein aggregates is not completely clear. In addition, the specific pathological context seems to be critical. In most neurodegenerative diseases, the formation of the autophagosomes appears unaffected, rather hyperactivated. Our data support the hypothesis that the dysregulation of autophagic pathway might contribute to PARK20 pathogenesis. Moreover, our findings indicate that Sac1 domain is necessary for the regulation of the autophagic pathway and its activity should be at least 50% to ensure correct functionality.

Recently, the membrane trafficking has emerged to have a crucial role in the pathogenesis of Parkinson's disease. In particular, both endosomal trafficking and lysosomal-degradative pathway seem playing a critical role (Perret 2015;

Schreij 2016). The fine balance between de novo synthesis, recycling and degradation (e.g. synaptic proteins, neurotransmitters or receptors for growth factors) is essential for maintaining the neuronal plasticity and functionality. Thus, together with the alterations of homeostasis and the functions of early endosomes (Fasano 2018), the dysfunction of autophagy could contribute to the onset of the disease.

Role of Synj1 in membrane trafficking

With respect to other inositol phosphatases, Synj1 has two consecutive phosphatase domains, Sac1 and 5'-phosphatase, through which dephosphorylates various substrates and for this peculiarity it might play multiple roles.

While the role of Synj1 in synaptic vesicle endocytosis and recycling is well established long time ago (McPherson 1996, Cremona 1999, Mani 2007, Haffner 1997, Harris 2000), only recent studies have highlighted an involvement of Synj1 in membrane trafficking. Loss of Synj1 has been shown to lead to defects in the endolysosomal pathway in photoreceptor neuron of zebrafish (George 2014, George 2016). In humans, our recent findings demonstrated that Synj1 plays a key role in the homeostasis and functions of early endosomes in different cell types, including neuronal cells (Fasano 2018). Moreover, our results reveal a novel role for Synj1 in the regulation of membrane trafficking at the ER-Golgi boundaries (Amodio 2019). It is likely that these regulatory functions might be associated to its diverse phosphatase activities. By modulating the levels of PI(3)P and PI(4)P Synj1 could control, respectively, the properties and functions of early endosomes and ER and Golgi where these PIs are enriched (Di Palo and De Camilli 2006).

A variety of endosomal proteins contain PI(3)P-binding modules, such as EEA1 and Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), and therefore the proper recruitment, both in spatial and temporal terms, to these compartments through PI binding could be crucial to exert their functions. Thus, it is likely that the imbalance of PI(3)P

levels might lead to the alteration of EEs. It is conceivable that the dysfunction of EEA1, which is a Rab5 effector and is required for endosomal tethering, could be responsible for the observed endosomal defects. Moreover, PI(3)P is also crucial for the biogenesis of autophagosomes (Dall'Armi 2013, Bento 2016).

The unbalance of the PtdIns4P content at ER membranes can lead to the impairment of carrier vesicles formation. Indeed, the dynamic control of PtdIns4P level is necessary to coordinate the progression of both ERES formation and COPII assembly (Nagaya 2002; Pathre 2003; Blumental-Perry 2006; Farhan 2008). In these events, a specific role is played by p125A that, upon PtdIns4P recognition, promotes the recruitment of Sec16 at the ERESs, which in turn favors COPII assembly and cargo export from the ER (Shimoi 2005; Iinuma 2007; Ong 2010).

All these findings pointed out multiple roles of Synj1 in different cellular pathways. This implies that defects of Synj1 activity might compromise different intracellular pathways. Consistently, defects of different trafficking pathways (for instance endosomal, early secretory and autophagy pathways) were observed in PARK20 fibroblasts.

Are these defects related to each other? Compelling evidence indicate that ER plays a critical role in the autophagy providing both the membranes for phagophore formation and the molecular machinery involved in the autophagy (Bento 2016). For instance Beclin-1, which is essential for autophagy initiation, is regulated at the ER membranes by members of the BCL-2 protein family, indicating that signalling events originating from the ER are essential for autophagy. Interestingly, some studies indicate that ER stress may induce the autophagy through the activation of UPR stress sensors such as IRE1α and PERK (Matus 2008, Kouroku 2007, Høyer-Hansen 2007).

One can imagine that the persistent activation of the PERK/eIF2a/ATF4 pathway of the UPR might induce an upregulation of autophagy. On the other hands, the persistent

ER stress by the overload of cargo protein within the ER could prevent the proper autophagy induction. Hence ER stress might concur to the observed dysregulation of autophagy. Further studies will be needed to elucidate these aspects.

As aforementioned, a role of early endosomes in autophagy has emerged in the last years. During maturation step, autophagosomes form hybrid vesicles (amphisomes) through fusion with different types of endocytic membranes, including early endosomal membranes (Berg 1998; Liou 1997). In addition, inhibition of early endosomes functions gives rise to the accumulation of autophagosomes (Razi 2009; Rusten 2009). Based on these findings, we can hypothesize that the impairment of endosomal trafficking caused by the loss of Synj1 activity might perturb the autophagy pathway.

The recent evidence that ER can establish membrane contacts with endosomal membranes (Eden 2016; Henne 2017) points out a more complex scenario. The defects of the three pathways observed in Synj1 knockdown cells and in PARK20 fibroblasts might be strictly interconnected. The proposed role of these ER-endosome contact sites in the autophagosome formation (Nascimbeni 2017) further supports this hypothesis.

Further studies will be important to better understand how these pathways are related to each other and how Synj1 could act as “glue” between them. There remain some interesting open questions: Synj1 resides at ER-EEs contact sites? How do these contacts help Synj1 activity to maintain PI(3)P and PI(4)P homeostasis? This will allow getting new insights in the molecular mechanisms of PARK20 pathogenesis and in unraveling new key pathways in Parkinson’s disease.

6. CONCLUSIONS

Two main aims of my PhD project were to study the role of Synj1 i) in the secretory pathway and ii) in the autophagy pathway.

First, our data indicate that Synj1 is involved in the regulation of the early secretory pathway, presumably by regulating the levels of PtdIns4P levels. Interestingly, the loss of Synj1 activity induces the persistent activation of the PERK/eIF2a/ATF4 branch of the unfolded protein response with consequent induction of oxidative stress and mitochondrial dysfunction.

Second, our study highlighted a role of Synj1 in the autophagy pathway likely by controlling the PI3P levels, which can be directly or indirectly critical for autophagosomes formation and maturation, respectively.

Overall, our findings pointed out multiple roles of Synj1 in different cellular pathways. They also corroborate its role as key regulator of membrane trafficking.

On the side of pathogenetic mechanism, our data highlight that defects of endosomal system, early secretory pathway and a dysregulation of autophagy could concur to PARK20 pathogenesis.

All together our data emphasize the link between membrane trafficking and Parkinson Disease.

7. LIST OF PUBLICATIONS

The data regarding the first aim of my project have been published on the journal Frontiers in Neuroscience:

Amodio G, Moltedo O, Fasano D, **Zerillo L**, Oliveti M, Di Pietro P, Faraonio R, Barone P, Pellecchia MT, De Rosa A, De Michele G, Polishchuk E, Polishchuk R, Bonifati V, Nitsch L, Pierantoni GM, Renna M, Criscuolo C, Paladino S*, Remondelli P*. *co-corresponding authors.

PERK-Mediated Unfolded Protein Response Activation and Oxidative Stress in PARK20 Fibroblasts. Front Neurosci 2019. 13:673.

Thanks to the strict collaboration between the research groups of Simona Paladino and Lucio Nitsch, during my PhD program I had the opportunity to participate to a project focused to study the molecular defects underlying down syndrome in order to unravel novel therapeutic approaches for attenuating the cognitive disability.

In particular, in this study we have shown that the treatment with pioglitazone restores the mitochondrial dysfunction of fetal trisomic-derived fibroblasts. Specifically, proper mitochondrial network and ATP production were re-established upon drug incubation.

These results have been published on the journal Frontiers in Genetics:

Mollo N, Nitti M, **Zerillo L**, Faicchia D, Micillo T, Accarino R, Secondo A, Petrozziello T, Calì G, Cicatiello R, Bonfiglio F, Sarnataro V, Genesio R, Izzo A, Pinton P, Matarese G, Paladino S, Conti A, Nitsch L.

Pioglitazone Improves Mitochondrial Organization and Bioenergetics in Down Syndrome Cells. Front Genet 2019. 10:606

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