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Investigating the involvement and the regulation of autophagy in Parkinson's disease

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Abbreviations

α-syn α-synuclein

4E-BP1 4E-binding protein 1

6-OHDA 6-hydroxydopamine

Aβ Amyloid beta

AD Alzheimer's Disease

AKT Protein kinase B

ALP Autophagy Lysosomal Pathway

AMPK AMP-activated kinase

AR Androgen receptors

ATG Autophagy-related genes

CAMKKβ Calcium/calmodulin-dependent protein kinase kinase

CAT Catalase

Cdc42 Cell division control 42

CLEAR Coordinated lysosomal expression and regulation

CMA Chaperone-mediated autophagy

CQ Chloroquine

CREB cAMP response element-binding protein

DA Dopamine

DEPTOR DEP domain containing mTOR-interacting protein

DHE Dyhydroethydium

DKO Double Knockout

ER Endoplasmic reticulum

ERK Extracellular regulated kinases

FIP200 Family interacting protein of 200 kDa)

GCase Glucocerebrosidase

GPX Glutathione peroxidase

GR Glutathione reductase

GSK3β Glycogen synthase kinase-3 β

HD Huntington's Disease

HEK Human embrionic kidney

HLH Helix-loop-helix

HO-1 oxygenase-1

HSP70 Heat shock cognate 70 kDa protein

HTT Huntingtin

iPSC Induced pluripotent stem cells

KLF2 Krüppel-like factor 2

LAMP1/2 Lysosomal-associated membrane protein 1/2

LB Lewy bodies

LC3 Light chain 3B

LKB1 Liver kinase B1

LRRK2 Leucine reach repeat kinase 2

LSD Lysosomal Storage Disorders

MAPK Mitogen-activated protein kinases

MCLN1 Mucolipin1

MEF Mouse embryonic fibroblasts

mIST8 Mammalian lethal with Sec13 protein 8

MITF Microphthalmia-associated transcription factor

MPTP 1-methyl-4-phenyl-1, 2, 3, 6- tetrahydrodropyridine

mTORC1 Mechanistic target of rapamycin complex 1

NAC N-Acetyl-L-Cysteine

ND1 NADH dehydrogenase 1

NDUFA4 NADH-ubiquinone oxidoreductase MLRQ subunit

NRF2 Nuclear factor erythroid-2-related factor 2

PAK p21-activated kinase

PARAPLAY Poly-ADP-ribose assisted protein localization assay

PD Parkinson's Disease

PINK1 PTEN-induced kinase 1

PKCβ Protein kinase C beta

PP2A Protein phosphatase 2A

PPARα Peroxisome-proliferator-activated receptors-α

PRAS40 Proline-rich Akt substrate of 40 kDa

Rac1 Ras-related C3 botulinum toxin substrate 1

Rag Ras-related GTP-binding

REDD1 Regulated in development and DNA damage responses 1

Rheb Ras-homologue enriched in the brain

ROS Reactive oxygen species

S6K Ribosomal protein S6 kinase beta-1

SBMA Spinal-bulbar muscolar atrophy

SOD1 Superoxide Dismutase 1

TDP43 TAR DNA-binding protein 43

TFE3 Transcription factor E3

TFEB Transcription factor EB

TFEC Transcription factor EC

TH Tyrosine hydroxylase

TSC Tuberous sclerosis complex

ULK1 Unc-51-like autophagy activating kinase

VPS35 Vacuolar protein sorting ortholog 35

Abstract

Autophagy is a conserved process that allows the degradation of intracellular debris within specific organelles, called lysosomes. The correct function of this cellular mechanism is crucial for the survival of neurons. Accordingly, the detrimental contribution of autophagic defects in the onset and progression of neurodegenerative diseases is widely accepted. For these reasons, the study of the molecular mechanisms of autophagy that are involved in neuronal damage and the characterization of the regulatory pathways that affect this process in neurodegeneration represent an important field in the research.

In the last few years, it has emerged that autophagy and lysosomal functions are able to sense the stimuli that originate from other organelles and modulate their activity accordingly. This notion may be of great relevance in the context of neurodegenerative diseases, which are characterized by impairment at different cellular levels. In this scenario, the investigation of the crosstalk between organelles may offer new insights for a better understanding of these pathologies and eventually lead to novel therapeutic approaches.

The communication between mitochondria and lysosomes has been gaining increasing attention, due to the crucial role of these organelles in neurodegeneration, and in particular, in Parkinson's disease (PD). In this framework, it has been shown that in the familial forms of PD, mutations of lysosomal proteins may cause secondary perturbations in mitochondrial homeostasis. Conversely, PD-associated proteins involved in mitochondrial dynamics may participate in the regulation of the autophagic machinery. Among them, DJ-1 is a protein linked to PD, whose activity promotes in the maintenance of mitochondrial quality control and in the protection against oxidative stress. In addition, DJ-1 has also been associated with autophagic alterations, although its precise role is still elusive.

Therefore, we evaluated how DJ-1 affects the autophagy-lysosomal pathway exploiting *Drosophila melanogaster* as an *in vivo* system, and human cell models for a deeper molecular characterization. Then, we investigated one of the possible signaling pathways that mediate the crosstalk between mitochondria and lysosomes in DJ-1 loss of function models.

Overall, our data demonstrate that DJ-1 influences the autophagic process at different levels, through a signaling cascade that involves the modulation of the AMPK-mTORC1 pathway, which responds to the DJ-1-mediated increase of reactive oxygen species.

Since autophagy activation is considered a good therapeutic strategy to counteract neurodegeneration, in parallel with the study on DJ-1, we also investigated a novel neuronal-specific pathway possibly involved in the regulation of autophagy.

More specifically, our lab recently demonstrated the capability of the neuronal-enriched kinase PAK6 to phosphorylate the family of chaperone proteins 14-3-3s, affecting their interactome. Therefore, we considered the hypothesis that PAK6 may represent a modulator of TFEB, the major transcriptional activator of autophagy, whose function is highly dependent on the binding with 14-3-3s.

In accordance with this hypothesis, the results we obtained demonstrated that PAK6 activity promotes the nuclear translocation of TFEB and the induction of autophagy in different *in vivo* and *in vitro* models. Importantly, our data suggest that PAK6 may participate to the regulation of TFEB through different mechanisms, by phosphorylating 14-3-3s and preventing their binding with the transcription factor and by directly interacting with TFEB.

In conclusion, with these projects we investigated autophagy in the context of neurodegeneration, addressing this topic from different perspectives and exploiting multiple models.

We characterized the participation of DJ-1 in the autophagic pathway, defining a potential mechanism of communication between mitochondria and lysosomes, and, by analyzing the activity of PAK6, we contributed to provide novel insights on the mechanisms of TFEB and autophagy regulation in neuronal cells.

Chapter 1

Introduction

Autophagy

1 Autophagy

The term autophagy was used for the first time in the field of cell biology in 1963 by the Belgian biochemist Christian de Duve, who described the molecular mechanism by which cells degrade cytoplasmic material within lysosomes (1). More recently, in the early nineties, the discovery of autophagy in yeast and the isolation of several autophagy-defective yeast mutants led to a huge increase in the understanding of this important cellular process (1,2). Nowadays, an extensive knowledge of the autophagic process has been reached and the crucial role of autophagy in cell physiology is widely accepted. However, many questions remain unanswered, and the research in the field is still increasingly expanding. For example, the precise mechanisms of autophagy regulation in different cells and tissues are still under investigation, as well as the study of the role of autophagy in several human diseases (3).
The autophagic process is ubiquitously performed in every cell and is essential to promote the clearance of intracellular debris, such as defective or unfunctional organelles and misfolded or aggregated proteins (4). Three different forms of autophagy are described: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Together, the modulation of these three distinct pathways allows the catabolism of different substrates and the proper intracellular clearance (5). Even though these processes are independent of each other, they have all in common the final step, which culminates within the lysosomes, where the autophagic cargoes are delivered to be degraded. The products of this degradation are then recycled to sustain metabolic functions and maintain the intracellular energy balance (5,6).

Considering the mechanisms of cargo recognition and delivery to the lysosomes, the most peculiar type of autophagy is CMA (7). The study of this process led for the first time to the understanding that autophagic clearance can be performed with high specificity toward the substrate (Fig 1). Indeed, the only type of cargo degraded via CMA is represented by proteins that contain the KERFQ pentapeptide motif in their sequence (7). The KERFQ sequence is recognized by the heat shock cognate 70 kDa protein (HSP70) also known as HSPA8, a chaperone protein that binds the cargoes targeting them directly to the lysosomes (7). The translocation of the CMA cargo proteins to the lysosomal lumen is allowed by the lysosomal-associated membrane protein 2 (LAMP2-A), localized on the lysosomal surface. This protein performs an essential activity for CMA to occur (7), and its presence within the

lysosomal membrane represents a distinctive trait of the CMA degradation pathway. In fact, since LAMP2 is specifically recognized by HSP70, only the pool of lysosomal vesicles that contain this protein is involved in the degradation through the CMA. Another peculiar feature of CMA is its low level of conservation throughout evolution. In contrast to microautophagy and macroautophagy, which are performed in every eukaryotic organism, CMA has been observed only in mammals and birds (7). This aspect may suggest that this process has evolved in complex organisms to promote a more precise regulation of clearance functions to sustain higher metabolic demands.

The less investigated type of autophagy is microautophagy. This process is characterized by the invagination of the lysosomal membrane around the cytoplasmic materials to be degraded (Fig 1). The lysosomal-derived vesicle formed by the membrane protrusion is then released directly into the lysosomal lumen, where it is degraded together with the cargo (6,8). The target of this degradative process is mainly represented by proteins. However, in yeast, microautophagy involving the elimination of mitochondria, portions of the endoplasmic reticulum (ER), peroxisome, and nuclear fragments has also been reported. In mammals, only microautophagy of the ER has been observed so far (6). However, considering the high degree of conservation of the microautophagic machinery, a deeper analysis of this mechanism could lead to the discovery that also in mammals different targets are degraded through this process.

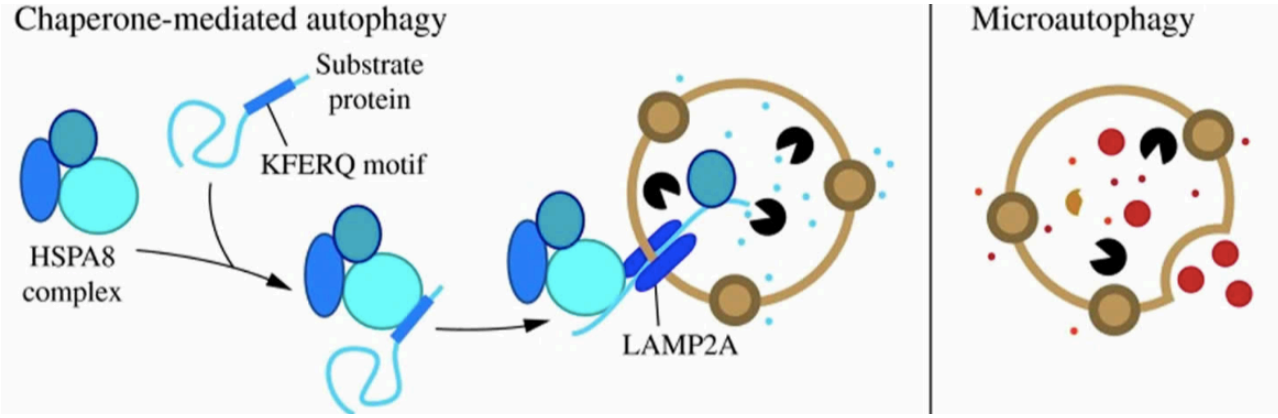


Fig 1: Schematic representation of the mechanisms of chaperone-mediated autophagy and microautophagy (5).

1.1 Macroautophagy

Macroautophagy (hereafter referred to as autophagy) is the most investigated degradation mechanism. This process is considered the main intracellular clearance pathway which allows either the bulk breakdown of non-selective material or the clearance of specific cargoes, like in the case of the degradation of organelles, such as mitochondria or ER, which is highly regulated and requires specific signaling pathways (1,5). The autophagy-Lysosomal pathway (ALP) consists of consecutive steps, performed by different proteins that cooperate all together. Most of these proteins are the transcriptional product of the so-called autophagy-related genes (ATG). To date, more than 40 ATG have been discovered and their role in the autophagic pathway is still under deep investigation (1).

The first stage of the autophagic process is the formation of the autophagosomes, double-membrane vesicles where the cargo is recruited to be delivered to the lysosomes (4,9,10). Autophagosome formation is one of the most crucial steps of the autophagic pathway. Therefore, the rate of autophagosome generation, as well as the number and size of autophagosomes are among the most important factors that determine the speed and the level of degradation. For this reason, it is not surprising that this first step of the process is highly and finely regulated. Autophagosomes are formed through the expansion of the phagophore, a bilayer membrane structure called omegasome that originates at the level of ER-mitochondria contact sites (9,11,12). Since the site of omegasomes gemmation represents an intracellular spot of intense metabolic activity, we can speculate that this localization may allow the rapid recruitment of metabolites and the formation of autophagosomes to maintain the correct homeostasis in these subcellular environments. The recruitment of a range of proteins in these sites is essential for the gemmation of the phagophore from the ER. Among them, the Unc-51-like autophagy activating kinase (ULK1), plays a crucial role in this process, and it is considered one of the major regulators of autophagy initiation (9). The elongation of the membrane of the phagophore and its closure leads to the formation of the autophagosomal vesicle, which allows the incorporation of the cargo. These steps are controlled by the activity of several ATG proteins, and, among them, ATG8 protein family is one of the most studied and characterized. ATG8 (also called Microtubule-associated proteins 1A/1B light chain 3B (LC3)) proteins are ubiquitously expressed, and they are mainly localized in the cytoplasm in a unlipidated form, usually

referred to as LC3-I. Upon autophagy induction, LC3-I is recruited at the membrane of the phagophore, where it gets lipidated by binding the amine headgroup of phosphatidylethanolamine (PE) present in the membrane. The lipidation of LC3 requires the coordinated function of several ATG proteins in a complex called the ATG8-conjugation system (13). The lipidated form of LC3 is indicated as LC3-II (10,13). LC3-II is involved in different important functions during the first stages of autophagy, including autophagosome maturation and cargo recognition. Indeed, LC3 activity promotes the remodeling of the phagophore, the regulation of its expansion, and the final closure of the phagophore membrane to form the autophagosome. LC3 proteins also serve as binding platforms for the recruitment of autophagic cargoes, by interacting with cargo receptors. These proteins are responsible for the delivery of the autophagic substrates to the autophagosome (10). The interaction between the cargo receptor proteins and LC3 is established through a specific motif, the so-called LC3-interacting regions (LIR), which is present in one or more copies in the sequence of cargo receptors (10). In addition to these functions, when autophagosome formation is completed, LC3 facilitates the fusion between autophagosomes and lysosomes (10), which represents the latest step of the autophagosomal cycle.

Autophagosome-lysosome fusion is another fundamental step of the autophagic machinery. The fusion pace is the principal parameter to define the autophagic flux, which represents a measure of the rate of autophagic degradation (3). Lysosomes are membrane-bound organelles and represent the central hub where autophagic cargoes are degraded. One of the most important lysosomal features is the acidic pH of its lumen (pH 4.5-5.5), which allows the well-functioning of more than 50 hydrolytic enzymes. Lysosomal enzymes have the role of catabolizing macromolecules, such as proteins, nucleic acids, lipids, and carbohydrates. (14). When the autophagosomes come in contact with the lysosomes, the outer autophagosomal membrane fuses with the lysosomal membrane, leading to the formation of the so-called autolysosomes, and allowing the incorporation of the autophagosomal cargo within the lysosomal lumen (15,16).

Importantly, at the end of the process, the products of degradation are exported back to the cytoplasm where they can serve as building blocks for the formation of new macromolecules, sustain metabolic activities, and maintain cell homeostasis (15,16). The overall autophagic pathway is schematized in Fig 2.

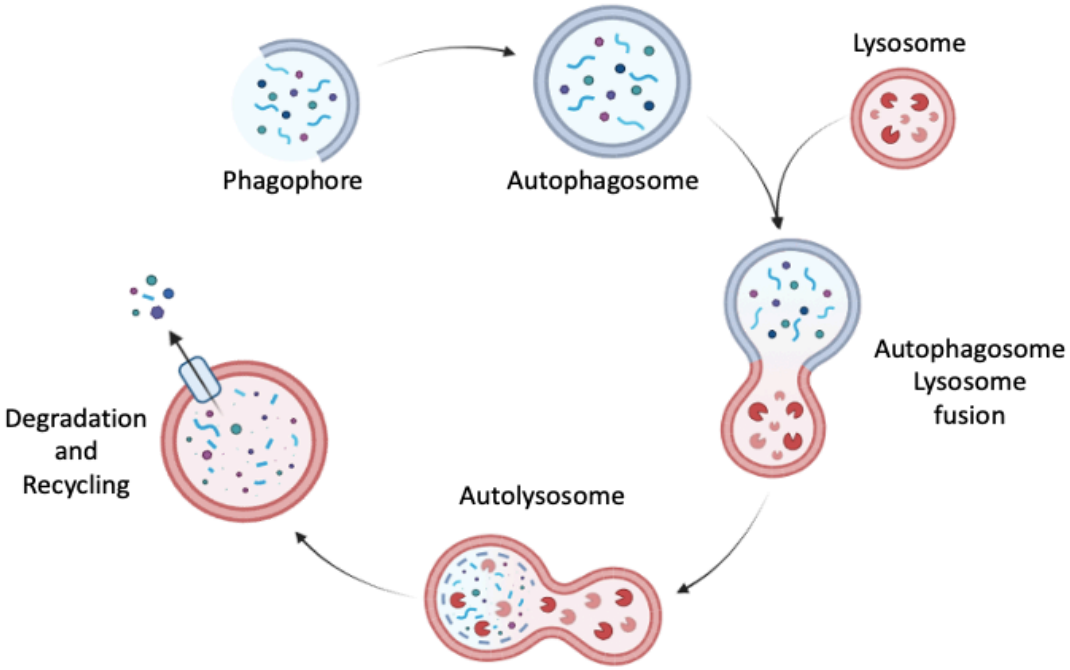


Fig 2: Schematic representation of the macroautophagy pathway.

1.2 Regulation of Autophagy

Autophagy is constitutively performed at the basal level in every cell, not only to promote the clearance of normal products of cell metabolism, but also to modulate different cellular processes, such as cell development, differentiation, and cell death (17,18). Autophagy becomes particularly relevant under stress conditions, ensuring the maintenance of cell homeostasis when the normal intracellular conditions are perturbed (19). It is important to underline that the rate of autophagic activity needs to be tightly controlled and alterations that increase or decrease the autophagic flux may be detrimental. For example, the uncontrolled degradation of intracellular material may lead to cell death; conversely, the downregulation of the degradation pathway may result in the intracellular toxic accumulation of undigested material. For these reasons, cells can rely on different mechanisms of autophagy regulation that cooperate in complex networks to guarantee the maintenance of the correct degradation rate.

Among the stimuli that modulate the autophagic flux, the most relevant is nutrient availability. Indeed, autophagy is strongly activated by starvation, when autophagic clearance is needed to produce new molecules and sustain intracellular metabolic activity.

1.2.1 The mTORC1 Complex

One of the key factors involved in the response to nutrient variation is the mechanistic target of rapamycin complex 1 (mTORC1). This protein complex is a crucial hub for different molecular pathways and is a nodal point between anabolic and catabolic processes. It can sense variations in the extracellular and intracellular environment and, in turn, its kinase activity can modulate downstream cell pathways (20). In physiological conditions, when the nutrient availability is sufficient to sustain cell metabolism mTORC1 is activated and represses autophagic activity. Conversely, in response to starvation, the kinase activity of mTORC1 is inhibited and autophagy is promoted (21–23).

The mTORC1 complex consists of 5 monomers: mTOR, which is the serine/threonine kinase catalytic subunit; the scaffolding subunit Raptor, which is essential for modulating the subcellular localization of the complex; the mammalian lethal with Sec13 protein 8 (mLST8), which stabilizes the catalytic subunits by interacting with mTOR; the proline-rich Akt substrate of 40 kDa (PRAS40) and DEP domain containing mTOR-interacting protein (DEPTOR), which are two inhibitory subunits (20,21).

mTORC1 is regulated by different upstream proteins, ensuring the sensing and integration of many cellular stimuli. Among them, one of the most important activators of mTORC1 is the small Ras-homologue enriched in the brain (Rheb) GTPase. When this protein is bound to GTP, it interacts with the catalytic domain mTOR activating the protein complex through its kinase activity (20). Even though the mechanism of mTORC1 activation has not been completely elucidated yet, recent crystallography studies demonstrated that Rheb-GTPases bind mTORC1 and promote a conformational change in the protein complex that induces its activation (24). Through the modulation of the activity of the Rheb-GTPases, mTORC1 is highly regulated by the heterotrimeric tuberous sclerosis complex (TSC) (25). By switching the Rheb GTPases from the GTP-bound active state into the GDP-linked inactive form, TSC inhibits mTORC1 (26,27). TSC-mediated mTORC1 regulation represents one of the most important mechanisms that allow mTORC1 to respond to metabolic cues, such as variations of growth factors and oxygen concentrations. Protein kinase B (AKT) and REDD1 (regulated in development and DNA damage responses 1) are two examples of proteins that participate in the modulation of mTORC1 activity by regulating TSC (28). More specifically, in response

to growth factors, AKT has been shown to phosphorylate the TSC at different phosphorylation sites to inhibit the protein and, consequently, activate mTORC1 (29,30). AKT has been demonstrated, both *in vitro* and in cell models, to regulate mTORC1 also through direct interaction with the protein complex by phosphorylating the mTORC1 subunit PRAS40 at the level of Thr246. This mechanism promotes the inhibition of the inhibitory PRAS40 subunit leading to the activation of mTORC1 (31). Conversely, REDD1 inhibits mTORC1 activity through the activation of TSC in response to the alteration of oxygen concentrations (29,32,33). Another extremely important negative modulator of mTORC1 is represented by the AMP-activated kinase (AMPK), which will be described in detail in the following paragraphs. AMPK is considered the main sensor of the intracellular energetic status and this protein has been shown to modulate mTORC1 activity both via direct interaction and through the modulation of TSC (Fig 3)(29,34).

All these data demonstrate that the regulation of the mTORC1 function is highly controlled by a complex network of proteins. Any impairment in these regulatory pathways may have a detrimental effect and significantly impact the maintenance of cell homeostasis. Thus, a deep understanding of the upstream stimuli that affect mTORC1 and the fine characterization of the downstream cellular processes modulated by this protein complex is crucial to improve the overall knowledge of cell physiology.

As previously mentioned, the main parameter that modulates mTORC1 activity and, consequently, the mTORC1-mediated regulation of the autophagic process is the availability of nutrients. In fact, this protein complex is primarily involved in the sensing of the amino acids concentration, the first nutrient source for cells to produce new proteins and promote cell growth (20,21). Although the precise mechanisms involved in the amino acid sensing by mTORC1 are still under investigation, several works demonstrated that some amino acids are more important than others in the signaling of the nutrient status: arginine, glutamine, and leucine are the amino acids whose levels mainly affect mTORC1 activity. Moreover, experiments performed in cells deprived of amino acids demonstrated that no single amino acid is sufficient for mTORC1 activation, which is rather promoted by the presence of a mix of different amino acids (21–23). Of note, mTORC1 can sense both cytosolic and lysosomal amino acids levels, in a way that allows cells to detect and finely regulate the availability of nutrients at different cellular compartments. In addition, in nutrient-rich conditions mTORC1

is recruited and activated at the lysosomal membrane. This localization may provide a quick response to variations of the lysosomal function suggesting that mTORC1 could be crucial not only in the modulation of autophagy but also in providing a feedback response to the lysosomal activity. Moreover, the importance of amino acid sensing within lysosomes supports the idea that lysosomes are not only the final hub for the cellular degradative pathways but also serve as important centers for metabolite storage representing, in such a way, crucial signaling compartments.

The lysosomal localization and the activation of mTORC1 at the lysosomal membrane are mainly mediated by the binding with a family of GTPases, the small Rag (Ras-related GTP-binding) GTPases. Importantly, these proteins are also involved in the pathway of amino acid sensing, suggesting that they may mediate the information about the nutrient availability and influence mTORC1 activity. These proteins are embedded in the lysosomal membrane and can bind the Raptor subunit of the mTORC1 complex. The presence of high amino acids levels promotes the dimerization of Rag GTPases, which is crucial for the stabilization of their GTP-bound form and the following binding with mTORC1. In this subcellular localization, mTORC1 is prone to be activated by the Rheb GTPases (35). In contrast, when the amino acids concentrations decrease, Rag GTPases undergo conformational modifications adopting a GDP-bound inactive state, which is unable to interact with mTORC1. In this condition, mTORC1 is released from the lysosomes and maintained in an inactivated state (20). Even though the study of the pathways involved in amino acid sensing is still ongoing, the proteins Sestrin1 and Sestrin2 were the first validated sensor proteins and represent two examples of the molecular mechanisms through which mTORC1 can respond to nutrient availability. Sestrin 1-2 can bind leucine, and, following this interaction, they regulate mTORC1, modulating autophagy. Sestrin 1-2 protein levels are tightly controlled, suggesting a fine mechanism of mTORC1 modulation determined by the balance between leucine and Sestrins concentration (Fig 3) (35).

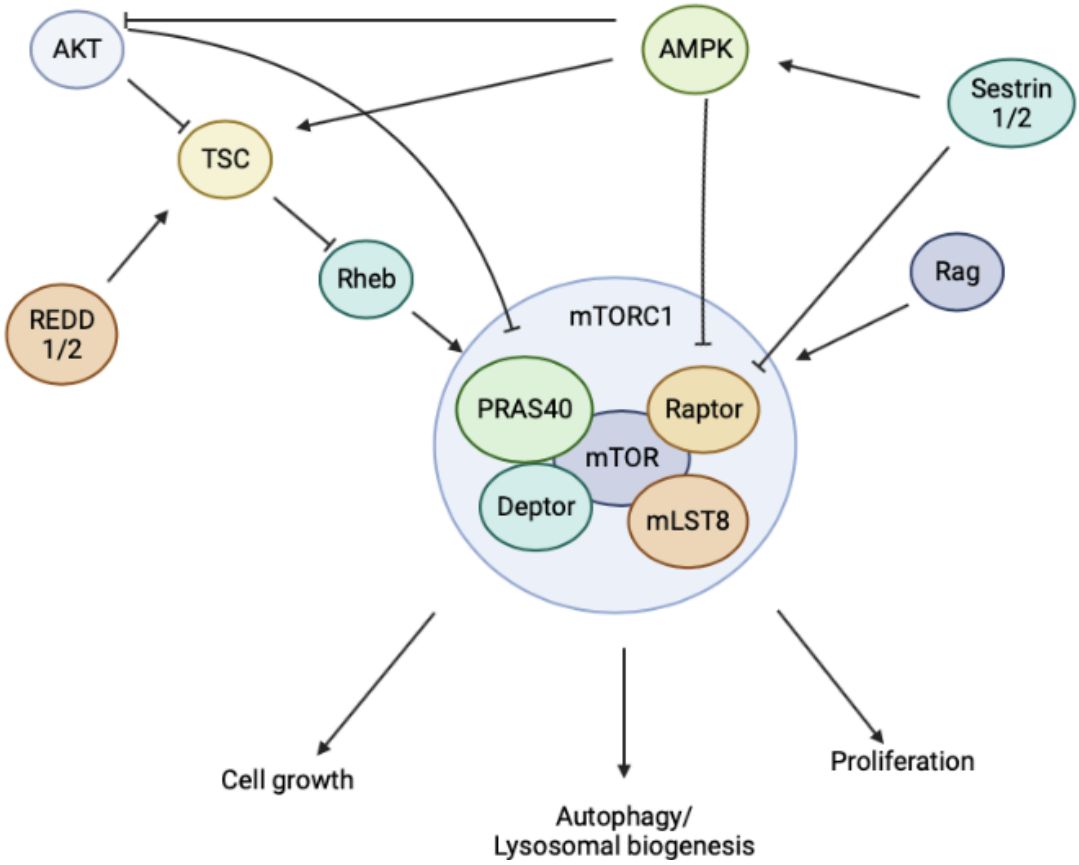


Fig 3: mTORC1 signaling pathways

The intricate network of proteins that affect mTORC1 activity allows the fine maintenance of the proper concentrations of amino acids. Downstream of mTORC1, autophagy is the most characterized process affected by variations of amino acid levels. The kinase activity of mTORC1 influences autophagy through at least two mechanisms. The former relies on the mTORC1-mediated phosphorylation of ULK1, which causes the inhibition of this protein, while the latter is linked to a transcriptional modulation of the ALP, being mTORC1 the main negative regulator of the transcription factor EB (TFEB)

1.2.2 ULK1-Dependent Activation of Autophagy

ULK1, the mammalian ortholog of the yeast Atg1, is a serine/threonine kinase and is considered one of the main regulators of autophagy. The crucial activity of this protein in the autophagic process is demonstrated by the fact that the loss of ULK1 in cells, or the inhibition of its kinase activity, is sufficient to impair the autophagic flux. Moreover, the knock-out (KO) of ULK1 together with its homolog ULK2 causes neonatal lethality in mice. ULK1 kinase

activity is essential for the proper function of the first steps of the autophagic cascade. The binding with ULK1 with FIP200 (family interacting protein of 200 kDa), ATG13, and ATG101 form the so-called ULK1 complex, which is necessary for the stabilization and stimulation of the kinase activity of ULK1. The importance of this complex for autophagy is highlighted by the observation that mouse embryonic fibroblasts (MEF) derived from FIP200 or ATG13 KO mice present impaired autophagy initiation. Interestingly, upon amino acid starvation, the ULK1 complex has been demonstrated to form punctate structures at the ER surface, at the level of the omegasomes, confirming the crucial involvement of ULK1 in the first phases of autophagosomes formation (36).

The aminoacidic sequence of the ULK1 is characterized by at least 30 phosphorylation sites, suggesting that its activity is tightly controlled by different kinases. This fine regulation allows to respond to several upstream stimuli. Among the kinases that affect ULK1 activity, mTORC1 and AMPK represent crucial factors involved in the modulation of autophagy in response to homeostasis alteration. They likely mediate opposite effects to maintain the correct balance of the intracellular catabolic functions. In nutrient-rich conditions, mTORC1 has been demonstrated to phosphorylate Ser757 of ULK1. This interaction strongly inhibits the protein's kinase activity, reducing autophagic functions (36,37). In contrast, AMPK phosphorylates different serine residues, such as Ser317 and Ser777, and activates the protein, promoting the initiation of autophagy (37). Considering that both AMPK and mTORC1 are constitutively expressed in almost every cell, the predominance of the effects of one over the other will highly depend on the energetic and metabolic conditions of the cell.

1.3 TFEB and the Transcriptional Regulation of Autophagy

As previously mentioned, mTORC1 can modulate autophagy also at the transcriptional level, by affecting the activity of one of its most characterized targets, TFEB. TFEB belongs to the microphthalmia/transcription factor E (MiTF/TFE) proteins family, which comprises other three transcription factors: transcription factor EC (TFEC), transcription factor E3 (TFE3), and microphthalmia-associated transcription factor (MITF). At the structural level, the members of the MiTF/TFE family, are characterized by the presence of a basic domain, which allows the binding to the DNA, a helix-loop-helix, and a leucine zipper domain which are crucial for dimerization and a transactivation domain, necessary for the transcriptional

activation. The most divergent member of the family is TFEC, which lacks the transactivation domain, and it has been reported to inhibit rather than enhance transcriptional activity (Fig 4).

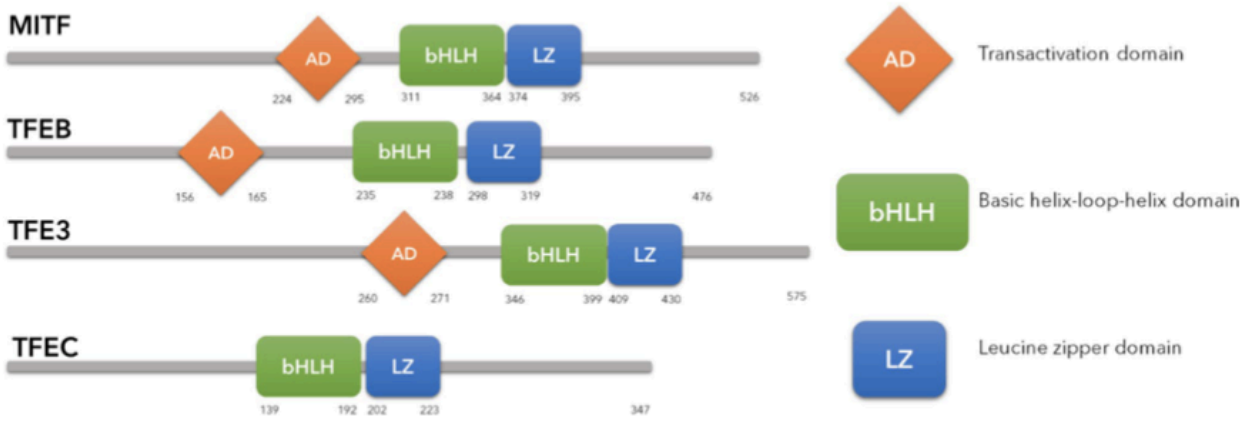


Fig 4: structure of the MiTF/TFE protein family (3).

The members of the family are crucial for the regulation of different cellular processes, such as mitophagy, lipid catabolism, and mitochondrial biogenesis. However, the main cellular pathway regulated by these proteins is autophagy. TFEB, in particular, is frequently regarded as the master regulator of this process. The effects of TFEB on the autophagic pathway have been deeply investigated in different models and are now well characterized. The overexpression of TFEB promotes bulk autophagy and increases the selective clearance of organelles, such as mitochondria. Moreover, TFEB has been found to induce lysosomal biogenesis and lysosomal exocytosis, a process that results in the fusion of lysosomes to the plasma membrane and the secretion of their content into the extracellular space. Accordingly, the overexpression of the *Caenorhabditis elegans* single orthologue of the MiTF/TFE proteins, Helix-Loop-Helix-30 (HLH-30), has been demonstrated to induce autophagy and increase worm lifespan. Conversely, in *Drosophila melanogaster*, the knock-down of the unique orthologue of these transcription factors, Mitf, causes autophagic impairments and autophagic substrates accumulation.

The genes under the control of TFEB are characterized by the presence in their promoter region of one or more copies of a specific DNA sequence, GTCACGTGAC, called the

Coordinated Lysosomal Expression and Regulation (CLEAR) motif. By recognizing this motif, TFEB can bind DNA beginning its transcriptional activity. In this way, TFEB modulates the transcription of a group of genes, known as the CLEAR network, which is involved in the functioning of every step of the autophagic machinery. The other MiTF/TFE proteins are also known to bind the CLEAR motif, suggesting a certain degree of redundancy in the function of the four transcription factors. This notion is also corroborated by the fact that the transcription factors of the MiTF/TFE family can form both homodimers and heterodimers interacting with each other. The dimerization is essential for the binding to the DNA, but the functional role of the different dimers is still unknown. The heterodimerization may depend on the relative expression of the four members in different cells or tissues and may represent one of the regulatory mechanisms to modulate the transcriptional activity of the MiTF/TFE family members.

TFEB is the most studied and characterized member of the MiTF/TFE family. Its essential role is also highlighted by the fact that this transcription factor is ubiquitously expressed in almost every tissue and cell. In mice, the KO of this protein is embryonically lethal and the impairment in TFEB regulation or activity is observed in several pathologies. Since autophagy must be tightly regulated, the modulation of TFEB activity is crucial as well. Even though the most characterized regulatory mechanisms of the transcription factor involve the post-transcriptional modification of the protein, TFEB can also be regulated at the transcriptional level. In this frame, several transcription factors have been shown to modulate the expression of TFEB. Among them, there are androgen receptors, peroxisome-proliferator-activated receptors-α (PPARα), cAMP response element-binding protein (CREB), and Krüppel-like factor 2 (KLF2), which have been shown to enhance TFEB activity. In addition, the *Tfeb* gene is characterized by the presence of the CLEAR motif in its promoter region, so that TFEB itself can also transcriptionally regulates its own expression. This feature allows a positive feedback loop that ensures the maintenance of high expression levels of TFEB in conditions in which its activity is required. These data clearly show that the level and the function of TFEB are two parameters that need to be regulated together to guarantee the correct activity of this transcription factor.

The levels of TFEB can also be affected through its degradation rate. The clearance of this protein is achieved through the ubiquitin-proteasome system. TFEB degradation rate is

particularly relevant for the modulation of the protein activity. Accordingly, the inhibition of the proteasome system not only causes the increase of the level of the transcription factor, but also promotes its nuclear localization and, consequently, the expression of its targets genes.

Besides the aforementioned mechanism of regulation, TFEB activity largely depends on its subcellular localization. In fact, TFEB exerts its transcriptional function in the nucleus, while its inactive form is maintained in the cytoplasm. For this reason, the activation state and the subcellular localization of TFEB are tightly controlled by posttranscriptional modifications and, in particular, phosphorylation. The amino acid sequence of TFEB is characterized by several phosphorylation sites, which are the substrate of different kinases that can stimulate or inhibit the protein activity (Fig 5).

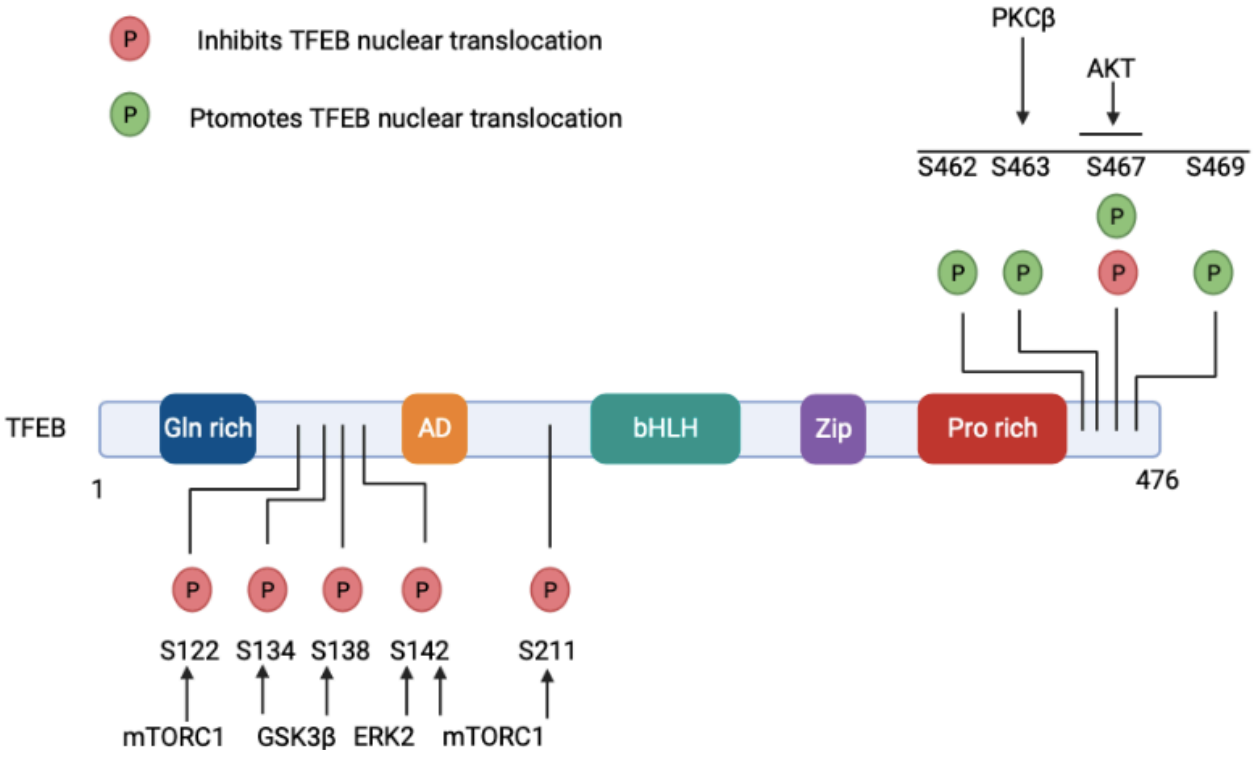


Fig 5: Schematic representation of TFEB structure with highlighted phosphorylation sites. Gln rich (glycine-rich motif), AD (activation domain), bHLH (basic helix-loop-helix domain), Zip (zip domain), Pro rich (proline-rich domain).

1.3.1 mTORC1 and TFEB

In 2011 TFEB has been demonstrated to be an effector of mTORC1 (38), linking for the first time the activity of the main intracellular nutrient sensor to the transcriptional regulation of autophagy. This discovery led, the next year, to the identification and characterization of the residue Ser211 as the most conserved and relevant phosphorylation site of TFEB, and one of the targets of mTORC1 kinase activity. mTORC1-dependent phosphorylation of Ser211 promotes the binding between TFEB and the family of the 14-3-3 chaperone proteins. Noteworthy, this interaction is crucial for the regulation of the transcription factor, as it is involved in the maintaining of TFEB in its inactive state in the cytoplasm. Accordingly, the mutation of Ser211 into an alanine residue, as well as the inhibition of the kinase activity of mTORC1, abolish the interaction between TFEB and 14-3-3s and promote a strong increase in TFEB nuclear translocation. The binding of 14-3-3 proteins with TFEB masks a putative nuclear localization signal present in the amino acidic sequence of the transcription factor, preventing the nuclear translocation of TFEB and, therefore, inhibiting its transcriptional function (39,40). In the same year also the Ser142 residue was demonstrated to be phosphorylated by mTORC1 contributing to the inhibition of the nuclear translocation of TFEB. Similar to Ser211, also the mutation of Ser142 reduced the mTORC1-mediated phosphorylation of TFEB and increases the nuclear localization of the protein. Interestingly, these phosphorylations are performed at the lysosomal membrane, where the Rag-GTPases, involved in the recruitment of mTORC1, can also bind TFEB to induce its relocalization from the cytoplasm to the lysosome in response to environmental cues (41). Another crucial residue exposed to the kinase activity of mTORC1 is the Ser122. Like the other target residues of mTORC1 kinase activity, mutations in Ser211 promote the nuclear translocation of TFEB (Fig 5; Fig 6) (42). In conclusion, mTORC1 can regulate TFEB activity by phosphorylating the protein in different residues. However, the relative importance of one residue compared to the other ones is still unclear, and, most probably, the three residues cooperate to ensure the fine control of TFEB activation. Interestingly, Ser211 appears to be the only one that mediates the binding with 14-3-3 proteins. Therefore, TFEB-14-3-3 interaction seems not to be essential to determine the cytoplasmic retention of TFEB, since the mutations of Ser122 or Sser142 have been shown to affect the localization of TFEB independently of 14-3-3 proteins binding.

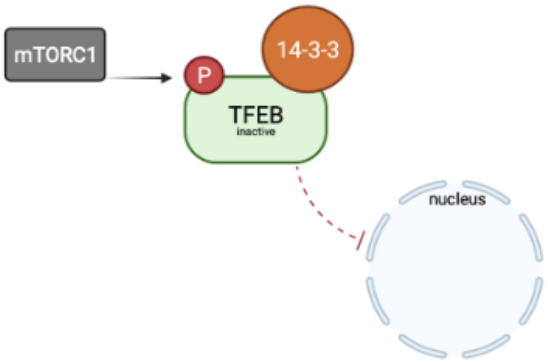


Fig 6 : mechanism of TFEB regulation by mTORC1. mTORC1 promotes TFEB phosphorylation and the consequent binding between the transcritpion factor and 14-3-3s. This interaction inhibits TFEB nuclear translocation

1.3.2 mTORC1- Independent Regulation of TFEB

Even though mTORC1 is considered the main regulator of TFEB, other kinases have been demonstrated to influence the transcriptional activity of this protein. The study of the posttranscriptional modifications of TFEB led to the identification of the extracellular regulated kinases (ERKs) as an upstream inhibitor of the transcription factor (43). ERK is a kinase belonging to the ERK-MAPK (Mitogen-activated protein kinases) pathway, which is involved in the functioning of several crucial cellular processes, such as cell survival, proliferation, and response to DNA damage (44). ERK was shown to phosphorylate Ser142 of TFEB, inducing its cytosolic localization (43). Accordingly, the serine to alanine mutation, abolishes the ERK-mediated phosphorylation of TFEB and stimulates its nuclear translocation, which is also observed in cells upon ERK downregulation (43). Interestingly, the site phosphorylated by ERK is also one of the targets of mTORC1, even though the functional link between the two kinases is still unknown. These two kinases likely compete for the same residue and the rate of phosphorylation mediated by one protein compared to the other one may depend on the cell type or cell environment. The coordination between ERK and mTORC1 in the modulation of TFEB activity may guarantee the optimal functional state of the transcription factor in the response to different stimuli (Fig 5).

More recently the serine/threonine Glycogen synthase kinase-3 β (GSK3β), whose activity is associated with several important cellular functions, including cell division, differentiation, proliferation, and apoptosis (45), has been observed to affect the subcellular localization of TFEB, inhibiting the nuclear translocation of the protein. Accordingly, the pharmacological

inhibition of GSK3β promotes an increase in the nuclear fraction of TFEB and the stimulation of autophagy. Site-directed mutagenesis experiments demonstrated that GSK3β phosphorylates the residues Ser134 and Ser138 of TFEB, resulting in the inhibition of the protein. Noteworthy, the GSK3β kinase activity also affects the phosphorylation level of Ser211 and, consequently, the binding between TFEB and 14-3-3 proteins, suggesting a functional interaction between the kinases that regulate TFEB, as a further layer of regulation of its transcriptional activity (Fig 5) (46).

1.3.3 Phosphorylation Sites in the C-terminus Domain of TFEB

The C-terminus of the TFEB amino acidic sequence is characterized by the presence of a stretch of serine residues (Ser462, Ser463, Ser466, Ser467, Ser469) which are the target of different kinases. Although these residues are far less investigated than the crucial sites mentioned before, they are increasingly recognized as important regulators of TFEB activity.

One of the proteins that phosphorylates TFEB in the C-terminal region is AKT. As previously mentioned, AKT has been described to modulate autophagy through the mTORC1 pathway, via TSC phosphorylation. However, the inhibition of AKT in a TSC KO background has been shown to promote TFEB nuclear translocation, proving that this kinase is able to modulate the subcellular localization of TFEB independently of the mTORC1 pathway (47). Furthermore, an *in vitro* kinase assay demonstrated that AKT phosphorylates the residue Ser467 of TFEB (47) suggesting that AKT can inhibit TFEB either through the phosphorylation of TSC and mTORC1 activation or through direct phosphorylation of the transcription factor. It is important to highlight that this redundancy in the regulation of TFEB, determined by the activity of multiple proteins and with the same kinase that can act through different pathways is probably required to ensure the fine regulation of TFEB preserving the possibility to adapt to prompt variations in cell conditions.

The serine residues of the C-terminal region of TFEB have been shown to be all possible targets of another important kinase, the protein kinase C beta (PKCβ), which likely promotes opposite effects on TFEB compared to AKT. The PKCβ function has been associated with several important cellular processes, such as cell proliferation, cell cycle, apoptosis, and autophagy (48). Indeed, PKCβ-mediated phosphorylation of TFEB promotes in osteoclasts the nuclear translocation of the transcription factor and the consequent induction of the

autophagy-related genes, as well as the stabilization and accumulation of TFEB (Fig 5) (49). This data highlights at least two important notions. The first one is that TFEB phosphorylation not only affects the activity of the protein but can also impact its stability, modulating its degradation rate. In this way, the regulation at different levels might act concomitantly to ensure the optimal function of the protein. In addition, in some cases, the same phosphorylation site has been shown to promote opposite effects, serving both as an activator or an inhibitor of TFEB. This controversial data has not been completely understood, it would be worth to analyze whether the phosphorylation of the same residue produces different effects based on the pattern of phosphorylation on other sites throughout the protein or, for example, depending on the cell type or the subcellular localization of the transcription factor. This data gives an idea of how complicated the molecular mechanisms that control the activity of TFEB are and how crucial it is to study in detail all the pathways involved in regulating this transcription factor. Importantly, as it will be described later, serine residues at the C-terminus have been recently demonstrated to be the target of another important protein, AMPK.

1.3.4 Phosphatases that Dephosphorylate TFEB

The search for a phosphatase able to dephosphorylate TFEB led to the identification of Calcineurin as a positive regulator of the transcription factor. Calcineurin has been demonstrated to promote the dephosphorylation of Ser142 and Ser211 disrupting the binding between TFEB and 14-3-3s and inducing TFEB translocation to the nucleus (50). Calcineurin function is particularly sensitive to variations of intracellular calcium levels, and is the protein has been demonstrated to become activated upon release of calcium from the lysosome through the lysosomal calcium channel mucolipin1 (MCLN1)(50). Calcineurin likely represents one of the mechanisms that allow the strict regulation of TFEB, and this dephosphorylation pathway may serve as a mechanism to anticipate and increase the rate of activation of TFEB. Moreover, the calcineurin-mediated TFEB regulation, may also increase the ability of the transcription factor to integrate and respond to the stimuli generated by lysosomes.

Recently, another phosphatase has been shown to be relevant for TFEB regulation. In cells treated with arsenic, a strong mitochondrial stressor that increases oxidative stress, protein

phosphatase 2A (PP2A) has been demonstrated to directly dephosphorylate TFEB (51). PP2A is a ubiquitously expressed serine/threonine phosphatase whose function, together with protein phosphatase 1 (PP1) accounts for up to 90% of the whole cellular phosphatase activity and is associated with the cellular pathways that regulate the cell cycle, migration, proliferation, cell survival, and cell metabolism (52,53). Mass spectrometry analysis experiments then showed that PP2A activity is exerted toward several serine residues in the amino acidic sequence of TFEB, including Ser109, Ser114, and Ser122 and Ser211. Consistently, *in vitro* experiments demonstrated that, under oxidative stress conditions, PP2A dephosphorylates TFEB, while a mutant lacking the catalytic subunit did not affect TFEB phospho-sites (51). In contrast to Ser122 and Ser211, the functional role of the serine residues 109 and 114 are not well characterized but they may participate in the fine regulation of TFEB subcellular localization (Fig 7).

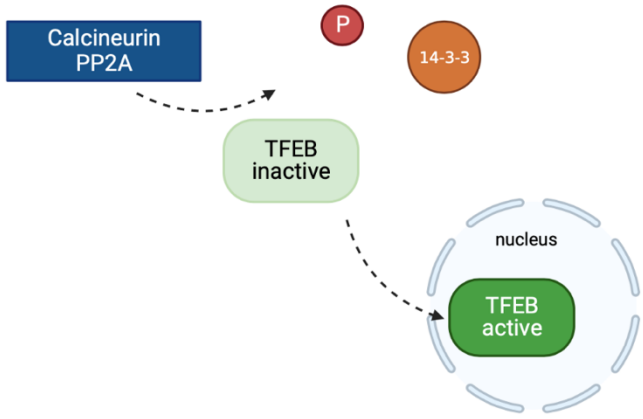


Fig 7: dephosphorylation of TFEB, Calcineurin, and PP2A mechanisms of action. The phosphatases dephosphorylate TFEB and cause the disruption of the TFEB-14-3-3s binding. In this condition, TFEB is free to translocate to the nucleus.

Altogether, these data demonstrate that TFEB activity is crucial to ensure the optimal rate of autophagic degradation. The importance of this transcription factor Is also confirmed by the high number of proteins involved in its regulation. The crucial role of TFEB suggests that defects in its activity may be associated with diseases. In this frame, some examples of the role and involvement of autophagic function and TFEB activity in human diseases will be reported in the following paragraphs with particular attention to neurodegenerative disorders.

1.4 Autophagy in Physiology and Disease

Autophagy not only promotes the degradation of unnecessary material but is also involved in the modulation of important cellular functions, such as cell development, differentiation, cell division, cell communication, and cell death. For this reason, it is not surprising that alterations of this process are linked to the onset and progression of several pathologies, such as cardiovascular diseases, cancer, and neurodegenerative disorders (Fig 8) (54,55). It is important to mention that both an uncontrolled activation of this machinery or a non-physiological inhibition can be detrimental, leading to different pathological consequences. Therefore, the autophagic process needs to be finely tuned in every cell and tissue.

Considering the role of TFEB as master regulator of autophagy, this transcription factor is frequently studied as a good target to modulate the autophagic pathway and restore the correct cell degradative functions. In this frame, several research lines recently investigated whether TFEB activity is affected in pathological contexts and how modulating its function may influence the disease phenotypes (3).

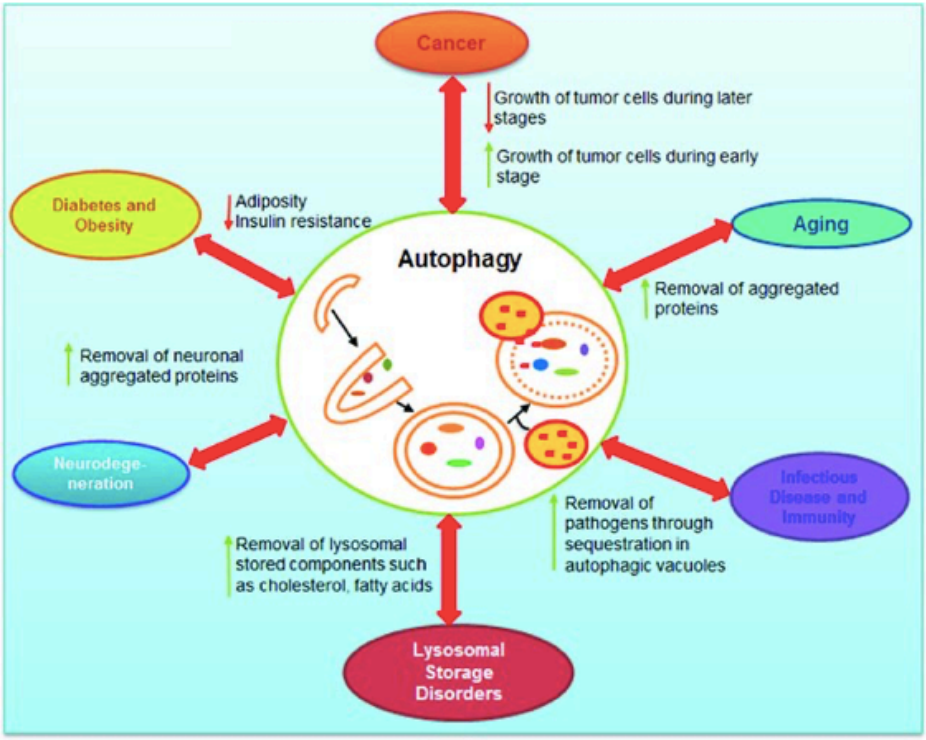


Fig 8: the role of autophagy in human diseases (56).

For the purpose of this research work, I will focus my attention on neurodegenerative disorders. Indeed, autophagic alterations have been demonstrated to have a crucial role in the onset and progression of this kind of pathologies.

1.4.1 Autophagy in Neurodegenerative Diseases

Neurodegenerative diseases are a group of pathologies affecting the nervous system characterized by the selective and progressive loss of neurons. They are classified according to clinical features and anatomic distribution of neuronal cell loss (55,57–59). Among neurodegenerative disorders, Alzheimer's Disease (AD), Parkinson's Disease (PD) and Huntington's Disease (HD) are the most studied and characterized. Actually, neuronal cell deficiency is responsible for the main phenotypic traits associated with these diseases, which frequently comprise both cognitive and motor impairments. A combination of environmental and genetic factors is considered to be behind these disorders. Although they are mainly characterized by sporadic origins, genetic forms are known as well, caused by mutations of specific genes, which are inherited according to mendelian laws. At present, only symptomatic treatments are available for these diseases and, in an aging society, the negative impact of these pathologies on public health is becoming increasingly high. Therefore, there is an urgent need for effective cures able to stop the progression of neurodegenerative disorders. To this aim, several research lines are currently investigating the cellular mechanisms underlying the different neurodegenerative diseases. Unfortunately, due to the complexity of the cellular pathways involved in these pathologies, a clear understanding of the processes that trigger neuronal cell death is a very challenging aim.

The link between autophagic defects and neurodegeneration was clearly demonstrated *in vivo* more than fifteen years ago, when two milestone papers described a relationship between the impairment of autophagic activity and neuronal cell loss in mice (60,61). Indeed, mice with brain-specific depletion of genes essential for autophagy, such as Atg5 and Atg7, showed a strong decrease in the lifespan as well as motor and behavioral deficits. Moreover, defects in neuronal autophagy were shown to cause an impairment in the degradation of ubiquitinated proteins that led to the accumulation of ubiquitin inclusions and finally to neuronal cell death (60,61). Nowadays, it is well accepted that autophagic impairments are among the most common features that are found in neurodegenerative disorders,

suggesting that tight control of this process is crucial to ensure neuronal cell health. The strong impact of the autophagic activity on the physiology of neurons may depend on the fact that neuronal cells need to sustain a huge metabolic demand and their survival depends on a tight regulation of cell homeostasis, including proteins and organelles quality control. Moreover, in neurons, autophagic machinery is further complicated by the peculiar morphology of these cells. Several studies demonstrated that the primary site of autophagic degradation in neurons is the cell body, where the majority of lysosomes are located. Conversely, autophagosomes are mainly generated in distal axons. Therefore, to fuse with lysosomes, cargo-loaded autophagosomes need to translocate from the axons to the soma, making the autophagic process more prone to defects.

In the last few years, several studies have investigated the role of autophagy in neurons and the molecular consequences of its dysfunctions for neurodegenerative disease. The impairments observed in the most common neurodegenerative diseases, involving different steps of the autophagic process, are reported in Fig 9. The importance of autophagic degradation in neurons is also confirmed by the fact that a regulated clearance of intracellular components is essential in non-dividing cells since the abnormal accumulation of cell debris, misfolded proteins, or damaged organelles can not be diluted through cell division and can lie at the basis of the toxicity that leads to neuronal loss. Therefore, it is not surprising that one of the major hallmarks of neurodegenerative diseases is the accumulation of proteinaceous inclusions in the soma of the surviving neurons (62). Several aggregation prone-proteins are known to generate toxic inclusion in neurons, the most studied being huntingtin (HTT), α-synuclein (α-syn), TAR DNA-binding protein 43 (TDP43), amyloid beta (Aβ), and hyperphosphorylated-tau. While defects in the autophagic pathway negatively impact on the accumulation of these proteins, at the same time, their aggregation can also negatively affect the autophagic process, further contributing to their toxicity and confirming the importance of an optimal degradation capacity to decrease the risk of neurodegeneration. The strong association between autophagy and neurodegeneration is further supported by the demonstration that several genes linked to the familial forms of neurodegenerative diseases have a role in the autophagic process (3).

As the master regulator of autophagy, TFEB plays a relevant role also in the context of neurodegeneration. Coherently, defects in the activity and in the regulation of this

transcription factor are observed in many neurodegenerative disorders (3). For instance, in a mouse model of HD, TFEB levels have been demonstrated to be reduced along with the expression of its target genes. Similarly, postmortem AD brains are characterized by a decrease in steady state and nuclear localization of TFEB (63). In accordance with this data, embryonic fibroblast from an AD mouse model and human AD neurons show inhibition of TFEB nuclear translocation, as indicated by hyperphosphorylation of the transcription factor and consequent downregulation in the expression of the CLEAR genes (64). However, the situation is not straightforward, and TFEB levels and activation may vary across different models. For example, in several mouse and human AD models, the levels of TFEB have been proven to be upregulated, with a mechanism that has been proposed as a compensatory response to balance the inhibition of the TFEB pathways (3). Moreover, a study in an AD mouse model characterized by the silencing of the AD-associated proteins presenilin1 and 2 revealed an increase in the CLEAR network in the brains (65). These contrasting results highlight the difficulty in analyzing autophagy and TFEB in neurodegenerative diseases. In this frame, it is crucial to consider that differences in TFEB levels may be determined by the fact that the regulation of this transcription factor seems to vary according to different developmental stages of the pathology, with an increase of TFEB expression and activity in the first phases followed by a reduction of TFEB pathway in the late stages of the disease. The picture is further complicated by the fact that TFEB regulation is highly cell-specific, and it may vary among the different classes of neurons (3).

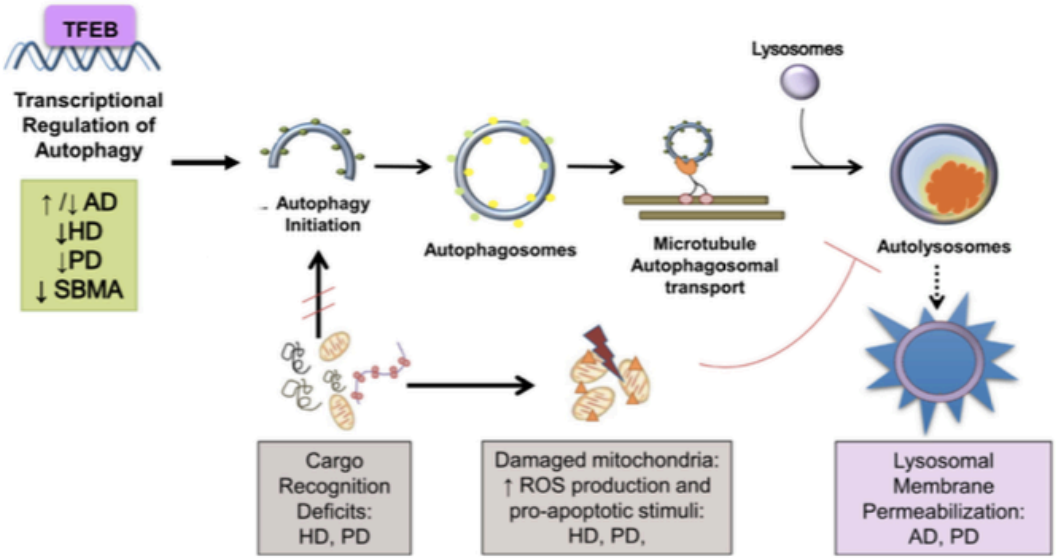


Fig 9: Schematic recapitulation of the defects that characterize different neurodegenerative diseases in every step of the autophagic process (56). AD (Alzheimer's Disease), HD (Hungtington's Disease), PD (Parkinson's Disease), SBMA (Spinal-Bulbar Muscolar Atrophy).

Given the essential role of autophagy in neuronal physiology, this process is considered a promising therepeutic target to modify the progression of neurodegenerative diseases. The induction of autophagic activity in neurons has been shown to be beneficial in several models, with particular emphasis given to the autophagy-dependent degradation and clearance of aggregated proteins. Several small compounds that can activate autophagy have been tested both *in vitro* and *in vivo* and they can be classified according to the pathway they affect into two subgroups: mTOR-dependent and mTOR-independent molecules, with the latter acting mainly through the activation of AMPK (66). The neuronal autophagy activators have to face two main issues. The former concerns the brain bioavailability of the compound: to reach the site of action and affect neuronal physiology, molecules need to pass the blood-brain barrier. In this regard, several molecules proven to activate autophagy in cell models failed to modulate this process *in vivo*, due to low central nervous system penetration (66). The latter problem is related to the specificity of the induced effects. As previously mentioned the potential therapeutic molecules need to be highly specific modulating only autophagy while maintaining the other cellular pathways unaltered. Moreover, autophagy activation must be directed specifically towards neurons, avoiding affecting other cells and/or tissues.

Even though the contribution of TFEB in neurodegenerative disease is still under investigation, this transcription factor has been intensively studied as a possible therapeutic target for the treatment of these disorders (Fig 10). For example, in different models of tauopathies, which are characterized by the accumulation of Tau, increasing TFEB expression has been demonstrated to be beneficial. More specifically, the adenovirus-mediated overexpression of TFEB reduced the level of the pathological form of tau and increased neuronal survival, improving also behavioral deficits. Moreover, the neuronal specific overexpression of TFEB in mice promoted the rescue of memory and learning skills, whose defects are associated with tau aggregation (67,68). Similarly, the increase of astrocyte TFEB expression in a mouse model of AD determined the reduction of Aβ accumulation in the brain interstitial fluid (69,70). In addition, TFEB injection in the striatum of HD mice reduces the level of the mutant and therefore pathological form of HTT. Despite the positive outcomes regarding the level of HTT, the overexpression of TFEB in this model also caused negative effects, such as ER stress and reactive gliosis, supporting the notion that TFEB levels and activity need to be tightly regulated in order to limit the possible negative consequences of its exogenous modulation (Fig 10) (71).

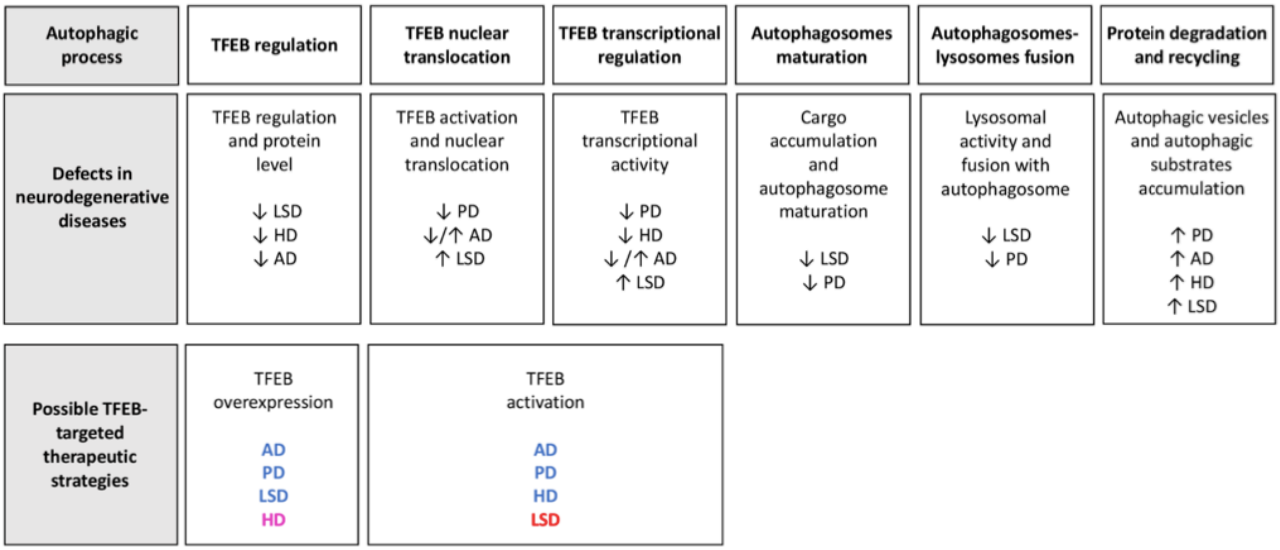


Fig 10: possible therapeutic strategies, based on TFEB regulation, to counteract the progression of neurodegenerative disorder. In the third line the arrow represents the direction of TFEB alteration in the different neurodegenerative diseases. In blue and red are reported the diseases in which the modulation of TFEB activity has been demonstrated to be, respectively, beneficial or detrimenta; in magenta the diseases in wiich the modulation of TFEB has no clear effect (3). AD (Alzheimer's Disease), PD (Parkinson's Disease), HD (Hungtngton's Disease), LSD (Lysosomal Storage Disorders).

1.4.2 Autophagy and Parkinson's Disease

Among the different neurodegenerative diseases, Parkinson's Disease (PD) represents the second most common disorder after AD. From the clinical point of view, the pathology is characterized by motor symptoms, such as resting tremors, postural instability, the rigidity of the skeletal muscles, and bradykinesia. These symptoms result mainly from the death of dopamine-producing neurons in the substantia nigra pars compacta, which project into the striatum, a region of the basal ganglia involved in the control of voluntary movements. In addition to motor symptoms, the disease is associated with a number of non-motor symptoms, including olfactory dysfunction and constipation, that can precede the manifestation of the motor dysfunction by more than a decade; as well as cognitive impairment and psychiatric disorders, that are usually associated with the late stages of the pathology (72,73).

Only a minority of patients, corresponding to the 5-10% of people suffering from this pathology, present a clearly familial origin, in which parkinsonism is inherited following the laws of mendelian inheritance. In fact, most PD patients have a sporadic disorder. The major causes of the idiopathic forms of the disease are still largely unknown, and the pathology is thought to be determined by a complex interaction of genetic and environmental factors. The genetics of PD has been marked by significant discoveries in more than 25 years of research, mainly throughout the study of the hereditary forms of the disease (72,73). These studies allowed the identification of several molecular players and pathways that are related to PD pathology. The first gene to be identified was the *SNCA*, whose transcriptional product is the α-syn protein. Importantly, along with dopaminergic neuronal loss, another pathological hallmark of PD is the accumulation of insoluble aggregates, mainly composed of misfolded α-syn in the somata of surviving neurons. This leads to the formation of round eosinophilic cytoplasmic inclusions, known as Lewy bodies (LBs). Under physiological conditions α-syn is degraded by the ALP, and the major cause of LBs accumulation seems to derive from the fact that misfolded or mutated α-syn fails to be processed and properly eliminated. The increase of aggregated α-syn in neurons results in neurotoxicity, eventually causing neuronal death. In this frame, the reduction of autophagic activity, one of the key features of PD, directly impacts the aggregation, limiting the degradation of the dysfunctional

cytoplasmic components. Therefore, the lack of an efficient degradation system for α-syn inclusions is considered a determining factor of PD severity (74,75).

Autophagic defects have been observed in the substantia nigra of PD patients, characterized by abnormal levels of LC3-II, decreased activity of lysosomal enzymes, such as cathepsin D and glucocerebrosidase (GCase), and accumulation of ubiquitinated LBs (76). In addition, deficits in the autophagosomal function, such as the recognition of autophagic cargo, have been observed in PD (77). The strong link between autophagic dysfunction and PD is further confirmed by the observation that several of the proteins associated with the genetic forms of PD have a role in ALP. Among them, α-syn overexpression has been observed to influence autophagic flux together with lysosomal hydrolytic defects. In addition, Leucine reach repeat kinase 2 (LRRK2), whose mutations represent the most frequent cause of familial PD, has been recently linked to autophagic-lysosomal function and the G2019S pathologic mutant causes in an abnormal increase of autophagy-related vesicles in SH-SY5Y cells (78). Moreover, age-dependent accumulation of enlarged lysosomes have been observed in kidneys of LRRK2 KO rodent models (79,80). Furthermore, other two proteins linked to familial PD, PTEN-induced kinase 1 (PINK1), and PARKIN are well known to modulate a particular type of autophagy, called mitophagy, which participates in the selective degradation of mitochondria (79,81). Noteworthy, the most common risk factor for PD is represented by heterozygous mutations in the *GBA1* gene, which encodes the lysosomal hydrolytic enzyme GCase, that hydrolyzes glucosylceramide into ceramide and glucose. Dysfunction in this enzyme determines impairment of basal autophagy and malfunction of lysosomal degradation (82–84). All these data underlie the primary involvement of autophagy in the onset and progression of PD pathology, also suggesting that this process may serve as a possible target to counteract the disease.

TFEB activity and regulation have been intensively investigated in the context of PD. The transcription factor appears to be excluded from the nucleus of dopaminergic neurons in the substantia sigra of PD patients and this aspect is correlated to the progressive decline in lysosomal markers, which can be observed in PD (85). This data suggests that defects in the regulation and the subcellular localization of TFEB may occur in PD. Interestingly, recent pieces of evidence highlight a possible link between α-syn toxicity and the dysfunctional activity of TFEB. In fact, α-syn has been demonstrated to share structural homology with

several regions of the 14-3-3 proteins. Furthermore, α-syn is able to bind 14-3-3s as well as to interact with some of the binding partners of these proteins (3).This data led to the hypothesis that there might also be a physical interaction between TFEB and α-syn, supported by the colocalization of TFEB in LBs, able to cause the inactivation of TFEB and its cytoplasmic retention. The aberrant α-syn-TFEB interaction may result in dysfunctional ALP and contribute to the onset of PD pathology, by compromising the clearance of toxic α-syn aggregates (86). Interestingly, the viral overexpression of TFEB in nigral dopaminergic neurons has been shown to rescue the lysosomal breakdown and enhance autophagic flux, resulting in the reduction of α-syn aggregates and in significative neuroprotection (87). Similarly, the activation of TFEB through the treatment with rapamycin, an inhibitor of mTORC1, promoted neuroprotection by enhancing autophagic flux and inducing the degradation of protein aggregates in PD animal models (87).

Chapter 2

Introduction

Crosstalk between Mitochondria and Lysosomes

2 Crosstalk between Mitochondria and Lysosomes

One of the main features of eukaryotic organisms is the presence of different compartments, called organelles, inside the intracellular space. This compartmentalization is crucial for cell viability, since often the different chemical reactions that take place within cells need particular conditions to properly occur. By allowing the temporal and spatial separation among the reagents, organelles guarantee that the cellular processes are performed in the most suitable environment (88). It is worth mentioning that, in a complex system like a cell, all the different compartments must work cooperatively so that it is not surprising that organelles communicate with each other and adopt different mechanisms to inform about their activity status and their conditions (88).

Due to the very important roles played by mitochondria and lysosomes the signaling pathways linking these organelles have gained increasing attention in recent years. Mitochondria and lysosomes dysfunctions are at the origin of several pathological conditions, including neurodegenerative disorders. While until a few years ago, researchers mainly focused their analyses at the level of a single organelle, more recently, it has become clear that these two compartments are tightly connected in their functions. This notion has led to the development of novel research field aimed at understanding whether and how mitochondria and lysosomes are linked and how they communicate with each other. Although the interaction mechanisms between mitochondria and lysosomes are still under investigation, several research lines proved that genetic defects of proteins involved in mitochondrial homeostasis may have secondary effects at the lysosomal level and, conversely, dysfunction of lysosomal proteins may affect mitochondria functionality (78,89). The study of the crosstalk between mitochondria and lysosomes is of great relevance for both physiological and pathological conditions and a better understanding of the signaling pathways involved in this communication may help develop good therapeutic strategies and identify novel targets to modulate their activity.

The most direct form of communication between mitochondria and lysosomes is represented by the physical interaction between the organelles. They have been observed to make contacts that seem to participate in the regulation of different processes, such as mitochondrial fission and lysosomal dynamics (90). Moreover, the contact sites appear to be spots of intense metabolic activity serving, for example, as regions for the transfer of ions

and molecules between organelles (91). Another type of direct communication is characterized by the selective lysosomal degradation of mitochondria performed through the process referred to as mitophagy. Mitophagy depends on the lysosomal activity and ensures the maintenance of a correct pool of mitochondria, eliminating the dysfunctional ones (89). Mitophagy defects may have detrimental effects and lead to pathological conditions. For example, mitochondria-degradation impairments are associated with several forms of genetic PD and some of the PD-linked proteins, such as PARKIN and PINK1, are involved in this process (92). Another type of crosstalk involves long-distance communications, realized through molecular signaling pathways that link one organelle to the other. Until recently, these pathways were poorly investigated and characterized, but they are currently gaining increasing attention. Recently, it has been showed that mitochondrial defects can modulate autophagy through the activity of AMPK, which appears to be a crucial protein able to sense mitochondrial functions and transfer the signals to the lysosomes (93).

2.1 AMPK: General Features

The protein kinase AMPK is considered a sensor of cellular energetic status because of its ability to respond to variations of cell homeostasis and to influence several important cellular processes. AMPK is a trimeric complex composed of three subunits, the catalytic subunit α, and the regulatory subunits β and γ. In vertebrates, each subunit can be found in more than one isoform, encoded by different genes. There are two isoforms for the α and β subunits (α1 and α2, β1 and β2) and three isoforms for the γ subunit (γ1, γ2, and γ3). All the combinations are allowed, resulting in twelve different AMPK complexes (94). It is likely that the different complexes have specific functions, even though this hypothesis has not been proved yet. In this regard, it was recently shown that the expression of the different subunits varies among tissues, corroborating the idea that they can be functionally distinct and that they may have tissue- or cell-specific roles. For example, it was shown that while the isoforms α1, β1, and γ1 are ubiquitously expressed, isoforms α2, β2, γ2, and γ3 are enriched in skeletal and cardiac tissues (95). Moreover, it has been demonstrated that the sensitivity of AMPK to ATP, ADP, and AMP, the most characterized feature of protein, depends on the γ isoforms. In particular, that the γ3-containing complexes seems to be more efficient in the response to variations in nucleotide levels.

Each subunit confers specific properties at the protein. The α subunit is composed of an N-terminal kinase domain (KD), which contains a residue of threonine (Thr172), which is crucial for the regulation and the activity of the protein, and a regulatory domain in the C-terminus (96). By binding the C- terminal region of the α subunit and the γ subunit, the β monomer acts as a scaffolding protein. The β subunit also contains a glycogen-binding domain (GBD), which allows the complex to act as a glycogen sensor (97). The γ subunit is characterized by the presence of four cystathionine-b-synthase (CBS) regions, which allow the binding with nucleotides (Fig 11, 12) (96).

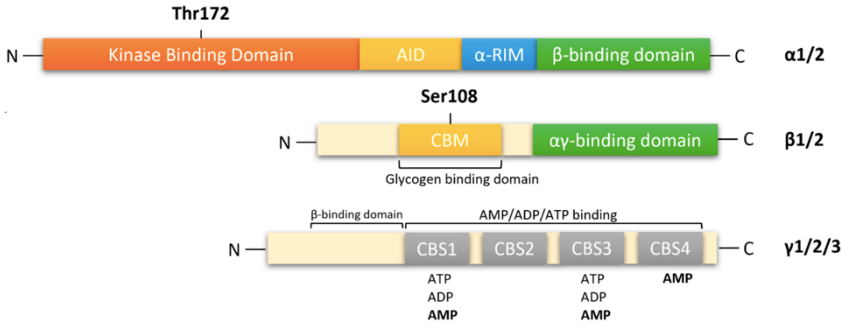


Fig 11: structure of the AMPK trimeric complex the figure shows the domains of the three AMPK subunits (98)

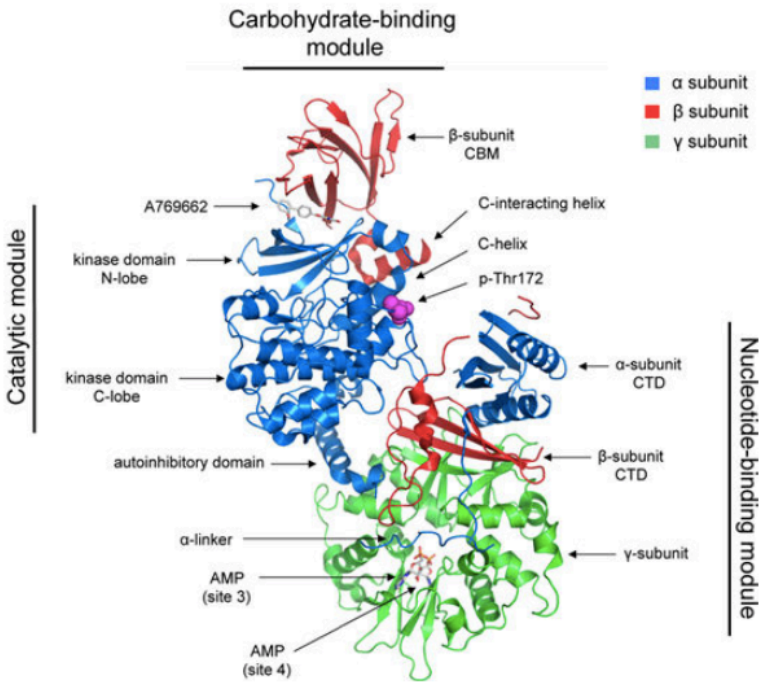


Fig 2: Christal struture of AMPK. the three subunits are depicted in different colors. The important sites for AMPK activity are highlighted with arrows (99).

2.1.1 Regulation of AMPK

The regulation of AMPK mainly depends on the phosphorylation state of Thr172, which is crucial for the activation of the complex, and the level of phosphorylation at this residue is frequently used as an indicator of protein activity. In mammals calcium/calmodulin-dependent protein kinase kinase (CaMKKβ) and liver kinase B1 (LKB1) are the principal kineses working upstream of AMPK (96). The level of AMPK phosphorylation is highly controlled by the binding of nucleotides to the γ monomer, which can bind both AMP and ADP, resulting in the allosteric activation of AMPK. There are three binding sites for nucleotides in the γ subunit, and AMPK becomes fully activated only after the binding with three AMP and/or ADP molecules. The precise mechanism of nucleotide-mediated activation of AMPK is still largely unknown but it has been shown that the first binding with AMP or ADP makes Thr172 prone to be phosphorylated by the upstream kinase LKB1. Subsequently, when two other AMP or ADP molecules bind AMPK the conformational change promoted by these interactions prevents the dephosphorylation of Thr172 ensuring a more stable and prolonged activation of the protein. Noteworthy, the binding with ATP completely abolishes these mechanisms inhibiting AMPK activity. In this way, the protein complex can promptly respond to variations in the energy status of the cells and modulate downstream processes accordingly (95). Nucleotides do not represent the only regulators of AMPK, which is also sensitive to changes in the intracellular concentration of calcium (Ca^{2+}). An increase in intracellular Ca^{2+} levels stimulates the other upstream kinase CaMKKβ, which phosphorylates AMPK, activating the protein (95). Among the different modulators of AMPK, mTORC1 has been described as a negative regulator of the protein. In particular, mTORC1 can phosphorylate AMPK at Ser347 and Ser345 of the α subunit, causing a decreased Thr172 phosphorylation (100). As previously mentioned, AMPK is also sensitive to glycogen. It has been shown *in vitro* that the protein binds glycogen at the GBD level and that this interaction inhibits AMPK phosphorylation, through a still undefined mechanism (97). Another stimulus that has been reported to modulate AMPK activity is the variation of reactive oxygen species (ROS) (95). How the concentration of ROS affects AMPK activity is still under investigation, but some pieces of evidence indicate that ROS can directly modulate AMPK function by the oxidation of specific amino acidic residues in the α-subunit, affecting the ability of AMPK to be phosphorylated by the upstream kinases. The effect of ROS on the protein function is likely cell-dependent as the data obtained in different models

are not always concordant. For example, it has been shown that in human embrionic kidney (HEK293T) and lung cells ROS can oxidize the cysteine residues Cys299 and Cys304 in the α-subunit, leading to increased activation of AMPK. In contrast, in cardiomyocytes H_2O_2 has been demonstrated to oxidize the residues Cys130 and Cys174, resulting in AMPK inhibition (95). Overall, these data demonstrate that AMPK is modulated by an intricated network of pathways that make this protein an important hub for the sensing of intracellular metabolic and energetic status. In turn, AMPK activity participates in the regulation of cell homeostasis by influencing important downstream processes: it is involved in the maintainance of glucose and lipid metabolism, modulation of transcriptional activity, cell growth, and, related to the purposes of this work, autophagy (Fig 13) (96,99).

In addition to the aforementioned factors involved in the modulatio of AMPK activity, it has been recently proposed that the subcellular localization of AMPK and the presence of distinct subcellular pools of AMPK can also influence its activity, allowing the protein to sense and respond to specific intracellular stimuli (101). For example, it has been shown that a fraction of AMPK localized at the level of lysosomes is particularly important for sensing variations in nutrient availability and for the fine tune of lysosomal functions (102). Similarly, the activation of the mitochondrial pool of AMPK has been shown to be crucial in responding to variations at the level of specific portions of the mitochondrial reticulum to modulate mitochondrial quality control with high spatial specificity (103). Considering the number of pathways able to alter AMPK function and the high specificity of its regulation, it appears evident that the modulation and the activity of AMPK are extremely complex. In spite of this complexity, it is increasingly clear that the role of AMPK is essential to maintain cell homeostatic balance. Moreover, because of its participation in a large number of cellular processes, AMPK may also be involved in different pathologies. For this reason, the detailed understanding of how AMPK activity is regulated in different conditions, and how it affects downstream processes is of crucial importance.

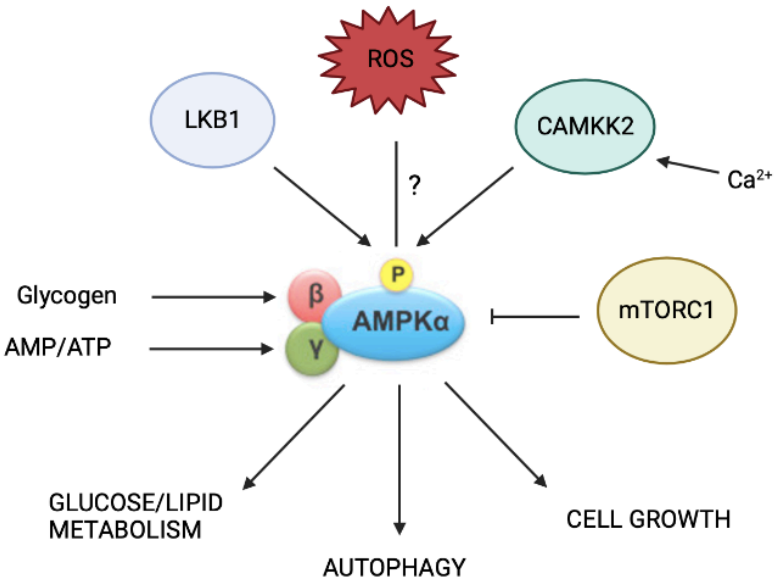


Fig 13: Schematic representation of AMPK signaling pathway

2.1.2 AMPK as a Modulator of Autophagy

As previously mentioned, among the important processes modulated by AMPK autophagy is the most relevant for the aims of this study. AMPK can affect ALP through different mechanisms. The first one involves the AMPK-mediated regulation of mTORC1. In this frame, it has been observed that the chemical activation of AMPK in rat skeletal muscle causes the reduction of the mTORC1 signaling pathway, as indicated by the decrease in the phosphorylation of two well-established downstream effectors of mTORC1: the Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and the Ribosomal protein S6 kinase beta (S6K) (104). One of the most characterized pathways that link AMPK activity to mTORC1 regulation involves the participation of TSC. AMPK directly phosphorylates TSC at the level of Ser1345, which, in turn, negatively regulates mTORC1 activity (105). In addition, AMPK can also modulate mTORC1 activity through direct phosphorylation of the protein complex, as suggested by the observation that the AMPK-mediated regulation of mTORC1 occurs also in TSC KO models as well as in organisms that lack the TSC protein, like *C. elegans* and *S. cerevisiae.* Consistently, it has been demonstrated that AMPK can phosphorylate the mTORC1 Subunit RAPTOR at the level of Ser722 and Ser792, resulting in the inhibition of the protein complex (106). More recently, another mechanism of AMPK-dependent regulation of autophagy was discovered based on the phosphorylation of TFEB. More specifically, AMPK has been demonstrated to phosphorylate TFEB in the stretch of

serine residues present in the C-terminal portion of the protein. Interestingly, although AMPK likely does not influence the nuclear translocation of TFEB, the absence of AMPK seems to strongly inhibit the transcriptional activity of the TFEB, even when the protein localizes in the nucleus, such as upon starvation or following mTORC1 inhibition (34). These important results uncover two important concepts about TFEB-AMPK interaction: first, the nuclear localization of TFEB does not necessarily correlate with transcriptional activation. Second, the phosphorylations at the C-terminal serine residues may not be involved in the determination of the subcellular localization of TFEB, but they likely tune the transcriptional activity of the protein. This notion is further supported by the observation that a mutant form of TFEB, non-phosphorylatable by AMPK on Ser466, Ser467, and Ser469, translocates into the nucleus in response to starvation but does not induce the expression of its target genes (34).

Among the mechanisms by which AMPK can influence autophagy, there is also the modulation of ULK1 activity. Indeed, it has been demonstrated that AMPK can phosphorylate ULK1 at Ser317 and Ser777 both *in vitro* and in cellular models (37). These phosphorylations are necessary for autophagic induction in MEF cells, as the non-phosphorylatable mutant of ULK is unable to activate ALP, even in response to pro-autophagic stimuli (37). Importantly, it was also shown that the interaction between AMPK and ULK1 is negatively modulated by TORC1 activity; coherently, active mTORC1 phosphorylates ULK1, resulting in the disruption of the ULK1- AMPK binding (Fig 14) (37).

All these data strongly highlight the tight connection between the function of AMPK and mTORC1 in the modulation of autophagy. Their activities need to be finely balanced to ensure the optimal regulation of ALP. Therefore a better understanding of how these proteins are influenced and regulated is crucial to characterize in detail the pathways that participate in the autophagic activity. Moreover, The modulation of these protein complexes activity it is likely dependent on the cell type and the cellular context. For these reasons, the study of mechanisms underlying the functional interaction between the two proteins in the modulation of the intracellular degradative pathways represents an open field in the current research.

Despite the experimental evidence presented until now on the active role of AMPK in promoting autophagy, it is worth mentioning that a recent work aimed at investigating the role of AMPK on neuronal autophagy demonstrated that the chemical activation of AMPK does not induce autophagy in neuronal cell models (107). This data, which needs further investigation, are open to different interpretations: on one side the result may suggest that the pathway modulated by AMPK activity in neurons is different compared to other cell types, on the other side, other mechanisms could be more relevant for the regulation of the ALP in this cellular population.

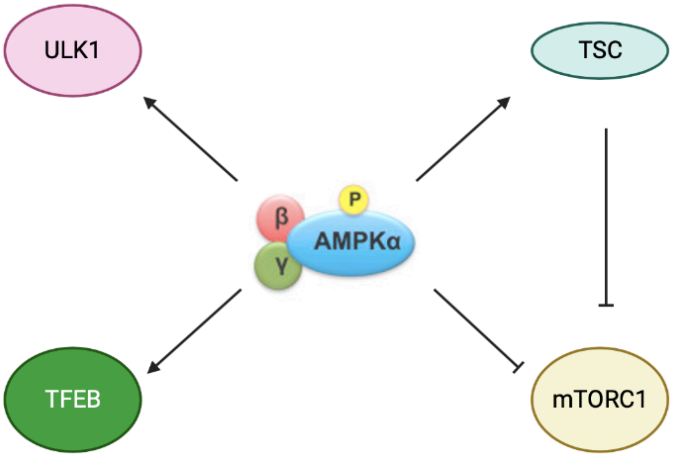


Fig 14: the most important mechanisms of AMPK-mediated regulation of autophagy

2.1.3 Role of AMPK in the Mitochondria-Lysosomes Crosstalk

Among the important roles ascribed to AMPK, its function as a mediator of the signals coming from mitochondria to the ALP has recently gained increasing attention. Being AMPK regulated by both ATP and ROS levels, this protein complex could likely be affected by variations in the activity of mitochondria. Actually, one of the main cellular functions of mitochondria is the production of ATP which is used as an energy-donor molecule to sustain the chemical reactions that occur within cells. Moreover, during the oxidative phosphorylation process, the electron carries of the electron transport chain are characterized by a continuous leakage of electrons that are prone to react with molecular oxygen, leading to the generation of ROS. Indeed, mitochondria are well known to represent one of the major cellular sources of ROS. Accordingly, it has recently demonstrated that AMPK can sense the stimuli related to mitochondrial functions and integrate them to

modulate the autophagic function. More specifically, using a cellular model characterized by mitochondrial defects, it has been shown that mitochondrial impairments result in autophagic alterations, including increased number of lysosomes containing undigested autophagosomes, reduction of lysosomal activity and decreased lysosomal pH (93). Importantly, in this cellular model mitochondrial defects were associated with a reduced amount of phosphorylated AMPK, suggesting a reduction in its activity. Noteworthy, the chemical activation of AMPK was able to restore autophagy and lysosomal functions (93). In the same study, AMPK activity has been described to respond differentially to specific mitochondrial insults. A reduced AMPK activity was observed in the presence of chronic mitochondrial defects while acute mitochondrial impairments promoted an increased AMPK activity, affecting its downstream pathways accordingly (Fig 15) (93). It is worth mentioning that the differential response of AMPK to the variation of the mitochondrial functions, may also provide a feedback mechanism that allows the maintenance of an overall stable mitochondrial network. Indeed, it has been suggested that, upon chronic mitochondrial insult, the induction of autophagy can improve mitophagy activity, facilitating the removal of dysfunctional organelles. Conversely, when the mitochondrial impairments are prolonged in time, the autophagic flux is slowed down, to avoid the autophagic degradation of the whole mitochondrial mass, most probably because it is better for the cell to cope with dysfunctional mitochondria than not to have mitochondria at all. Considering all these data, we can conclude that the activity of AMPK is necessary to maintain the proper function at the level of mitochondria and lysosomes, by influencing the dynamics of both organelles and allowing the coordination of their activities.

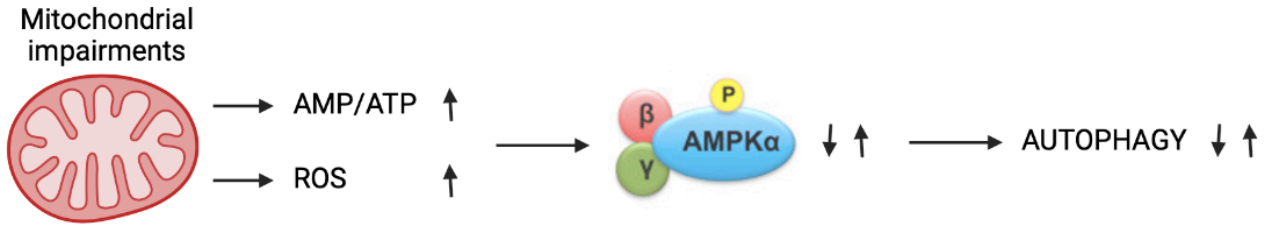


Fig 15: AMPK activity in response to mitochondrial defects. Different mitochondrial impairments may lead to variation in the AMP/ ATP ratio, as well as alteration of the ROS levels. These defects may affect the activity of AMPK, which is likely affected based on the extent of the impairment (acute or chronic), but also on the cell type and on the overall cell homeostatic situation. AMPK, in turn, can modulate downstream processes, including autophagy.

2.2 Crosstalk between Mitochondria and Lysosomes in PD

As previously mentioned, at the subcellular level PD phenotypes include impairments in mitochondrial dynamics and defects in autophagic-lysosomal functionality (108). Moreover, considering the preferential degeneration of dopaminergic neurons observed in PD, this neuronal population seems to be particularly sensitive to mitochondrial dysfunction, and defects in the electron transport chain activity, as well as reduction of mitochondrial dynamics, and increase of ROS production are thought to participate in dopaminergic neuronal loss (108). The first evidence of the strong link between mitochondria dysfunction and PD onset came from studies performed on animal and cellular models in the presence of toxins known to promote PD manifestations in humans, such as the toxin 1-methyl-4-phenyl-1, 2, 3, 6- tetrahydrodropyridine (MPTP) and the pesticide rotenone. By inhibiting complex 1, these molecules affect the mitochondria electron transport chain, indicating that the respiratory activity and especially the functions of complex I are crucially involved in the pathology. Consistently several proteins linked to the genetic forms of PD have a direct or indirect role in mitochondrial dynamics. For instance, the proteins PINK1 and PARKIN, associated with genetic autosomal recessive forms of PD (109), have been demonstrated to play a fundamental role as regulators of the mitochondria quality control and their dysfunctions cause severe mitochondrial impairments and accumulation of dysfunctional mitochondria, due to defects in the clearing activity (108).

As described in the previous sections, autophagy and lysosomal activity have also been frequently linked to PD onset and progression. Accordingly, several PD-related genes are somehow involved in the ALP (76). For example, the *ATP13A2* gene, which is linked to autosomal recessive PD, encodes for a lysosomal enzyme that regulates lysosomal cation homeostasis (109). Similarly, The *VPS35* gene encodes the vacuolar protein sorting ortholog 35 (VPS35), which is related to an autosomal dominant form of PD and is involved in the protein trafficking within the lysosomal and endosomal compartments (76,78). In addition, heterozygous mutations in the *GBA1* gene, which codifies for the hydrolytic lysosomal GCase, are the most common risk factor for developing PD (76,78,109). These examples confirmed that both mitochondria and lysosomal dysfunctions represent prominent factors in the pathogenesis of PD and strongly contribute to neuronal degeneration.

As already emphasized, mitochondria and lysosomes do not function independently but communicate with each other. Therefore, it is not unexpected that the characterization of a number of PD-associated proteins revealed that their activity is not limited to one organelle, but they often manifest pleiotropic effects altering both mitochondria and autophagic homeostasis. For example, even though the silencing of ATP13A2 has been associated with strong changes in lysosomal functions, impairments in this protein activity have been also linked to mitochondrial deficiencies, including the reduction of mitochondrial respiration in human fibroblast, and the increase of mitochondrial mass in SH-SY5Y cells, due to mitochondrial clearance defects (76,78). Along this line, a decrease in GCase enzymatic activity is primarily involved in lysosomal defects, causing the reduction of lysosomal degradation, accumulation of autophagic substrates, and autophagic impairments. However, GCase silencing also leads to compromised mitochondrial activity, with the reduction of membrane potential, impairment of mitochondrial respiration, and mitochondrial fragmentation (78). On the other side, mitochondrial proteins linked to PD have been also associated with lysosomal defects. For instance, it has been shown that PINK1 loss of function leads also to inhibition of the lysosomal activity and enlargement of lysosomal compartments, suggesting that the protein may influence the ALP through different mechanisms. Interestingly, another indication of the strong relationship between mitochondria and lysosomes in PD comes from experimental models of the disease based on rotenone treatment. As aforementioned, this molecule primarily affects mitochondrial function, leading to a reduction in complex I respiration, and this effect is thought to be the major contributor to the PD-inducing activity of this compound. However, exposure of MEF to rotenone demonstrated that this molecule also impacts lysosomal biogenesis and causes alteration of lysosomal genes transcription (78). All these data clearly underly the relevance of communications among organelles and confirm the importance to deeply investigate the molecular pathways involved in the crosstalk between mitochondria and lysosomes in PD pathology. An increased knowledge in this research field may be very relevant not only for a better understanding of cellular physiological processes, but also to understand how defects in one organelle can negatively impact the other with pathological consequences that can lead to human diseases.

Among the proteins linked to the genetic forms of PD that might be exploited to investigate the molecular mechanisms underlying the crosstalk between mitochondria and lysosomes,

DJ-1 appears to be quite interesting. While its function has been mainly associated with the maintenance of mitochondria homeostasis, a few reports suggested its involvement in the regulation of autophagy. Therefore, a clear assessment of the contribution of DJ-1 to ALP and the characterization of the molecular mechanisms underlying the effects of DJ-1 at the mitochondria level and on the autophagic pathway could be helpful not only to understand whether the functions of this protein in different cellular compartments are connected, but also to gain information on the general mechanisms involved in the crosstalk between these organelles (Fig 16).

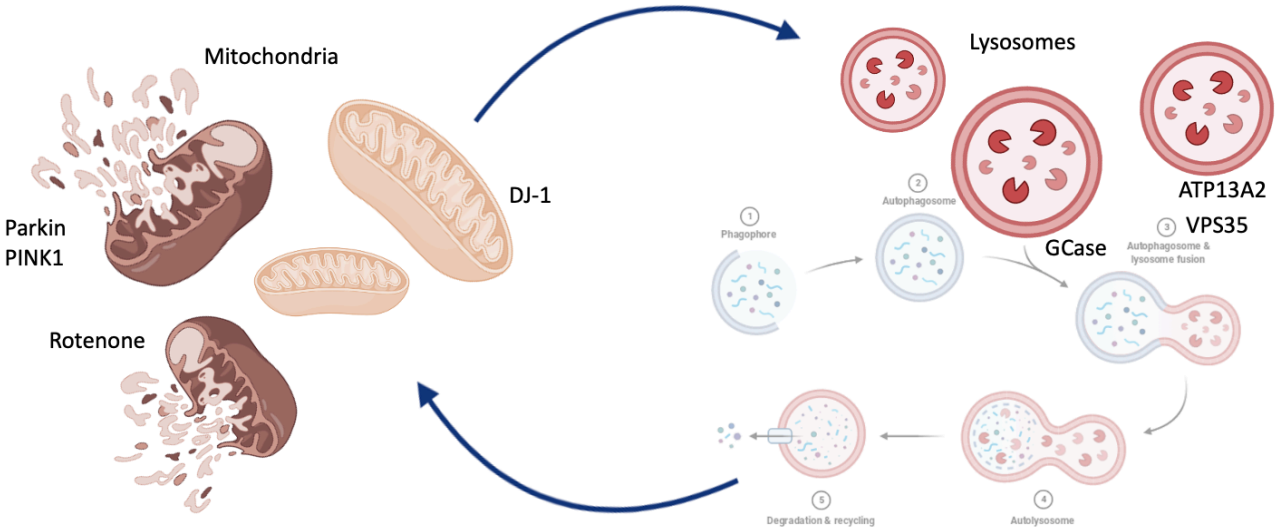


Fig 16: Mitochondria-lysosomes crosstalk in PD.

Chapter 3

Introduction

Crosstalk between Mitochondria and Lysosomes: The role of DJ-1

3 DJ-1: General Features

DJ-1 is a small homodimeric protein whose monomeric units are constituted by 198 amino acids and interact through hydrophobic bonds. The capability to form dimers seems to strongly impact the protein function and its structural stability, as mutations that affect the dimerization have been associated with rapid protein degradation through the proteasome system (110). DJ-1 is highly conserved throughout evolution, and orthologs of DJ-1 with a very conserved structure have been found in eukaryotes, prokaryotes, and plants (110,111). The first characterization of DJ-1 dates back to 1997 when the group of Daisuke Nagakubo identified the protein as a ubiquitously expressed oncoprotein able to transform mice cells alone or in cooperation with other oncogenes (112,113). In agreement with this data, DJ-1 was successively observed to be overexpressed in different types of tumors, including prostate cancer, ovarian carcinoma, breast cancer, and acute leukemia (113). Moreover, the expression levels of DJ-1 have been suggested to correlate with the rate of tumor progression, aggressiveness, and cancer recurrence with increased DJ-1 levels associated with survival reduction of patients with different types of tumors (112). Then in 2003 the group of Prof. Bonifati found out that homozygous mutations in the gene encoding the protein DJ-1, called, for this reason, *PARK7*, were linked to a recessive form of PD, characterized by early onset age and slow progression rate (114). The first two mutations that have been characterized were a 14 kilo base pair deletion that results in the absence of the gene product, and a missense mutation (T497C) leading to the substitution of a leucine with a proline at the residue 166 (L166P). Nowadays, about 20 mutations in the *PARK7* gene have been demonstrated to be linked to PD onset (115). In agreement with their recessive nature, all these mutations lead to the loss-of-function of the protein. Altogether these results highlight how DJ-1's level needs to be finely balanced, since the overexpression of the protein may promote cancer onset, while its deletion or the loss of its function can lead to neurodegeneration.

DJ-1 is ubiquitously expressed and has been found in most body tissues (112). At the subcellular level, DJ-1 is mainly localized in the cytoplasm even though a fraction of the protein has been shown to translocate into the nucleus, upon an increases production of ROS, as in the case of the exposure of cells to 6-hydroxydopamine (6-OHDA), suggesting that DJ-1 may have a role in this cellular compartment (116). Moreover, the mitochondrial

translocation of a fraction of the protein has been observed under stressful conditions, such as the treatment with compounds that increase ROS levels (117). However, the sub-mitochondrial localization of DJ-1 is still poorly defined and contrasting results have been described. In fact, the protein has been observed both in the outer mitochondrial membrane and in the intermembrane space. Recently, a novel and highly sensitive assay to assess proteins localization, called poly-ADP-ribose assisted protein localization assay (PARAPLAY), has been used to analyze DJ-1 localization at the mitochondrial level. With this technique, a fraction of DJ-1 has been observed in the mitochondrial matrix, both in physiological conditions and, to a greater extent, as a consequence of stressors (118). Even though the precise role of DJ-1 and its functional relevance in the different cellular compartments are still under investigation, these data clearly suggest a link between DJ-1 function and mitochondria. The translocation of DJ-1 into the mitochondria upon exposure to stressful conditions might represent a mechanism that allows cells to respond to variations in their homeostatic parameters by modulating the mitochondrial dynamics via DJ-1 activity.

3.1 DJ1: Physiological Functions

DJ-1 appears as a multifunctional protein, whose activity has been associated with several different processes, including cell death, proteasome activity, inflammation, and cell division (reviewed in (119) and in figure 18). Some of the important roles ascribed to DJ-1 that are relevant in the frame of this Ph.D. work will be reported and described in the following sections.

3.1.1 ROS Sensing and Antioxidant Activity

The changes in the subcellular localization of DJ-1 and the increase of DJ-1 protein level in response to the increased of ROS strongly support the hypothesis that DJ-1 activity may be affected by altered concentrations of free radical species (117,120). In addition, this protein has been shown to exert a protective function against oxidative stress, promoting a reduction of ROS in different experimental models, such as SH-SY5Y neuroblastoma cells, and HeLa cells, but also fruit flies and mice. On the contrary, the silencing of the protein causes cell hypersensitivity to oxidative stress, enhanced levels of ROS , and ROS-induced cell death (117,120–124). Noteworthy, DJ-1 antioxidant properties seem to be particularly relevant for dopaminergic neurons, as demonstrated by the protective role exerted by the protein

against ROS-mediated apoptosis in dopamine-producing neurons of rodents (125,126). This data, along with the observations that PD-related mutants of DJ-1 fail to rescue the detrimental effect of excessive ROS, suggest that the antioxidant activity of this protein may represent one of the most important factors that link DJ-1 to PD pathology (120).

One of the characteristic features of DJ-1 that can confer the protein a ROS-sensitive function is the presence of three conserved cysteine residues Cys43, Cys53, and Cys106. These amino acids are highly responsive to variations in oxygen levels and can undergo post-translational modifications due to their reactivity with ROS. In particular, the Cys106, which has been described to be highly relevant for the protective function exerted by the protein, seems to be the most affected by ROS (111). Coherently, the replacement of this residue with serine, alanine, or aspartate abolishes the protective effect of DJ-1 in response to oxidative stress (127). Cys106 can be easily oxidized to form three different species following the consecutive addition of oxygen: cysteine-sulfenic (-SOH), -sulfinic (-SO2 H), and -sulfonic (-SO3H) acid (128). Therefore, this Cysteine residue is thought to be the major contributor to the ROS-sensing ability of DJ-1 (127). More specifically, the sulfinic form has been proposed to be the active form of the protein, which participates in antioxidant functions. On the contrary, when ROS concentrations become too high, the further oxidation of this residue to the sulfonic form results in the destabilization of the protein and loss of its function (129). Importantly, hyperoxidized DJ-1 has been found in idiopathic PD patients' brain tissue, suggesting that there may be a link between excessive oxidation of the protein and the disease (Fig 17) (129).

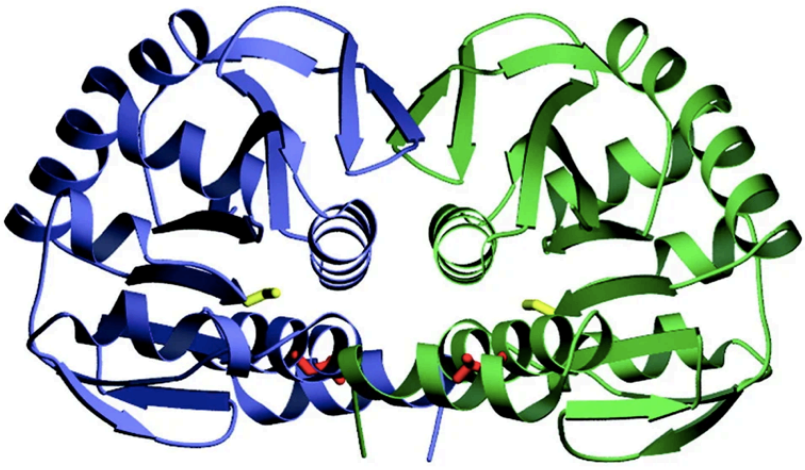


Fig 17: Crystal structure of human DJ-1. the highly conserved Cys106 and Leu166, which is mutated in PD, are highlighted in yellow and red, respectively (129).

Although the molecular mechanisms that confer DJ-1 its antioxidant properties are not clearly defined, it has been shown that the protein can eliminate hydrogen-peroxide *in vitro* and in cells, suggesting a direct scavenging activity of DJ-1, which still need further investigation (120). Moreover, DJ-1 could regulate ROS levels also through the modulation of the activity of some transcription factors. Among them, DJ-1 has been described, in human and mouse cell models, to increase the activity of the nuclear factor erythroid-2-related factor 2 (Nrf2), which is involved in the transcriptional regulation of antioxidant genes, such as the ones encoding heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT) (130). DJ-1 has been also proposed to participate in the antioxidant response through the interaction with the Copper-Zinc Superoxide Dismutase 1 (SOD1), one of the major antioxidant enzymes that converts superoxide anions into oxygen and hydrogen peroxide (131). The enzymatic activity of SOD1 relies on with the presence of two metal ions, Zinc and Copper, at its active site. Importantly, DJ-1 has been shown to bind copper, and several reports have hypothesized the ability of DJ-1 to transfer copper to SOD1, participating in such a way in its maturation. However, *in vivo* experiments performed in our group using *Drosophila melanogaster*, suggest that the activity of DJ-1 is exerted independently from SOD1 and that the two proteins participate in different antioxidant pathways (132).

Overall, these data indicate that DJ-1 participate in the antioxidant response, even though the elusive information regarding DJ-1 activity limits our comprehension of the precise molecular mechanisms adopted by DJ-1 to exert this role.

3.1.2 Mitochondrial Homeostasis

One of the most investigated functions of DJ-1 is its participation in the maintenance of mitochondrial homeostasis. In particular, DJ-1 seems to be involved in sustenance of mitochondrial respiration and prevention of mitochondrial-mediated ROS increase, although the exact mechanisms responsible for these activities are not fully elucidated (133,134). The absence of DJ-1 has also been demonstrated to cause mitochondrial fragmentation and reduction of mitochondrial membrane potential in mouse primary cortical neurons and MEF cells as well as in the human dopaminergic BE(2)M17 neuroblastoma cell line (133,134). Furthermore, DJ-1 has been proposed to directly influence mitochondrial activity. More

specifically, through a co-immunoprecipitation assay performed in HEK cells DJ-1 has been shown to bind the proteins NDUFA4 (NADH-ubiquinone oxidoreductase MLRQ subunit) and ND1 (NADH dehydrogenase 1), two subunits of complex I. Interestingly, the level of interaction increases upon oxidative stress, suggesting that this binding has a functional role in the DJ-1-mediated response to hyper oxidation. Through this interaction, DJ-1 seems to sustain the activity of complex I , as further supported by results obtained in SH-SY5Y cells, where the silencing of DJ-1 has been shown to cause a reduction of about 30% of complex I activity (135,136). Furthermore, in mouse-derived dopaminergic neuronal cells the loss of complex I activity has been associated with defects in complex I assembly, suggesting that DJ-1 may participate in the process of stabilization of the mitochondrial complex I subunits, to guarantee the correct assembly and functionality (137). Besides the binding with complex I, DJ-1 has been recently observed to interact with the β subunit of the mitochondrial ATP synthase to ensure the proper enzymatic activity of this protein (138).

Another proposed role of DJ-1in the control of mitochondrial dynamics is its involvement in mitophagy. In particular, it has been shown that DJ-1 deficiency causes mitophagy impairments in human fibroblasts and human iPSC-derived neurons. Furthermore, this effect has been associated with a possible role of DJ-1 as a downstream effector of the PINK1-PARKIN pathway that leads to mitochondrial degradation. In particular, it has been suggested that DJ-1 translocates to depolarized mitochondria in a PINK1/PARKIN-dependent manner to allow mitophagy to properly occur (139). Accordingly, It has been reported that DJ-1 upregulation protected PINK1 KO cells upon rotenone treatment and that PINK1 or PARKIN overexpression rescued mitochondrial fragmentation in DJ-1- null cells (134). However, there is no complete agreement with these data and whether DJ-1 belongs to the PINK1-PARKIN pathway or whether it acts in parallel to participate in mitochondrial clearance is still an open question. In fact, in another work, the presence of two distinct parallel pathways has been proposed to explain the observation that DJ-1 upregulation protects PINK1 KO cells upon rotenone treatment and that PINK1 or PARKIN overexpression rescues mitochondrial fragmentation in DJ-1-null cells (134).

Even though the literature about the role of DJ-1 in mitochondrial homeostasis is vast, the data mentioned in this section underly that the precise function of the protein at the mitochondrial level is still not completely understood. In this frame, several questions remain

unanswered, regarding the precise sub-mitochondrial localization of DJ-1 as well as the exact molecular pathways that allow DJ-1 to participate in mitochondrial functions.

3.1.3 Autophagy

The multifunctional role of DJ-1 is also reflected in its purported participation in the autophagic pathway. However, although different reports suggested that DJ-1 affects ALP, the pieces of information about the role of this protein at the autophagic level are often inconsistent. More than ten years ago it has been observed that in SH-SY5Y characterized by complex I deficiency, the silencing of DJ-1 reduces the level of the autophagosomal marker LC3 and causes hyperactivation of mTORC1, linking for the first time the activity of DJ-1 to the canonical pathway that modulates autophagy (140). Accordingly, a lysotracker-based assay together with the monitoring of LC3 lipidation confirmed a decreased autophagic activity in the absence of DJ-1 in MEF cells (141). Moreover, the silencing of DJ-1 has been recently shown to impair autophagy in microglia, reducing the clearance of aggregated proteins, including α-syn (142). Despite these data that all together seem to corroborate a correlation between DJ-1 deficiency and reduction in ALP, other research works reported opposite effects. For example, the group of Mark Cookson observed increased LC3 lipidation in DJ-1-null dopaminergic neuroblastoma cell lines, suggesting that the absence of the protein induces autophagy (134). Along the same line, the overexpression of DJ-1 in prostate cancer cells has been observed to inhibit autophagy (143).

These data clearly point out that the precise role of DJ-1 in autophagy needs to be further characterized. The contrasting results might be due to the different models used in the experiments, and it is possible to hypothesize that DJ-1 functions differ in a cell- or tissue-dependent manner. Another important consideration is that autophagy is a highly complex process and monitoring LC3 lipidation is not sufficient to clearly assess the direction of autophagic flux, so that other experimental parameters need to be taken into consideration to clarify the involvement of DJ-1 in the autophagic pathway. It is worth mentioning that also the molecular mechanisms associated with the modulation of the autophagic flux by DJ-1 activity are still unknown. In this frame, it has been suggested that the autophagic alterations caused by DJ-1 represent a secondary effect that derives from DJ-1-dependent variations

of ROS levels (144). This appealing hypothesis needs to be confirmed in order to understand whether and how DJ-1 activity impacts ALP.

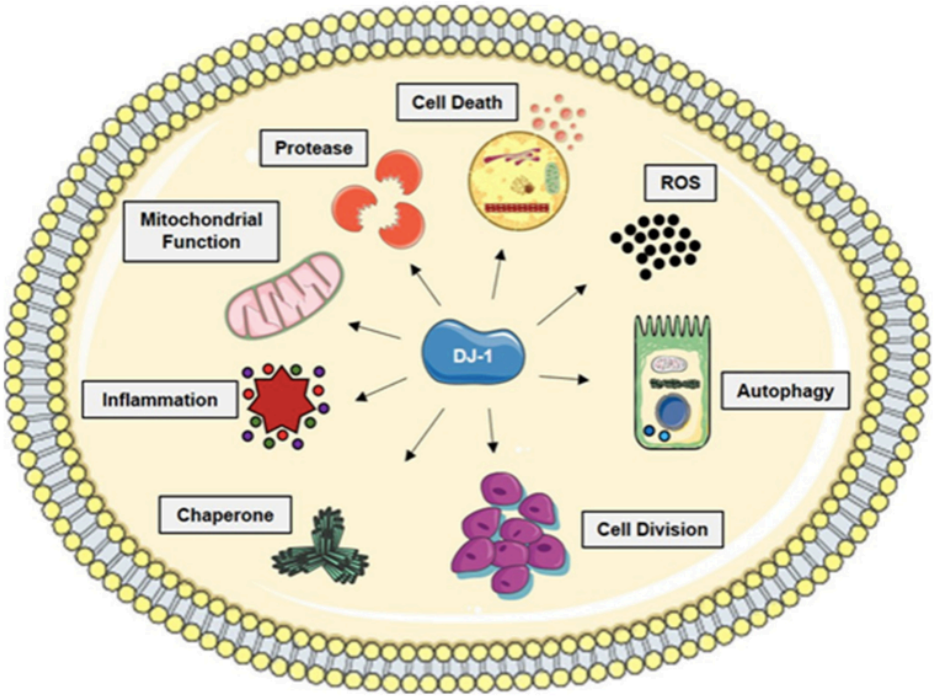


Fig 18: purported involvement of DJ-1 in different cell processes (119).

Chapter 4

Aim of the Project

4 Aim of the Project

The maintaining of a finely tuned autophagic activity is crucial to preserve cell homeostasis. This is particularly relevant in neurons, whose survival depends on a tight proteins and organelles quality control. Indeed, the contribution of autophagic dysfunction in neurodegeneration is widely accepted (59,145). In this frame, the detailed characterization of autophagy in neurodegenerative diseases and the analysis of the pathway of regulation of this process represent a highly investigated, but still open research field. Importantly, in the last few years it has emerged that the regulation of ALP may also occur as an effect of alterations in other cellular processes. In this regard, it is now clear that autophagic activity can be affected by mitochondrial function (78,89).

Noteworthy, neurodegenerative diseases, such as PD, are frequently determined by a broad pattern of defects, that leads to impairments at the level of different cellular compartments and are often characterized by mitochondrial function alterations and autophagic activity impairments (76,108). Therefore, the investigation of the pathways that link the activity of mitochondria and lysosomes may be of great relevance in the context of neurodegenerative disorders. The understanding of how mitochondria and lysosomes influence each other activity could shed new light on the molecular pathways involved in neurodegeneration, and it may eventually help in finding new targets to beneficially intervene in the modulation of both organelles' activity. As mentioned in the introduction, the most established mechanisms of mitochondria-lysosomes interaction regard the direct contact between these organelles and the lysosomal-mediated degradation of mitochondria through mitophagy (91,92).

However, it has been recently demonstrated that a long-distance communication between mitochondria and the ALP can be achieved through the activation of specific signaling cascades. Among them, it has been shown that the modulation of the AMPK pathway can ensure the crosstalk from mitochondria to the lysosomes (89,93).

The study of DJ-1 activity may provide a good opportunity to analyze the involvement of autophagy in PD and to study whether mitochondrial functions influence basal autophagy in neurodegeneration. Accordingly, DJ-1 is a multifunctional protein whose activity has been mainly associated with the regulation of mitochondrial dynamics and protection against excessive ROS; nevertheless, the protein has also been linked to autophagic alterations,

pointing to DJ-1 as a possible good target to investigate the mechanisms of communication between mitochondria and the autophagy-lysosomal machinery (146).

In light of these considerations, this Ph.D. project was focused on the analysis of DJ-1 function, exploiting *Drosophila melanogaster* for the *in vivo* characterization of the protein activity, and a human neuronal-derived induced pluripotent stem cells (iPSC) model.

The first aim of this work was to confirm that DJ-1 participates in the regulation of ALP and investigate in detail how the protein affects the autophagic machinery.

After confirming the involvement of DJ-1 in autophagy regulation, the second aim of this project was to assess whether the mitochondrial dysfunctions caused by the absence of DJ-1 may represent one of the stimuli contributing to the alteration of the autophagic process. In this frame, we investigated whether DJ-1 is involved the modulation of the AMPK signaling pathway that links mitochondrial and lysosomal functions.

Chapter 5

Results

Drosophila Melanogaster

5 Results

As previously described, DJ-1 activity has been linked to the regulation of mitochondrial dynamics, the maintenance of stable ROS levels, and the modulation of autophagic activity. Despite years of intensive research on this protein, its specific function and the underlying mechanism of action remain unclear. Some of the obstacles to the precise understanding of the physiological functions of DJ-1 could be represented by the multifunctional nature of the protein, which likely participates in several molecular pathways and has multiple subcellular localizations.

Our lab is currently characterizing the effects of DJ-1 deficiency *in vivo* exploiting *Drosophila melanogaster*. Fruit flies represent a very useful model organism not only for the analysis of this protein but, in general, for studies related to neurodegenerative processes. Indeed, many genes and molecular pathways associated with neuronal physiology are highly conserved between flies and humans. In addition, flies can be easily genetically manipulated to generate relevant mutant lines or express useful exogenous constructs.

The fruit fly genome encodes two *DJ-1* orthologues, *dj-1α* and *dj-1β*. While *dj-1α* is mainly expressed in male testes, the *dj-1β* pattern of expression is ubiquitous. At the protein level, dj-1β shares a high degree of conservation with the human ortholog (70% of similarity). Furthermore, likewise the human counterpart, dj-1β seems to be primarily involved in antioxidant response and regulation of mitochondrial homeostasis. Accordingly, we have demonstrated that *dj-1β KO* flies are characterized by mitochondrial defects at the ultrastructural level and reduction of complex 1 activity (Fig 19 and Fig 20) (146). Starting from these observations, my work focused on the analysis of dj-1β physiological function using both *dj-1β* knock-out (KO) and overexpressing (OE) flies.

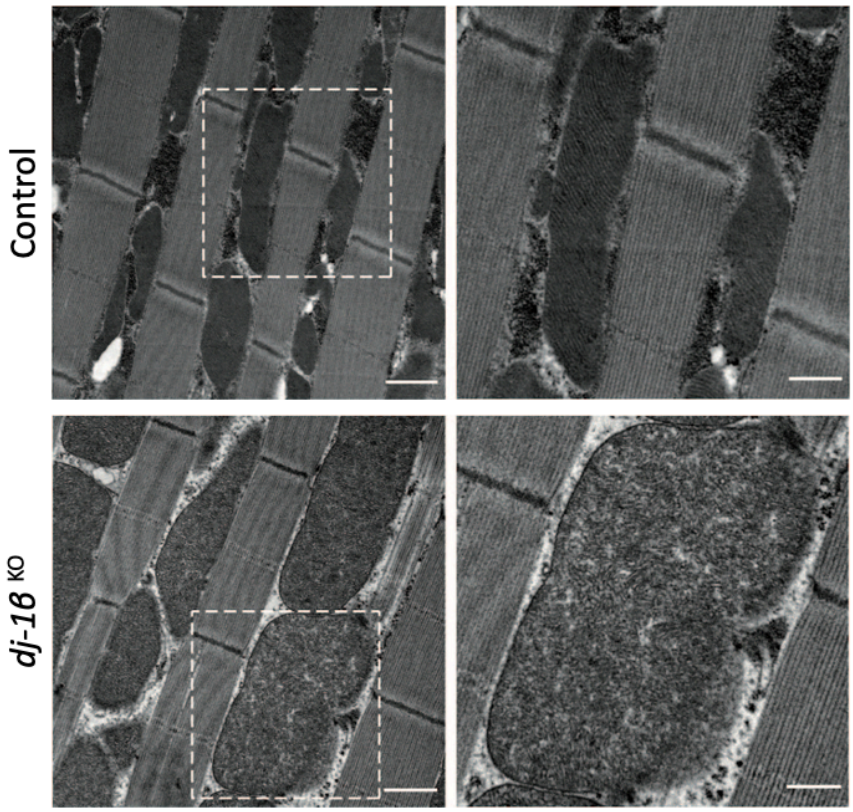


Fig 19: Electron microscopy images representing thoracic muscle mitochondria, left panel (scale bar: 1 µm). Magnified pictures, right panel (scale bar: 500 nm). Differences in mitochondrial dimension and morphology, as well as in the organization of mitochondrial cristae are clearly visible from these images. (Adapted from (146))

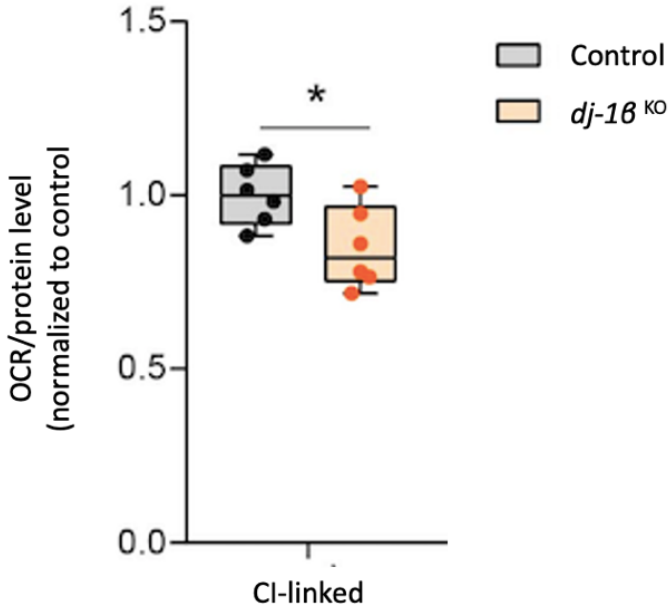


Fig 20: The oxygen consumption rate (OCR) of complex I shows a decrease in complex I activity in *DJ-1β*^{KO} flies. (Adapted from(146))

In light of the involvement of DJ-1 in Parkinson's disease, we were particularly interested in the role of the protein at the brain level and in neuronal physiology. Therefore, whenever possible, we performed experiments using fly brains.

In addition to the fly model, we also deepened these aspects in a very relevant model of the pathology, represented by human induced pluripotent stem cells (iPSC) carrying a loss-of-function base pair deletion in the PARK7 gene, which encodes the DJ-1 protein. Because of the possibility to differentiate iPSC in dopaminergic neurons, these cells represent an extremely valuable tool to study the molecular mechanisms associated with DJ-1 activity, allowing us to compare and validate the data obtained in flies in a human model.

5.1 DJ-1 Alters the Autophagic Degradation in *Drosophila Melanogaster*

Cell degradation processes allow the clearance of cell debris, such as old and damaged organelles and dysfunctional or misfolded proteins. By analyzing the level of degradation substrates, it is possible to get a preliminary indication of the clearance activity of a biological system. In this frame, ubiquitin is a small protein that can covalently bind to other proteins to target them to degradation (147). Consequently, the level of ubiquitin and ubiquitinated proteins varies according to the rate of their breakdown. The immunoblot analysis using an anti-ubiquitin antibody allows the detection of a signal derived from all the proteins that are post-transcriptionally modified following the binding with ubiquitin. In a first series of experiments, we collected lysates from whole-body *dj-1β* mutant and wild-type (WT) control flies and evaluated the level of these clearance substrates. The result shown in Fig 21a demonstrates that *dj-1β* KO flies are characterized by a higher level of ubiquitin and ubiquitinated proteins compared to control flies. This data may suggest that the clearance processes are impaired upon *dj-1β* loss-of-function, resulting in the accumulation of proteins that are not correctly degraded. Although interesting, this data does not give a precise information on autophagic clearance; indeed, ubiquitinated proteins may be degraded both through the autophagic process and through the proteasomal system (147). To rule out the possibility that the data obtained may be completely dependent on proteasomal degradation defects, we analyzed the amount of the autophagic substrate p62, a conserved protein that acts as a specific autophagic adaptor (148). Like ubiquitin, the western blot analysis of p62 revealed increased levels of this protein in *dj-1β* null flies, confirming that the clearance activity may be affected and suggesting that autophagy is altered upon *dj-1β* absence (Fig 21b). Considering our interests in the role of DJ-1 in the brain, we repeated similar experiments using cell lysates from fly heads. Also in this body region, we obtained comparable results (Fig 22a-b).

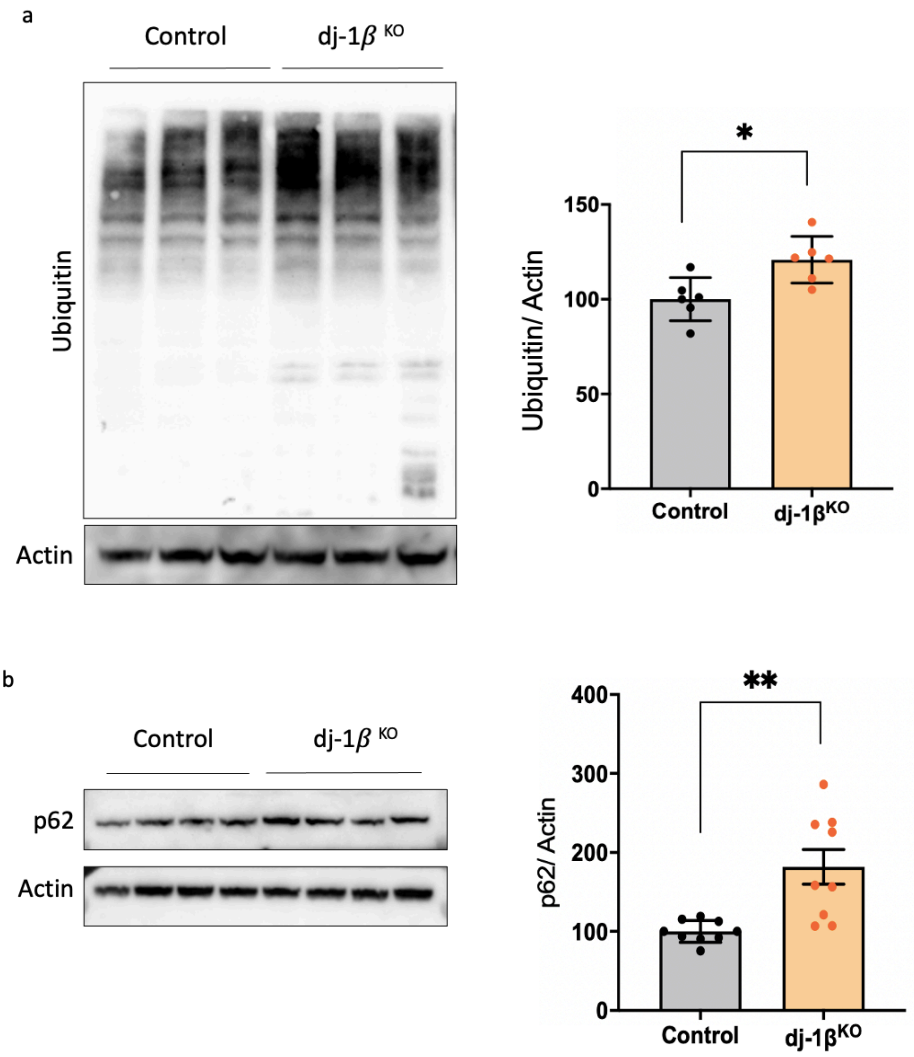


Fig 21: Western blot analysis for the clearance activity evaluation. Representative images and quantifications relative to the immunoblots of (a) Ubiquitin and (b) p62 levels in control and *dj-1β*^{KO} flies. Samples were obtained from whole-body fly lysates. Actin was used as a loading control. At least 6 biological replicates were analyzed per genotype. Data were analyzed with t-test, * $p<0.05$, ** $p < 0.01$.

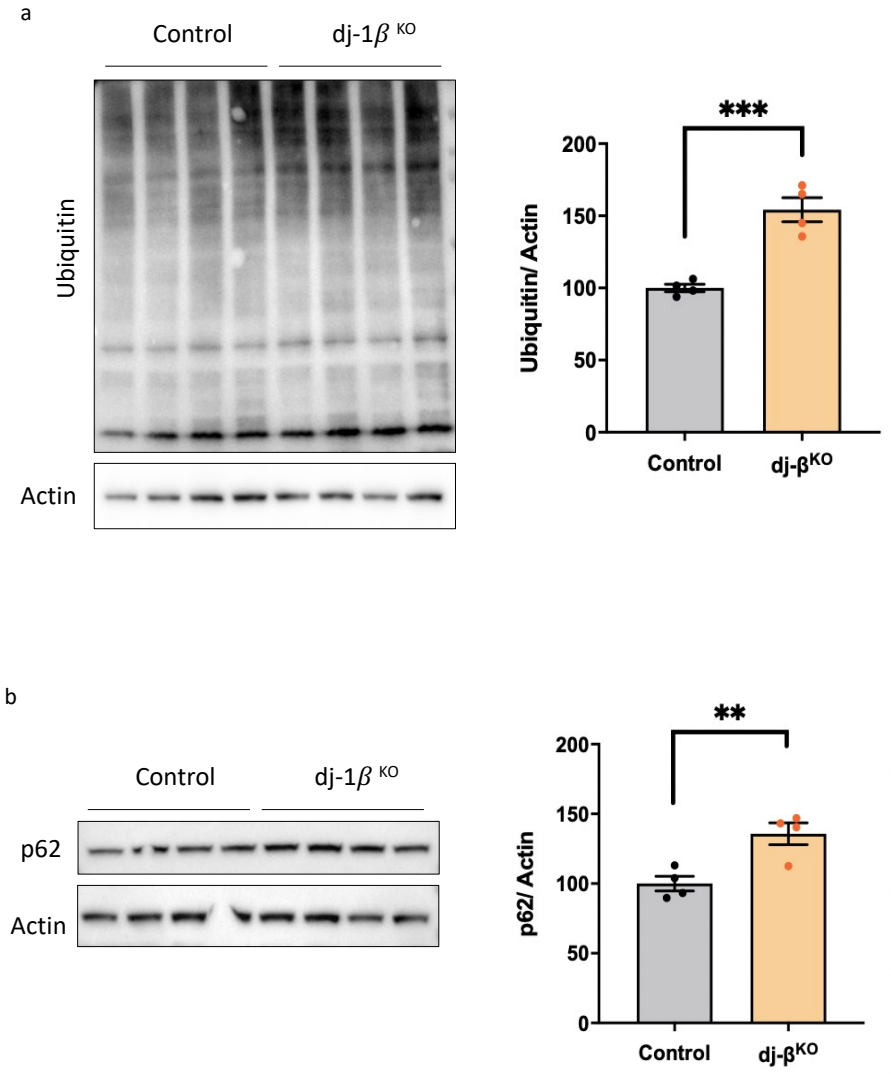


Fig 22: Western blot analysis for the clearance activity evaluation. Representative images and quantifications relative to the immunoblots of (a) Ubiquitin and (b) p62 levels in control and *dj-1β*^{KO} flies. Samples were obtained from fly heads. Actin was used as the loading control. 4 biological replicates were analyzed per genotype. Data were analyzed using t-test, * $p<0.05$, ** $p < 0.01$.

5.2 The Absence of DJ-1 Modulates the Number of Autophagic-Related Vesicles

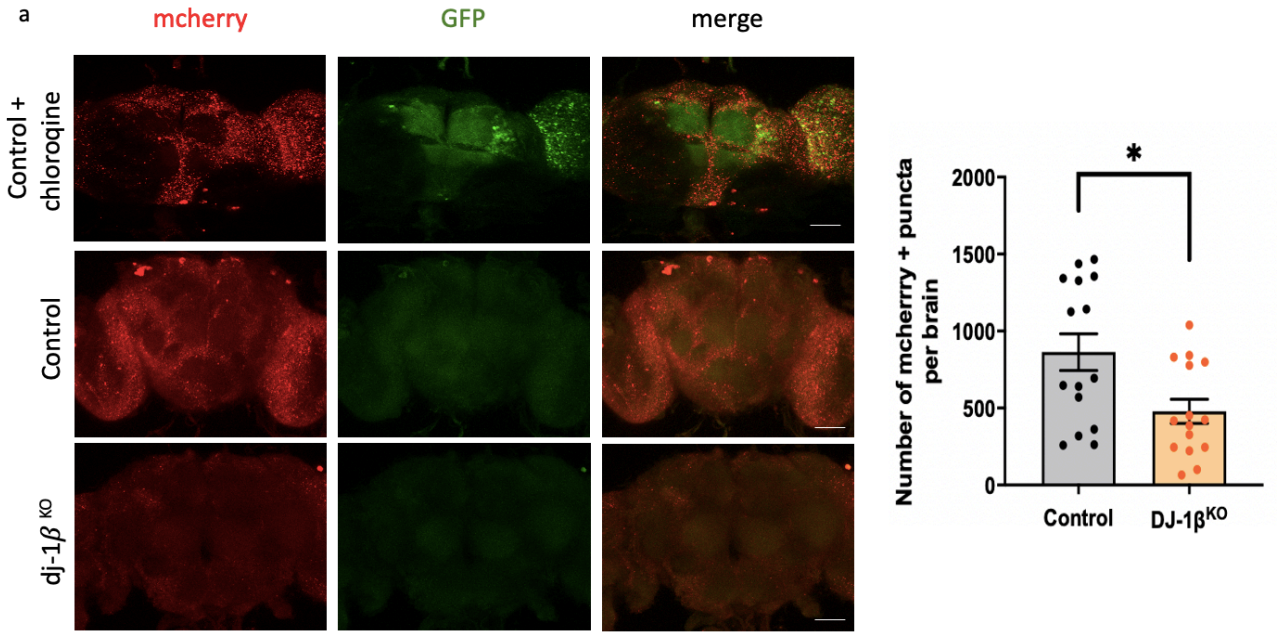
The data presented in the previous section link DJ-1 level to reduced degradative activity in *D. melanogaster,* which may depend on an alteration of autophagy. One of the parameters to evaluate the efficiency of this process is the analysis of the autophagic flux, which is defined by the ratio of autophagosomes-lysosomes fusion and measures the speed of autophagic degradation. To analyze the autophagic flux in flies we exploited a *Drosophila*

line overexpressing the Atg8-GFP-mcherry construct. This autophagy reporter is constituted by the autophagosomal membrane protein Atg8 (LC3 ortholog) tagged with GFP and mCherry. This construct allows the visualization of autophagosomes and autolysosomes independently. Indeed, when Atg8 is localized at the autophagosomal membrane, mcherry and GFP emit red and green fluorescence respectively, producing a yellow signal. In contrast, when autophagosomes fuse with lysosomes, the acidic pH of the autolysosomes quenches the GFP fluorescence, generating a mCherry-red signal. By calculating the ratio between autolysosomes (red) and autophagosomes (yellow) it is possible to evaluate the autophagic flux. We overexpressed the Atg8-GFP-mCherry construct in control and *dj-1β* KO flies and assessed the autophagic flux in fly brains. Importantly, to rule out the possibility that defects in the construct may affect the outcome of the experiment, we exposed control flies to chloroquine (CQ), a compound that inhibits autophagosome-lysosome fusion by increasing the lysosomal pH. In the brain of treated flies, autophagosomes are clearly visible, demonstrating that the construct works as expected (Fig 23a). Then, we compared the Atg8-GFP-mcherry-signal between wild-type and *dj-1β* null flies. Interestingly, we could not detect any autophagosome in controls nor in mutant flies as no GFP-positive structures are visible (Fig 23a). This result may be determined by the fact that autophagic flux in the brain proceeds at a high rate in both genotypes and autophagosomes immediately fuse with lysosomes as soon as they are produced. Overall, these data suggest that there are no differences in the autophagic flux between the two genotypes. However, we observed a significant decrease in autolysosomes in *dj-1β* KO flies compared to controls, as indicated by the reduction of red structures (Fig 23a), suggesting that autophagy is somehow affected by the absence of DJ-1.

To have a more detailed picture of the autophagic process in *dj-1β* KO flies we then evaluated whether other autophagic vesicles are affected by the absence of the protein. To this aim, we analyzed the number of acidic compartments in fly brains, by staining tissues with the pH-sensitive dye Lysotracker red. Since acidic structures comprise both lysosomes and autolysosomes, the evaluation of these cell compartments may be highly informative to get better insights into the autophagic machinery. Interestingly, in basal conditions, we detected an abnormal lysotracker staining in *dj-1β* KO flies, characterized by numerous enlarged lysotracker-positive structures (Fig 23b). Considering that *dj-1β* mutant flies showed a reduction of autolysosomes, we can assume that the increase of the lysotracker-

derived signal is determined by an enhanced number of lysosomes. To increase our ability to interpret these data, we decided to assess how the number of lysosomes varies upon autophagic induction. Therefore, we performed the lysotracker staining in flies subjected to starvation. Interestingly, we observed that while in control flies the number of lysotracker-positive structures remained low upon autophagy induction, *dj-1β* KO flies responded to starvation by further increasing the number of lysosomes in the brain (Fig 23b).

Overall, these results demonstrate that the autophagic process is affected by the loss of function of dj-1β, that causes an increase of lysosomes and a reduction of autolysosomes. Importantly, the increased number of lysosomes upon starvation suggests that the absence of dj-1β may impair the turnover of these structures. Indeed, the induction of autophagy should fasten the lysosomal degradative activity. Conversely, the accumulation of these organelles observed in *dj-1β* KO fly brains after starvation, indicates that lysosomes accumulate, likely due to defects in the clearance of their cargoes and decrease in the rate of turnover of these organelles.



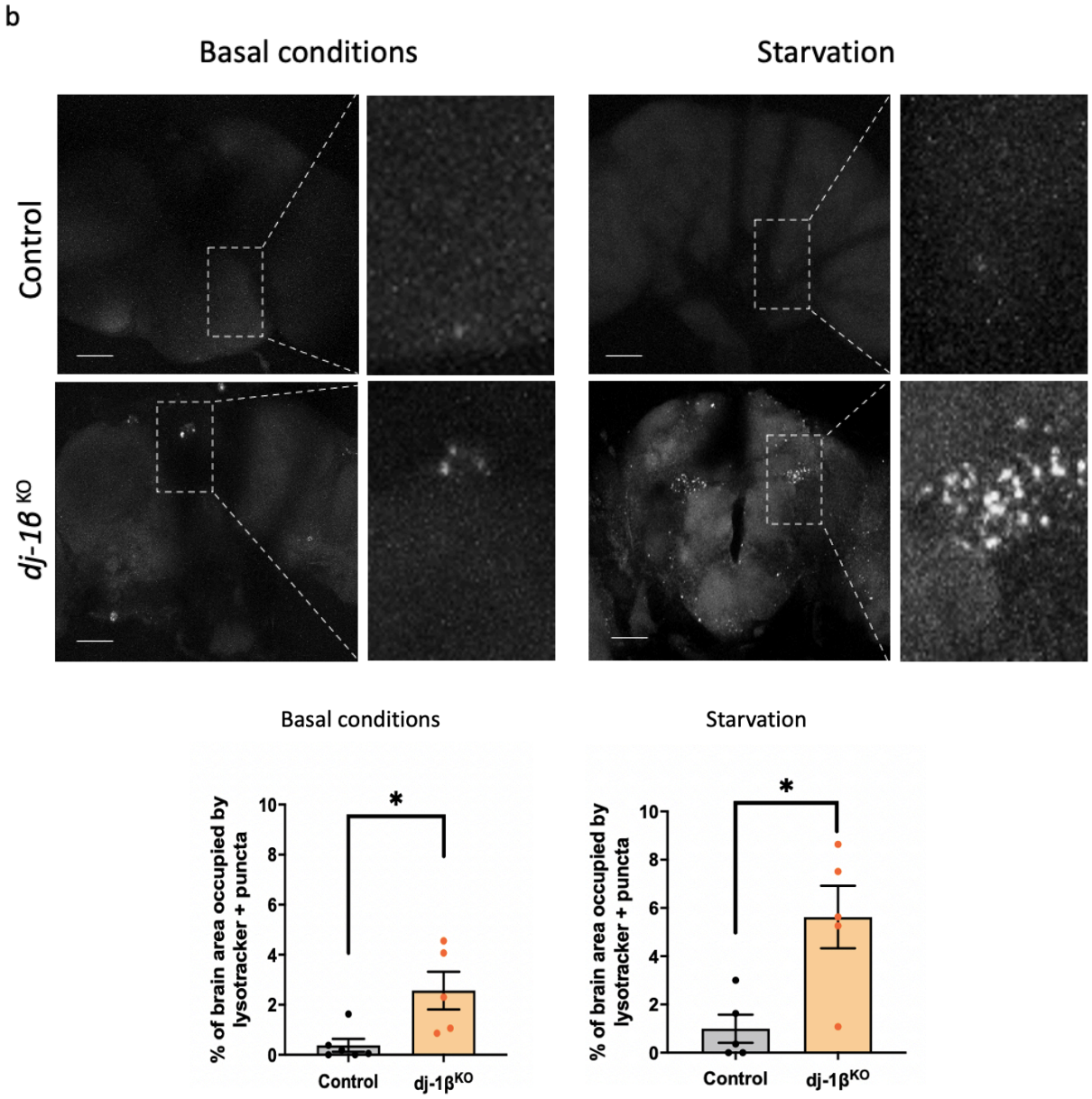


Fig 23: (a) Representative images (scale bar 50 μm) of fly brains expressing the Atg8-GFP-mcherry autophagy reporter. The upper panel is relative to control flies treated with 10 mM of CQ for 34 hours. The quantification is relative to the amount of mCherry signal, corresponding to autolysosomes. Fifteen fly brains per genotype were analyzed. Data have been compared using the t-test. * $p < 0.05$. (b) Pseudocolor representative images (scale bar: 50 μm) and relative quantification of *Drosophila* brains stained with the LysoTracker Red probe under basal conditions and after 3 hours of starvation. Five Drosophila brains per genotype were analyzed. Data have been compared with t-test. * $p<0.05$.

5.3 DJ-1 Affects Lysosomal Activity in *Drosophila Melanogaster*

The impairment of the degradative activity and the variation in the number of autophagic structures described above may depend on the alteration of lysosomal function. Indeed, lysosomes are the sites where degradation occurs, and impairments of their activity may lead to clearance defects and accumulation of autophagic substrates. To test this hypothesis, we carried out two independent experiments to evaluate the activity of lysosomal proteins. We first performed the DQ-BSA assay. This experiment allows the evaluation of lysosomal proteases activity through imaging techniques, giving us the possibility to specifically focus on the brain. This assay relies on the exploitation of a protease substrate, that emits fluorescence when it gets cleaved by proteolytic enzymes. Therefore, the fluorescence intensity after exposure to the substrate directly correlates with the degradation activity. By exploiting this technique, we observed that mutant flies are characterized by a decreased activity of lysosomal proteases in the brain compared to control animals, confirming an impairment of degradative functions caused by the absence of dj-1β (Fig 24a).

Then, to have independent confirmation of the DJ-1-mediated lysosomal impairment, we performed an enzymatic assay to assess the function of the lysosomal hydrolytic enzyme GCase. GCase catalyzes the breakdown of glucocerebroside into ceramide and glucose (149). Importantly, this protein is linked to PD, as heterozygous mutations in the *GBA1* gene are the most common risk factor for the development of the pathology. For this reason, the analysis of this protein activity may not only be used as an approximation of lysosomal function but is also relevant in the context of PD. The enzymatic assay showed that the activity of GCase in whole fly body lysates is significantly decreased upon dj-1β absence. Importantly, we clearly linked the GCase function dj-1β, as its activity is completely restored when dj-1β is overexpressed (Fig 24b).

The same assay was performed using lysates from WT and *dj-1β* KO fly heads, and we confirmed a reduction of GCase activity (Fig 24c), suggesting that brains are also affected by the dj-1β-linked reduction of GCase activity.

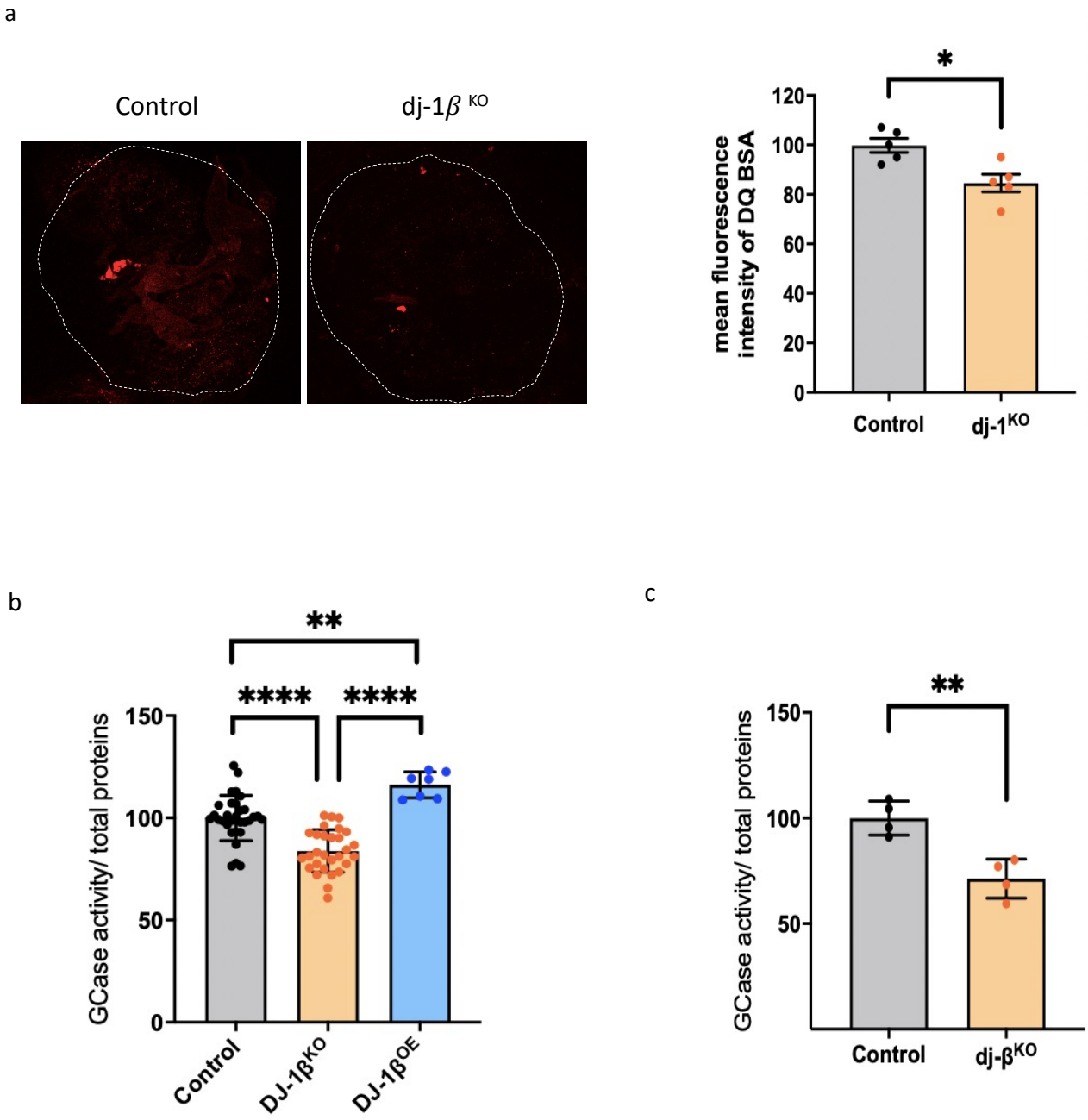


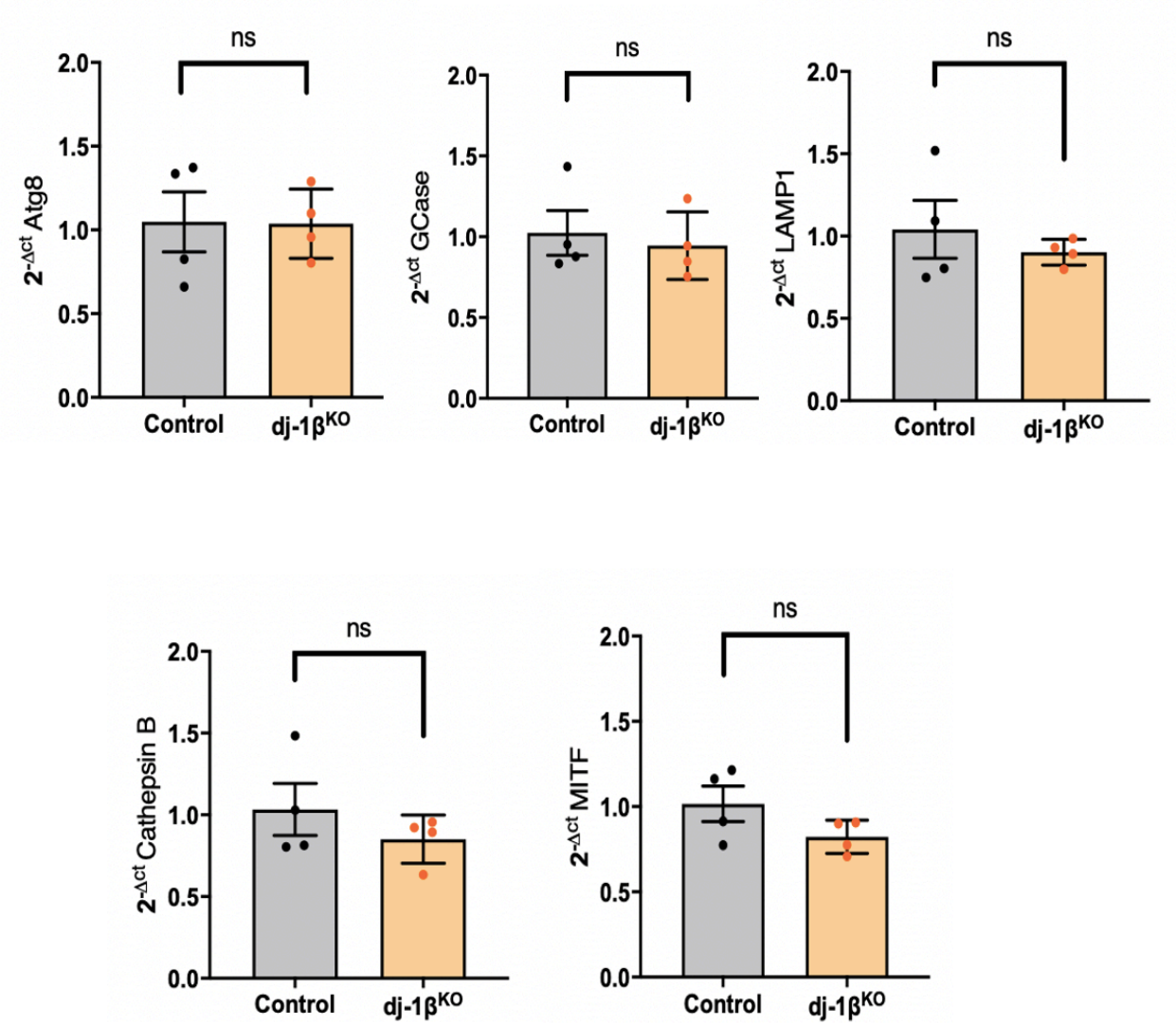
Fig 24: assessment of lysosomal activity in *dj-1β* KO and control flies. (a) Representative images (scale bar 50 μm) and quantification of brains stained with red DQ-BSA for the evaluation of lysosomal proteases. The assay was performed on 5 brains per genotype. Data were analyzed using t-test. * $p < 0.05$. Enzymatic assay for the activity of GCase performed in lysates obtained from fly whole bodies (b) and from fly heads (c). The assay was performed at least on 4 biological replicates per genotype. Data were analyzed with one-way ANOVA with Turkey's multiple comparisons test (a) and t-test (b). ** $p < 0.01$, **** $p < 0.0001$.

5.4 Assessing the Transcriptional Activity of TFEB

One of the possible explanations for the reduction of autolysosomes and the impairment of lysosomal activity may be found in the alteration of autophagic induction. For this reason, we investigated whether the silencing of *dj-1β* affects the transcriptional activity of Mitf, the *Drosophila* ortholog of TFEB. As mentioned in the introduction, TFEB is considered the master regulation of autophagy at the transcriptional level since it enhances the expression of several genes involved in the autophagic process (150). To assess Mitf transcriptional function we performed real-time (RT) quantitative (q) PCR on whole-body mRNA extracted from WT and *dj-1β* KO flies, evaluating the levels of genes that are transcriptionally controlled by Mitf. The PCR showed no significant differences in the mRNA levels of these genes between the two genotypes, although a general trend toward a reduction of the analyzed transcripts was observed in mutant flies compared to controls (Fig 25a). This result suggests that TFEB activity may be mildly affected by the level of dj-1β but it is likely that other mechanisms participate in the dj-1β-dependent regulation of autophagy. In this regard, more investigations are necessary to understand in detail the molecular pathways that allow DJ-1 to participate in ALP modulation. Noteworthy, the RT qPCR performed on mRNA samples extracted from fly heads revealed no differences in the levels of genes regulated by TFEB (Fig 25b), further confirming the necessity to look for other mechanisms involved in the regulation of autophagy.

Overall, the data presented so far indicate that autophagy is affected by altering the level of DJ-1. More specifically, as represented in Fig 26, the loss of DJ-1 might cause impairments at different levels of the autophagic process

a


ns
2.0
1.5
1.0
0.5
0.0
2-Δct Atg8
Control
dj-1βKO
ns
2-Δct GCase
Control
dj-1βKO
ns
2-Δct LAMP1
Control
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ns
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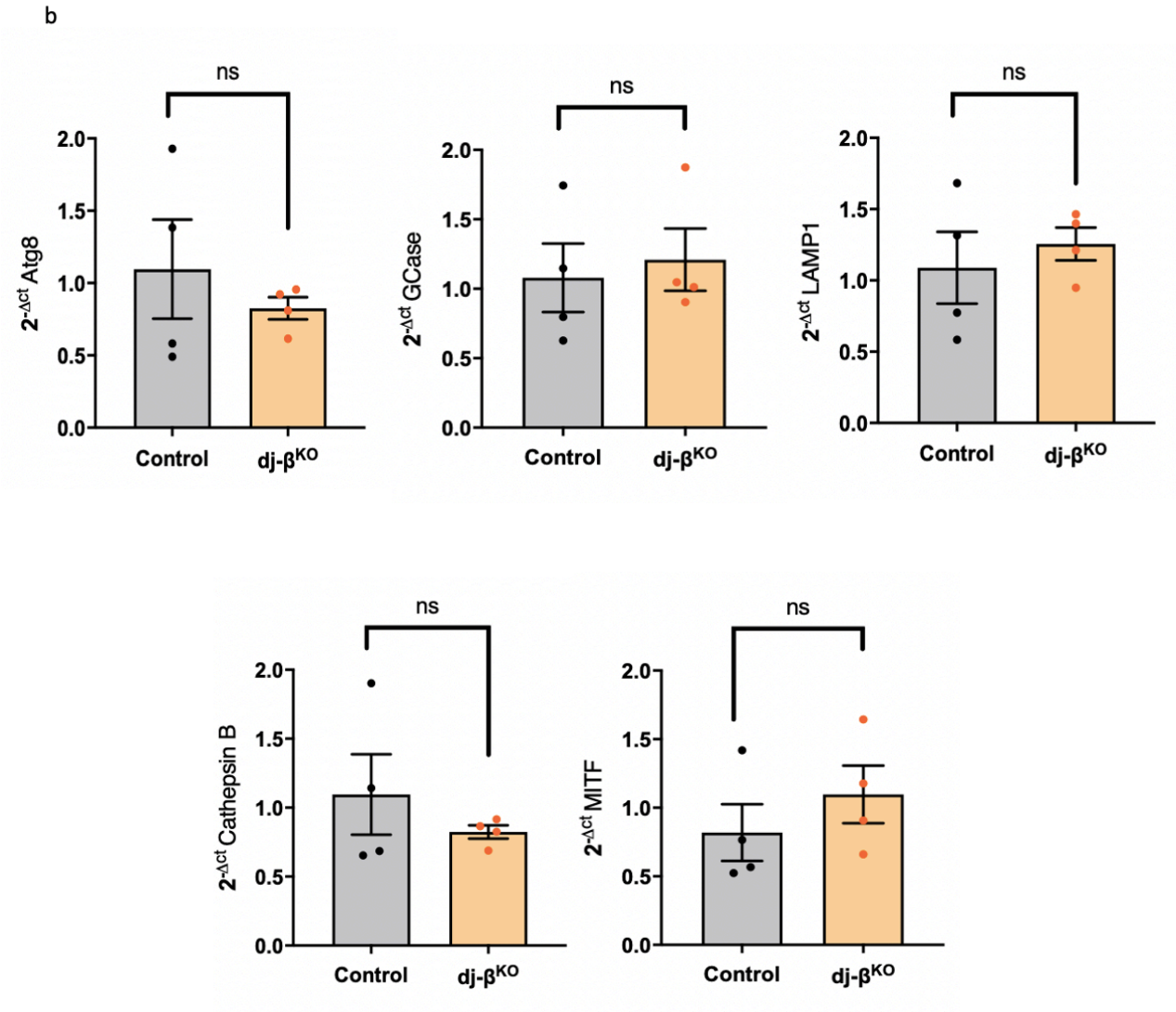


Fig 25: RT qPCR analysis relative to a number of genes regulated by Mitf (TFEB). Analyses were performed using RNA extracted from the whole body (a) and fly heads (b). For each genotype, 4 biological replicates were analyzed. Data were compared with t-test ns = not-significant.

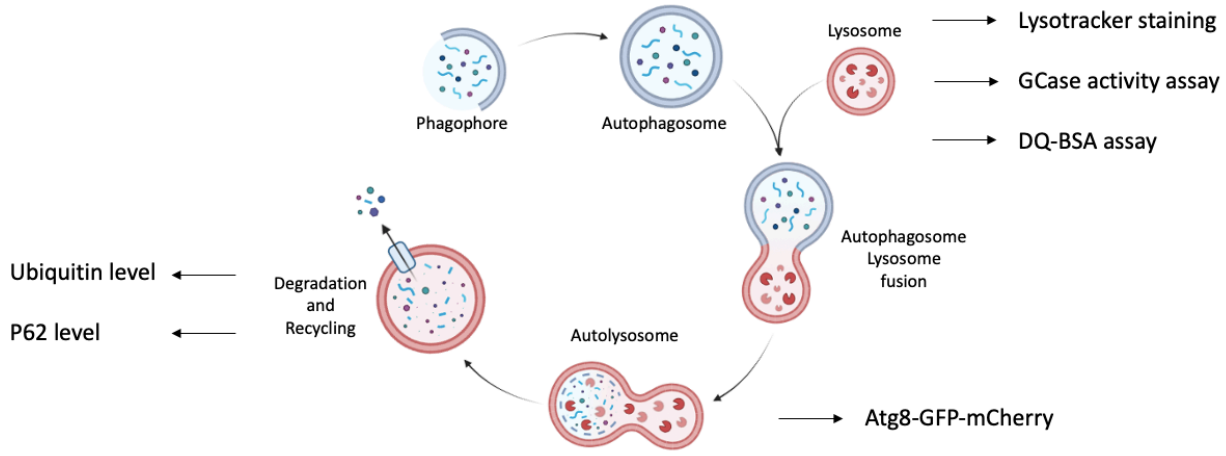
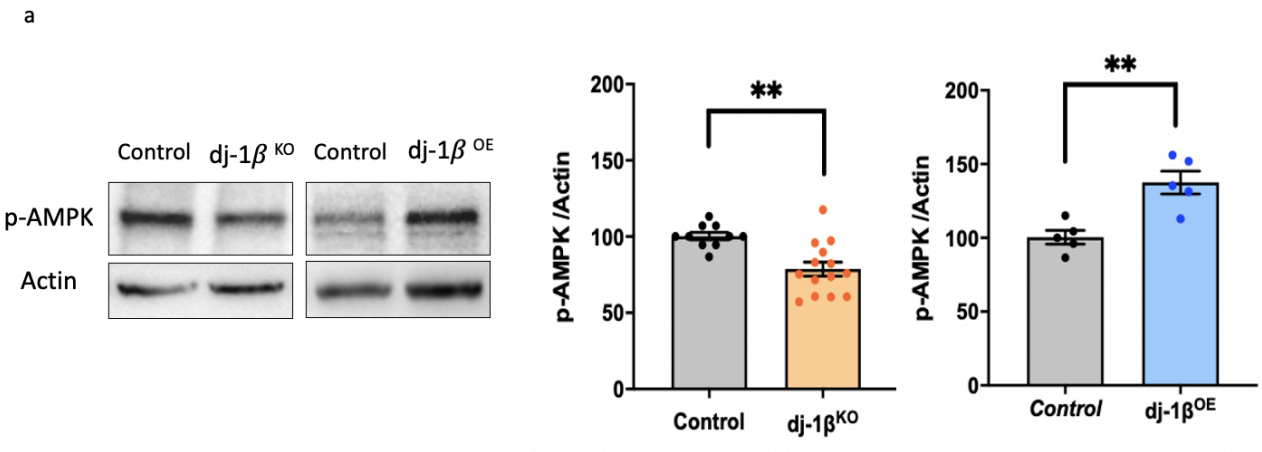


Fig 26: Schematic representation of the autophagic process. Arrows highlight the experiments that allowed the detection of impairments at the different levels of the autophagic machinery.

5.5 DJ-1 Affects the Activity of AMPK

Most of the data available in the literature about DJ-1 point to its role as a modulator of mitochondrial dynamics and ROS homeostasis. Considering the tight link between mitochondria and autophagy-lysosomal function, we hypothesized that a possible explanation for the observed ALP dysfunctional phenotypes in *dj-1β* KO flies is that they represent secondary effects determined by DJ-1-mediated mitochondria alterations. Considering that the protein AMPK has been recently described as a mediator in the communication between mitochondria and lysosomes (93), we decided to assess whether AMPK could modulate its activity in response to the variation of DJ-1 levels and participate in the regulation of the autophagic machinery. Most specifically, we evaluate the activity of AMPK by analyzing the level of its phosphorylation at the Thr172 residue, which is well known to correlate to the activation of the protein. The result obtained from whole-body protein lysates demonstrated a reduction of the active form of AMPK in *dj-1β* null flies. Importantly, this effect was completely reverted upon *dj-1β* overexpression, demonstrating the link between AMPK activity and dj-1β level (Fig 27a). The differences observed in the level of phospho-AMPK may be explained either by a direct inhibition of the protein or by a general decrease in the total protein amount. Unfortunately, there are no total-AMPK antibodies available for drosophila; so, to assess whether dj-1β alters the transcription levels

of the protein, we evaluated the mRNA expression of AMPK. We did not detect any difference in the AMPK mRNA level, suggesting that the decrease of phospho-AMPK is caused by a reduction of protein activation (Fig 27b). To address whether the AMPK activity plays a role in the modulation of the autophagic pathway, we generated a fly strain overexpressing a constitutively active form of AMPK (AMPK CA) in a *dj-1β* KO background. Taking advantage of this mutant fly line, we observed the effect of AMPK CA overexpression on our most strong and confirmed dj-1β-associated phenotype, the GCase activity. Interestingly, the exogenous activation of AMPK completely rescued the GCase impairment in *dj-1β* KO flies (Fig 27c). Overall, these data suggest that the absence of dj-1β causes a reduction of AMPK phosphorylation, leading to ALP alteration. Noteworthy, the overexpression of the active form of AMPK appears to be beneficial by reverting the lysosomal impairments observed in *dj-1β* null flies. After the confirmation of the link between DJ-1 and AMPK, we assessed whether the functional correlation between these two proteins is relevant also in the brain. To this aim, we evaluated the level of phospho-AMPK in fly-heads lysates obtained from *dj-1β* mutant and control flies. This analysis revealed that, in contrast with what was observed in the whole body, the absence of dj-1β in heads leads to the hyperactivation of AMPK (Fig 27d). This unexpected result suggests that DJ-1-mediated alteration of AMPK function is not ubiquitously achieved in every tissue or that the effect of DJ-1 on AMPK phosphorylation is tissue- or cell-specific.



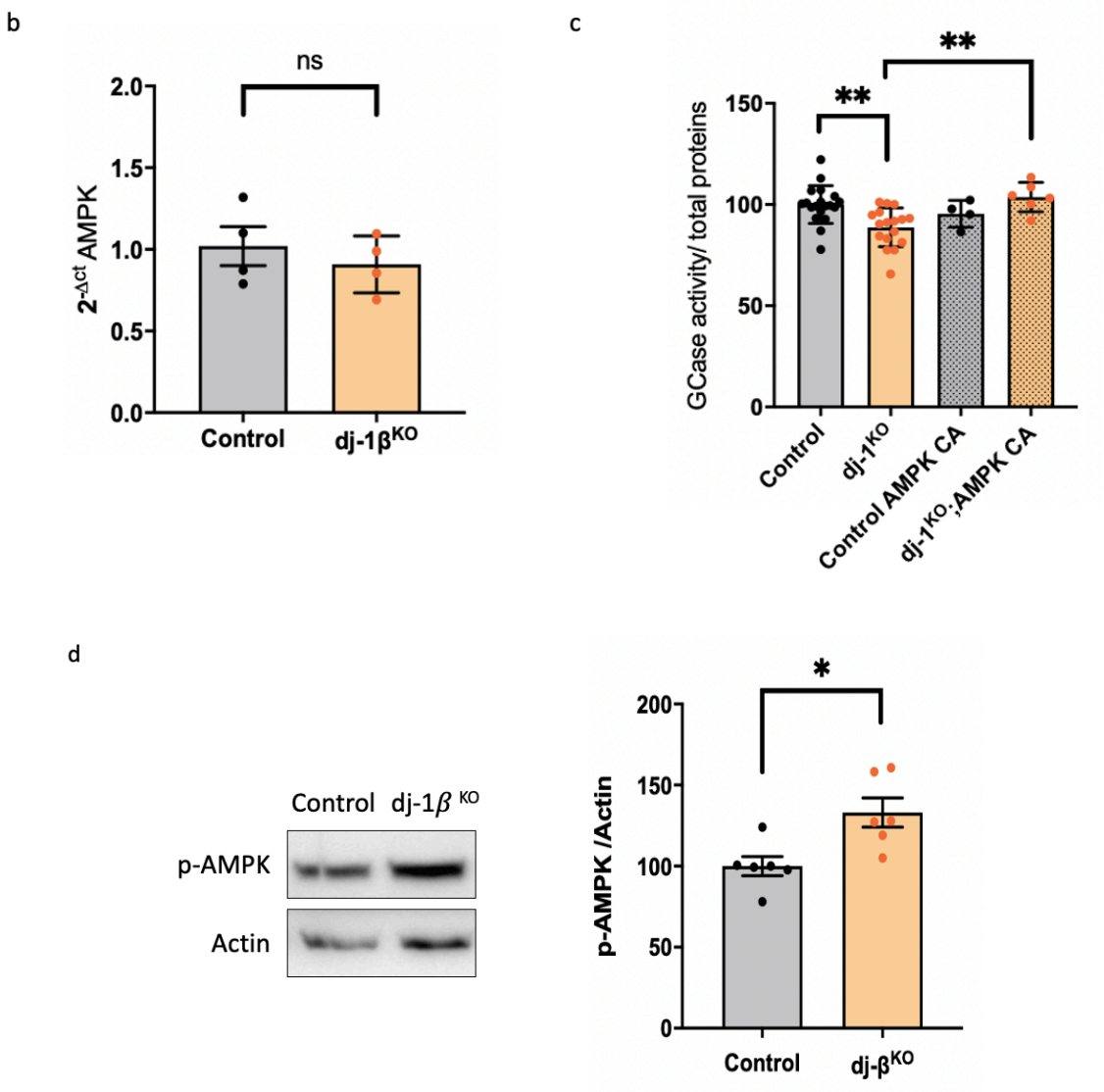


Fig 27: (a) Western blot analysis to evaluate the phosphorylated form of AMPK (Thr172). *dj-1β* KO and OE flies were compared with genetically-matched controls. Data corresponding to at least 5 biological replicates per genotype were compared with t-test. ** $p < 0,01$. (b) RT qPCR of AMPK mRNA level. Four biological replicates were analyzed using t-test. ns = not significant. (c) GCase enzymatic activity assay of *dj-1β KO, dj-1β KO* overexpressing AMPK CA flies and matching controls. At least 4 biological replicates were used for the experiment. Data were analyzed with one-way ANOVA with Turkey's multiple comparisons test. ** $p < 0,01$. (d) Western blot of p-AMPK (Thr172) from fly heads lysates. Six biological replicates were used for each genotype. Data were analyzed using t-test. * $p<0.05$

5.6 DJ-1 Alters mTORC1 Activity

Among the substrates of AMPK, mTORC1 is one of the most relevant for the modulation of autophagy. As reported in the introduction, mTORC1 is negatively regulated by AMPK to promote autophagic induction through different pathways. Through a western blot analysis, we indirectly assessed the activity of mTORC1, by evaluating the Thr389 phosphorylation level of the ribosomal protein S6K, one of mTORC1 well-established targets. The experiment performed on whole-fly body protein lysates indicated higher levels of phospho-S6K in *dj-1β* KO flies compared to the control, suggesting that mTORC1 is hyperactivated upon dj-1β absence. The overexpression of dj-1β is sufficient to decrease the mTORC1 activation (Fig 28a). This effect may account for the ALP impairment observed in dj-1β mutant flies, as mTORC1 hyperactivation may result in autophagy inhibition, both transcriptionally, via the downregulation of Mitf (TFEB), but also through the negative modulation of ULK1. We further confirmed the correlation between AMPK and mTRC1 activity by observing that the overexpression of AMPK CA in *dj-1β* KO flies causes the reduction of mTORC1 activity to the level of the control (Fig 28b). As for the case of AMPK, we then investigated the activation of mTORC1 in fly heads. Interestingly, our analysis indicated that the loss-of-function of dj-1β causes an increase in the level of phospho-S6K also in this body region, suggesting that the effect of DJ-1 on m-TORC1 is consistent among different tissues and not always dependent on AMPK activity (Fig 28c). Altogether these data may lead to several speculative interpretations. First, the hyperactivation of AMPK in dj-1β KO fly heads may be a compensatory mechanism, which, however, is not sufficient to inhibit mTORC1. Alternatively, the pathways affected by (or down-stream) AMPK are not identical in every tissue so that the activity of this protein complex in the brain may not affect mTORC1 regulation, which in contrast is likely achieved by other cell mechanisms.

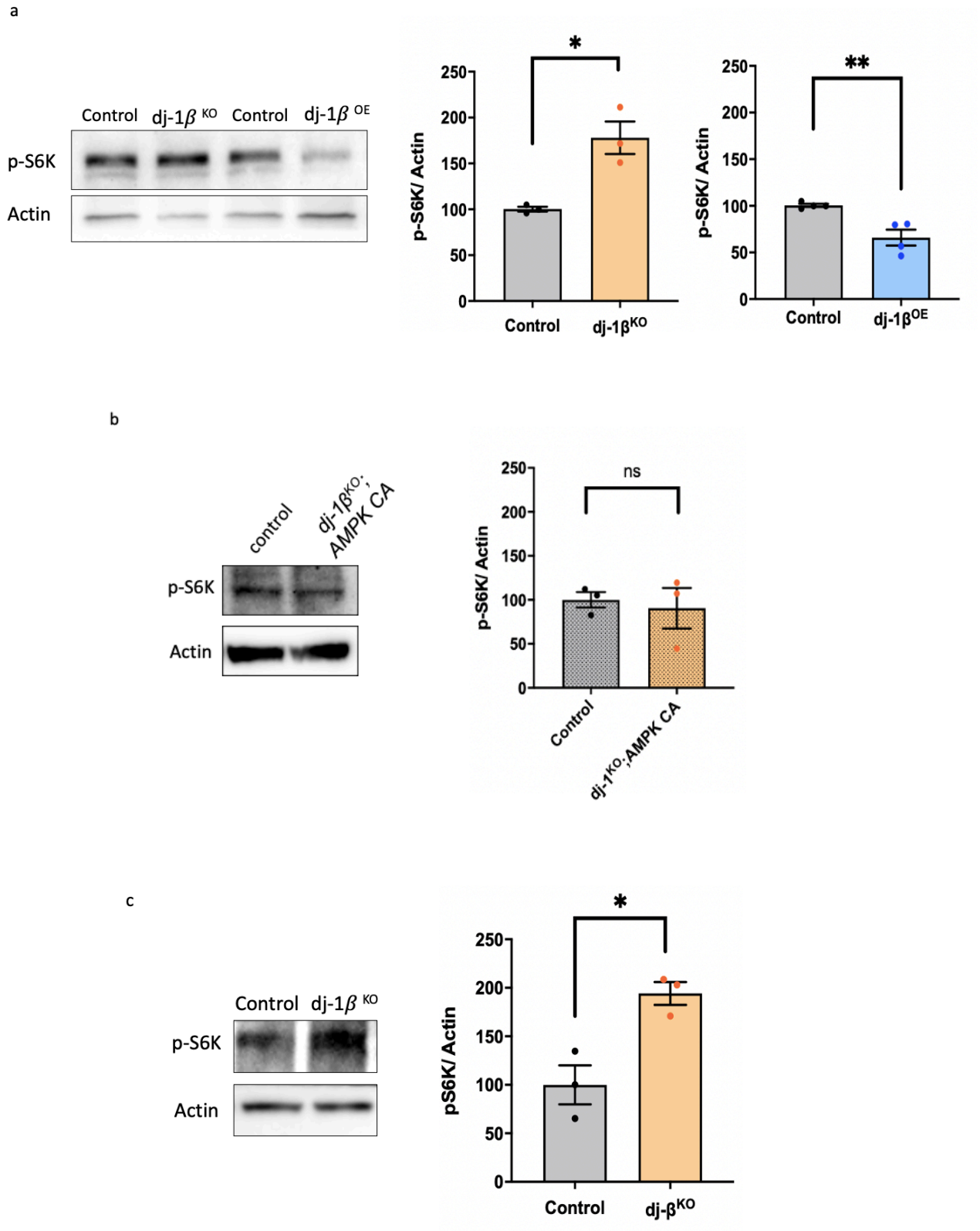


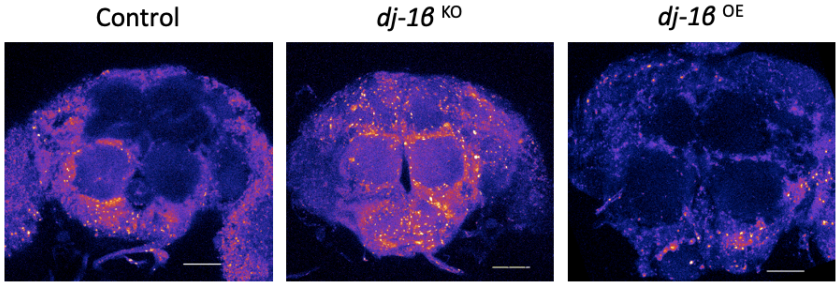
Fig 28: Western blot analysis of phospho-S6K (Thr369). (a) Whole-body lysates of *dj-1β* KO, *dj-1β* OE flies, and matching controls. (b) Whole-body lysates of *dj-1β* KO, AMPK CA flies, and WT control. (c) Head lysates of *dj-1β* KO flies and control. At least 3 biological replicates were used per each genotype. Data were analyzed with a t-test. ns = not significant, * $p < 0.05$, ** $p < 0.01$.

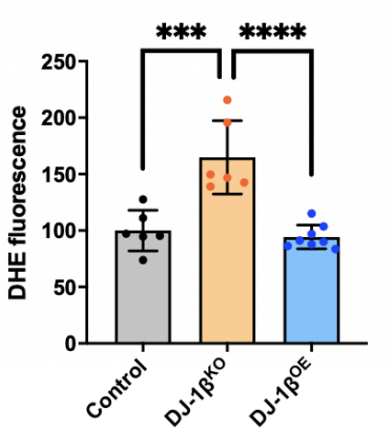
5.7 The Increase of ROS Triggers the Autophagic Defects in *dj-1β* KO Flies

The data collected so far suggest that DJ-1 participates in autophagic regulation, by affecting in some way both the AMPK and mTORC1 pathways. At this point, we wondered what may be the upstream stimulus that modulated AMPK activity in *dj-1β* mutant flies. Among the factors known to affect AMPK phosphorylation, the intracellular concentration of ROS is known to be linked to DJ-1 function. Therefore, we tested whether the alteration in ROS level mediated by DJ-1 may be the trigger stimulus that influences the AMPK-mTOR1 pathway. To this aim, we used the dyhydroethydium (DHE) fluorescent dye, that detects the superoxide anion and hydrogen peroxide, to evaluate the level of ROS in fly brains. In agreement with previously published data, our result confirmed that dj-1β loss-of-function causes a significant increase of ROS in the brain, which is completely rescued upon dj-1β overexpression (Fig 29a). Finally, to validate the hypothesis that the levels of ROS represent the factor leading to ALP alterations, we treated *dj-1β* KO flies with a ROS scavenger, N-Acetyl-L-Cysteine (NAC), and evaluated some of the autophagic phenotypes that we found to be affected by the absence of dj-1β. Interestingly, we observed that ROS scavenging in *dj-1β* null flies produces a trend toward the increase in the number of autolysosomes (Fig 29b). Moreover, the lysotracker red staining upon ROS scavenging confirmed the partial of the lysosomal phenotype, with the decrease of lysotracker-positive structures in *dj-1β* KO flies (Fig 29c). Altogether, these data suggest that the ALP alterations observed in *dj-1β KO* flies are at least partially mediated by the increase in ROS concentration.

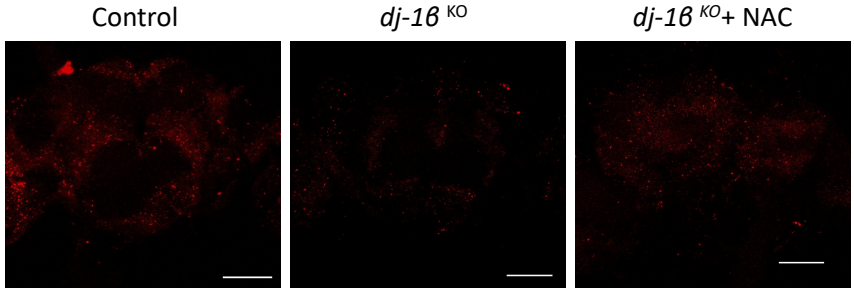
In conclusion, as represented in Fig 30, a plausible hypothesis is that the loss-of-function of DJ-1 impacts on mitochondrial dynamics, resulting in the abnormal increase of ROS concentration. This alteration in the oxidative homeostasis may modulate the AMPK-mTORC1 pathway, eventually leading to defects in the autophagic process.

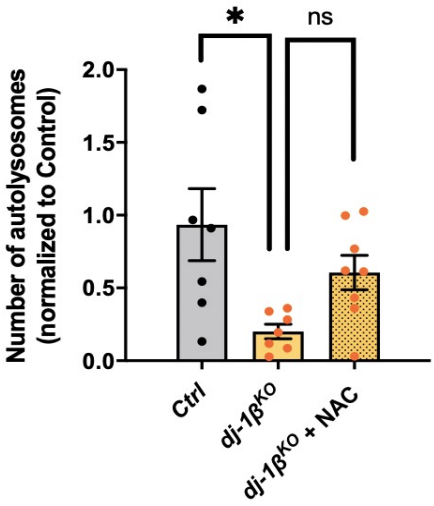
a


Control
dj-1β KO
dj-1β OE

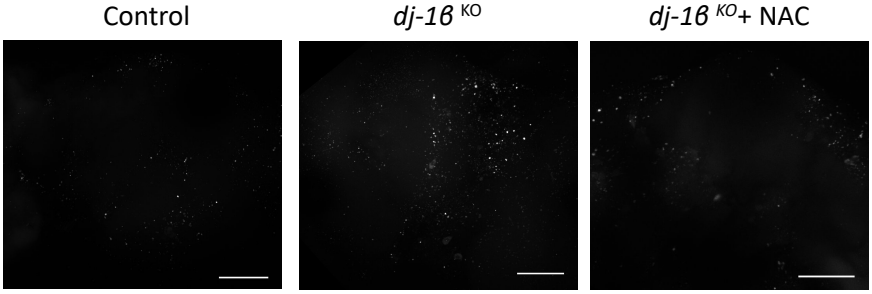

***
****
DHE fluorescence
250
200
150
100
50
0
Control
DJ-1βKO
DJ-1βOE

b


Control
dj-1β KO
dj-1β KO+ NAC


*
ns
Number of autolysosomes (normalized to Control)
2.0
1.5
1.0
0.5
0.0
Ctrl
dj-1βKO
dj-1βKO + NAC

c


Control
dj-1β KO
dj-1β KO+ NAC

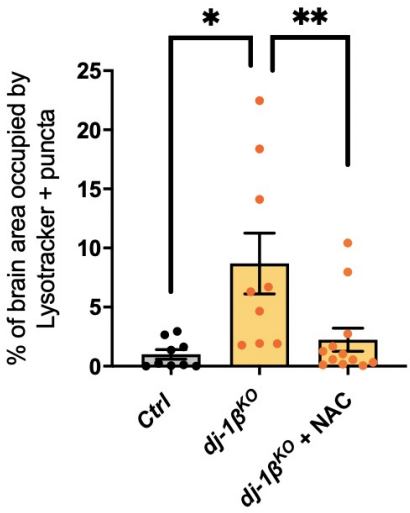

*
**
% of brain area occupied by Lysotracker + puncta
25
20
15
10
5
0
Ctrl
dj-1βKO
dj-1βKO + NAC

Fig 29: (a) Pseudocolor representative images (scale bar 50 μm) and quantification of *dj-1β KO, dj-1β OE* and control fly brains stained with DHE for the evaluation of ROS. At least 6 brains were analyzed for each genotype. Data were analyzed with one-way ANOVA with Turkey's multiple comparisons test. (b) Pseudocolor representative images (scale bar 50 μm) fly brains expressing Atg8-GFP-mCherry construct relative quantification. Images show mCherry signal of *dj-1β KO, dj-1β KO* treated with NAC flies and WT control. (c) Pseudocolor representative images (scale bar 50 μm) of fly brains stained with LysoTracker Red and quantification of LysoTracker area. *dj-1β KO, dj-1β KO* treated with NAC and control flies were used. Flies were treated with 1mM of NAC for 7 days. Analyses were performed using one way ANOVA with Turkey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

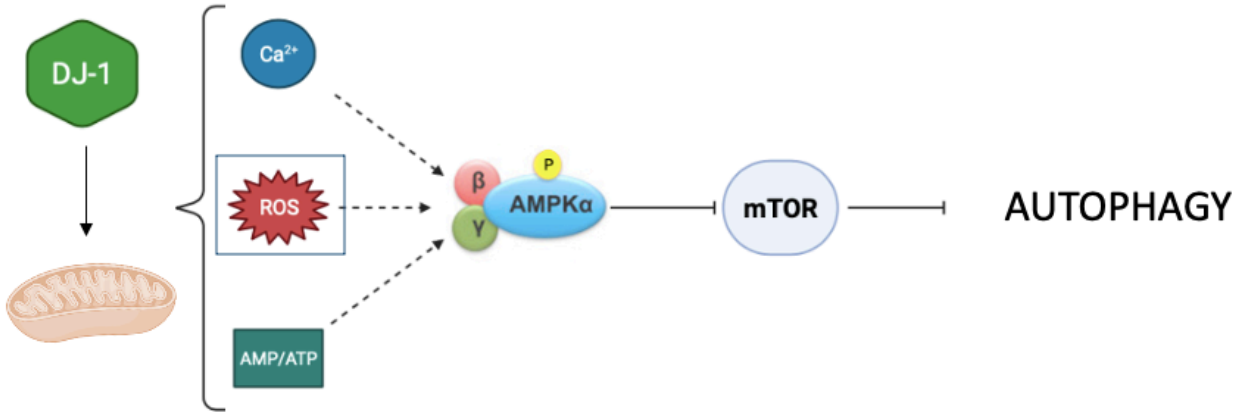


Fig 30: Schematic representation of the molecular pathway investigated through our analysis.

Chapter 6

Results

iPSC

6 Results

The results obtained in *Drosophila melanogaster* showed that DJ-1 participates in both mitochondrial and lysosomal homeostasis. Moreover, we observed that the DJ-1-mediated autophagic alterations may be achieved through the modulation of the signaling pathway regulated by AMPK and/or mTORC1. Based on our data and on some pieces of evidence reported in literature (93,151) a plausible hypothesis is that AMPK may act as a sensor of mitochondrial function and, in turn, modulate autophagy in *dj-1β* KO flies. Overall, our results may be very relevant to shed light on the function of DJ-1, whose precise activity has not been fully characterized to date. In addition, the use of fruit flies as an experimental model offered some advantages in the analysis of the protein function. Indeed, one of the main strengths of our results is that they were performed *in vivo*, taking advantage not only of the genetic versatility of *Drosophila*, but also of the level of integration and complexity that a living system may offer. Nevertheless, it is important to take into account some relevant limitations that characterize *D. melanogaster* as an organism for neurodegeneration modeling. First, the evolutionary distance between humans and flies may account for significative differences in the pattern of expression and the function of some proteins, or the regulation of signaling pathways; second, the limited availability of reagents and antibodies suitable for analysis in fruit flies reduces the possibility to dissect in detail complex molecular mechanisms.

For this reason, we established a collaboration with Dr. Mark Cookson's lab, with the aim to deepen the study of DJ-1 in a relevant model for the pathology, which is represented by human induced pluripotent stem cells (iPSC). We exploited both wild-type cells and an isogenic line carrying a loss of function mutation in DJ-1 (A111L) that eliminates steady-state DJ-1 and results in the complete knockout of the protein (152). Because of the possibility to differentiate iPSC in neuronal cells, they represent an extremely relevant and valuable tool to study the mechanisms associated with DJ-1 activity in the neuronal context.

6.1 Differentiation of iPSC into Dopaminergic Neurons

Dopamine (DA)-producing neurons are the main neuronal population affected in PD (153); therefore, the analysis of DJ-1 in this specific cell type may allow us to obtain very relevant pieces of information for the understanding of DJ-1-associated PD pathology. Following a protocol already established in the lab, we differentiated iPSC into DA neurons. After differentiation, we assessed through western blot the knock-out of DJ-1 in the mutated cell line, and we checked for the presence in our cell culture of the dopaminergic neuronal marker tyrosine hydroxylase (TH) (Fig 31). These results confirm that the differentiation protocol worked and that we generated DA neurons. It is important to underly the fact that the efficiency of differentiation is not 100%, and only a portion of cells in culture is represented by TH-positive neurons. The staining of cells with a TH antibody and the visualization at the confocal microscope confirmed that the dopaminergic differentiation corresponds to about the 50% of cells. The remaining 50% of cells are most likely cells differentiated in other neuronal populations and/or other non-differentiated cells.

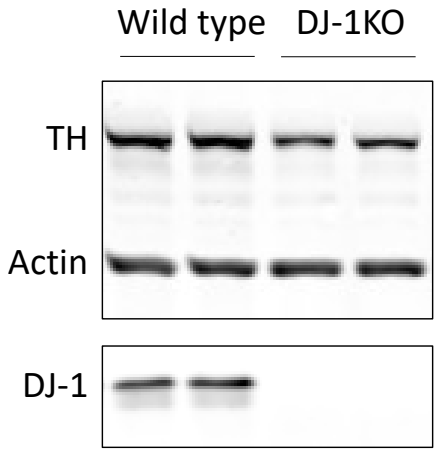


Fig 31: Representative immunoblot analysis showing that DJ-1 A111L mutation causes the complete knockout of the protein. The dopaminergic neuronal marker TH confirmed that the differentiation of iPSC into DA neurons was successfully achieved.

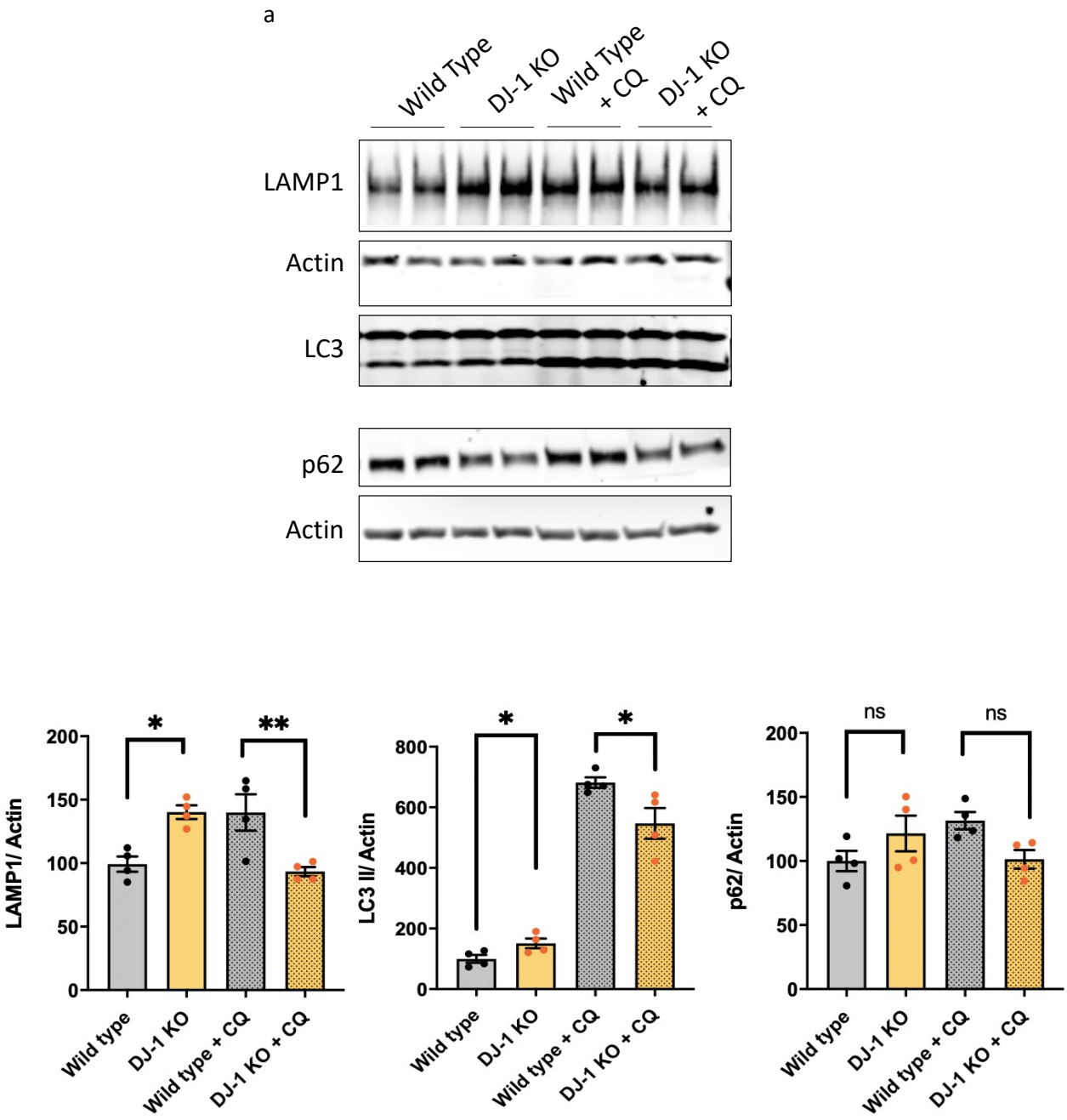
6.2 DJ-1 Loss-of-Function Affects Autophagy in Human iPSC

To test the assumption that DJ-1 affects autophagy in human cells we performed immunoblot analysis evaluating the level of some autophagic markers. Importantly, we confirmed that the absence of DJ-1 alters the autophagic flux, since we detected an increase in the level of the lysosomal protein LAMP1 and the level of lipidation of the autophagosomal marker LC-3 (fig 32a). The increase of these two autophagic markers may be explained by an increase

in the autophagic flux, but may also be due to defects in their degradation, leading to the accumulation of undigested lysosomal cargoes. To better clarify these aspects, we evaluated the level of the autophagic substrate p62 to have a preliminary indication of the rate of degradation. The western blot showed a trend toward the increase of p62 level in DJ-1 KO cells, although not statistically significant, this data suggests that the loss of function of the protein may impair the autophagic degradation activity, in line with what we observed in *Drosophila* (Fig 32a).

When studying autophagy, is frequently difficult to uncontrovertibly assess the direction of the autophagic impairment. Indeed, it may happen that different alterations lead to the same outcomes. For example, an increase in lysosomes may be determined by an induction of lysosomal biogenesis or an accumulation of lysosomes due to defects in the degradation of the cargoes. To understand the direction of the autophagic flux, we exposed cells to chloroquine (CQ), a compound that inhibits autophagosomes-lysosomes fusion by increasing the lysosomal pH (154). If the autophagic flux has a high rate, the blockage of autophagy should lead to an increased accumulation of substrates. Conversely, if the autophagic process proceeds at slow rate, the accumulation of autophagic substrates upon autophagy inhibition should be lower. Interestingly, we observed that the treatment with CQ leads to higher accumulation of LAMP1, LC3 II, and p62 in wild type compared to DJ-1 KO flies (Fig 32a). These results may indicate that the rate of autophagic flux is higher in control cells, suggesting that the loss of function of DJ-1 leads to a general decrease in autophagic activity.

Finally, to assess the lysosomal activity in DJ-1 mutant cells, we performed the DQ-BSA lysosomal proteases assay. Consistent with the data obtained in fruit flies, we observed that the absence of DJ-1 decreases the activity of lysosomal proteolytic enzymes, confirming the impairment of autophagy (Fig 32b).


a
Wild Type
DJ-1 KO
Wild Type + CQ
DJ-1 KO + CQ
LAMP1
Actin
LC3
p62
Actin
LAMP1/ Actin
LC3 II/ Actin
p62/ Actin
Wild type
DJ-1 KO
Wild type + CQ
DJ-1 KO + CQ
*
**
ns

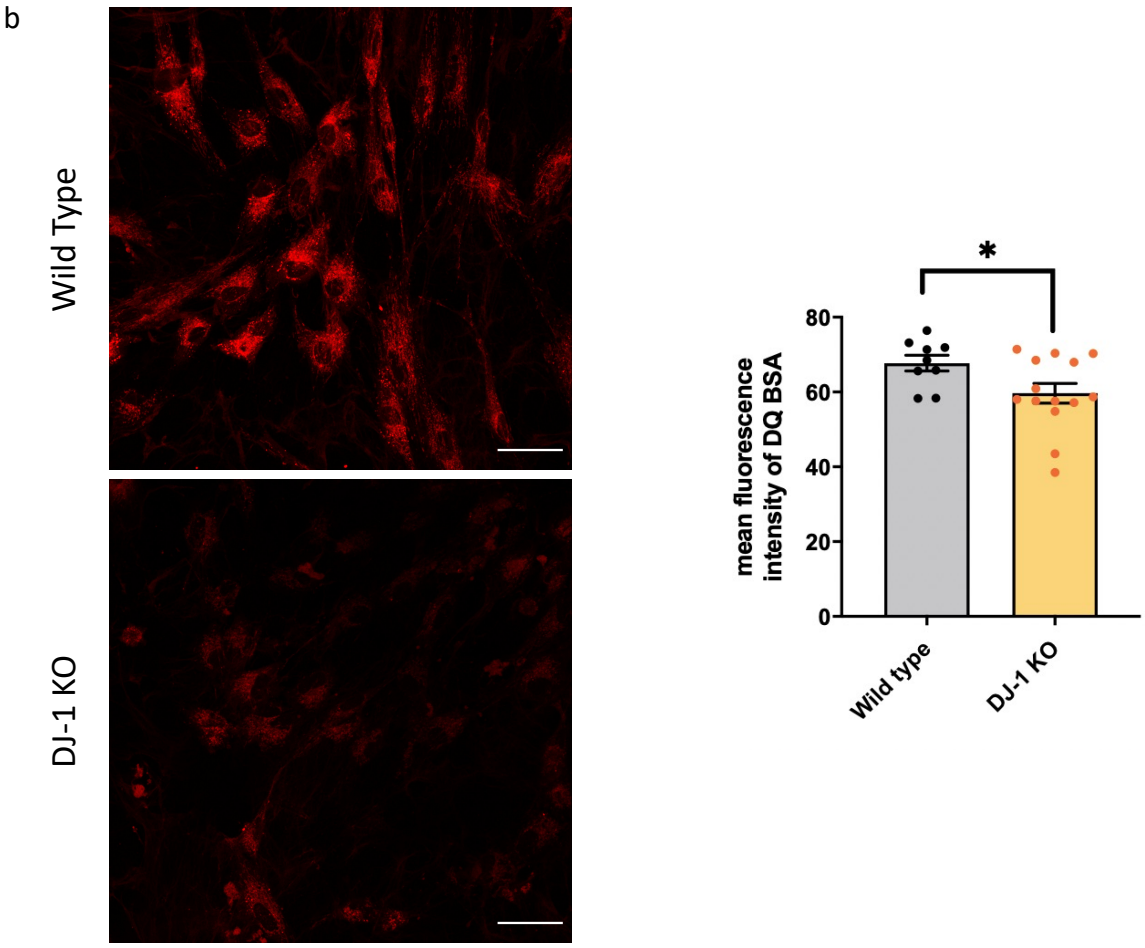


Fig 32: (a) Western blot analysis and quantification of the lysosomal markers LAMP1, LC3, and p62 in wild-type and DJ-1 KO cells. Actin was used as loading control. The experiment was performed 4 times and the analysis was performed using One-way ANOVA with Turkey's multiple comparisons test. We blocked autophagic flux by treating cells with CQ (50 μM for 3 hours) (b) Representative images (Scale bar 50 μm) and quantification of the DQ-BSA assay for the evaluation of lysosomal protease activity. The analysis was performed on 2 independent cell cultures after differentiation. Each dot in the graph represents the mean fluorescence of DQ-BSA dye in the area occupied by cells in each microscopic field. Analysis was performed with t-Test. ns = not significant, * $p<0.05$, ** $p < 0.01$.

6.3 DJ-1 Influences the AMPK/mTORC1 Pathway in Human Cells

As suggested by the results obtained in *D. melanogaster*, DJ-1 may participate in the autophagic regulation through the modulation of AMPK and/or mTORC1 activity. In *Drosophila* we observed that the absence of DJ-1 causes a decrease of AMPK phosphorylation in the whole fly body, while we observed higher levels of active AMPK in fly

heads. Since one limitation of our analysis in the fly model was the lack of a working antibody to detect the total amount of AMPK, we repeated the analysis in the human cell model. More specifically, using cell lysates from wild-type and DJ-1 null cells, we assessed whether the loss of function of DJ-1 in differentiated iPSC affects the phosphorylation of AMPK, and in which direction. To answer this question, we performed an immunoblot analysis evaluating the ratio between the phosphorylated form and total AMPK. Interestingly, we observed that the level of phospho-AMPK increases in DJ-1 KO cells compared to the control (Fig 33a). Although the cell lysates we used for the experiment are composed of a mixture of cells, they mainly include neurons. Therefore, these data may suggest that the loss of DJ-1 function leads to the activation of the AMPK pathway in neuronal cells. This result is consistent to the one obtained in fly heads, indicating that AMPK may be activated in the brain both in DJ-1 KO *Drosophila* and in humans.

As thoroughly explained in the introduction, AMPK can modulate autophagy via the modulation of mTORC1 (106). Interestingly, our experiments in *Drosophila*, showed that the loss of DJ-1 causes an increase in mTORC1 activation, independently of the phosphorylation state of AMPK. As performed in fruit flies, we indirectly checked the activity of mTORC1 also in human cells, by evaluating the phosphorylation of S6K. In line with the results obtained in *Drosophila*, in DJ-1 null human cells the phosphorylation level of S6K is higher compared to wild-type cells, even though the difference is not significant (Fig 33b). Importantly, the treatment of cells with the specific inhibitor of mTORC1, torin1, completely abolishes the phosphorylation of S6K, confirming that the level of phosphorylation of this protein is a reliable approximation of mTORC1 activity (Fig 33b). Overall, the mTORC1 hyperactivation detected in DJ-1 loss of function models may account for the autophagic impairment that we assessed.

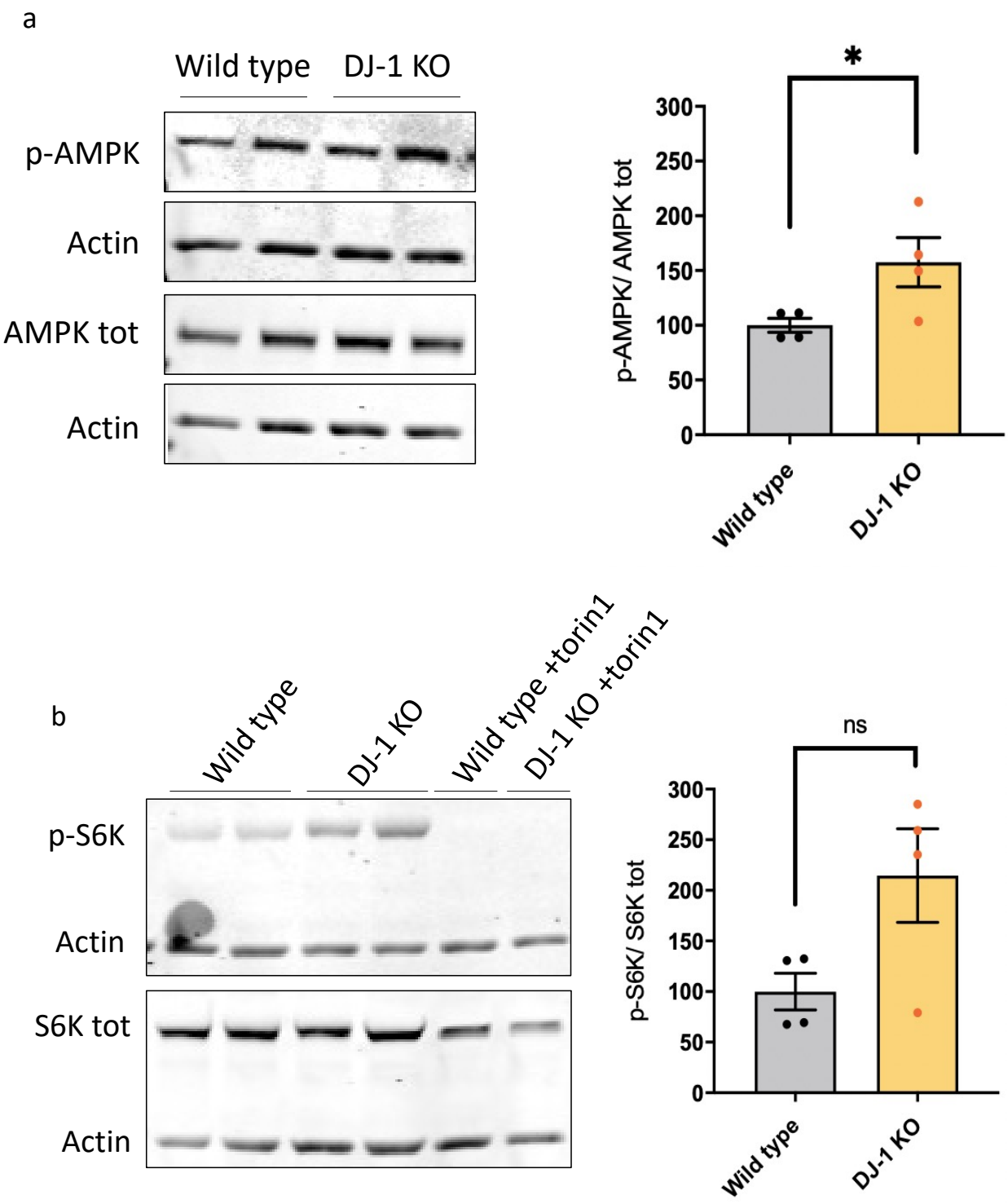


Fig 33: Western blot analysis of human cells for the evaluation of p-AMPK and total AMPK (a) and p-S6K and total S6K (b). Actin was used as the loading control. In the blot relative to S6K levels, we treated cells with the specific inhibitor of mTORC1, Torin1 (2.5 µM, 3 hours). We analyzed the data using t-Test based on 4 biological replicates. ns = not significant, * $p<0.05$.

6.4 DJ-1 Regulates the Level of ROS in Human Cells

Dj-1 activity has been widely studied as a regulator of ROS level (124); coherently we showed that the absence of DJ-1 causes an increase of ROS in *Drosophila melanogaster* brain. We checked the levels of ROS in DJ-1 KO cells by exposing them to the oxygen peroxide reporter DHE. Also in this model we confirmed that the loss of function of DJ-1 leads to an increase in ROS (Fig 34).

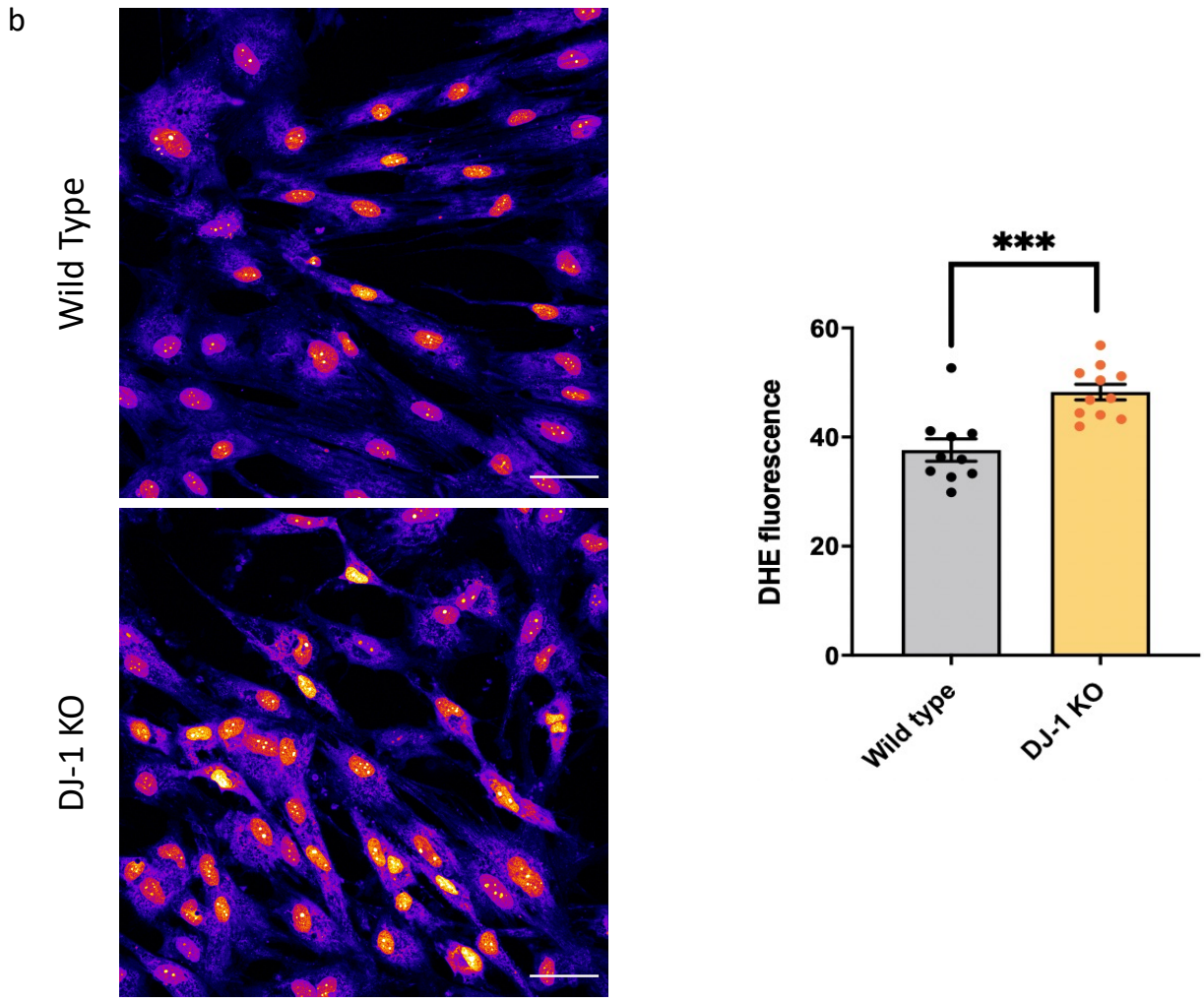
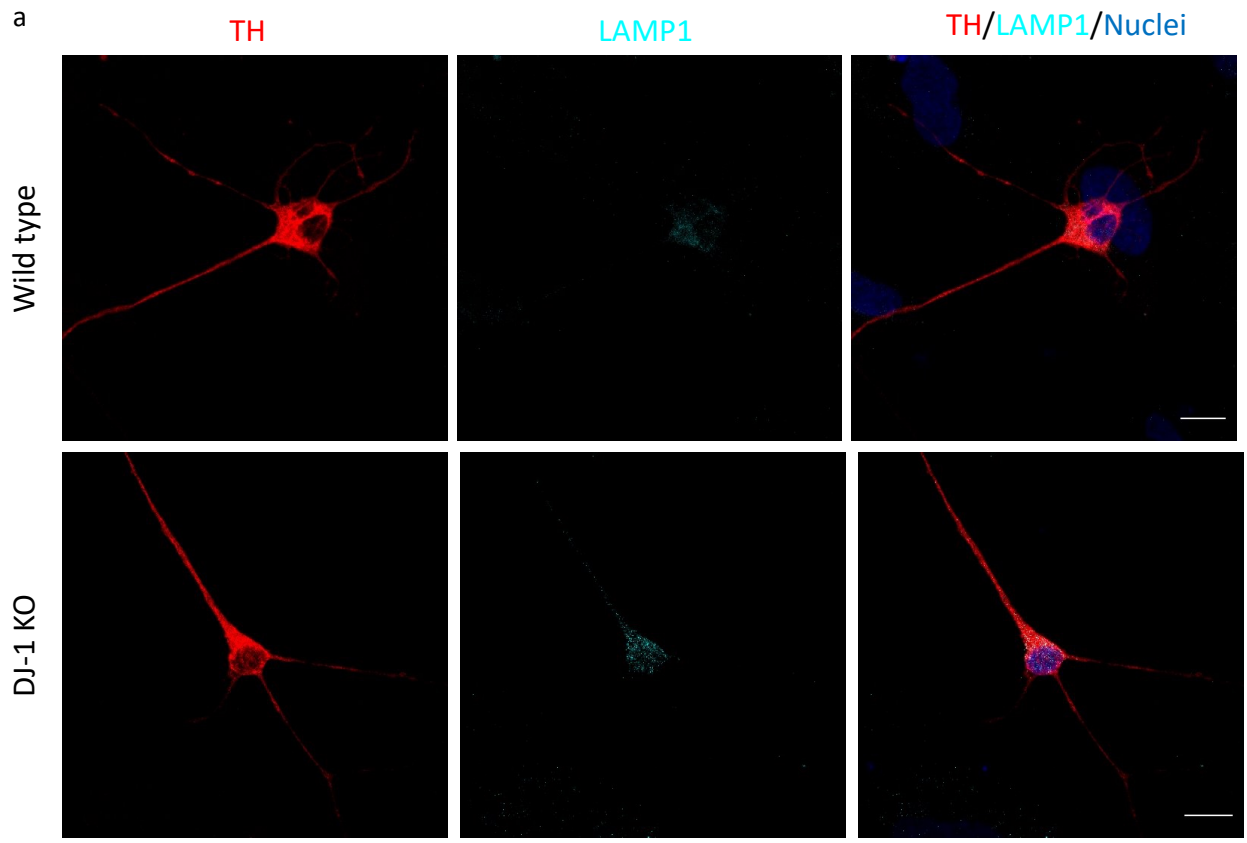
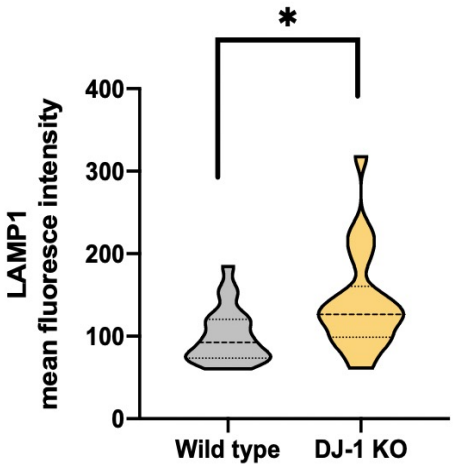


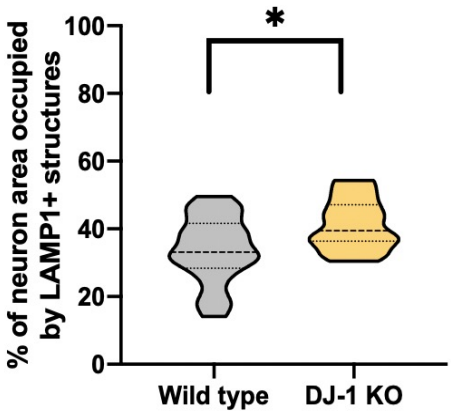
Fig 34: Representative images (Scale bar 50 μm) and quantification of the mean fluorescence DHE intensity. The analysis was performed on 2 independent cell cultures after differentiation. Each dot in the graph represents the mean fluorescence emitted by DHE dye in the area occupied by cells in each microscopic field. Analysis was performed with t-test. *** $p < 0.001$.

6.5 DJ-1 Promotes Autophagic Alterations in Dopaminergic Neurons

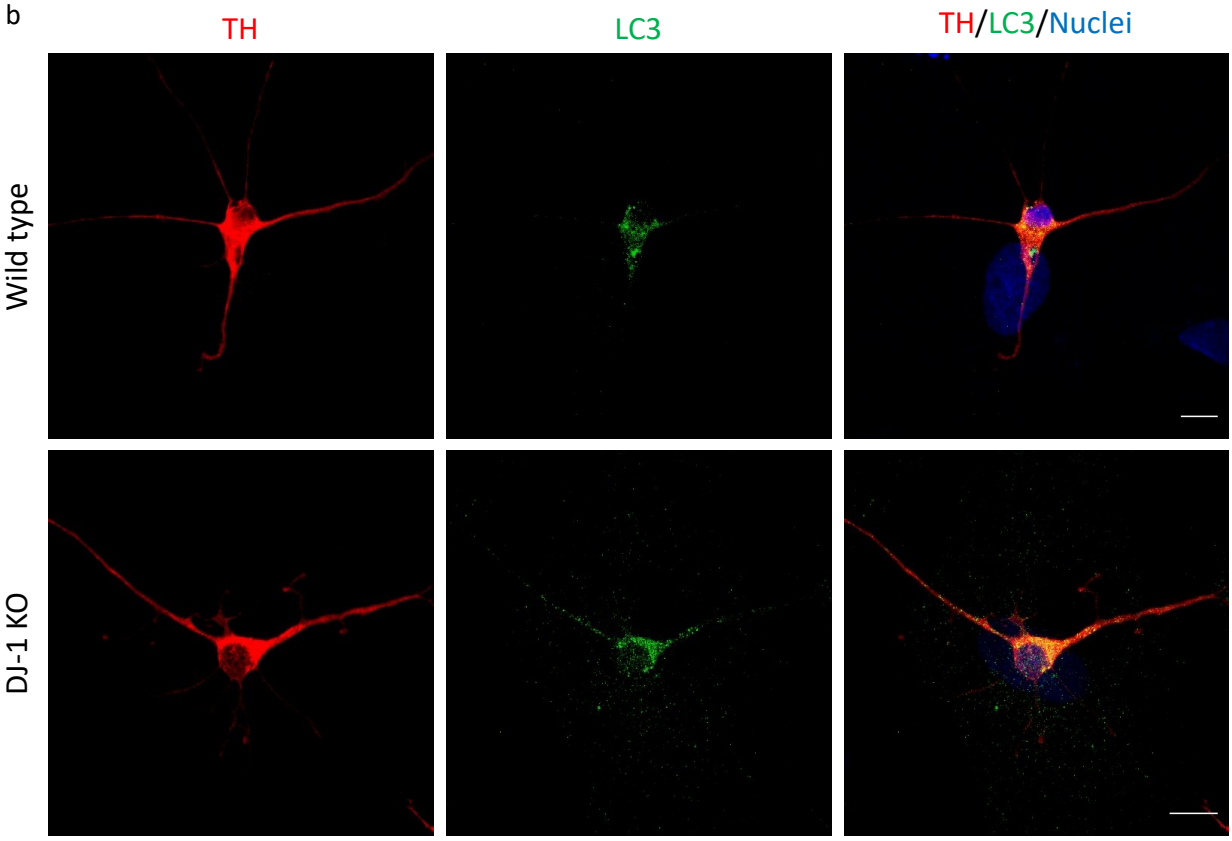
As mentioned, the differentiation of iPSc into DA neurons is not 100% efficient, leading to a mixed culture that includes different neuronal populations and other cells. Since DA neurons are the neuronal type mostly involved in PD, we were particularly interested in studying the effect of DJ-1 loss-of-function in this population of neurons. Through the confocal imaging technique, it is possible to focus the analysis of the level of some crucial autophagic markers at the level of dopaminergic neurons, visualized through the endogenous expression of TH Interestingly, in TH-positive cells, we detected a significant increase in the mean fluorescence intensity of the lysosomal marker LAMP1 together with an enhancement of the area of dopaminergic neurons occupied by LAMP1 positive structures. This result is in line with the ones obtained in the mixed cell culture as well as in *Drosophila* brains, further corroborating the hypothesis that DJ-1 activity influences the rate of accumulation and degradation of lysosomes (Fig 35a). In addition, in this cell type, we confirmed that the absence of DJ-1 leads to an increase of the area occupied by the autophagosomal markers LC3, even though the LC3-dependent mean fluorescence intensity is not statistically different between the two genotypes (Fig 35b). In contrast, the fluorescence intensity and the area occupied by p62- positive structure is not affected by DJ-1 (Fig 35c). These results support the hypothesis that the absence of DJ-1 in DA neurons causes a mild alteration of autophagy, but that the clearance functions are overall preserved. This outcome may be explained by some compensatory mechanisms that allow autophagy to perform its degradative activity despite the absence of DJ-1. In this scenario, we can assume that the differences in p62 observed in cell lysates may be caused by other types of cells present in the culture.

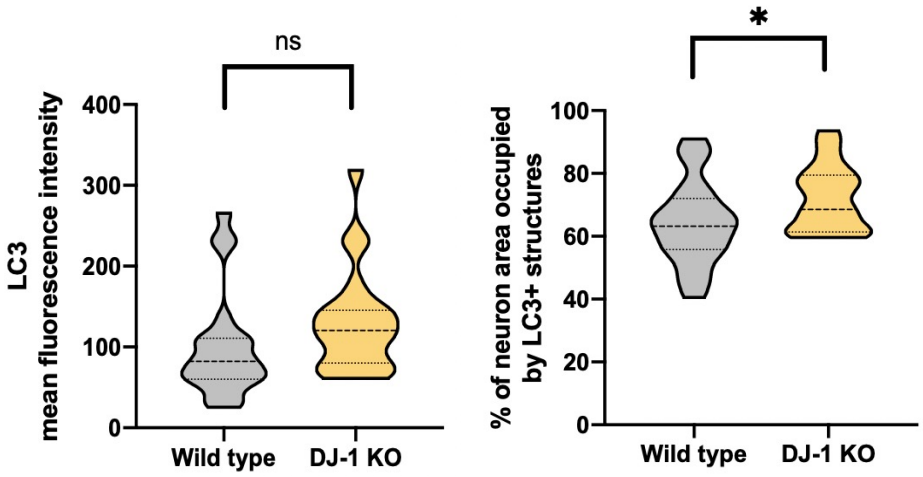

a
TH
LAMP1
TH/LAMP1/Nuclei
Wild type
DJ-1 KO


*
LAMP1
mean fluoresce intensity
400
300
200
100
0
Wild type
DJ-1 KO


*
% of neuron area occupied
by LAMP1+ structures
100
80
60
40
20
0
Wild type
DJ-1 KO

b


TH
LC3
TH/LC3/Nuclei
Wild type
DJ-1 KO


ns
LC3
mean fluorescence intensity
400
300
200
100
0
Wild type
DJ-1 KO
*
% of neuron area occupied
by LC3+ structures
100
80
60
40
20
0
Wild type
DJ-1 KO

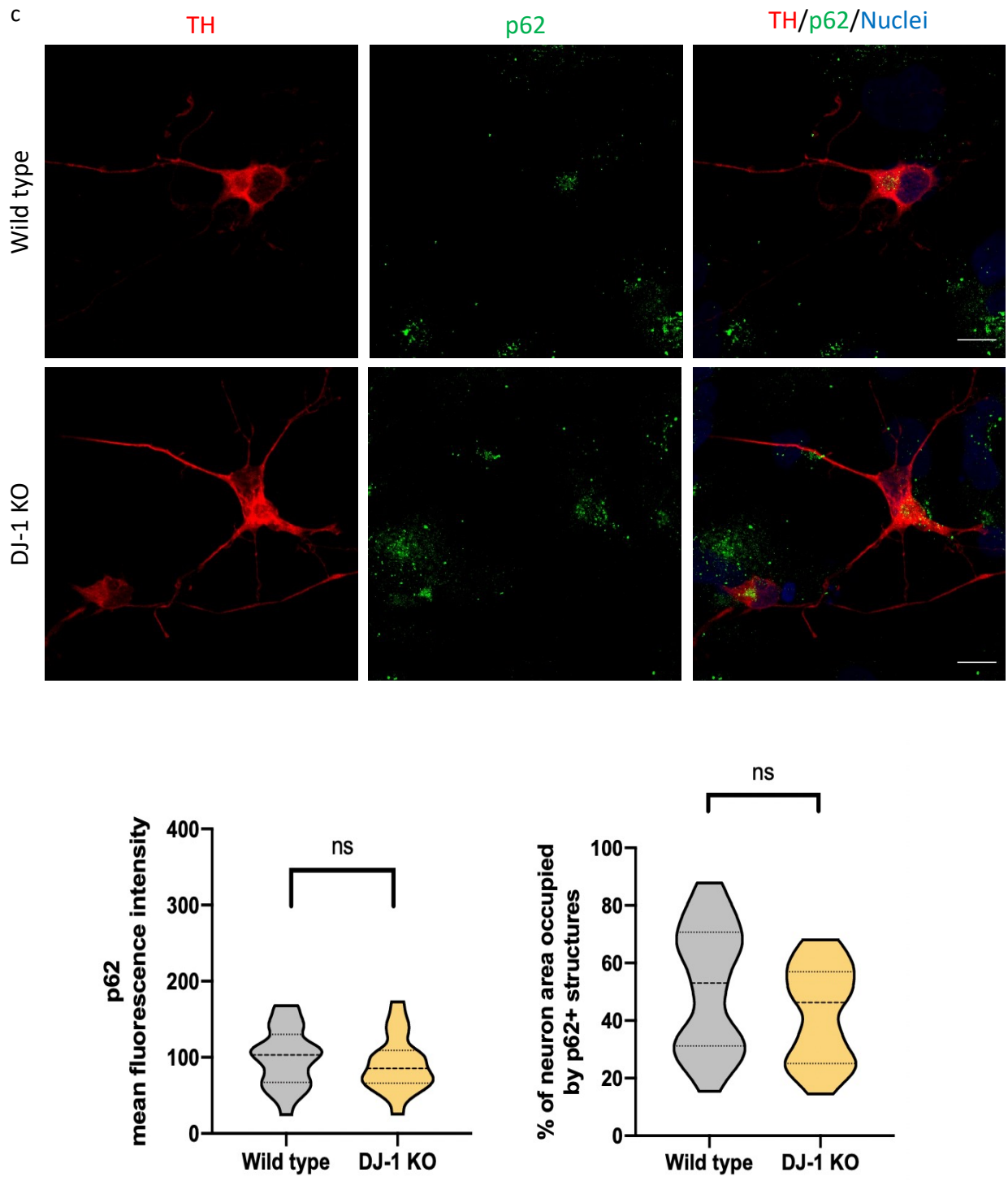


Fig 35: Representative images (Scale bar 10 µm) and quantifications of the mean fluorescence and the % of TH-positive cells area occupied by LAMP1 (a), LC3 (b), and p62 (c). The analysis is relative to at least 40 cells per genotype, deriving from two independent cell differentiation procedures. The intensity of the lysosomal markers considered was limited to the area occupied by the TH-positive fluorescence. Data were analyzed with a t-test. ns = not significant, * $p<0.05$.

6.6 DJ-1 Regulates the Activity of AMPK in Dopaminergic Neurons

We previously characterized the effect of DJ-1 on AMPK showing that the absence of DJ-1 causes alterations in the protein activity both in *Drosophila melanogaster* and in human cells. The data obtained in flies concerning the link between DJ-1 levels and AMPK activity, highlight the possibility that the AMPK is differently regulated in different tissues and that its response to DJ-1 loss of function may be cell-specific.

Therefore, we also evaluated the level of phospho-AMPK in DA neurons by analyzing the fluorescence intensity derived by the staining with a phospho-AMPK antibody in TH-positive cells. Interestingly, our data suggest that the mean fluorescence intensity relative to the phosphorylation of AMPK decreases in DJ-1 KO DA neurons with respect to wild-type cells (Fig 36). These data differ from those obtained in the cell lysates and in fly heads, suggesting that in the brain the regulation of AMPK activity is highly cell-dependent. The increased amount of phosphor-AMPK observed in fly heads and in the mixed cell cultured may be caused by the effect in non-DA neurons.

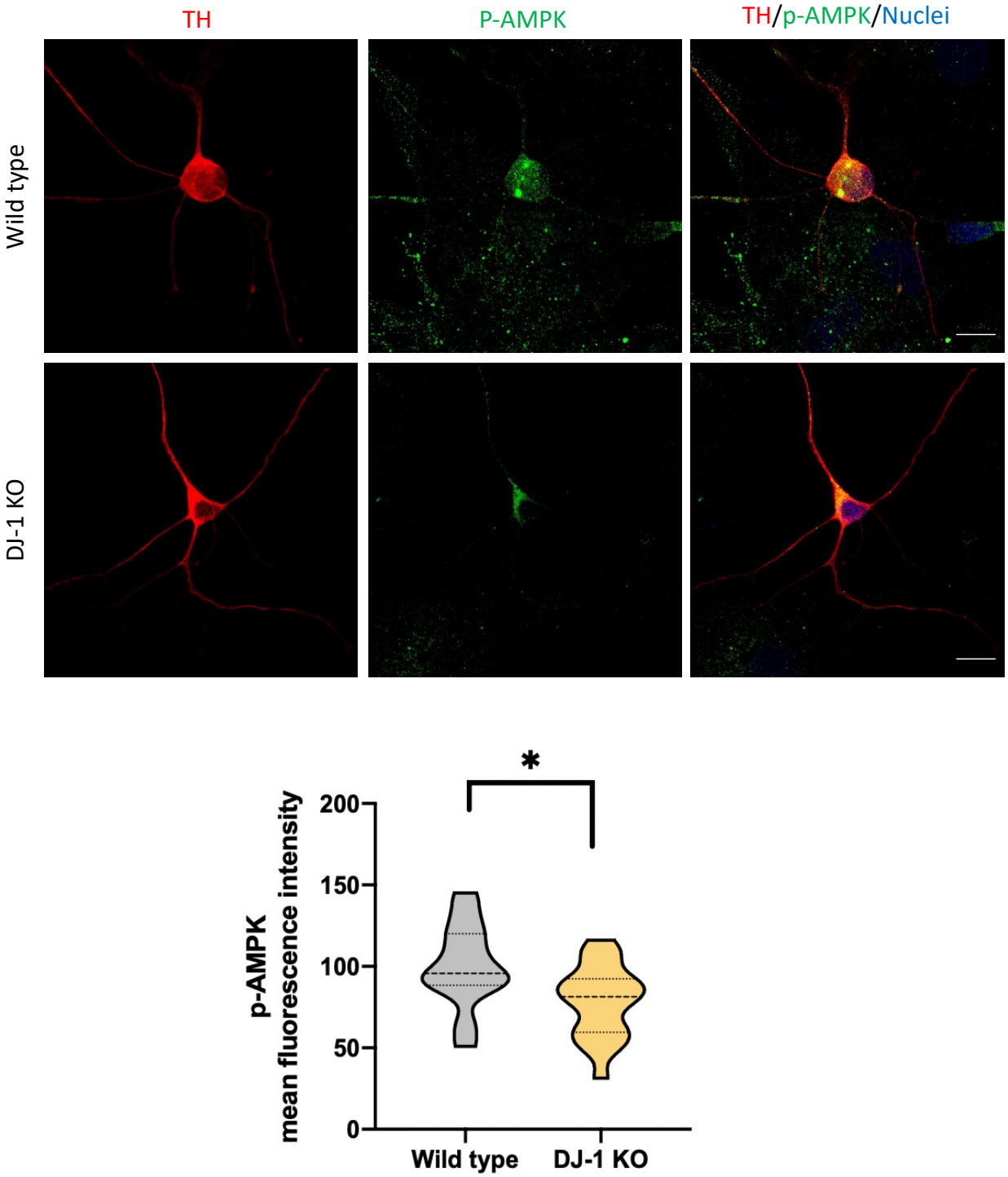


Fig 36: Representative images (Scale bar 10 μm) and quantifications of the mean fluorescence relative to phospho-AMPK. The analysis is relative to at least 40 cells per genotype, deriving from two independent cell differentiation protocols. The intensity of the phospho-AMPK fluorescence was limited to the area occupied by the TH-positive cells. Data were analyzed with t-test. * $p<0.05$.

Chapter 7

Results

Mouse Model

7 DJ-1 Modulates the AMPK/mTORC1 Pathway in Mouse Brain

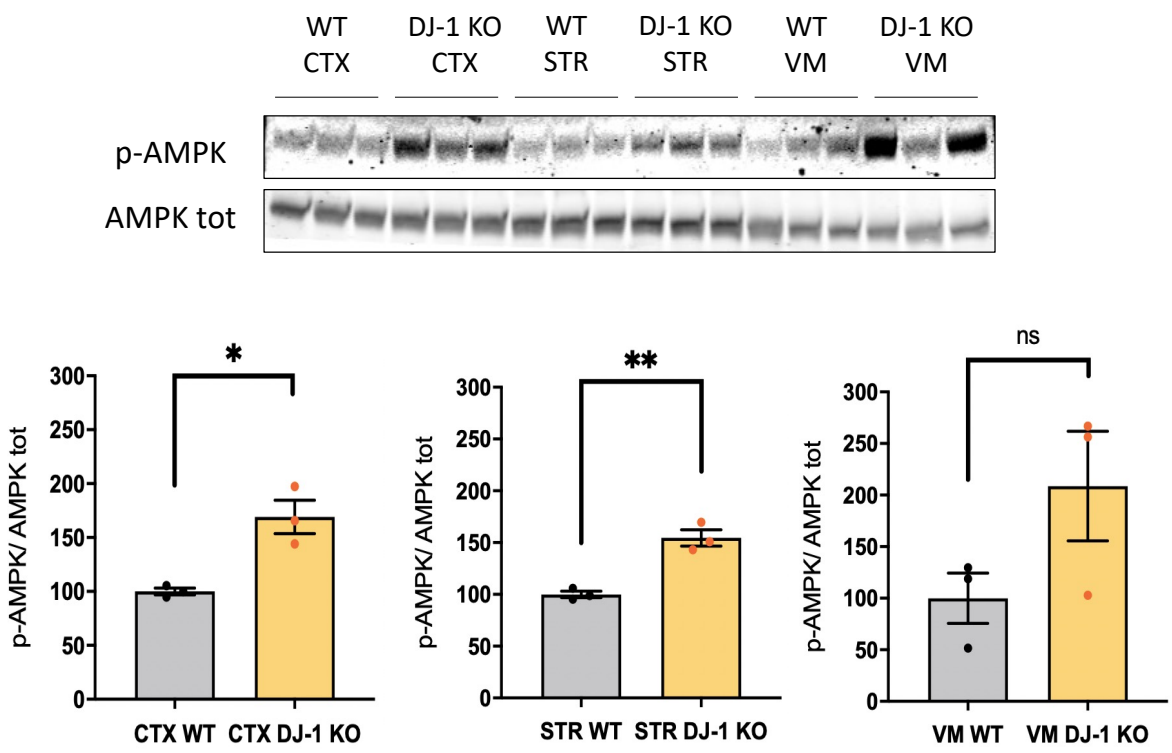
The DJ-1- mediated alteration of AMPK and mTORC1 may be highly relevant for the characterization of the role of DJ-1 in neuronal physiology and in PD. Indeed, AMPK and mTORC1 are the hub of several important pathways and can modulate, through their activity, crucial processes, including autophagy, apoptosis, and cell differentiation (40,104). Therefore, we took advantage of another animal model represented by DJ-1 KO mice, to confirm the link between DJ-1 function and the AMPK-mTORC1 pathway. We used lysates from three brain regions, namely cortex, ventral midbrain, and striatum, derived from wild-type and DJ-1 KO mice and we evaluated the activity of AMPK and mTORC1 by western blot. Since our previous data suggested that the regulation of the AMPK pathway is highly tissue- or cell-specific, we analyzed different brain regions to evaluate how the absence of DJ-1 affects this signaling pathway.

Similar to fly heads, mouse brain is characterized by a strong increase of AMPK phosphorylation in all the brain regions considered (Fig 37a). This result represents a further corroboration of the effect of DJ-1 on AMPK, suggesting that in the brain tissue, AMPK responds to the loss of DJ-1 by increasing its activity. Once again it is worth mentioning that brain is characterized by the presence of different populations of neurons and non-neuronal cells, therefore we cannot exclude that different effects could be highlighted by cell-specific analyses.

Moreover, we evaluated the activity of mTORC1 by using an antibody against the phosphorylated form of S6K. In mice brain regions we confirmed the results obtained both *Drosophila* and in human cells, which showed a significative increase of mTORC1 activity (Fig 37b).

In conclusion, the data presented here, obtained in different *in vivo* and *in vitro* models, suggest that DJ-1 can alter the activity of AMPK and mTORC1. Interestingly, the hyperactivation of mTORC1 was observed in every model. This effect may determine the DJ-1-mediated impairment of autophagy. Conversely, the effect of DJ-1 on AMPK activity seems to be highly tissue- and cell-dependent and other experiments will be needed to fully characterize the link between the two proteins.

a


WT CTX
DJ-1 KO CTX
WT STR
DJ-1 KO STR
WT VM
DJ-1 KO VM
p-AMPK
AMPK tot
p-AMPK/ AMPK tot
*
CTX WT
CTX DJ-1 KO
**
STR WT
STR DJ-1 KO
ns
VM WT
VM DJ-1 KO

b

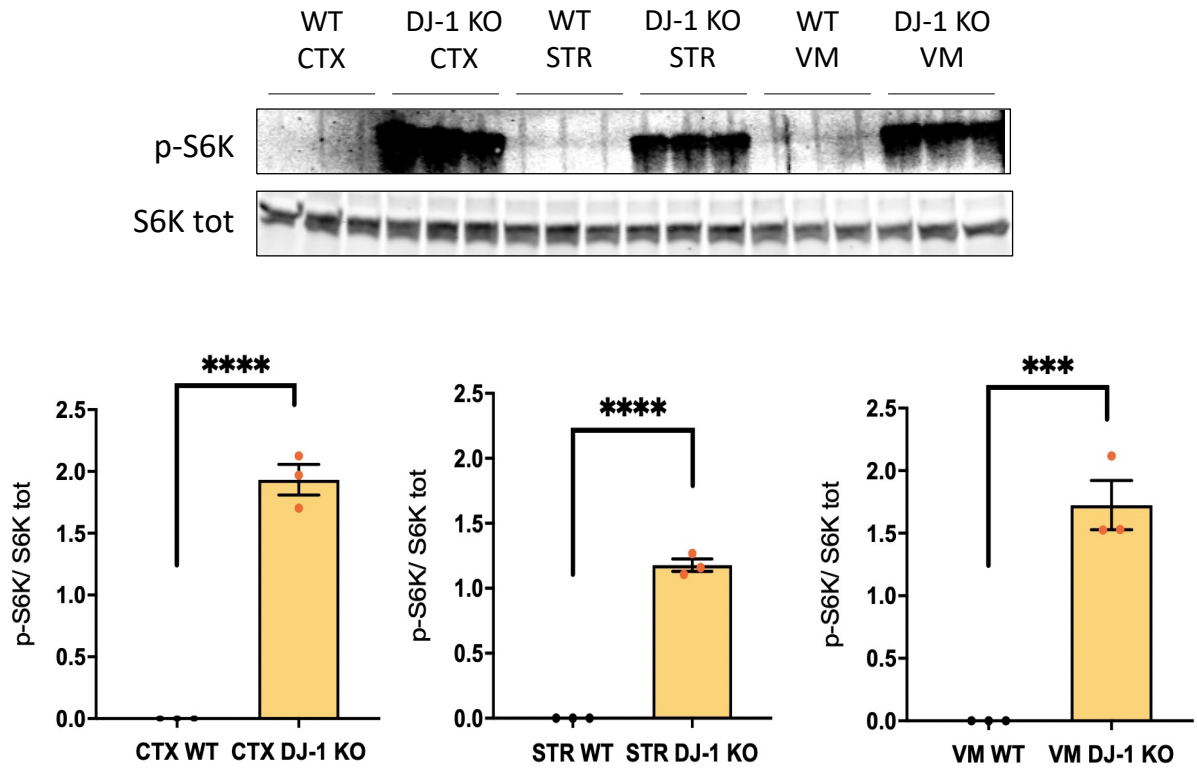

WT CTX
DJ-1 KO CTX
WT STR
DJ-1 KO STR
WT VM
DJ-1 KO VM
p-S6K
S6K tot
p-S6K/ S6K tot
****
CTX WT
CTX DJ-1 KO
****
STR WT
STR DJ-1 KO
***
VM WT
VM DJ-1 KO

Fig 37: western blot analysis of mouse brain regions of wild type and DJ-1 KO mice (cortex -CTX-, striatum-STR-, ventral midbrain -VM) and evaluation of p-AMPK and AMPK total (a) and p-S6K and S6K total (b) levels. We analyzed brain regions deriving from 3 animals per genotype. The data were analyzed using t-test. ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Chapter 8

Discussion and Conclusions

8 Discussion and Conclusions

After twenty years of intense investigation into DJ-1 activity, the precise function of the protein has not been fully characterized yet. DJ-1 has been described as a multifunctional protein, involved in the regulation of several cellular processes, including the maintainance of mitochondrial homeostasis, the protection against excessive accumulation of ROS, and the modulation of autophagy (133,146,155). Most of the data regarding DJ-1 activity have been obtained in cellular models; therefore, to gain more information about the physiological role of DJ-1 in an *in vivo* system, our lab has been widely investigating the effects of DJ-1 loss of function in *Drosophila melanogaster* (146). Fruit flies represent a valuable tool to characterize several aspects of human diseases, especially in the context of neurodegenerative disorders. Exploiting this model organism, our lab demonstrated that DJ-1 participates in the maintenance of mitochondrial quality control. Accordingly, *dj-1β* mutant flies display mitochondrial ultrastructural modifications and mild alteration of mitochondrial activity (146). Recently, mitochondrial dysfunctions have been linked to autophagic defects, and the activation of specific signaling pathways seem to ensure the communication between organelles and cell processes.

In our *dj-1β* null model, we have demonstrated that the resistance to starvation is reduced compared to wild-type controls (146). This data represented a first hint of the possible participation of DJ-1 in the modulation of autophagy. Indeed, starvation is well-known to induce autophagy and the hypersensitivity of *dj-1β KO* flies to this stimulus, has prompted us to investigate the role of DJ-1 at the autophagic level in *Drosophila*. Moreover, we characterized the possible molecular mechanisms that allow the protein to modulate both mitochondrial and autophagic functions.

By evaluating the level of autophagic substrates, like ubiquitin and p62, we demonstrated that the degradative pathways may be affected by the absence of DJ-1. Coherently, we observed an accumulation of these proteins in whole fly body lysates. Nevertheless, considering the link between DJ-1 and PD, our main interest was to evaluate the function of this protein in the brain. To this aim, we investigated whether the level of ubiquitin and p62 is altered also in lysates obtained from heads of wild-type and *dj-1β* KO flies. It is worth mentioning that the main constituent of fly heads is represented by the brain tissue; therefore, the immunoblot analysis performed in fly heads may represent a good

approximation to investigate the role of DJ-1 in the brain. Importantly, also in *dj-1β* null fly heads we observed an accumulation of these autophagy substrates, confirming the effect of DJ-1 on the degradation pathways. These results, and in particular the one relative to p62, point to autophagy as the degradative process affected by DJ-1 loss of function. Therefore, we characterized more in detail the autophagic pathway in *dj-1β* null flies.

Autophagic flux is one of the most important parameters, that needs to be evaluated to assess autophagic activity. The autophagic flux can be estimated by the speed of autophagic degradation, which is approximately calculated by the rate of autophagosomes-lysosomes fusion. In this frame, the Atg8-GFP-mCherry construct is widely used in different experimental systems to assess the autophagic flux. This autophagic reporter allows the visualization of autophasosomes (yellow) and autolysosomes (red). By calculating the ratio between red and yellow structures it is possible to evaluate the rate of autophagosomes-lysosomes fusion. We generated fly strains overexpressing the autophagic reporter Atg8-GFP-mCherry in wild-type and *dj-1β KO* animals and analyzed the differences in the autophagic flux inside the brains. Interestingly, we could not detect any autophagosomes in the fly brains of both genotypes. This result may be due to the high rate of fusion between autophagosomes and lysosomes in the brain that leads to the immediate generation of autolysosomes as soon as autophagosomes are produced. This assumption is supported by the fact that the treatment of flies with CQ and the blockage of the autophagosomal-lysosomal fusion led to the accumulation of autophagosomes in control fly brains. Even though we did not detect any autophagosomes and therefore we cannot assume differences in the autophagic flux, we observed a significative alteration in the number of autolysosomes, with a reduced number of these vesicles in *dj-1β KO* flies. The decreased number of autolysosomes may derive from two opposite effects: it may be due to faster degradation of these structures, but it could also be caused from defects in the autophagic process determined by an overall reduction of autophagic flux. This second hypothesis may also explain the data regarding the observed accumulation of autophagic substrates, suggesting an impartment of degradation.

To get better insight into the autophagic machinery in *dj-1β* null drosophila, we investigated the number of lysosomes in fly brains. Lysosomes are the major degradative organelles and alteration of their number or activity may lead to overall autophagic defects. We performed

lysotracker staining of fly brains to assess the number of acidic compartments, namely lysosomes and autolysosomes, and we observed an increase in the lysotracker-positive structure in *dj-1β* KO fly brain. Conversely, control fly brains were characterized by a barely visible lysotracker signal, even upon starvation-mediated autophagic induction. Considering that the brains of *dj-1β* mutant flies are characterized by decreased autolysosomes, a plausible explanation is that the enhanced lysotracker signal is determined by an accumulation of lysosomes. This result suggests that the absence of DJ-1 affects the autophagic process, influencing the number of structures involved in the degradation functions. The abnormal accumulation of lysosomes may be explained by defects in their degradation activity, which could result in the increase of lysosomes whose cargoes are not degraded. Accordingly, lysosomal impairments have been already associated with an increase in the lysotracker signal in other fly models (156,157).

To better understand the DJ-1-associated lysosomal phenotype, we evaluated lysosomal function through DQ-BSA assay for the activity of lysosomal proteases. This experiment confirmed that the absence of *dj-1β* causes a reduction of lysosomal activity in the fly brain. After this first indication of a decrease in lysosomal functions we performed a GCase activity assay. We assessed a reduction in the activity of this hydrolytic lysosomal enzyme both in *dj-1β* KO fly bodies and heads. Importantly, we directly linked the activity of DJ-1 to GCase, since the overexpression of *dj-1β* in flies reverted the phenotype, resulting in an increased enzymatic activity. It is worth mentioning that GCase mutations and are the most common risk factor for PD; therefore, the analysis of this enzyme activity may not only be used as an approximation of lysosomal activity, but may be very informative also in the context of PD (149). As a future perspective, it would be interesting to analyze more in detail how DJ-1 and GCase are associated and understand whether the reduction of GCase activity in a PD model may represent a factor that exacerbates the PD phenotypes and speed up the progression of the pathology.

Overall, our data clearly suggest that *dj-1β* impacts the autophagic process at different levels, altering the number of autophagic-related structures, impairing the lysosomal function, and reducing the autophagic degradation rate.

At this point, we hypothesized that the DJ-1-mediated phenotypes in the ALP are caused by defects in the transcriptional activity of TFEB, the master regulator of autophagy (150). To assess the activity of this transcription factor in flies we performed RT qPCR evaluating the expression of genes controlled by Mitf, the fly ortholog of TFEB. We assessed the activity of Mitf on the whole fly bodies and in heads, but we were not able to detect any significative difference between wild-type and *dj-1β* KO flies. This result may suggest that other pathways are responsible for the autophagic alteration caused by the absence of DJ-1. In this frame, it is important to highlight that autophagy is controlled and regulated at different levels and TFEB activity represents just one of the possible ways to modulate autophagic functions. Moreover, the autophagic machinery regulation is highly cell- and tissue-dependent and in our experiments, we could not discriminate among different cell types, since different tissues and cells were all pulled together. This experimental approach may represent one of the limitations of the use of *Drosophila* with respect to cell models, because the outcomes may be the approximation of different phenotypes that are cell- or tissue-specific. It would be interesting to evaluate whether the activity of TFEB varies in different DJ-1 KO cell models to understand whether the protein exerts different effects on autophagy in different cell types.

After having demonstrated the link between *dj-1β* and autophagy, we hypothesized that the phenotype observed may represent a secondary effect caused by the DJ-1-mediated alteration of mitochondria. This assumption is supported by two major notions. First, it is widely accepted that DJ-1 affects mitochondrial dynamics (144,146); In addition, it has been recently demonstrated that alterations of mitochondrial homeostasis lead to the modulation of the AMPK pathway that in turn, may impact autophagic activity and lysosomal functions (89,93). AMPK is a protein complex that can sense variation in the intracellular energetic status and modulates several downstream pathways, including autophagy (94,99). For this reason, we evaluated the activity of AMPK in *dj-1β* null flies by analyzing the level of phosphorylation at the level of Thr172 residue, which correlates with the protein activation.

Our results showed that DJ-1 alters the phosphorylation level of AMPK, suggesting that this protein may be involved in the DJ-1-associated phenotypes. Indeed, we observed a reduction of phospho-AMPK in the whole *dj-1* KO fly bodies while the activity of the protein increases in mutant fly heads. AMPK is a crucial protein for the maintaining of cell

homeostasis and can respond to different stimuli, such as variation in the AMP/ATP ratio and alteration of the ROS levels (99). It is likely that the protein complex regulation is different in different tissues or that it is subjected to different stimuli based on the body regions. However, it is crucial to highlight the fact that both deregulation and uncontrolled hyperactivation of the protein could lead to negative effects and impact the ALP. Therefore, in the future it would be very relevant to precisely understand how the tissue-specific DJ-1-associated modulation of AMPK is achieved and how these two proteins are linked in different cells.

Since our result showed that in the brain AMPK is activated upon DJ-1 loss of function, this effect may represent a compensatory mechanism to enhance autophagic functions in this tissue. However, we cannot exclude that, within the brain, the activity of AMPK is differently regulated in different brain cells.

In spite of some elusive results, our data clearly confirmed that AMPK activity affects autophagy in *Drosophila*. In fact, when we overexpressed a constitutively active form of AMPK in *dj-1β* mutant flies, we observed that the activation of the protein is sufficient to rescue the GCase-associated lysosomal defect in the whole body. It would be crucial to perform the same experiments also in fly heads, where endogenous AMPK results hyperactivated. If the overexpression of AMPK-CA rescues the autophagic alterations also in this tissue, this would suggest that the increased phosphorylation of AMPK observed in this body region is probably a compensatory mechanism, which is however not sufficient to counteract the autophagic alterations promoted by the loss of DJ-1.

The assumption that AMPK activation represents a compensatory effect in the brain of *dj-1β* KO flies is supported by the observation that the activity of mTORC1 seems to be upregulated both in fly bodies and heads. In fact, since the activity of mTORC1 is dependent on the AMPK function, we expected to observe a negative correlation between the activity of the two proteins. Accordingly, when we assessed the activation of mTORC1 by evaluating the level of phosphorylation of one of its well-established targets, S6K, we detected an increase of S6K phosphorylation in the whole *dj-1β* null fly bodies, as a response to AMPK deregulation. Coherently, phospho-S6K levels decreased both upon *dj-1β* overexpression and upon AMPK exogenous activation, confirming the functional link between AMPK and

mTORC1. However, mTORC1 appeared hyperactive in fly heads, where also AMPK activation is higher. This result may be due to the fact that AMPK activation is not sufficient in fly heads to compensate for the increase mTORC1 activity, leading to autophagic impairment. Indeed, mTORC1 hyperactivation may account for the autophagic phenotype observed in *dj-1β* KO flies, since this protein complex is known to inhibit autophagic induction through different mechanisms, including the regulation of TFEB and ULK1(40,47,158). One of the possible interpretations of these data is that, in the absence of DJ-1, other cellular processes are activated to increase AMPK activity, trying to restore the proper autophagic function. Interestingly, a recently published paper suggests that in neurons the activation of AMPK is not efficient in the modulation of autophagy, while mTORC1 activity represents one of the main factors that influence the correct progression of the autophagic process (107). Therefore, it is likely that in the brain autophagy mainly responds to mTORC1 function and the hyperactivation of AMPK is not sufficient to reduce the activity of mTORC1 and promote autophagic activation.

Having our data demonstrated the participation of DJ-1 in the modulation of autophagy and its influence on the AMPK-mTORC1 pathway, we finally investigated the upstream stimulus that could promote the alteration of this signaling cascade and lead to autophagic modulation. Among the factors that can influence the activity of AMPK and mTORC1, the alteration of ROS level is a parameter that is well-known to be affected by DJ-1(144). Accordingly, we confirmed that *dj-1β* KO flies are characterized by increased levels of ROS in the brain, which decreased upon *dj-1β* overexpression. Importantly, the effects of ROS on AMPK are not fully understood yet, and it is possible that different reactive species promote opposite responses, and that the AMPK is differently influenced depending on the cell types. Therefore, it would be highly informative in the future to completely characterize whether and how the DJ- mediated increase of ROS alters AMPK function in different tissues and cells. At present, what we demonstrated with this work is that the scavenging of ROS rescued the autophagic phenotypes associated with the number of lysosomes and autolysosomes in the brain, suggesting that the DJ-1-mediated variation of ROS levels is in fact at least partially responsible for the alteration of autophagy.

Collectively, our results point to DJ-1 as a modulator of both mitochondrial and autophagy pathways. Indeed, the protein appears to participate in the control of mitochondrial

dynamics, in the regulation of ROS, as well as in autophagic modulation. Importantly, our data suggest that the effect of DJ-1 on autophagy partially depends on the cellular redox state. These pieces of information may be of high relevance for the neurodegenerative diseases, which are frequently characterized by both mitochondrial and autophagic impairment (78). In this frame, our results confirmed that autophagy and mitochondrial functions are linked in PD and may together participate in the development and progression of the pathology.

As previously mentioned, *Drosophila melanogaster*, albeit representing a valuable tool to untangle some important aspects of neurodegeneration, presents some limitations, which must be consider. Besides the evolutionary differences between *Drosophila* and humans that must be taken into account when performing translational research on human pathologies, the limited availability of antibodies and reagents reduces the possibility to deeply investigating the molecular mechanisms associated with DJ-1 activity. Therefore, it is fundamental to corroborate the data obtained from fruit flies using other tools, such as mouse or human models.

For these reasons, we established a collaboration with Dr. Mark Cookson at the National institute of Health (NIH) and I had the possibility to spend a period in his lab, where I worked with human wild-type and DJ-1 KO iPSC differentiated into DA neurons. Using this model, we confirmed that the silencing of DJ-1 affects autophagic activity, promoting alterations in the number of autophagosomes and lysosomes, and impairing lysosomal activity. In line with the data obtained in fruit flies, we detected increased levels of LAMP1 and LC3. In addition, we corroborated that DJ-1 levels influence the concentration of ROS. Importantly, we also observed that DJ-1 KO cells are characterized by modulation of the AMPK-mTORC1 pathway, with the hyperactivation of both proteins, as observed in fly heads. As already mentioned, the alteration of this pathway may have great relevance for cell physiology, since these protein complexes are at the crossroad of several crucial processes, including not only autophagy, but also cell differentiation and apoptosis (21). Therefore, to further increase the soundness of these important results, we validated these data in another mammalian model. More specifically, we evaluated the activity of AMPK and mTORC1 in different mouse brain regions, namely, cortex, ventral midbrain and striatum, of both wild-type and DJ-1 KO mice, and we confirmed that DJ-1 KO mouse brains are characterized by hyperactivation of

AMPK and mTORC1. As mentioned above, we would expect that the hyperactivation of AMPK should result in a reduction of mTORC1 activity. In contrast, our data indicate that the absence of DJ-1 may alter the functional link between the two proteins, suggesting that mTORC1 activation may be responsible for the ALP defects caused by DJ-1.

A piece of information that we need to consider is that different subcellular pools of AMPK have been recently described to affect specific cell compartments (103). In this scenario, it is possible that mTORC1 hyperactivation is directly linked by a lysosomal fraction of AMPK and the contrasting results that we obtained may depends on other pools of the protein, which are involved in other signaling pathways. As a future perspective, it would be very interesting to investigate whether and how DJ-1 specifically affects different subcellular fractions of AMPK and what are the consequences of their modulation at different cellular levels.

Finally, it is worth mentioning that the differentiation of iPSC does not result in a pure DA neuronal culture. Actually, the differentiation protocol leads to a cell culture composed by about 50% of dopamine producing neurons, together with other neuronal cell types and, likely, non-differentiated cells. Since the regulation of the pathway that we investigated appeared highly tissue- and cell-dependent, we then specifically focused on DA neurons, which are the main class of neurons affected in PD and, therefore, the most relevant cell type for our research (73).

With this neuronal population, we further corroborated the alteration of the ALP, since we detected an increase of LAMP1 and LC3 amount in DJ-1 KO DA neurons. However, we did not confirm the activation of AMPK observed in drosophila and mouse brains, since we detected a decreased AMPK phosphorylation in DJ-1 KO dopaminergic cells compared to the control. These data further confirm the assumption that these molecular signaling pathways are differently affected in different cell types and highlight the fact that it would be crucial in the future to finely characterize the effect of DJ-1 in different neuronal populations as well as in non-neuronal brain cells. Indeed, it is likely that the data obtained in fly and mouse brains, and in the mixed human cell culture are determined by non-DA neuronal cells.

In conclusion, our data demonstrated that in different *in vivo* and *in vitro* models DJ-1 participates in the modulation of the crucial signaling cascade regulated by AMPK and

mTORC1, being, in such a way, a modulator of autophagic activity. Although, as underlined throughout the discussion, several questions remain unanswered, our work shed some light on DJ-1 physiological function and may lay the basis for a better characterization of the protein as a multifunctional factor able to affect several important cellular processes.

Chapter 9

Introduction

The Neuronal Regulation of Autophagy: The Role of PAK6

9 14-3-3 Proteins: Function and Regulation

14-3-3s belong to a family of highly conserved proteins, which in mammals includes seven isoforms (β, γ, ε, η, σ, τ, ζ). The main function of 14-3-3s is to bind phosphorylated proteins and act as chaperones and scaffolding molecules, regulating the function of their interactors through different mechanisms. By interacting with their binding partners, 14-3-3s may affect their stability, structural conformation, subcellular localization, and interactions with other molecules (159,160).

14-3-3s exist as homo- or hetero-dimers, the dimeric form allows these proteins to assume a characteristic conformation with a concave groove that facilitates the binding with other proteins (161). Proteomic analysis revealed that these proteins are involved in the regulation of hundreds of different target binding partners, supporting the critical role of 14-3-3s in many biological processes, including regulation of cell metabolism, apoptosis, and gene transcription (162). 14-3-3s are ubiquitously expressed and high levels of these proteins have been detected in the brain, where they participate in neuronal functions, influencing neurite outgrowth, neuronal migration, differentiation, and neurotransmitter release. Due to their essential contribution to many cellular pathways, 14-3-3 proteins are genetically linked to several neurodegenerative disorders, including PD (159,160,163). In this frame, 14-3-3 phosphorylation has been observed to be altered in lysates from PD patients brains and their possible association with the pathology is also supported by the observation that 14-3-3s can interact with some of the proteins linked to the familial forms of PD, such as LRRK2, PARKIN, and α-syn (163). In addition, 14-3-3s overexpression has been proven to exert neuroprotective effects in PD models, including the one induced by the exposition to toxins such as rotenone and (MPTP) (163).

Even though the main role of 14-3-3s is to modulate the function of other proteins, their activity is also subjected to regulation, which is mainly achieved through post-transcriptional modifications. The most known and characterized modification that affects 14-3-3 activity is phosphorylation and different kinases are known to target these proteins. Phosphorylation of Ser58/59 causes the disruption of 14-3-3 dimer and loss of interaction with binding partners. Other phosphorylation sites are present in the amino acidic sequence of 14-3-3, such as Ser/Thr232 and Ser184/186 and their state seems to affect 14-3-3s interactome (163). This parameter has been proposed to be affected also via the acetylation of lysine

residues within the 14-3-3 sequence. Furthermore, 14-3-3s binding partners can also act as regulators, serving as “sink” molecules that sequester 14-3-3s, preventing their interaction with other molecules (Fig 37) (163).

9.1 TFEB-14-3-3 Interaction

As already mentioned, among 14-3-3 interactors, TFEB is particularly relevant for the purposes of this work. 14-3-3s bind phosphorylated TFEB, modulating its nuclear translocation, and, therefore, affecting its transcriptional activity (164). In this frame, it has been observed that the dimeric structure of 14-3-3s can simultaneously capture two TFEB molecules phosphorylated in Ser211. This site undergoes conformational changes after the binding with 14-3-3s and the region of the nuclear translocation signal, which is essential for the transport of the protein to the nucleus, is masked (164). Even though the TFEB-14-3-3 interaction has been intensively characterized, several questions remain still unanswered. For example, it is not clear whether TFEB can equally bind the seven 14-3-3 proteins or whether it preferentially interacts with a specific isoform or a subset of them.

Another point that would be very relevant to assess is whether and how the phosphorylation sites of TFEB that are not the canonical targets of mTORC1 can affect its binding to 14-3-3s and its activity. Furthermore, the binding between 14-3-3 and TFEB has been investigated only in the cytoplasm, however, it has been shown that 14-3-3s can have also nuclear localization and can participate in the regulation of the nuclear dynamics of their targets (i.e. the sub-nuclear localization, the rate of nuclear export or the binding with DNA)(165). In this frame, it would be very worthwhile to understand whether also the nuclear fraction of TFEB is influenced by the interaction with these proteins. Additionally, it is still not clear whether TFEB-14-3-3s interaction only depends upon TFEB phosphorylation or whether the phosphorylation state of 14-3-3s, which has been shown to influence interactome of these proteins(163,166), is also relevant for this binding.

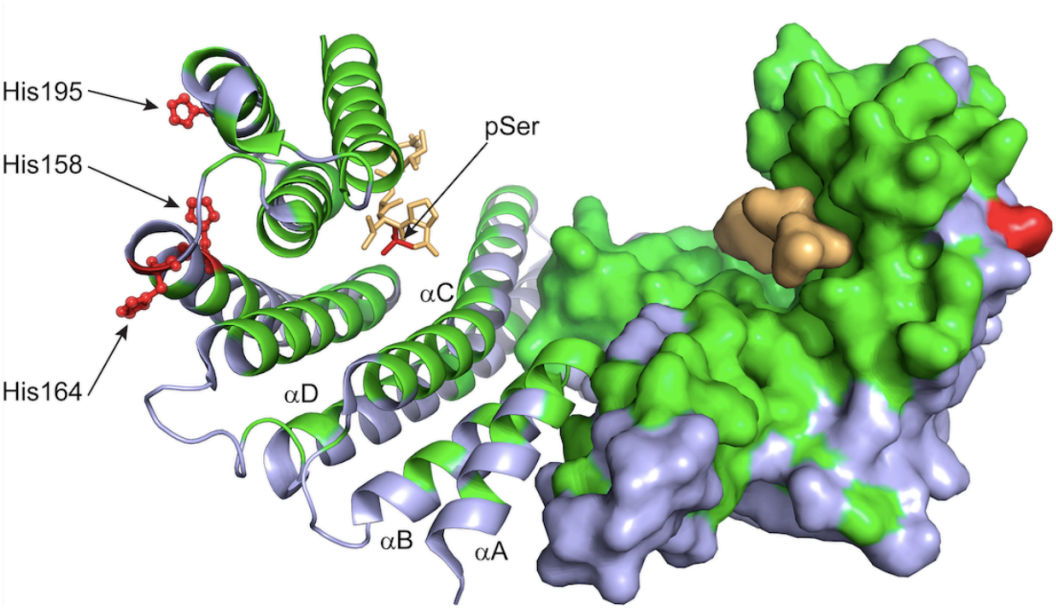


FIG 37: Structure of human 14-3-3γ isoform. Important residues for the binding of the protein to its partners are shown in red, in yellow is highlighted the phosphorylation site, Ser58/59. The dimeric structure of the protein is represented in backbone ribbon (left subunit) and surface representation (right subunit) (167).

Among the proteins that interact with 14-3-3s, our group recently demonstrated that the members of the p21-activated kinase (PAK6) protein family can phosphorylate 14-3-3s, affecting their activities and the interaction with other proteins (166).

9.2 PAK Protein Family: General Features and Activity

The PAK family comprises a group of serine/threonine kinases regulated by the binding with small GTPases, such as cell division control 42 (Cdc42) protein and Ras-related C3 botulinum toxin substrate 1 (Rac1) protein. PAK proteins are highly conserved throughout evolution; indeed, the gene sequence and the protein structure are similar in all eukaryotic cells. In mammals, the PAK family consists of two groups (1 and 2) that differs in the structure, functions and activation mechanism (168).

PAKs of group 1 (PAK1, PAK2 and PAK3) share high homology at the level of their sequence and their structure. At the amino-terminus, these proteins are characterized by a GTP-

binding-domain (GBD) and an autoinhibitory domain (AID), while the kinase domain is localized at the C-terminus. In the inactive state, group 1 PAKs form homodimers. This conformation leads the AID of one monomer to interfere with the kinase domain of the other monomer, inhibiting the protein activity. The binding of small GTPases to the GBD triggers important conformational changes causing the dissociation of the AID from the kinase domain, activating the proteins. Importantly, to get activated, these proteins undergo autophosphorylation in crucial phospho-sites (Thr423(PAK1)/ Thr402(PAK2)/ Thr421(PAK3)), which are essential for protein stability and kinase activity (169,170).

Group 2 PAKs (PAK4, PAK5, and PAK6) are slightly divergent compared to the proteins of group 1, presenting differenced in the kinase and in the AID-GPB domains. These features may account for the alternative activation mechanisms that characterize the two subfamilies. Indeed, the function of the second group of PAK proteins does not depend upon the binding with GTPases, which, instead, is relevant for their subcellular localization. On the contrary, it has been reported that the release of the kinase domain and the consequent activation of these proteins is promoted by the binding with local partners. Similar to the PAKs of group 1, upon activation, these proteins undergo auto-phosphorylation at specific serine residues in the kinase motif (Ser474 (PAK4), Ser602 (PAK5), and Ser560 (PAK6)). The level of phosphorylation at these sites is crucial for the function of PAKs and directly correlates with their activity (169–171) (Fig 38).

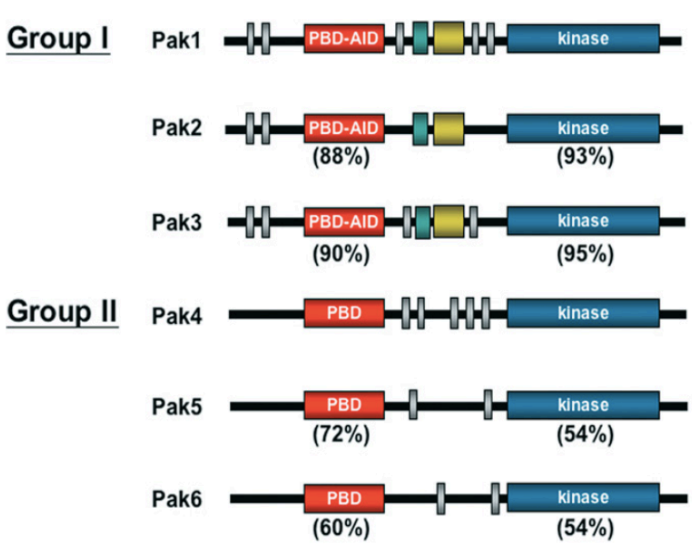


Fig 38. Structural organization of PAKs domains (168). Proline-rich region (grey), PIX binding site (Yellow), acidic region (green).

9.2.1 PAK Protein Family: Pattern of Expression and Function in the Brain

In mammals PAK proteins are present in almost every cell-type, but there are differences in the expression pattern among the family members. PAK of group 1 have been widely found in most of the tissues, with PAK1 and PAK3 predominantly observed in the brain. Regarding the group II PAKs, PAK4 is ubiquitous, while the distribution of PAK5 and PAK6 is more limited. These two proteins are mainly found in the brain, even though low expression levels of PAK6 have been observed also in the testis, prostate, and kidney (Fig 39) (170).

PAK proteins have been associated with different cellular pathways, such as the regulation of cytoskeletal dynamics, which represents their best-characterized role, but they are also involved in the modulation of apoptosis, gene transcription, and autophagy (172). The high level of expression of these proteins in the brain suggests that they may have important functions in this tissue. Accordingly, their activity has been linked to the remodeling of cytoskeletal apparatus, influencing not only neuronal plasticity, axonal guidance, and outgrowth, but also the maintenance of synaptic activity and neuronal spine function (170). Among the proteins of group 1, PAK1 is involved in actin cytoskeleton polymerization during the developmental stages of neuronal differentiation and neuronal migration, and it has also been found to modulate dendritogenesis and neuronal spine formation. Group II PAKs is also highly involved in brain development and function. The most characterized protein of this subgroup, PAK4 modulates neuronal plasticity, but it has also a role in a variety of functions, such as astrocytic activation, viral infection and modulation of transcription factors involved in the expression of inflammatory cytokines (170,173). In addition, PAK5/PAK6 KO mice present shorter neurite, confirming that these proteins are important for neuronal network development, influencing neuronal morphology, the ability to form and sustain synaptic contacts, and synaptic activity (174). These data clearly link the activity of these proteins to brain function, and it is not surprising that alteration in the level of these proteins has been observed in different neurodegenerative diseases.

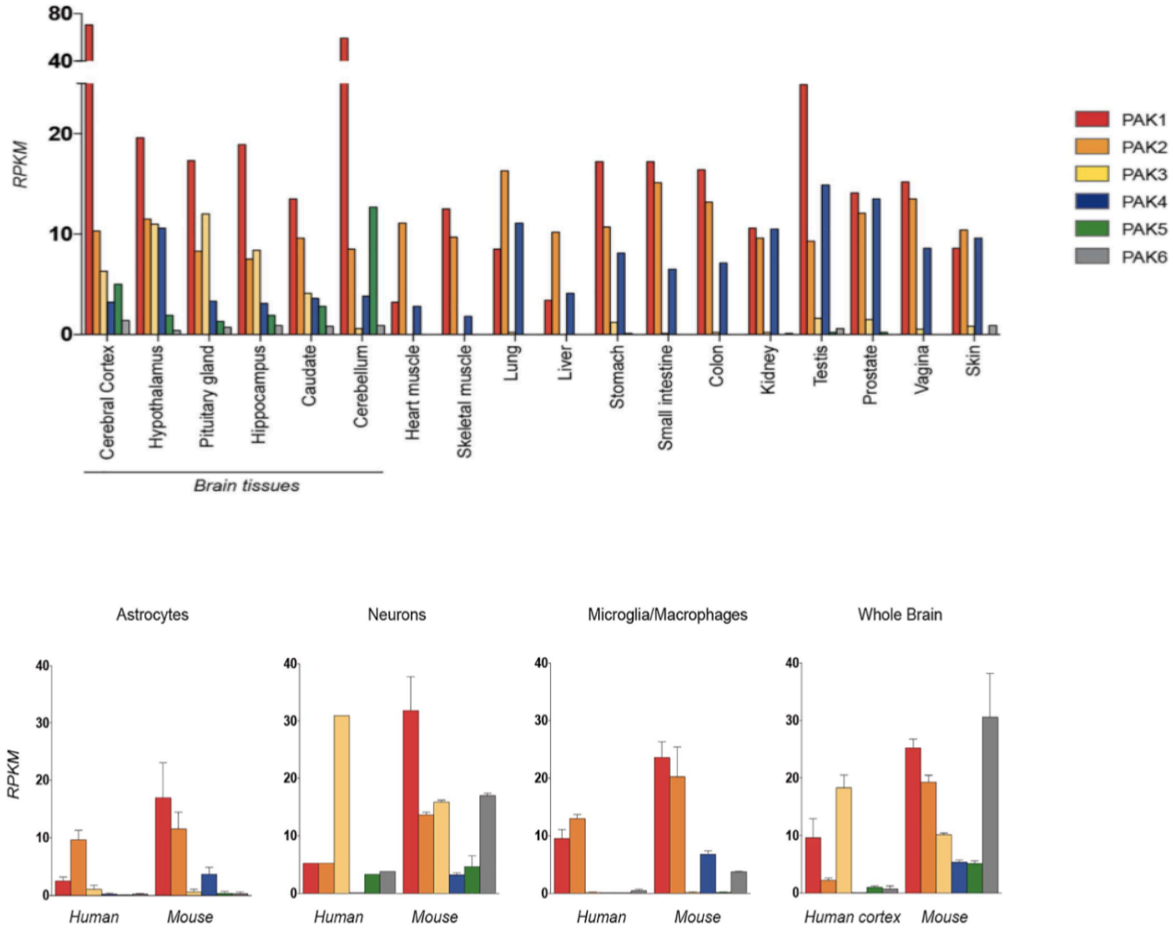


Fig 39: Pattern of expression of PAK proteins. In the upper panel is represented the level of expression of PAK proteins in different tissues; in the lower graphs the level of expression pf PAK proteins in the cell populations of the brain (170).

9.3 PAK6

Among the PAK proteins, p-21- activated kinase 6 (PAK6) has been originally studied as a binding partner of the androgen receptor (AR) (175). This binding results in the PAK6 nuclear translocation, which represses the steroid hormone-dependent transcriptional activity (175). Given the implication of AR transcriptional function in the development of some forms of cancer, the role of PAK6 has been mainly investigated as a kinase able to regulate tumor development and progression (176,177). Despite its crucial role in cancer, the function of PAK6 in the brain is also of great interest for the purposes of this research work. Interestingly, human PAK6 was shown to be prominently expressed in neuronal cells in brain, suggesting that its main function may be associated to the specific regulation of neuronal physiology (170). In this cell type PAK6 has been found in the cytoplasm of the soma and of the neurites; however, given its nuclear localization in other cell types, where PAK6 regulates transcription factors activity, it is not to be excluded that a fraction of PAK6 may also have nuclear subcellular localization in neurons. In neuronal cells PAK6 regulates actin polymerization and cytoskeleton reorganization, influencing neuronal complexity as well as pre-and post-synaptic morphology. Thanks to these roles PAK6 promotes synaptic efficiency, increases the size of dendritic spines, and induces the formation of new synapses. Moreover, since the actin cytoskeleton in synaptic structures is also essential for the correct motility of vesicles from pre- to post-synaptic terminals, PAK6 has also been shown to coordinate the traffic of vesicles (170). The participation of PAK6 in the maintenance of neuronal physiology is also confirmed by the link of this protein with PD. In this regard, hyperactive PAK6 has been observed in the postmortem brains of patients carrying the PD-related G2019S LRRK2 mutation as well as in idiopathic PD patients. Moreover, PAK6 is an established interactor of LRRK2 (166,170,178), this interaction, in physiological conditions, seems to be crucial for the correct activation of PAK6, which undergoes autophosphorylation and activation after the binding with LRRK2 (178). At the functional level, the binding of LRRK2 to PAK is necessary to promote dendrite development, branching, and spine formation. Interestingly, PD-associated LRRK2 mutations may affect the activity of PAK6, resulting in an aberrant overactivation of the kinase that can lead to neuronal defects and, eventually, to pathological conditions (166). These data link the activity of this protein to PD pathology and confirm the potential relevance of investigating PAK6 in neurodegeneration.

9.3.1 PAK6-14-3-3 Interaction

The analysis of the binding between LRRK2 and PAK6 led to the discovery that this interaction is influenced by the PAK6 kinase activity toward 14-3-3 proteins. In this context, it has been demonstrated that PAK6 can phosphorylate 14-3-3s on Ser59 with the highest affinity for the γ isoform. As already reported, this amino acidic residue is located at the dimer interface and its phosphorylation causes a shift from the dimeric to the monomeric state of 14-3-3s. Monomeric 14-3-3s are incapable of interacting with their binding partners; therefore, the phosphorylation of this residue may cause a loss of affinity of 14-3-3 to their client proteins. This mechanism, which may be very relevant for the activity of several known 14-3-3 protein interactors, has been proven to be crucial for LRRK2. By the phosphorylation of 14-3-3γ Ser59, PAK6 promotes the dissociation of this proteins from LRRK2, resulting in a decrease of LRRK2 phosphorylation and activity (166). In this scenario, PAK6 may represent an important kinase in the brain, able modulate 14-3-3s interactome in neurons, potentially affecting a number of cellular processes that are regulated by the chaperone proteins.

Chapter 10

Aim of the Project

10 Aim of the Project

The data available in the literature point to autophagy as one of the major processes altered in neurodegenerative disorders (66). Coherently, our study about DJ-1 further supported the idea that autophagic impairments are involved in PD pathology (146).

Defects of the clearance pathways may detrimentally affect neuronal homeostasis by promoting the accumulation of dysfunctional organelles and impair the neuronal protein quality control. Accordingly, one of the main features of neurodegenerative disorders is the accumulation of protein and the formation of cytoplasmic proteinaceous aggregates, that may result in neuronal toxicity and, eventually, neuronal death. These protein inclusions are known to be autophagic substrates; therefore, ALP defects may impact on the rate of their degradation and accumulation, exacerbating their toxic effects (3).

These pieces of evidence suggest that maintaining of functional clearance mechanisms is crucial for neuronal homeostasis and for preventing neuronal death. In this frame, it is not surprising that autophagic induction is thought to be a promising therapeutic approach to counteract neurodegeneration (179).

Importantly, the enhancement of autophagic activity, albeit may be positive for neuronal cell survival in certain conditions, could also have negative effects if not properly controlled or if targeted to other cell types. Therefore, in the effort to regulate autophagy, is crucial to take particular care not only to the extent of the modulation but also to the cells or tissue in which the external manipulation is directed. Given these premises, the main objective of this project is to unravel a neuronal-specific mechanism of autophagy modulation. This would lead to a better understanding of the physiological pathways that affect autophagy in neuronal cells and, eventually, open the way to the discovery of novel targets for neurodegenerative diseases therapies.

Among the possible mechanisms to regulate autophagy, the induction of TFEB transcriptional activity may be a good strategy to increase the expression of genes involved in the ALP (180). As previously described, TFEB level and activity has been reported to be altered in several neurodegenerative disorders, confirming the importance of this protein in this context. The inverse correlation between TFEB nuclear localization and

neurodegeneration is supported by several research works that observed a decrease of TFEB activity and reduced CLEAR genes expression in HD, AD and PD models and postmortem human brain samples. In this frame, the modulation of TFEB to restore the homeostatic activity of this protein in neurons would represent a great opportunity for the beneficial regulation of the whole ALP (3,71,181,182).

As detailed in the introduction, TFEB activity and subcellular localization is highly dependent on the binding with 14-3-3 proteins. 14-3-3s are known to bind TFEB in the cytoplasm to prevent its nuclear translocation and, consequently, its transcriptional activity (150). We specifically focused on this interaction, with the hypothesis that modulating TFEB-14-3-3 binding would represent a relevant way to regulate TFEB function. In this framework, our lab recently identified the neuronal-enriched kinase PAK6 as a modulator of 14-3-3s interactome. More specifically, it has been demonstrated that PAK6 can phosphorylate Ser59 of 14-3-3 γ isoform preventing its binding with the PD-associated protein LRRK2 (166).

In light of this, the two most relevant aims of this project were to test whether PAK6 is able to induce the nuclear translocation of TFEB by phosphorylating 14-3-3s and reducing their binding affinity for TFEB.

Accordingly, in the first part of the project we confirmed that PAK6 is involved in the modulation of TFEB subcellular localization and autophagic activity.

Then we focused on the analysis of the molecular mechanisms that determines the effect of PAK6 on TFEB regulation, unravelling a direct TFEB-PAK6 interaction, and shedding lights on potential new TFEB regulation mechanisms, specifically in neurons, based on the nuclear binding between TFEB and 14-3-3s.

Chapter 11

Results

11 Results

As mentioned in the previous sections, the possibility of modulating autophagy in neurons may represent a valuable therapeutic tool to intervene in the onset and progression of neurodegenerative disorders. In light of this, the aim of my project was the characterization of a novel mechanism to regulate the most important clearance pathway specifically in neurons. Considering that our lab identified the neuronal kinase PAK6 as a modulator of 14-3-3s interactome (166), we evaluated the possible role of PAK6 in the control of the subcellular localization and activity of TFEB, which are highly regulated by the binding with 14-3-3s (183).

To unravel the involvement of PAK6 in neuronal autophagy, we took advantage of different relevant *in vivo* and *in vitro* models. For the evaluation of the role of the kinase *in vivo*, we mainly exploited *Drosophila melanogaster.* As previously described, fruit flies represent a valuable model for the study of neuronal physiology. Moreover, being autophagy highly conserved in all eukaryotic organisms, *Drosophila* provides an excellent system to study this process, being characterized by a high degree of integration, but with a relatively low level of complexity. Importantly for this project, fruit flies possess only one gene corresponding to the group II PAKs, the mushrooms bodies tiny (*mbt*), as well as a single orthologue for the MiTF/TFE protein family, Mitf. This feature allows to characterize the physiological role of PAK6, and its possible interaction with TFEB in a relatively easy model, avoiding the redundancy caused by the presence of different homologs of the same protein. Mbt was found in *Drosophila* as a protein necessary for the correct development of mushroom bodies, brain areas involved in the formation of memories (184). The study of mbt sequence revealed a high homology with the proteins of the group II PAKs (184–186). Even though the role of this protein in *Drosophila* is still poorly investigated, it was demonstrated that the downregulation of mbt causes reduction of neuroblasts, defects in the central brain and eye impairment, with a decrease in the number of photoreceptors. Recently, the loss of mbt in *D. melanogaster* has been associated with PD-like features, such as motor defects and alterations in neuronal development (187).

To characterize mbt activity, we took advantage of the *Drosophila melanogaster* GAL4-UAS system. This technique involves the crossing between a fly line expressing the yeast transcription factor GAL4 under the regulation of a specific promoter, and a strain containing

a gene of interest in frame with the upstream activating sequence (UAS), which is bound by GAL4. The progeny originating from crossing these parental lines will express the gene of interest in the cell lines affected by the promoter gene linked to GAL4. In this way, we had the possibility to express an shRNA against *mbt*, silencing the protein in different cell types, i.e. neurons and glial cells, as shown in figure 40 (a-b). Moreover, we took advantage of a Pak5/Pak6 knockout (KO) mouse model to complement our result in a vertebrate system. Thanks to the valuable collaboration with other laboratories (Simone Martinelli lab, Istituto Superiore di Sanità, Rome, Italy), we had the chance to corroborate important results in *Caenorhabditis elegans* using an overexpressing approach. The biochemical experiments were performed using different human immortalized cell models, such as SH-SY5Y cells, Hela cells and HEK293T cells.

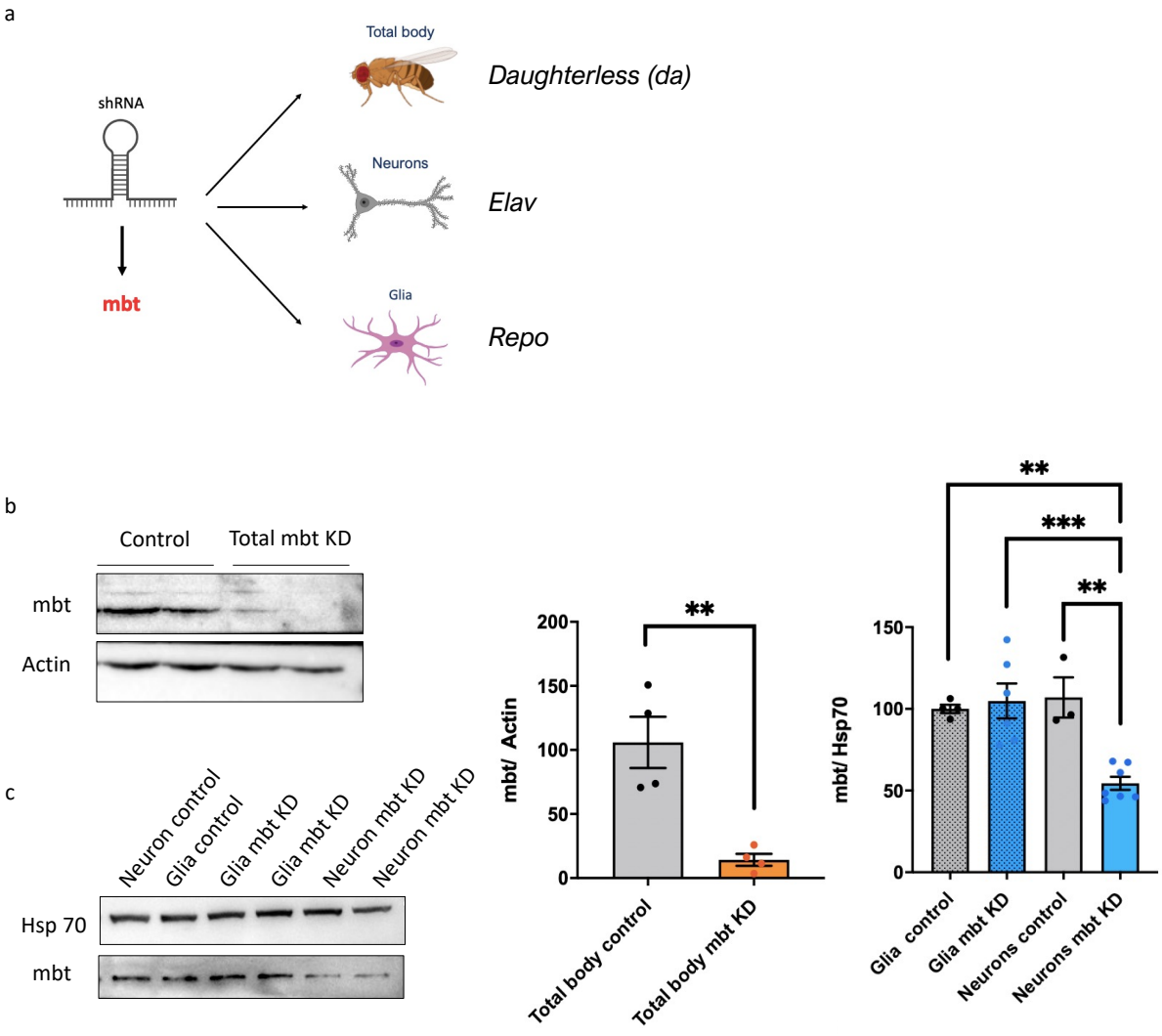


Fig 40: (a) Schematic representation of the mechanisms used for the targeted downregulation of mbt. Through the GAL4-UAS system, expressed the shRNA against mbt in the whole body, or specifically in neurons or in the glia. The cell-targeted expression was achieved through the use of the specific promoters Daughterless, Elav and Repo. Western blot analysis was performed to check the level of mbt downregulation in the whole-body (b), in neurons and in glial cells (c). At least three independent experimental replicates were analyzed. Data were analyzed using t-test (b) and one way ANOVA, Turkey's multiple comparisons test (c). ** $p < 0.01$, *** $p < 0.001$.

11.1 mbt Silencing Impairs Fruit Fly Lfespan

To characterize the effect of *mbt* downregulation, we expressed the shRNA directed against the protein ubiquitously in the whole fly body, using the promoter daughterless. It is important to underly that for each fly strain used, we also generated a genetically-matched wild type control. The silencing of *mbt* strongly impacts fly development, causing a decrease in the hatching rate (Fig 41a). Then, we assessed the longevity of hatched flies and observed that the knock down (KD) of *mbt* significantly reduces fly lifespan, suggesting that the kinase plays a critical role for survival processes (Fig 41b). Finally, we tested the motor ability of surviving flies, by performing a negative geotaxis assay, which allows to evaluate fly climbing ability. *Mbt* KD flies are characterized by strong motor impairments (Fig 41c). Overall, these results confirmed the data reported in (187), showing robust developmental and phenotypic defects of flies downregulating *mbt*. The severe phenotypes displayed by total-body-*mbt* KD flies indicate that the ubiquitous silencing of mbt may be too detrimental, causing defects in different important cellular processes. In this scenario, our ability to evaluate the involvement of mbt in autophagy may be affected by the alteration of other pathways. For this reason, we decided to limit the silencing of this protein in the brain, where the characterization of the protein activity is more relevant for the purposes of this work. Specifically, we exploited flies that downregulate *mbt* in the glia, using the Repo promoter (Repo-Gal4), and in neurons, thanks to the Elav promoter (Elav-Gal4).

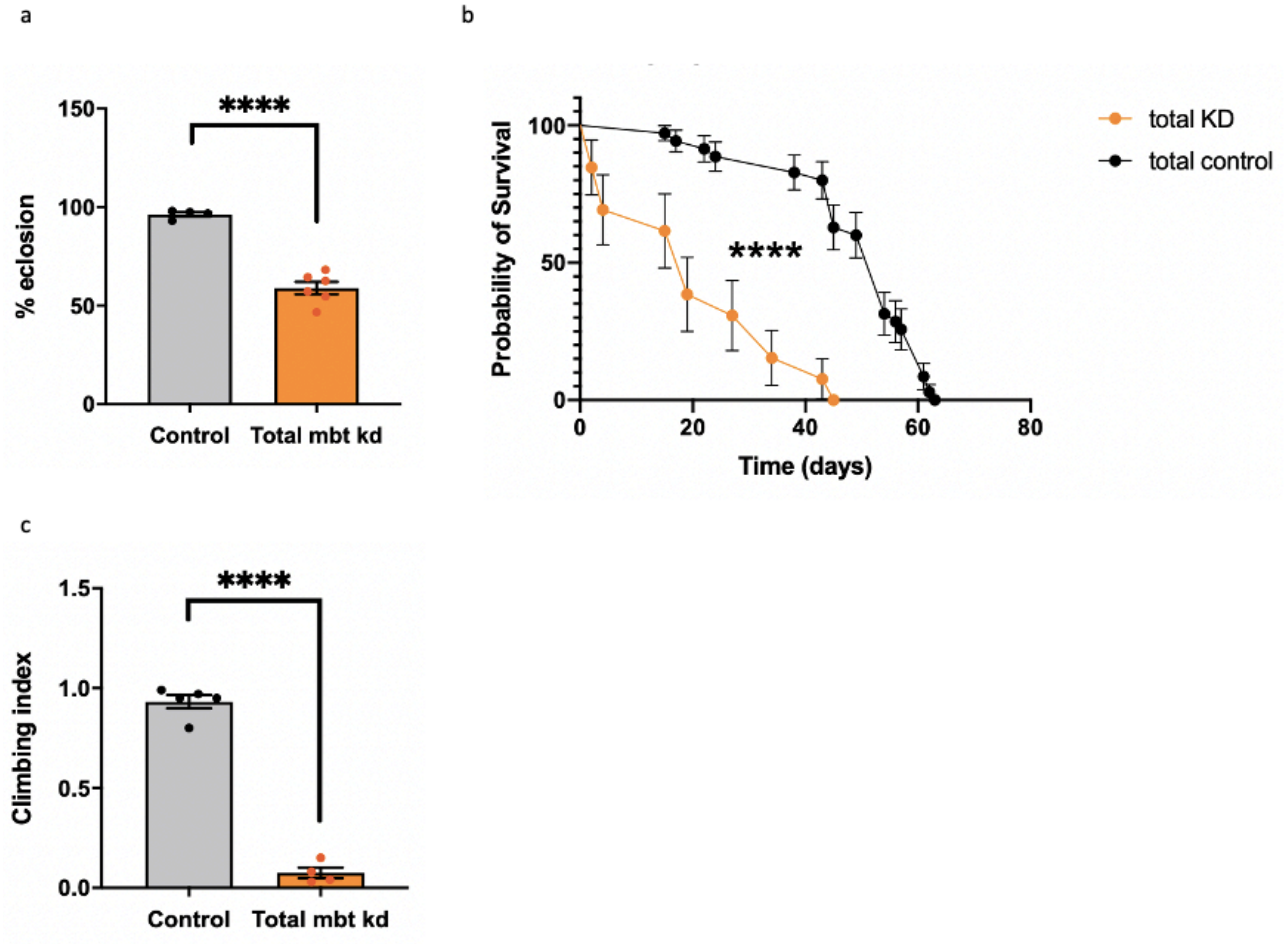


Fig 41: Phenotypic characterization of ubiquitous *mbt* downregulation. (a) Ratio of fly eclosion. Six independent experiments were performed and analyzed using t-test. (b) Probability of survival of control and total-body *mbt* KD flies. At least 40 flies per genotype were used in the experiment. Log-rank (Mantel-Cox) test. (c) Climbing ability of control and *mbt* KD flies. Five independent climbing analyses were performed, and the data were analyzed using t-test. **** $p < 0.0001$.

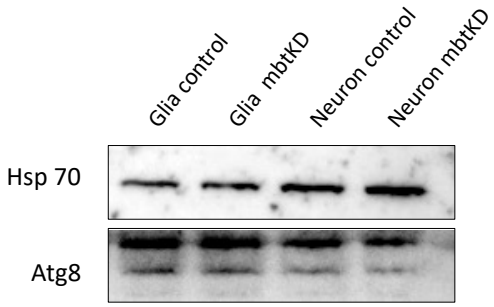
11.2 Neuronal Downregulation of mbt Affects Autophagy in *D. melanogaster*

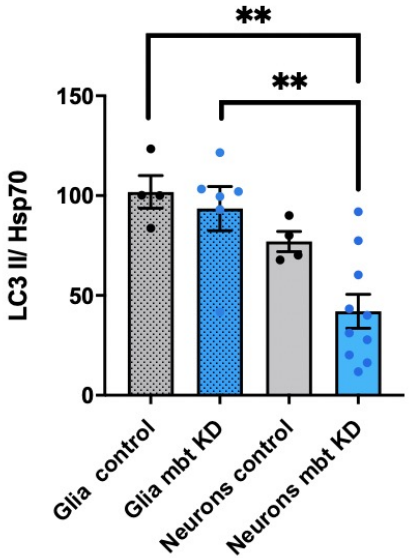
PAK6 in mammals is highly enriched in neurons (170); therefore, we wanted to investigate the role of mbt in this cell type. Moreover, to assess the pattern of expression of *mbt* in fly brain, we also exploited a fly strain downregulating *mbt* in glial cells. As a preliminary test for the possible involvement of mbt in autophagy, we evaluated the level of Atg8 (LC3) in the heads of neuronal- and glial-*mbt* KD flies. Interestingly, we observed that the neuronal-targeted downregulation of *mbt* causes a reduction in the level of the lipidated form of Atg8.

Conversely, by expressing the shRNA against *mbt* in glial cells, not only we did not detect any effect on Atg8 lipidation (Fig 42a), but we could not observe any downregulation of mbt level, which may be due to the low expression of the protein in this cell types (Fig 40c). To confirm the involvement of mbt in the ALP, we measured lysosomal activity by performing GCase assays of samples obtained from neuronal- and glia- mbt KD fly heads. Similar to the previous result, we observed a decreased in the activity of the lysosomal enzyme only in flies with neuronal specific silencing of *mbt* (Fig 3b). These data suggest that mbt may promote autophagic alteration in neurons, but not in glial cells. This result highlights the possibility that the pattern of expression of *mbt* in Drosophila brain is similar to the one observed for PAK6 in mammals, characterized by neuronal enrichment. Finally, we stained neuronal-mbt KD fly brains with lysotracker red, to evaluate whether the downregulation of mbt affects the number of acidic compartments, namely lysosomes and autolysosomes. Noteworthy, we detected a significant decrease of lysotracker-positive structures in mbt-KD flies compared to matching controls, further supporting the hypothesis that mbt may participate in autophagy modulation (Fig 42c).

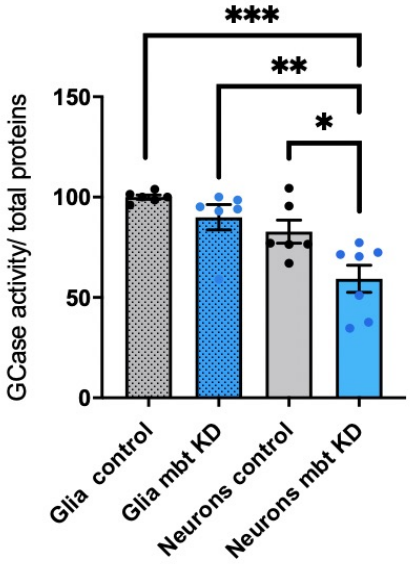
Having observed a clear link between mbt and ALP in neurons, we next decided to assess whether this protein affects the transcriptional activity of the *Drosophila* ortholog of TFEB (Mitf). We performed RT qPCR of genes modulated by Mitf in samples obtained from the brain of neuronal-targeted *mbt* KD and control flies. As shown in figure 42d, we could not detect any significative difference between the two genotypes. This data may indicate that mbt modulates the ALP independently of Mitf activity. Another possible explanation is that the mbt regulates Mitf specifically in neuronal cells. In this case, the neuronal effect of mbt is likely diluted by the signals derived from non-neuronal brain cells.

a

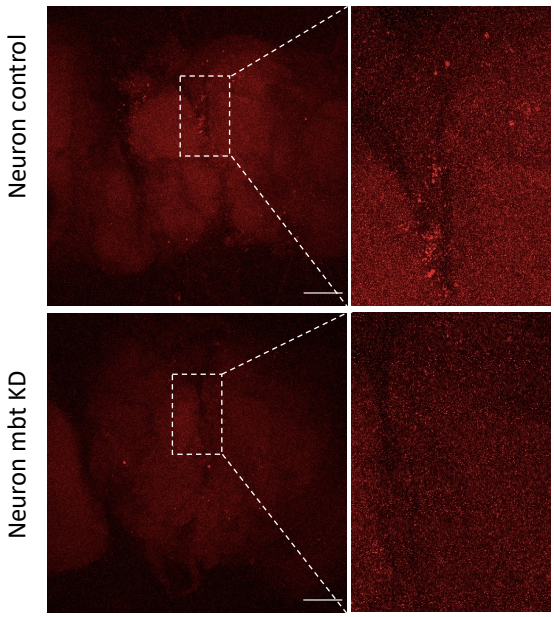

Glia control
Glia mbtKD
Neuron control
Neuron mbtKD
Hsp 70
Atg8

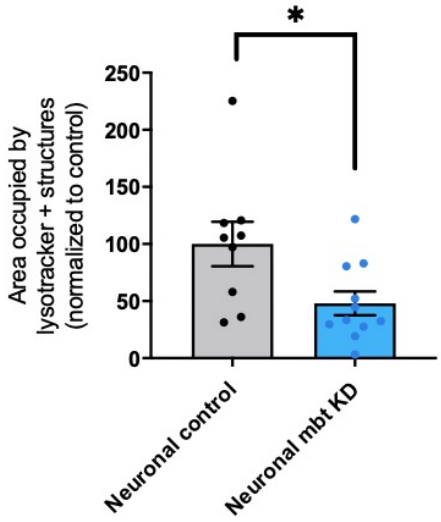

**
**
150
100
50
0
LC3 II/ Hsp70
Glia control
Glia mbt KD
Neurons control
Neurons mbt KD

b


***
**
*
150
100
50
0
GCase activity/ total proteins
Glia control
Glia mbt KD
Neurons control
Neurons mbt KD

c


Neuron control
Neuron mbt KD


*
250
200
150
100
50
0
Area occupied by lysotracker + structures (normalized to control)
Neuronal control
Neuronal mbt KD

d

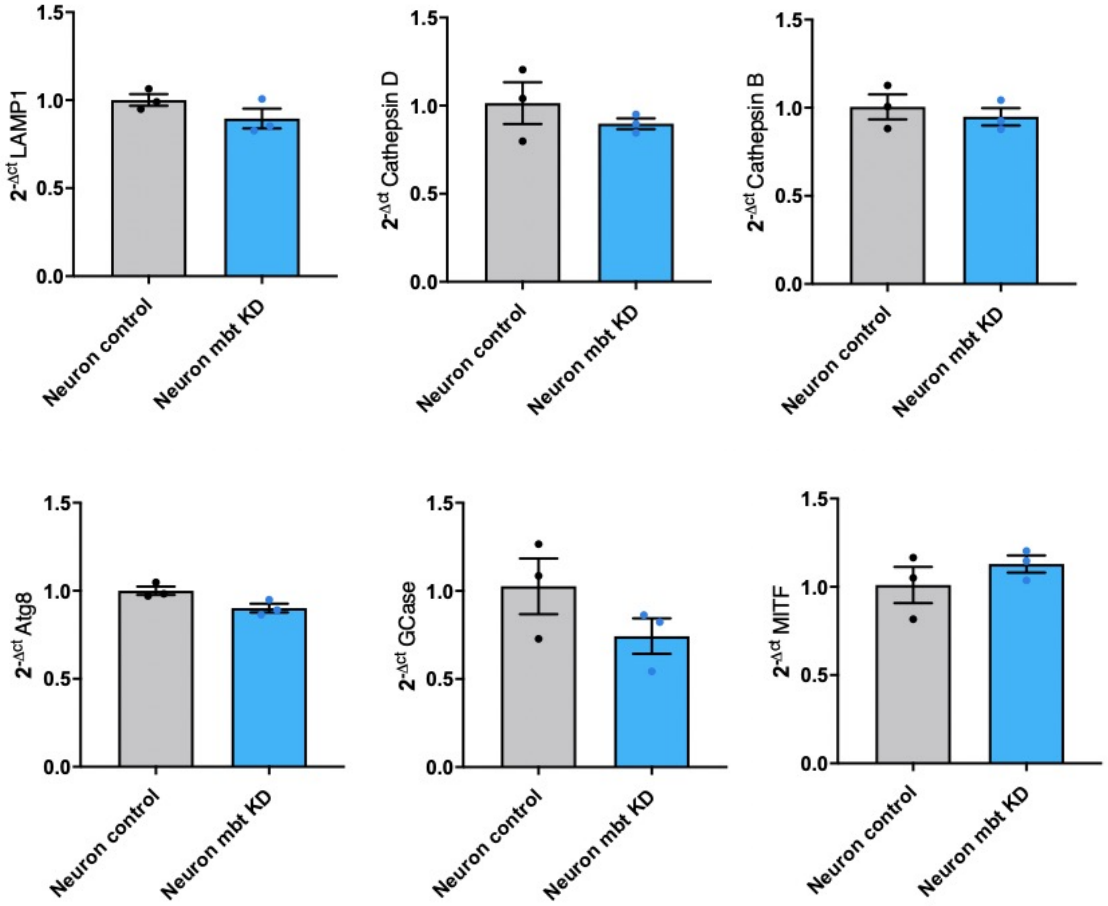
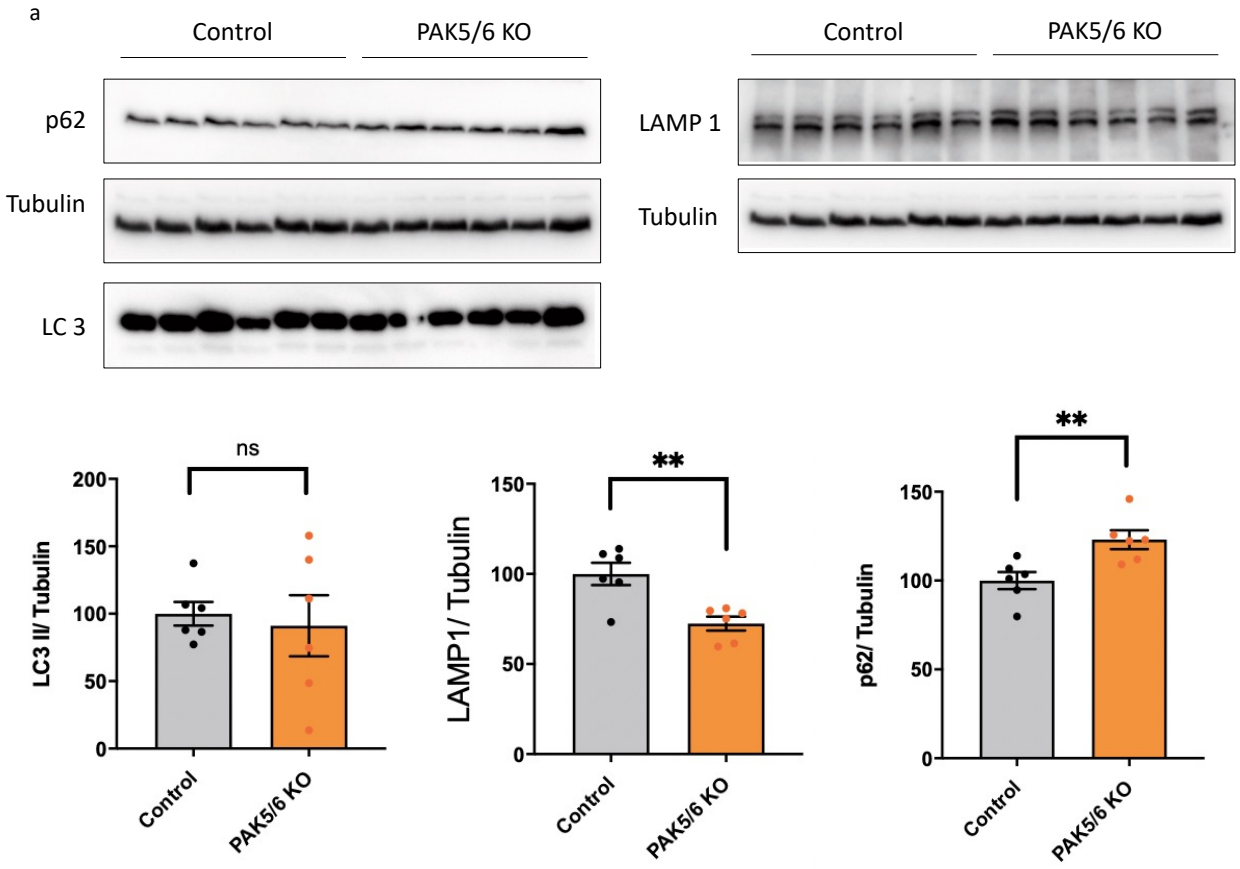


Fig 42: Characterization of *mbt* silencing in neurons and in glial cells. (a) western blot for the evaluation of LC3 lipidation level of neuronal and glia mbt-KD fly heads. At least 4 replicates were used for each genotype. Data were analyzed with a one way ANOVA with Turkey's multiple comparisons test. (b) GCase enzymatic assay of fly heads downregulating *mbt* in neurons and in the glia and matching controls. At least 5 biological replicates were used. Data were analyzed using one way ANOVA, Turkey's multiple comarisons test (c) Fly brains were stained with lysotracker red. In the graph the area occupied by lysotracker-positive structures was quantified. Data were analyzed using t-test on at least 9 brains per genotype. (d) RT qPCR of samples from neuronal targeted *mbt* KD and control flies. Experiments were performed three times. Data were analyzed with t-test but no differences were found. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

11.3 Autophagy is Altered in Pak5/Pak6 Knockout Mice

The results obtained in fruit flies point to mbt as a modulator of autophagic activity. However, being mbt the only *Drosophila* ortholog of PAK4, PAK5, and PAK6, it is not possible to discriminate whether this function is performed by a specific member of the mammalian group II PAKs or whether it is a shared feature of all the kinases of this subfamily. Since we were particularly interested in studying neuronal specific mechanisms to regulate autophagy, we focused on the two members of the group II PAKs whose expression in mammals is enriched in neurons, PAK5 and PAK6 (170).For this reason, we evaluated lysosomal and autophagic functions in Pak5/Pak6 KO (DKO) mice. We collected lysates from the cortex of DKO mice and matching controls and analyzed the level of different autophagic markers. Interestingly, we observed alterations in the level of LAMP1 and p62, suggesting a possible involvement of PAK5 and PAK6 in the autophagic pathway in mice (Fig 43a). To confirm the ALP alterations, we performed the GCase assay on the same lysates and, similar to fruit flies, we detected a decrease in GCase activity in DKO mouse brain compared to WT controls (Fig 43b).



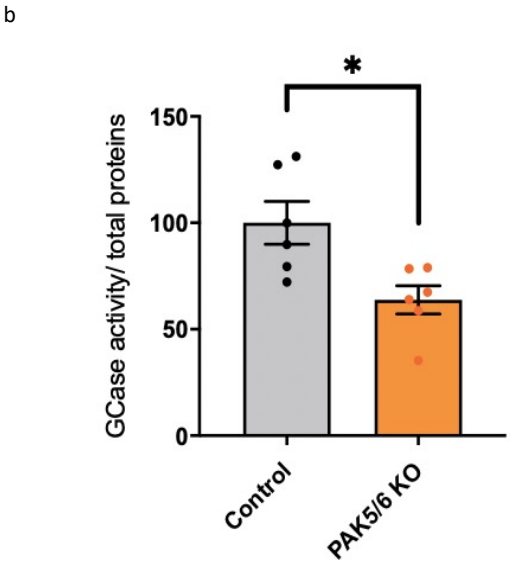


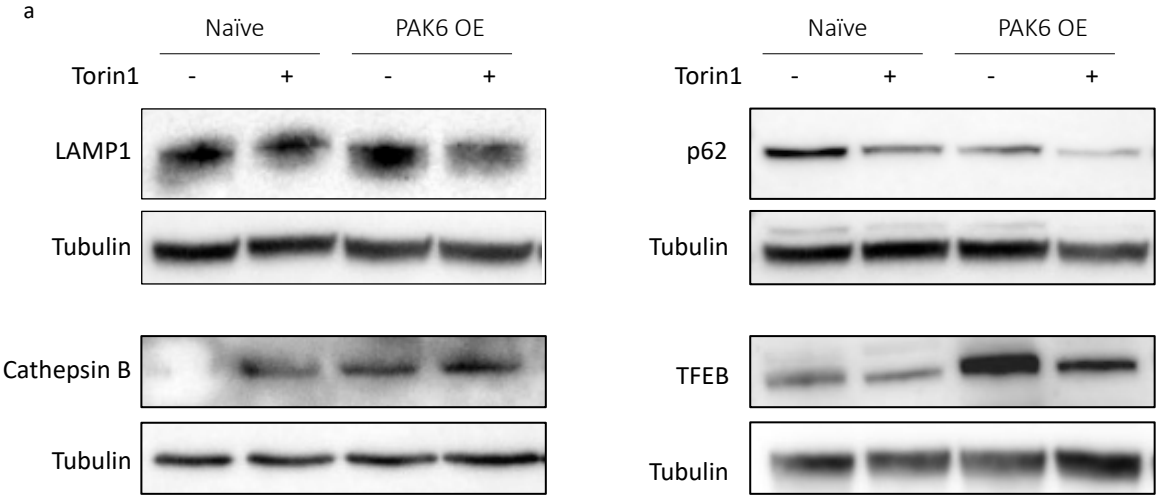
Fig 43: (a) western blot of PAK5/6 KO mouse cortex. 6 mice were used per genotype. Data were analyzed with a t-test. (b) GCase enzymatic assay of doble KO and control mice cortex. 6 biological samples were analyzed using t-test. ns = not significant, * $p < 0.05$, ** $p < 0.01$.

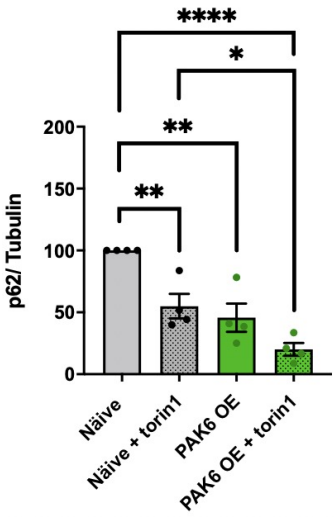
11.4 PAK6 Modulates Autophagy in SH-SY5Y Cells

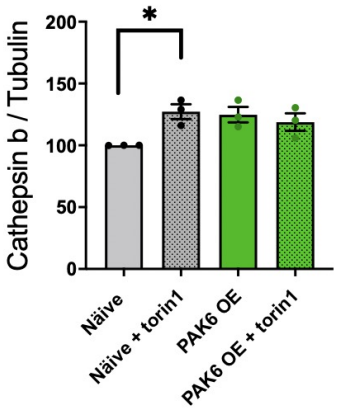
Among the members of the group II PAKs in human, PAK6 has the most restricted pattern of expression in the brain, where it is present mainly in neuronal cells. For this reason, we next focused on the activity of this kinase in relation to ALP. To characterize whether PAK6 plays a role in autophagy regulation in neurons, we took advantage of two SH-SY5Y neuroblastoma stable cell lines previously generated by Dr. Susanna Cogo and available in the laboratory. We used naïve cells, with PAK6 endogenously expressed, and a stable cell line overexpressing WT PAK6 (hereafter PAK6 OE). In these cell lines we evaluated a number of autophagic markers and found significant alterations in the levels of the autophagic substrate p62, with reduction of this autophagic substrate in cells overexpressing PAK6, and variation of the lysosomal enzyme cathepsin B level (Fig 44a). Importantly, the level of these autophagic-related proteins in cells overexpressing PAK6 are comparable to the ones observed in Naïve cells treated with the autophagy inducer Torin1, suggesting that PAK6 activity results in the activation of autophagy. Noteworthy, upon PAK6 overexpression, we detected a significative increase in the level of TFEB (Fig 44a). These data suggest that PAK6 may modulate the ALP through the alteration of the activity or the steady state level of TFEB (Fig 44a).

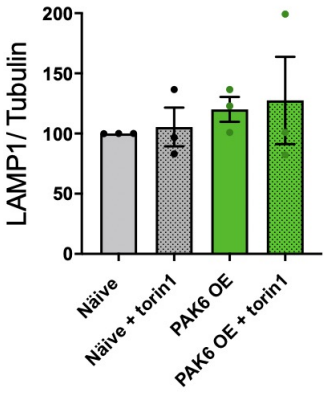
Given the well-established role of 14-3-3 proteins in the activation of TFEB (183), and the involvement of PAK6 in 14-3-3s phosphorylation and regulation reported in our laboratory (166), we assessed whether the level of 14-3-3s phosphorylation varies upon PAK6 overexpression. Coherently with the data reported in literature, we detected an increase in phospho-14-3-3s in ser58/59, which is the target site of the kinase activity of PAK6 (Fig 44b). This result suggests the possibility that PAK6 modulates TFEB activity and ALP, through the phosphorylation of 14-3-3 proteins.

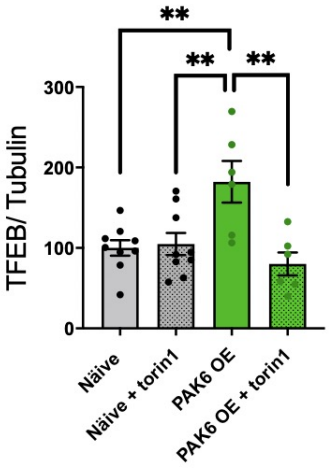
a


Naïve
PAK6 OE
Torin1
- + - +
LAMP1
Tubulin
Cathepsin B
Tubulin
Naïve
PAK6 OE
Torin1
- + - +
p62
Tubulin
TFEB
Tubulin


p62/ Tubulin
****
*
**
**
Naïve
Naïve + torin1
PAK6 OE
PAK6 OE + torin1


Cathepsin b / Tubulin
*
Naïve
Naïve + torin1
PAK6 OE
PAK6 OE + torin1


LAMP1/ Tubulin
Naïve
Naïve + torin1
PAK6 OE
PAK6 OE + torin1


TFEB/ Tubulin
**
**
**
Naïve
Naïve + torin1
PAK6 OE
PAK6 OE + torin1

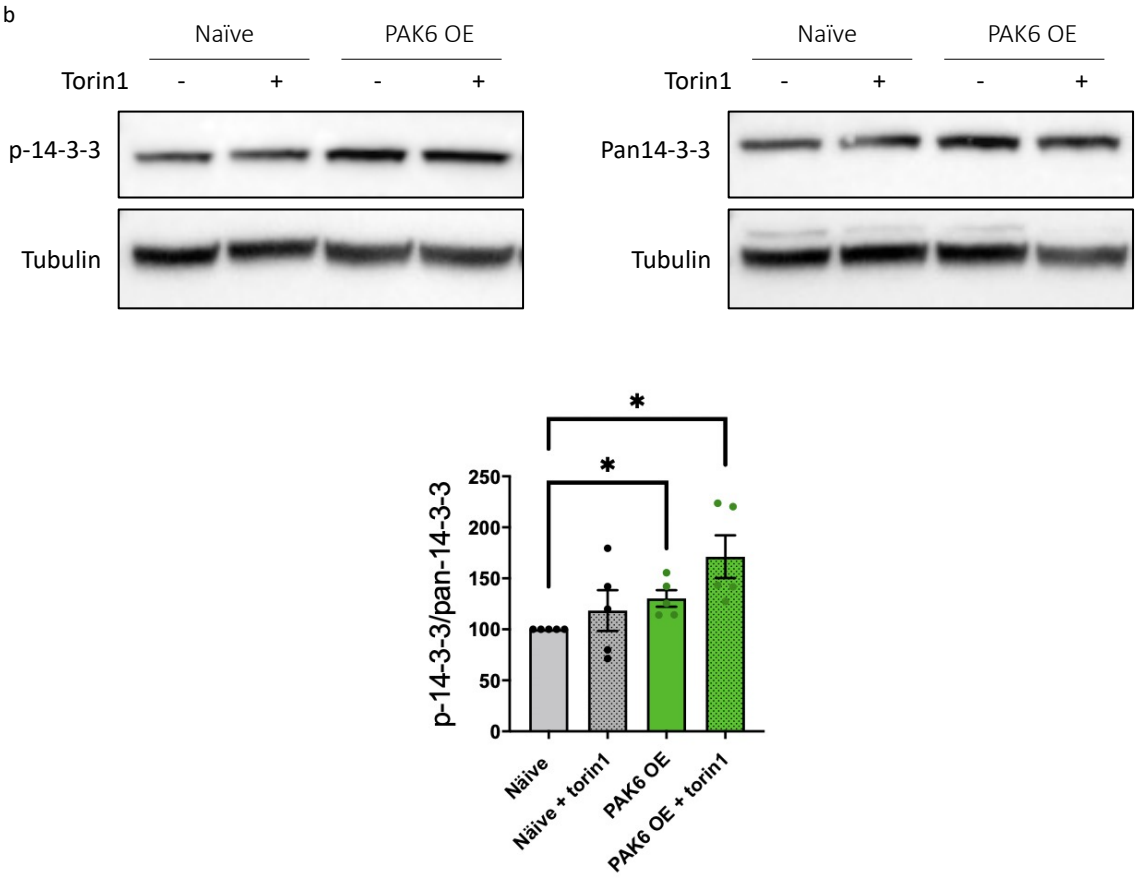


Fig 44: Western blot to evaluate autophagic activity (a) and the phosphorylation level of 14-3-3s (b) in Naïve and PAK6 OE cells. Cells were treated with 2.5 μM of Torin1 for 3 hours. Each experiment has been performed at least three times. Data were analyzed using one-way ANOVA, Turkey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, **** 0.0001.

11.5 PAK6 Participates in the mTORC1 Pathway

P62 is an autophagy receptor that is degraded by the ALP (148). A decrease in p62 levels in PAK6 OE cells suggests that autophagy may be enhanced, similar to the effect of the mTORC1 inhibitor torin-1 (Fig. 44). Thus, we next assessed whether PAK6 exerts its regulatory function on autophagy participating in the mTORC1 pathway, which represents the canonical signaling cascade that leads to autophagy modulation. To test this hypothesis, we treated Naïve and PAK6 OE cells with the specific mTORC1 inhibitor torin1. The impact of the torin1 treatment on PAK6 activity was evaluated by performing an immunoblot experiment to detect the level of the phosphorylated (active) form of PAK6. Notably, the antibody that we used is not specific for PAK6, since it recognizes phospho-Ser560 of PAK4, PAK5, and PAK6. However, the observation that the signal of the antibody is barely visible in Naïve cells, while is highly evident in the cell line overexpressing PAK6 allows to conclude

that the differences observed are determined by PAK6 activity. Interestingly, we detected a significant increase in the level of phospho-Ser560 upon treatment with torin1, both in Naïve cells and in cells overexpressing PAK6 (Fig 45a). The same result was confirmed through confocal imaging, by staining the cells with an anti-phospho-PAK4/5/6 ser560 antibody (Fig 45b). These data strongly suggest that PAK6 is involved in the mTORC1 pathway and point to PAK6 as a downstream effector of mTORC1, since its activity is enhanced upon the inhibition of the protein complex.

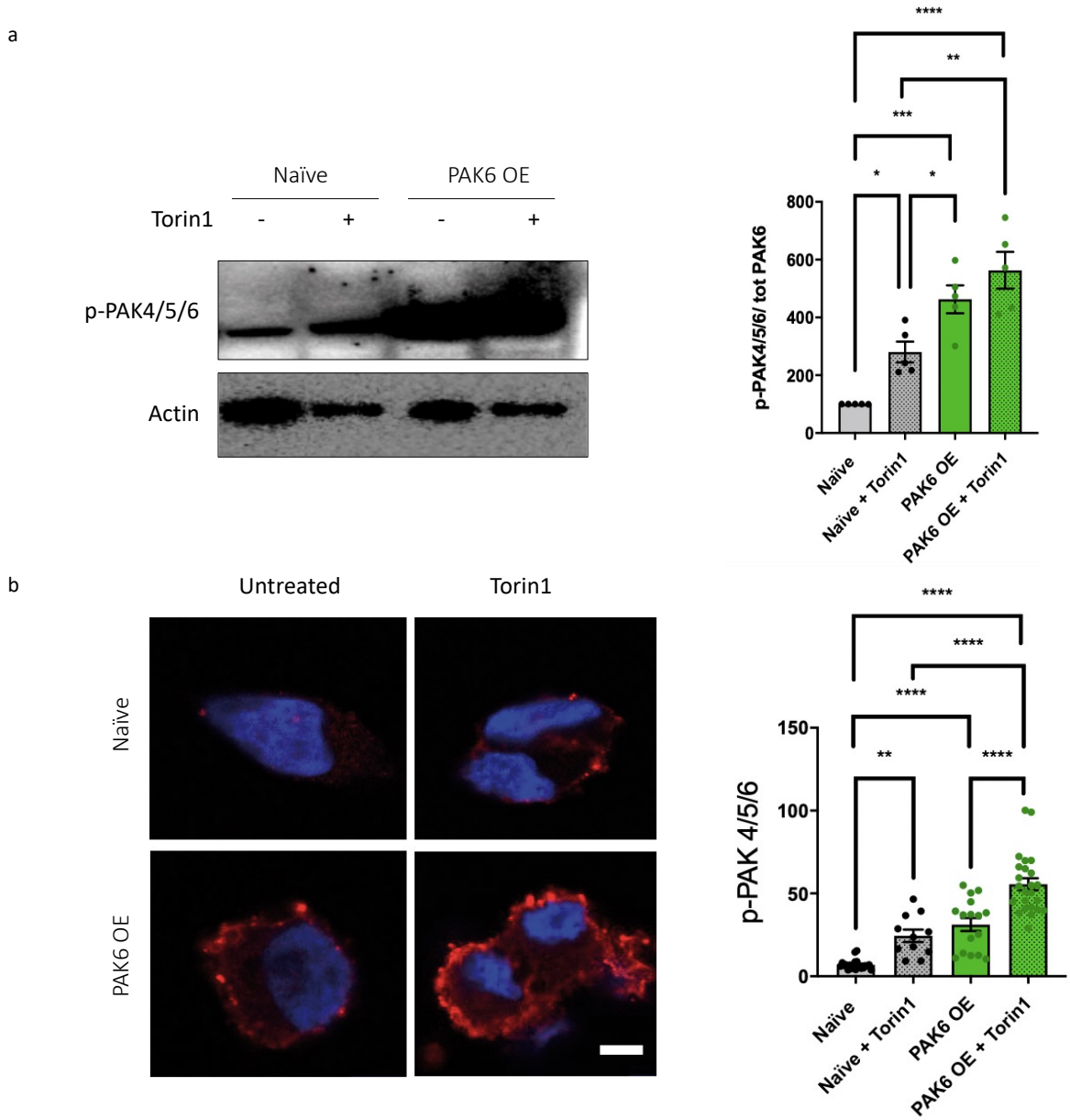


Fig 45: Evaluation of PAK4/5/6 phosphorylation upon inhibition of mTORC1 via Torin1 treatment (2,5 μM for 90 minutes). The level of p-PAK6 has been evaluated through western blot (a) and confocal imaging (scale bar: 10 μm) (b). Data were analyzed with one-way ANOVA with Turkey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

11.6 PAK6 Activity Promotes TFEB Nuclear Translocation

So far, we demonstrated that PAK6 influences autophagic activity and that participates in the signaling cascade regulated by mTORC1, that leads to TFEB modulation (150); moreover, we confirmed that 14-3-3s, which have an established role in the activation of TFEB (39), represent phosphorylation targets of PAK6 (166). In this scenario, we characterized whether the kinase activity of PAK6 affects TFEB subcellular localization. By using the same cell lines exploited in the previous experiments, we assessed whether PAK6 overexpression promotes variations in the level of nuclear TFEB. Through nuclear cell fractionation we observed that the level of TFEB in the nucleus positively correlates with the level of PAK6. Accordingly, nuclear TFEB amount in cells overexpressing PAK6 is higher compared to naïve cells (Fig 46a). This data suggests that PAK6 may promote TFEB nuclear localization.

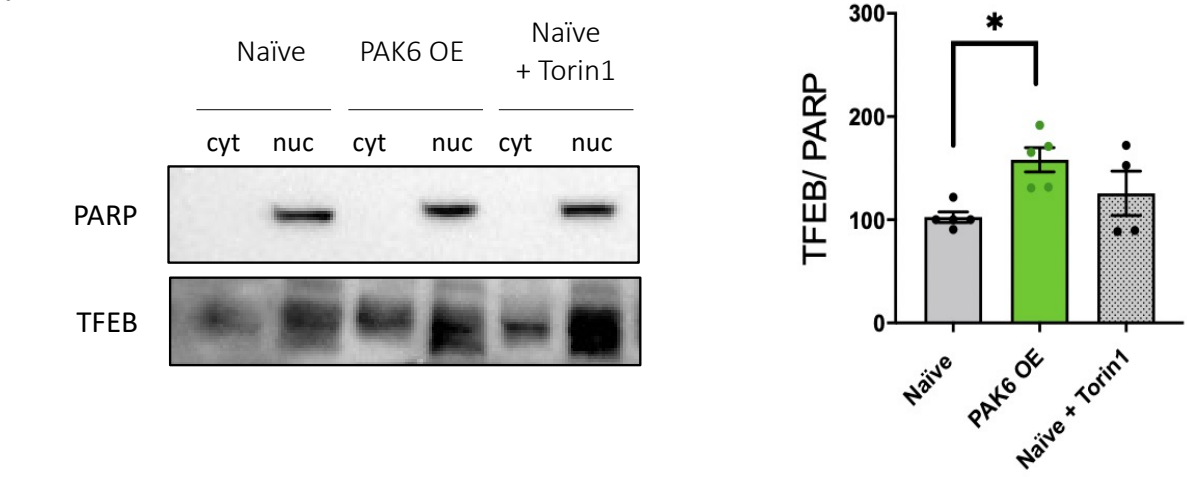
Then, to get better insight into the activity of PAK6 we cotransfected HeLa cells with TFEB wild-type (WT) and different mutants of PAK6. We used PAK6 WT, a kinase dead mutant of PAK6 (K436M) and a constitutively active mutant of the protein (PAK531N) (166). In these cells we assessed the rate of TFEB translocation to the nucleus through confocal imaging. TFEB nuclear translocation for each cell was calculated as the ratio between the difference of TFEB in the nucleus and TFEB in the cytoplasm and the total amount of TFEB in the cell. Interestingly, we observed that while in cells expressing PAK6 WT and PAK6 K436M, TFEB is mainly in the cytoplasmic fraction, in cells transfected with the constitutively active form of PAK6, TFEB almost completely localizes in the nucleus (Fig 46b). Notably, the rate of nuclear translocation of TFEB is comparable to the one observed in cells treated with Torin1, that was used as positive control (Fig 46b). This data strongly suggests that the kinase activity of PAK6 induces the shuttling of TFEB from the cytoplasm to the nucleus (Fig 46b).

To confirm these data with endogenous TFEB, our collaborator Prof. Diego Medina at TIGEM confirmed that PAK6 kinase activity promotes endogenous TFEB nuclear translocation in HeLa cells. In addition to PAK6 S531N, PAK6 WT is also able to triggers TFEB translocation in the endogenous context (Fig 46c)

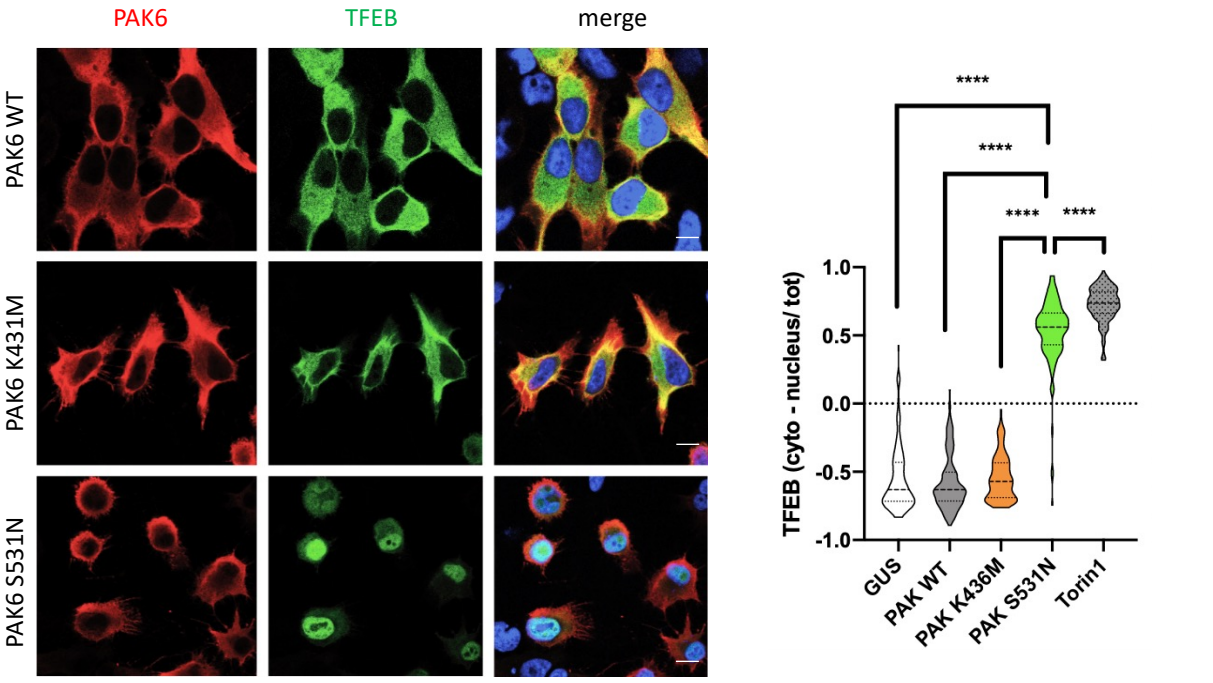
Given the relevance of TFEB for the modulation of the autophagic process, the finding of a novel neuronal regulator of its activity may have a high impact in the overall knowledge of

neuronal physiology. For this reason, we decided to increase the soundness of these data by corroborating the result in an *in vivo* system. To this aim we established a collaboration with the lab of Prof. Simone Martinelli at the Istituto Superiore di Sanità in Rome. Martinelli is an expert in the exploitation of *C. elegans* as a model organism. In this organism Martinelli's group analyzed the nuclear translocation of Helix-loop-helix-30 (HLH30), the worm ortholog of TFEB, in animals overexpressing human PAK6 WT and PAK6 S531N. Interestingly, upon starvation we observed that the activity of PAK6 anticipates the nuclear translocation of HLH-30. Being starvation a stimulus that inhibits the mTORC1 pathway, this result further confirms that PAK6 may participates in this signaling cascade (Fig 46d). This interesting result confirms that the activity of PAK6 is involved in the modulation of TFEB *in vivo*. However, it is important to underly that in basal condition the overexpression of PAK6 S531N in *C. elegans* is not sufficient to induce HLH-30 translocation to the nucleus. This result may be due to the fact that we used a human protein in *C. elegans*; therefore, we cannot exclude that the mutation of Ser531, which in human results in the constitutive activation, does not have the same effect in worms.

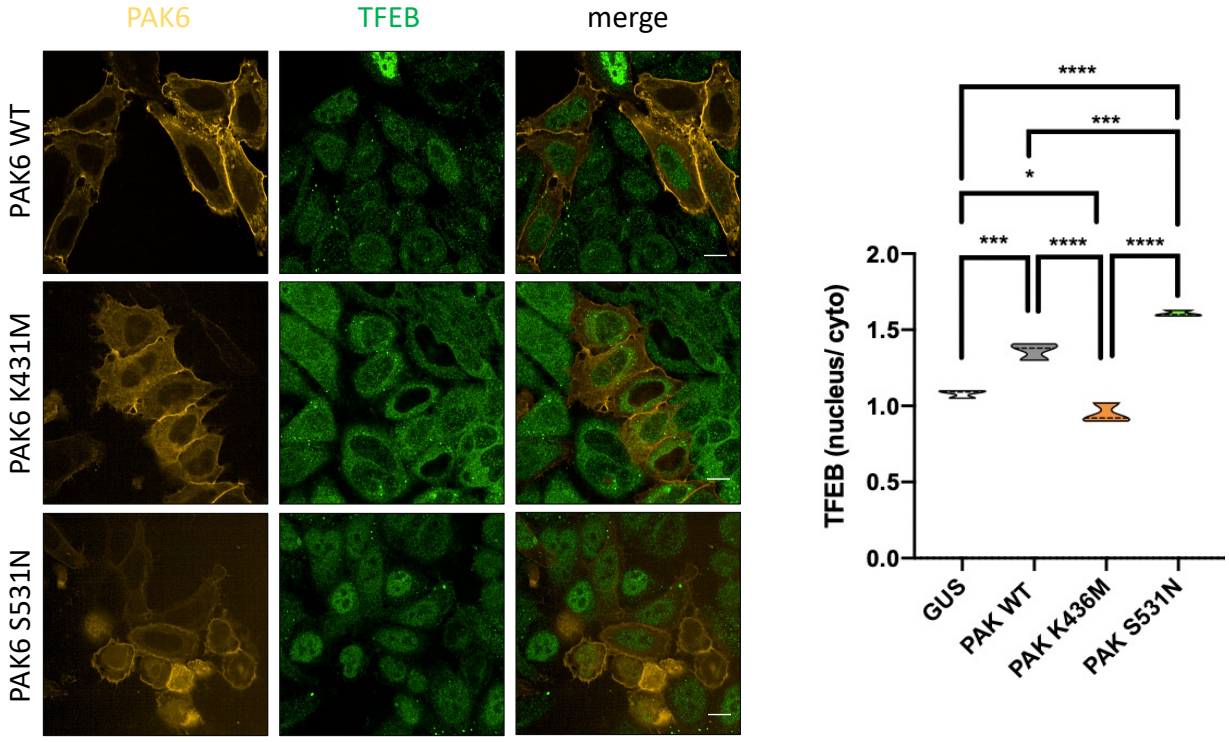
a



b


PAK6
TFEB
merge
PAK6 WT
PAK6 K431M
PAK6 S531N
TFEB (cyto - nucleus/ tot)
1.0
0.5
0.0
-0.5
-1.0
****
****
****
****
GUS
PAK WT
PAK K436M
PAK S531N
Torin1

c


PAK6
TFEB
merge
PAK6 WT
PAK6 K431M
PAK6 S531N
TFEB (nucleus/ cyto)
2.0
1.5
1.0
0.5
0.0
****
***
*
***
****
****
GUS
PAK WT
PAK K436M
PAK S531N

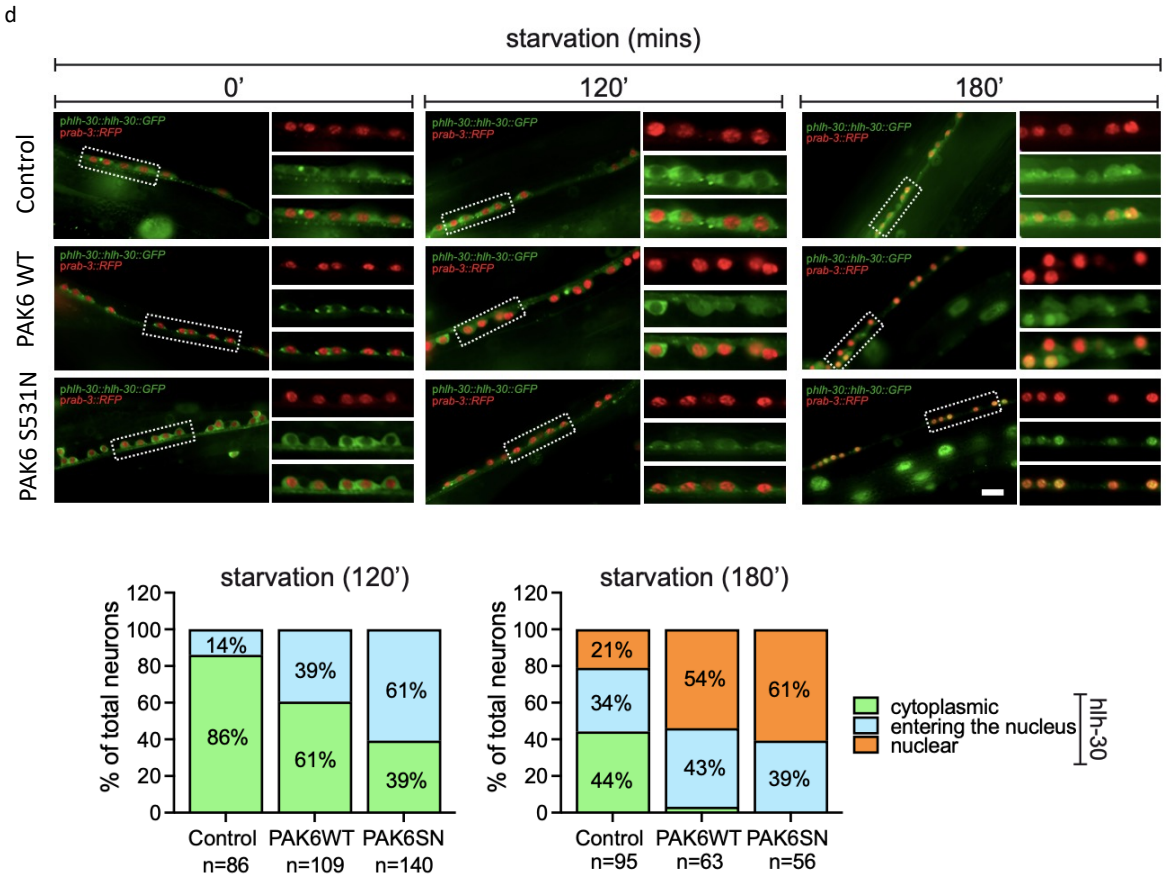


Fig 46: Evaluation of PAK6 role in the nuclear translocation of TFEB. (a) western blot analysis of samples obtained from cell nuclear fractionation. In the graph we evaluated the amount of TFEB in the nuclear fraction. Naïve cells were treated with Torin 1 (2,5 μM for 90 minutes) as positive control. The experiment was performed 5 times. Data were analyzed using one way ANOVA with Turkey's multiple comparisons test (b) TFEB nuclear translocation was assessed in Hela cells cotransfected with TFEB and different mutant of PAK6 (Scale bar 10 μm). Cells cotransfected with PAK6 WT were treated with 2,5 μM of Torin1 for 90 minutes to induce TFEB activation. Minimum 120 cells per genotypes were evaluated. Data were analyzed using one-way ANOVA, Turkey's multiple comparisons test. (c) Endogenous TFEB nuclear translocation was analyzed by the lab of Diego Medina in Hela cells transfected with PAK6 WT, PAK6 S531N, and PAK6 K431M (Scale bar 10 μm). At least 550 cells per genotype were screened. Data were analyzed using one-way ANOVA, Turkey's multiple comparisons test. (d) HLH-30 nuclear translocation was assessed in *C. elegans* overexpressing human PAK6 WT and S531N (Scale bar 50 μm). The percentage of neurons (prab-3 positive) with nuclear TFEB were quantified. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

11.7 PAK6 Directly Interacts with TFEB and Promotes TFEB-14-3-3 Binding

We demonstrated so far that PAK6 activity induces autophagy by promoting the nuclear translocation of TFEB. We then wanted to get better insight into the interaction between TFEB and PAK6 and the possible involvement of 14-3-3 proteins. To this aim, we performed a TFEB pulldown assay in cells cotransfected with TFEB WT and different mutant of PAK6. We used PAK6 WT, PAK6 S531N and PAK6 S531N/S113A, which is constitutively active, but unable to bind to 14-3-3 proteins (166). These mutants allow to understand not only whether there is a binding between PAK6 and TFEB, but also to assess whether this interaction is modulated or affected by PAK6 kinase activity and by the binding between PAK6 and 14-3-3s. After cell transfection, we immunopurified TFEB and, through western blot, assessed the presence of PAK6 and 14-3-3s bound to the purified transcription factor. Importantly, we detected a signal relative to PAK6 and in each sample, excluding the negative control (cells cotransfected with TFEB and Gus) (Fig 47). These data suggest that PAK6 and TFEB directly interact, however, it seems that PAK6 kinase activation causes a partial dissociation from the transcription factor (Fig 47). One of the possible explanations may be that PAK6 binds TFEB in the cytoplasm under resting conditions and dissociates from the transcription factor when it translocates to the nucleus, or that the conformational change induced by the S531N causes the dissociation of the proteins. When we checked for the presence of 14-3-3 proteins we obtained a very surprising result: even if we could not detect any signal with the anti-pan-14-3-3s antibody, we observed that the kinase activity of PAK6 strongly increases the binding between TFEB and the phosphorylated form of 14-3-3s at the PAK6 phosphorylation site Ser58/59. Notably, this interaction seems to be prevented when PAK6 cannot bind 14-3-3 proteins (S113A) (Fig 47). This result was highly unexpected, given that 14-3-3 proteins are reported to bind TFEB in the cytoplasm, to prevent its nuclear translocation. Conversely, our data demonstrates that PAK6 promotes the shift of TFEB from the cytoplasm to the nucleus. Therefore, we hypothesized that PAK6 kinase activity, by inducing the translocation of TFEB to the nucleus, promotes the binding between the transcription factor and a nuclear phosphorylated 14-3-3 isoform. This binding may be important for the regulation of TFEB nuclear dynamics, such as its subnuclear localization or its binding with the DNA. Importantly, the binding between PAK6 and 14-3-3s seems to be necessary for this interaction to occur (Fig 47).

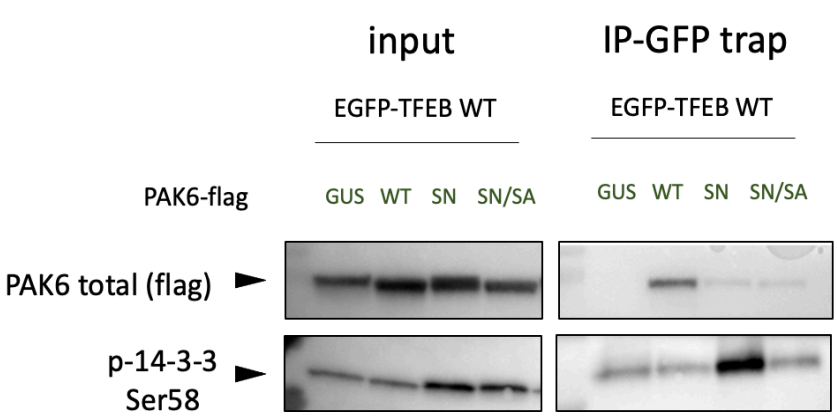
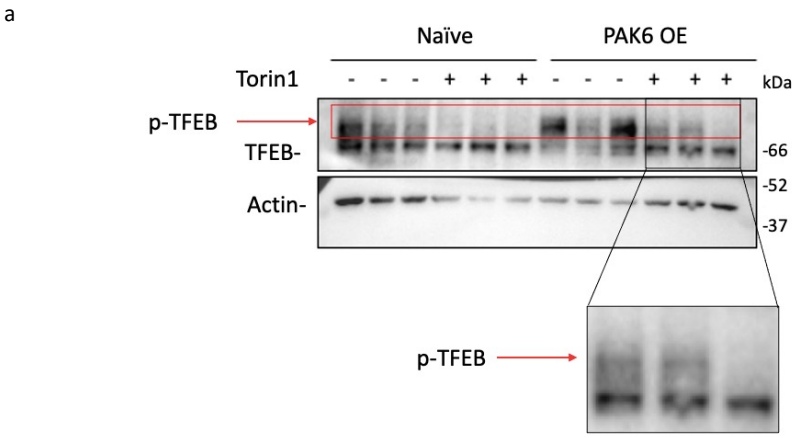


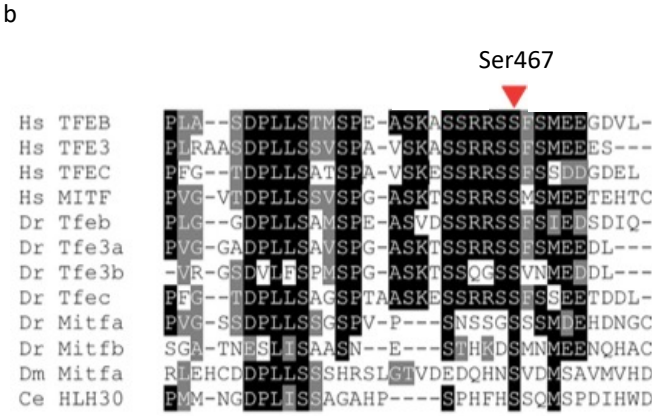
Fig 47: Pulldown assay of TFEB WT. We purified TFEB through GFP-trap. The image is a representative western blot of samples obtained from TFEB purification. The experiment was confirmed 3 independent times, but the quantification was hindered by the variability across the replicates.

11.8 Ser467 of TFEB is the Target of PAK6

The pulldown assay suggested a physical binding between TFEB and PAK6. To gain further evidence about PAK6-TFEB interaction, we evaluated the level of TFEB phosphorylation upon differential expression of PAK6. We treated half of the samples with torin1, to exclude the possible confounding effect of the mTORC1-mediated phosphorylation of TFEB. Importantly, the phosphorylation state of TFEB can be investigated via western blot. Indeed, the phosphorylated form of the transcription factor has a different pattern of migration in SDS-PAGE. Coherently, phospho-TFEB migrates more slowly compared to the unphosphorylated form of the protein. This technique, although very useful, does not allow to discriminate the phosphorylation at different phospho-sites. Still, it provides a general indication about the phosphorylation state of the protein and a rough proportion of the phospho- versus non phospho-fraction. Taking advantage of this feature of TFEB, cells were analyzed using an antibody specific against TFEB. As showed in Fig 8a, both naïve and PAK6 OE untreated cells show a clear band relative to the phosphorylated form of the protein. As expected, upon Torin1 treatment, the upper TFEB band is abolished in naïve cells, indicating the complete loss of phosphorylated TFEB. Importantly, the treatment does not cause the total loss of the upper band of TFEB in PAK6 OE cells, suggesting that the overexpression of the kinase may induce an mTORC1-independent phosphorylation of TFEB (Fig 48a). These data suggest that PAK6 may directly affect TFEB phosphorylation. To further explore this possibility, we performed a bioinformatic analysis to seek the putative site(s) that may be the targets of PAK6 direct phosphorylation in the amino acidic sequence of TFEB. Since we demonstrated the positive effect of PAK6 on TFEB, we looked for a phospho-site that can induce the activation of the transcription factor. In this frame, the

serine residues at the C-terminal of the protein are positive regulator of TFEB (150). Among these serine residues, Ser467 is one of the most conserved throughout evolution, being present in most of the animal organisms, including worms, fruit flies, and mammals. Since we reported the effect of PAK6 in all these animal models we hypothesized that this residue may represent a possible target of PAK6 (Fig 48b). Additionally, Ser467 is located in a PAK6 consensus site (Fig 48c), supporting this residue to be relevant in the PAK6-mediated regulation of TFEB. Based on these considerations, we generated a TFEB non-phosphorylable mutant in Ser467 (TFEB S467A) and evaluated the ability of this mutant to respond to PAK6 activity.





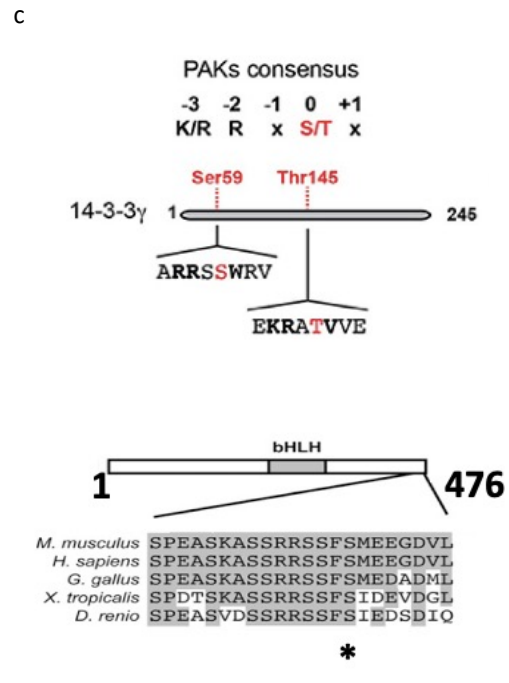


Fig 48: (a) representative western blot to analyze the phosphorylation state of TFEB. Using an antibody anti total TFEB it is possible to have a preliminary indication of TFEB phosphorylation, the phosphorylated form of the protein migrates slower in SDS-page, as represented by the red box. (b) Alignment of the c-terminal motif of TFEB in different species. The arrow highlight Ser467, which is conserved in every species. (c) PAK6 consensus site compared to the motif of TFEB containing Sr467.

11.9 Ser467 is involved in the in the PAK6-Mediated Nuclear Translocation of TFEB

To test whether PAK6-dependent TFEB nuclear translocation depends on S467 phosphorylation, we assessed how the subcellular localization of mutant TFEB varies according to PAK6 activity. We cotransfected HeLa cells with TFEB WT or S467A and PAK6 S531N and compared the nuclear translocation of the two TFEB forms. Interestingly, we observed that in the negative control (cells cotransfected with TFEB WT or S467A and Gus), the mutant TFEB shows a higher rate of nuclear translocation compared to the WT form. This data, even though difficult to rationalize, has been already reported in literature (47) and explained by a reduced affinity of the mutant of TFEB for 14-3-3s (47), moreover, it suggests that Ser467 has a role in the modulation of TFEB subcellular localization. Conversely, upon PAK6 constitutive activation, TFEB-S467A is less efficient in its nuclear translocation compared to TFEB WT (Fig 49). These data confirm that the serine residue in position 467 is important for the shuttling of TFEB from the cytoplasm to the nucleus promoted by PAK6. Importantly, since the mutation in Ser467 does not completely prevent TFEB nuclear translocation, the experiment highlights that this residue may represent a site for the fine modulation of the process, whose regulation, however, is mainly achieved through other mechanisms.

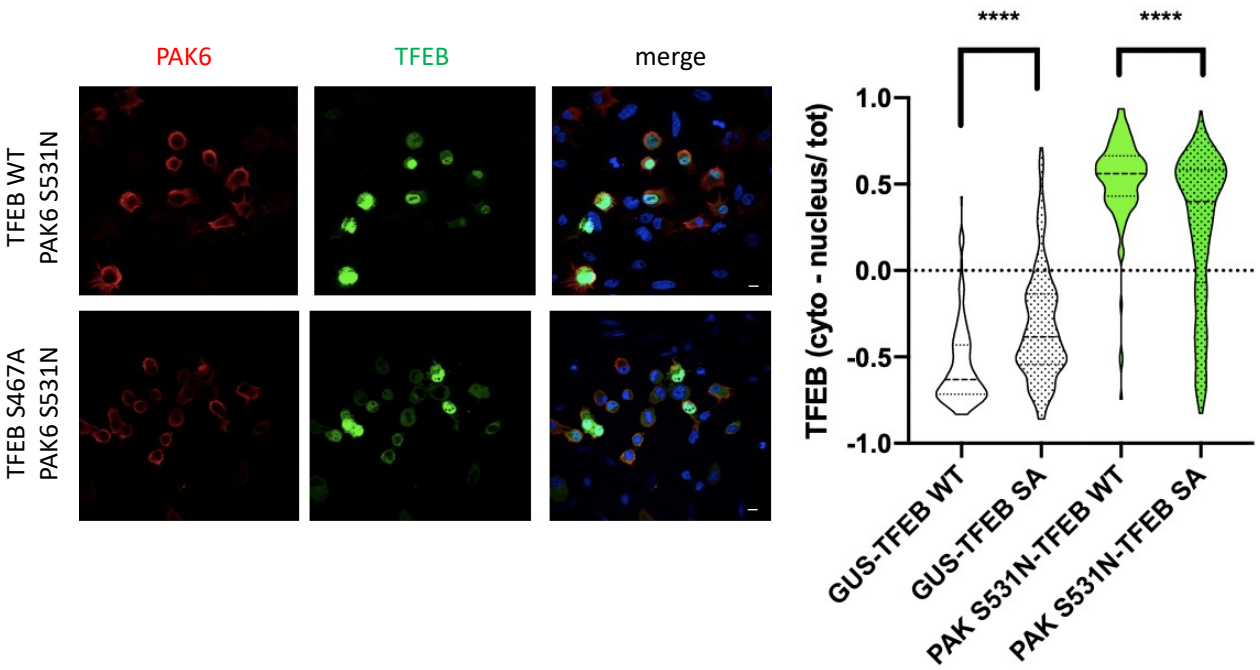


Fig 49: TFEB nuclear translocation was assessed in Hela cells cotransfected with TFEB WT and TFEB S457A and different PAK6 S531N (Scale bar 10 μm). Cells cotransfected with Gus were used as negative control. At least 150 cells were analyzed per each genotype. Data were analyzed using one-way ANOVA with Turkey's multiple comparisons test. **** $p < 0.0001$.

11.10 Ser467 Mutation Prevents TFEB-p14-3-3 Binding

Considering the potential role of Ser467 in the PAK6-mediated TFEB regulation, we performed GFP-TFEB pulldown assay with GFP trap resin, cotransfecting cells with TFEB S467A and the mutants of PAK6 (PAK6 WT, PAK6 S563N, PAK6 S564NS113A). The immunoblot with the samples obtained from the immune-isolation of mutant GFP-TFEB revealed that the binding between PAK6 and TFEB is preserved even upon the mutation of Ser467 (Fig 50). However, the mutation of the serine residue completely abolishes the interaction of TFEB with phospho-14-3-3s (Fig 50). This result suggests that PAK6 binds TFEB in a region that does not comprise Ser467, however this residue is crucial for the PAK6-mediated regulation of TFEB. It is likely that this Serine site is the target of PAK6 phosphorylation.

These data represent preliminary results that cannot be completely explained based on our available data. Future characterization of the interaction between TFEB and p-14-3-3 will be performed, as well as the analysis of the role of Ser467 in the regulation of TFEB mediated by PAK6. In this frame, we recently established a collaboration with Professor Elizabeth Inde

at the University of Melburne, to characterize how the activiy of PAK6 influences the nuclear dynamics of TFEB.

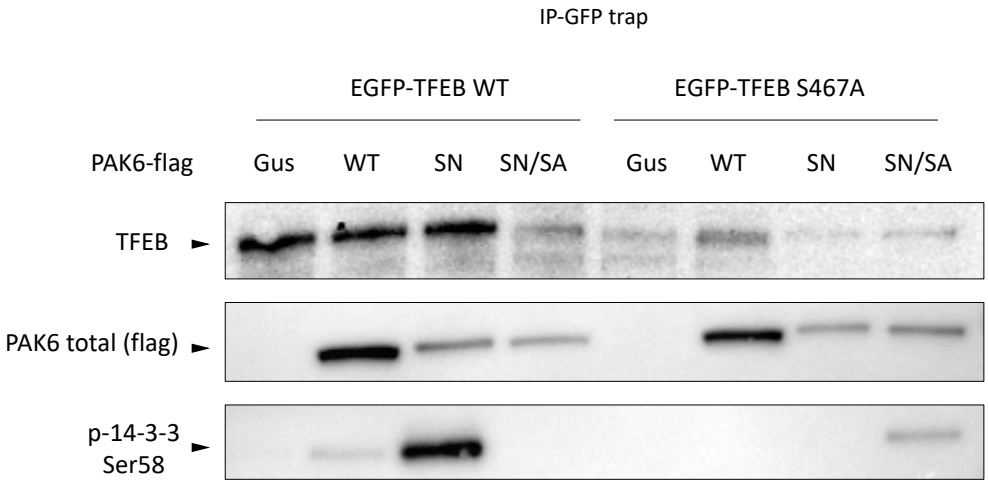


Fig 50: Representative image of the pulldown assay of TFEB WT and TFEB S467A. Immunopurification of TFEB through GFP-trap. The assay was performed 3 times.

11.11 mbt Downregulation Affects α-syn Aggregation in Fruit Fly

Our results so far demonstrated that PAK6 can induce autophagy through the induction of TFEB nuclear translocation. We then investigated whether PAK6 activity could be relevant in a pathological contest, promoting the clearance and aggregation of the PD-associated protein α-syn. With this purpose, we take advantage once again of the versatility of the fruit fly genetics. Indeed, we generated a fly line overexpressing human α-syn and silencing mbt in DA neurons through the specific dopaminergic neuronal driver TH. We first assessed the survival of these flies and observed that the DA-specific downregulation of *mbt* causes a significant decrease of the lifespan of flies overexpressing α-syn. This result suggests that mbt variation affects the phenotype associated to α-syn (Fig51a). Then, using the same model, we analyzed how mbt influences DA-neurons survival and aggregation of α-syn in this class of neurons, by staining fly brains with the DA-neuronal marker TH and a specific antibody for the aggregated forms of α-syn. Interestingly, we observed that the silencing of *mbt* causes an increase of DA neuronal loss and the enhancement of the number of DA-neurons positive for the aggregated form of α-synuclein (Fig 51b). This result confirms the possible role of mbt in the neuronal-specific clearance of aggregated proteins through autophagy.

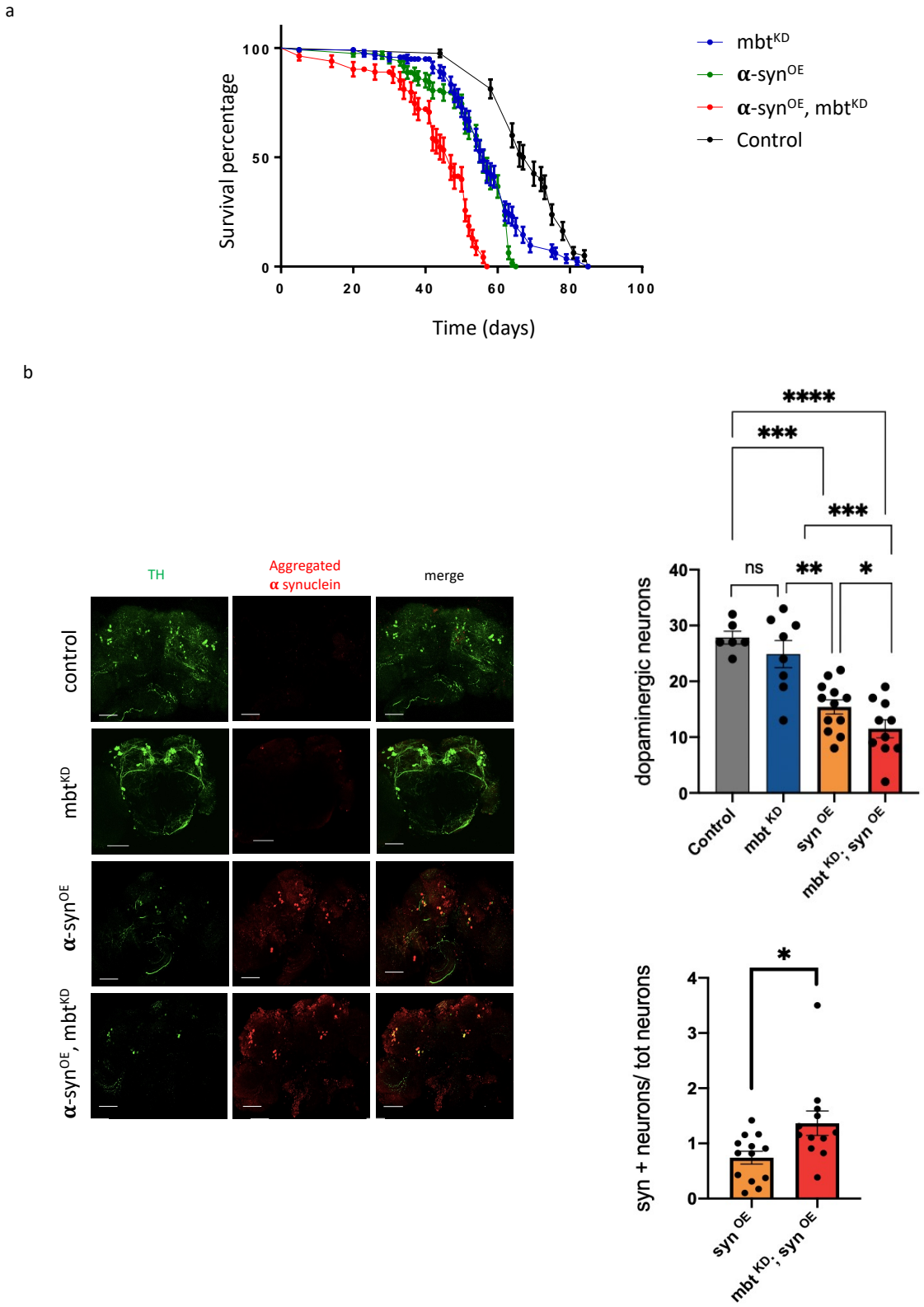


Fig 51: (a) probability of survival of flies with da-specific overexpression of α-synuclein, downregulation of mbt, and WT control. At least 40 flies per genotype were used in the experiment. Log-rank (Mantel-Cox) test was used as a test for the survival probability. (B) Representative images (Scale bar 50 μm) and quantification of fly brains stained with TH and aggregated α-synuclein. The number of da neurons and the number of da-neurons positive for α-synuclein were quantified. Data were analyzed with one way ANOVA, Turkey's multiple comparisons test. ns = not significant, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

Chapter 12

Discussion and Conclusions

12 Discussion and Conclusions

As extensively explained, autophagy plays an essential function in the preservation of cellular homeostasis (17). Impairments of this process may have highly detrimental outcomes for cell survival, and they have been investigated as a contributor of neuronal degeneration (61,188). Indeed, neuronal cell viability depends upon a strict proteins and organelles quality control leading to the general accepted hypothesis that activating autophagy in these cells may be beneficial to treat neurodegeneration. In this project, we characterized a novel role for the neuronal enriched protein PAK6 as an activator of autophagy. Specifically, we investigated the effect of PAK6 kinase activity in the regulation of the master regulator of autophagy, TFEB.

Our group previously demonstrated that PAK6 can phosphorylate 14-3-3 γ to modulate the interaction with its binding partners, such as LRRK2 (166). Being TFEB a well-established interactor of 14-3-3 proteins (164), we tested the hypothesis that PAK6 could influence the activity of the transcription factor, by affecting the binding between TFEB and 14-3-3s. Our interest in the PAK6 activity was mainly due to its pattern of expression; in fact, in humans this kinase is highly enriched in neuronal cells, making it a putative suitable target to impact on neuronal activity (170). In addition, the possible involvement of the protein in the regulation of autophagy may lead to great advances in the characterization of this process in neurons.

In the first part of the project, we confirmed that PAK6 activity influences the autophagic process. We took advantage of different *in vivo* and *in vitro* models to assess the role of PAK6 in the ALP. The experiments performed in *Drosophila melanogaster* revealed that the neuronal silencing of the fly ortholog of the group II PAKs, *mbt*, causes a reduction of the level of the autophagosomal markers Atg8 (LC3 orthologue), a decrease in the number of acidic compartments in the brain, together with an impaired GCase activity. These data strongly suggest that *mbt* downregulation affects the homeostatic regulation of autophagy in neurons.

Interestingly, the decrease in mbt level were observed in flies that expressed the shRNA directed against the protein in neurons, but not in glial cells. Moreover, we detected alterations in the ALP pathway in fly heads only with the neuronal expression of the shRNA

against mbt. These data provide us a strong hint about the pattern of expression of *mbt* in fly brains, suggesting that the distribution of the PAK4/5/6 ortholog is higher in neurons than in other brain cells. This notion supports the idea that *mbt* expression pattern in fly brain is comparable to the one of PAK6 in the same tissue in mammals, and that fruit flies may represent a good tool to perform translational study on the activity of PAK6 in a simpler *in vivo* model. The restricted expression pattern of *mbt* may also explain the results obtained in the RT qPCR analysis. Indeed, we could not observe any difference in the mRNA level of genes involved in the autophagic process between flies downregulating mbt in neurons and control animals. This may be determined by the fact that the role of mbt is limited to a specific cell type in the brain and by analyzing the whole tissue, the effect of its downregulation is strongly diluted by the other cell types and becomes barely detectable.

Importantly, the ubiquitous expression of the shRNA causes a strong downregulation of mbt and a very severe phenotype, indicating that, besides neurons, mbt function is exerted in other tissues in *Drosophila*. This is not surprising considering that *mbt* represents the only ortholog of human Pak4, Pak5 and Pak6 and is likely that the single fly protein recapitulates the functions and the pattern of expression of all the three mammalian counterparts.

The presence of a single ortholog of group II PAKs in fruit flies represents a limitation for the characterization of PAK6 activity; for this reason, we took advantage of different mammalian models.

In mice lacking PAK5 and PAK6 we obtained results comparable to the ones observed in *Drosophila*. This *in vivo* model was available in our lab and provide us a very advantageous tool to rule out the possible contribution of PAK4 in autophagy modulation. Indeed, among the proteins of group II PAKs, PAK4 has the broadest pattern of expression, being ubiquitously present in most cell types. Conversely, PAK5 and 6 distribution in the brain is more limited to neurons (170). Using this model, we confirmed that the PAK proteins influence autophagy in mammalian brain, since the knockout of Pak5 and 6 causes alterations in the level of the autophagic reporter proteins LAMP1 and p62, as well as a reduction of GCase activity in cortex lysates.

After having confirmed the involvement of group II PAKs in neuronal autophagy in flies and in mice, we switched to a human cell model, SH-SY5Y cells. This system allowed to

specifically focus on PAK6 activity through the stable overexpression of the protein and the characterization of its effect on the autophagic pathway. Interestingly, in these cells we confirmed that PAK6 participates in the ALP modulation. Moreover, we demonstrated that the kinase is involved in the canonical pathway that modulate of autophagy, regulated by mTORC1 activity. Coherently, we evaluated the activation of PAK6 upon mTORC1 inhibition by monitoring its phosphorylation in Ser531. The strong increase of phospho-PAK6 after exposing cells to the autophagic activator Torin1 suggests that this protein represents a downstream effector of mTORC1 and that it gets activated by stimuli that leads to the induction of autophagy.

In addition, we observed that the overexpression of PAK6 influences the subcellular localization of TFEB, causing an increase in the nuclear form of the transcription factor. This result linked for the first time PAK6 level to TFEB subcellular regulation. We confirmed this data and our hypothesis that PAK6 kinase activity regulates TFEB intracellular localization in HeLa cells. Accordingly, we detected a strong TFEB nuclear translocation upon constitutive activation of PAK6. Interestingly, our results revealed that the nuclear localization of TFEB is prevented not only in cells overexpressing the kinase dead mutant of protein, but also the wild type form. This result may suggest that the level of PAK6 is not sufficient to exerts its role as an inducer of TFEB, but it needs to be activated. Another possible explanation to this result may be that HeLa cells do not express endogenous PAK6 and may lack the signaling pathway that leads to its activation; this would also explain the differences regarding the level of nuclear TFEB observed in SH-SY5Y cells overexpressing PAK6 WT. Indeed, SH-SY5Y neuroblastoma cells are characterized by endogenous expression of PAK6, even if at low level. However, the experiment performed in the lab of Professor Diego Medina at The Telethon Institute of Genetics and Medicine (TIGEM) in Hela cells showed that the overexpression of the WT form of PAK6 is sufficient to induce the translocation of endogenous TFEB, suggesting that the differences observed among our experimental approaches may be due to the exogenous overexpression of the transcription factor.

Given the relevance of our results, demonstrating a novel mechanism of autophagy regulation in neurons, we wanted to confirm the effect of PAK6 also *in vivo*. We exploited *C. elegans* overexpressing human WT or S531N mutant PAK6 and assessed the nuclear translocation of the worm ortholog of TFEB, HLH-30. In this model we demonstrated that

PAK6 anticipates the starvation-induced translocation of HLH-30 to the nucleus. Importantly, in *C. elegans* we observed that the overexpression of PAK6 alone, both in the WT and in the constitutive active form, is not sufficient to exert its effect on the localization of the transcription factor. Conversely, an autophagic-inducing stimulus, like starvation, is needed to promote the PAK6-mediated activation of HLH-30. This result may be determined by the fact that we overexpressed the human PAK6 protein in worms and, albeit the S531A mutant is constitutively active in human, we cannot exclude that this mutation does not promote the same effect in *C. elegans*. Moreover, being food deprivation a stimulus that affects mTORC1 activity (41), the starvation-mediated activation of PAK6 in *C. elegans* corroborates the hypothesis that the kinase participates in the pathway controlled by this protein complex.

Overall, the data obtained in the first part of the project confirmed that PAK6 regulates TFEB and modulates autophagy.

TFEB localization and activity are controlled by several pathways and proteins. Coherently, PAK6 likely participates in the fine regulation of the transcription factor together with other proteins, rather than being alone a major contributor of its activity. This may seem a limiting factor to the aim of regulating autophagy, however, it also has positive sides, considering that PAK6 may represent a specific target to precisely tune the process.

Another major concept that arises from our data is that the function of PAK6 in regulating autophagy seems to be highly conserved throughout evolution. Accordingly, we confirmed that this kinase modulates autophagy in different models, from worms to human cells, passing through flies and mouse models. The high conservation of a pathway is an indication of its relevance. Indeed, signaling pathways or proteins that are not crucial tend to get lost or modify their functions in the course of evolution. Conversely, important molecular mechanisms are likely to be maintained. In the case of group two of PAK proteins, during evolution there has been a gene duplication that determined the generation of three proteins starting from a single ancestor. This may have been relevant to separate the functions of the proteins in different cells according to their pattern of expression. In this scenario, we cannot exclude that other PAK proteins have similar effects on autophagy, but we can speculate that PAK6 has evolved to serve as a neuronal-specific modulator of this process.

After having demonstrated the function of PAK6 on autophagy, we investigated the molecular mechanisms that may be involved in its activity. Our initial hypothesis was that PAK6 modulates TFEB subcellular localization by phosphorylating 14-3-3s and modulating their binding. In line with this, we confirmed that the overexpression of PAK6 in SH-SY5Y cells increases 14-3-3 proteins phosphorylation in Ser59. Then, we performed a pulldown assay, to investigate the interaction among PAK6, TFEB and 14-3-3 proteins. We were particularly interested in understanding whether PAK6 directly interacts with TFEB. For this reason, we immunopurified TFEB and checked for the presence of PAK6 after purification. Importantly, we co-expressed TFEB with different mutants of PAK6, to understand if the activity of the kinase influences its binding with the transcription factor. We used a WT and a constitutively active mutant of the kinase. Noteworthy, PAK6 gets purified together with TFEB in all the samples, suggesting that there may be a direct interaction between the two proteins. Interestingly, it seems that the activation of PAK6 reduced its binding with TFEB. Considering that upon PAK6 activation, TFEB is almost completely nuclear, we speculate that the binding between PAK6 and TFEB occurs in the cytoplasm and is prevented upon TFEB entry to the nucleus.

Moreover, when we checked for the presence of 14-3-3s after TFEB purification we could not detect any signal. This data is likely determined by an experimental artifact. Indeed, based on the data in literature, we expect to observe a binding between TFEB and 14-3-3s, at least in the sample derived from cells overexpressing PAK6 WT, when TFEB is mainly in the cytoplasm (150,164). It is important to note that we used a pan-14-3-3 antibody, that should recognize all the 14-3-3 isoforms. However, it was previously observed in our group that the efficiency of the antibody toward the different isoforms varies. In this frame, it is not known yet whether TFEB binds all the 14-3-3 isoforms with equal affinity or if it prefers a subgroup of these proteins. It will be highly crucial to investigate this aspect in the future, to increase our knowledge of TFEB physiology. There is another aspect that has not been investigated yet, which is whether the phosphorylation state of 14-3-3s affects their binding to TFEB. As we already mentioned, the 14-3-3s phosphorylation may influence their interactome, however, there are no information in literature about TFEB on this specific topic (166).

For this reason, in the pulldown experiment we also checked for the presence of phosho-14-3-3s, using an antibody against the phosphorylated Ser59, which is a conserved residue in all the isoforms and is the target of PAK6 (166). Surprisingly, we observed that the constitutive activation of PAK6 strongly promotes the binding between TFEB and phosho-14-3-3s. Unfortunately, we were not able to discriminate which of the different 14-3-3 isoforms bind to TFEB. Considering the TFEB activation induced by PAK6, we hypothesized that, by promoting the nuclear translocation of TFEB, the kinase causes the binding of the transcription factor to a nuclear phosphorylated form of 14-3-3s. In this context, it is known that 14-3-3 proteins may have both cytoplasmic and nuclear localization and there is evidence in literature about their nuclear chaperone functions (165,189). Therefore, we can speculate that the nuclear binding between TFEB and 14-3-3s is involved in the modulation of TFEB subnuclear dynamics, such as its localization in the nucleus or its binding with DNA.

To date, all the information available in literature states that the binding between the transcription factor and 14-3-3s occurs only in the cytoplasm and is crucial for the inhibition of TFEB. Therefore, our data may represent a novel discovery about TFEB activity and may lead to a great advance in the characterization of its regulatory mechanisms. To rule out the possibility that the presence of 14-3-3s in our samples is mediated by the binding between 14-3-3s and PAK6, we repeated the experiment adding a condition in which we overexpress together with TFEB a mutant of PAK6 characterized by constitutive activation but unable to bind 14-3-3 proteins (PAK6 S531N/S331A) (166). In this circumstance, we detected a signal relative to PAK6 comparable with the band observed in the PAK6 S531N mutant sample, suggesting that, upon PAK6 activation, the binding of the kinase with 14-3-3 proteins does not influence its interaction with TFEB. This result also indicates that the binding between PAK6 and 14-3-3 alone is not involved in the modulation of nuclear translocation of TFEB. Accordingly, the laboratory of prof. Diego Medina confirmed that the overexpression of PAK6 S531N/S331A promotes TFEB nuclear translocation, similar to what observed in cells overexpressing PAK6 S531N, and this occurs also when monitoring endogenous TFEB.

On the contrary, the binding between PAK6 and 14-3-3s seems to be essential for TFEB interaction with phosho-14-3-3s, as the overexpression of PAK6 S531N/S311A completely abolishes the signal relative to phosho-14-3-3 proteins after TFEB purification. These data need further investigations to be precisely understood and rationalized; however, we can

hypothesize that the PAK6 mediated phosphorylation of 14-3-3s, but not the direct binding between these two proteins, is necessary to induce the nuclear interaction between TFEB and phospho-14-3-3s. This results further confirmed that the interaction between TFEB and phospho-14-3-3s is direct and is not mediated by PAK6.

Considering these results, we asked how TFEB and PAK6 could interact. We focused in particular on the c-terminal region of TFEB. Indeed, this domain is characterized by the presence of a stretch of serine residues that are known to induce the nuclear translocation of TFEB (150). Among them, Ser467 is highly conserved and is present in all the organisms in which we observed the effect of PAK6, such as *C. elegans*, *D. melanogaster*, mice, and humans. This serine residue has been already proved to be involved in the regulation of TFEB subcellular localization, as it is a target of both AMPK and AKT proteins (34,47). Moreover, we also assessed that Ser467 is localized in a consensus region for PAK6. All these indications led us to the idea that this residue may be relevant for the PAK6-mediated activation of TFEB. To test this hypothesis, we generated a TFEB non phosphorylable mutant in Ser467, TFEB S467A. Then, we assessed whether the mutation in this serine influences the translocation of TFEB to the nucleus determined by PAK6 activation. Interestingly, we observed that the mutation causes a reduction in TFEB nuclear translocation when TFEB S467A is co-transfected in cells together with constitutively active PAK6. This data confirms that Ser467 is involved in the PAK6-mediated TFEB activation. Importantly, the mutation does not completely prevent the nuclear relocalization of the transcription factor, suggesting that other mechanisms are involved in the PAK6-TFEB functional interaction. One possible explanation is that PAK6 modulates TFEB activity both indirectly, by phosphorylating 14-3-3s and regulating their binding with TFEB, and directly, by phosphorylating TFEB in Ser467.

Importantly, the pulldown assay performed using TFEB S467A allowed to investigate whether Ser467 is involved in the interaction among PAK6, TFEB and 14-3-3s. We assessed that the mutation does not prevent the PAK6-TFEB binding, indicating that PAK6 likely interacts with the transcription factor in another region. However, the mutation completely abolishes the binding between TFEB and phospho-14-3-3.

Overall, our data suggest that PAK6 binds TFEB, likely in the cytoplasm, in a site which does not comprise Ser467; however, this site may be a target of PAK6 kinase activity and the

posttranscriptional modification in this residue may promote the increase of TFEB nuclear translocation and the binding between the transcription factor and phospho-14-3-3s in the nucleus. We can speculate that this phosphorylation participates in the fine regulation of TFEB localization in the cytoplasm and in the nucleus, modulating the dynamic of the protein within the nuclear compartment.

Thanks to the collaboration with Professor Hinde at the university of Melbourne, we are now investigating how PAK6 influences TFEB activity within the nucleus, how PAK6 can impact on TFEB dynamic properties in the cytoplasm and in the nucleus, and how these mechanisms are affected by Ser467 phosphorylation. These experiments are performed using advanced fluorescence imaging methods, such as Number and Brightness and pair correlation analysis.

Despite very interesting, this result is not sufficient to completely characterize the activity of TFEB and further experiments would be needed to confirm our hypothesis; however, they provide further and novel understanding on TFEB regulation and on its interaction with 14-3-3 proteins. As mentioned, in the future it would be crucial to investigate whether TFEB localization is differentially affected by different 14-3-3 isoforms and how 14-3-3 phosphorylation influences the regulation of TFEB. Moreover, it would be necessary to confirm and deepen the information about the interaction between TFEB and 14-3-3s in the nucleus, which we observed for the first time.

Finally, to investigate the possible relevance of PAK6 activity in the context of PD, we took advantage of the genetic versatility of *Drosophila* to generate a fly strain overexpressing human α-syn and silencing mbt in dopaminergic neurons, the class of neurons mainly affected by the pathology (73). In this model, we confirmed that the downregulation of mbt strongly reduces the lifespan of flies overexpressing α-syn, suggesting that mbt may have a role in the toxicity caused by α-syn dishomeostasis. Accordingly, we observed that the silencing of mbt promotes the accumulation of aggregated α-syn in dopaminergic neurons as well as dopaminergic neuronal loss. This last piece of information provides a strong indication that the alteration of mbt activity influences α-syn rate of accumulation and degradation, likely through the modulation of autophagy. Importantly, this data was confirmed in the lab of Veerle Bekeland in a PD mouse model characterized by

overexpression of the G2019S mutation of LRRK2 (190). In these mice, the overexpression of α-syn together with the constitutive active mutant PAK6 demonstrated that the activity of PAK6 reduces α-syn level in dopaminergic neurons (data not shown), confirming the important role that PAK6 may have in the regulation of the clearance functions in neurons.

Overall, our data point to PAK6 as conserved neuronal inducer of TFEB and autophagy regulator both *in vivo* and in cellular models. Since the activation of autophagy may have great relevance for neurodegenerative diseases, PAK6 may represent a promising target to develop novel therapeutic strategies against neurodegeneration.

Chapter 13

Materials and Methods

13 Materials and Methods

13.1 Animals

13.1.1 Drosophila melanogaster

Flies were raised on agar, cornmeal and yeast food, at 25°C, in 12 hours light/dark cycles. Stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC, Dept Biology, Indiana University 1001 E. Third St. Bloomington, IN 47405-7005 USA).

Name	Description	Stock Number	Source
W^{1118}	Control	5905	BDSC
Yellow	Control	169	BDSC
dj-1$\beta^{\Delta 93}$	Deletion in *dj-1β* gene	33600	BDSC
*UAS-dj-1*β	UAS line to express *dj-1β*	33604	BDSC
UAS-Atg8a-mCherry-GFP	UAS line to express Atg8-mCherry-GFP reporter	37749	BDSC
UAS-AMPK T184D	UAS line to express constitutive active AMPK	32110	BDSC
UAS-shRNA mbt	UAS line to express shRNA against mbt	17453	BDSC
UAS-human α-syn	UAS line to express human α-synuclein	8146	BDSC
da-Gal4	Ubiquitous driver (*daughterless*)	8641	BDSC
Elav-Gal4	Neuronal driver (Elav)	5145	BDSC
Repo-Gal4	Glial cells driver (*Repo*)	7451	BDSC
ple-Gal4	Dopaminergic neuronal driver (TH)	8848	BDSC

13.1.2 Mus musculus

Male C57Bl6/J (wild-type [WT], n = 3 animals) and DJ-1 knockout (RRID: MMRRC_032090-MU from Dr. Huaibin Cai, n = 3 animals) were given access to food and water ad libitum and housed in a facility with 12 hours of light/dark cycles. Mice were sacrificed by CO2 inhalation at one month of age. All animal work was performed after approval of the National Institute on Aging (NIA) Animal Care and Use Committee.

13.1.3 Caenorhabditis elegans

The Bristol N2 (control animals), MAH240 [phlh-30::hlh-30::GFP + rol-6(su1006)] and OH10689 [prab-3::NLS::tagRFP] strains were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN). Culture, maintenance, germline transformation, and genetic crosses were performed using standard techniques (191)

13.2 Cell Models

13.2.1 SH-SY5Y

SH-SY5Y cells were cultured in a 1:1 mixture of Dulbecco's modified. Eagle's medium (DMEM, Life Technologies) and Ham's F12 medium (F12, Life Technologies) medium (ratio 1:1), supplemented with 10% fetal bovine serum (FBS, Life Technologies). Cell lines were maintained at 37°C in a 5% CO_2 controlled atmosphere. 0.25% trypsin (Life Technologies), supplemented with 0.53 mM EDTA, was employed to detach cells generate subcultures. Stable cell lines overexpressing PAK6 wild type were generated as described in (166). Briefly, the cDNA sequence encoding PAK6 was cloned into the lentiviral plasmid pCHMWS-MCS-ires-hygro and used to transduce cells. 500 ug/ml hygromycin was utilized for selection, while 100 ug/ml for maintenance.

13.2.2 HEK293T and HeLa

HEK293T and HeLa cells were cultured in DMEM supplemented with 10% FBS (Life Technologies), and 1 % trypsin was employed to detach cells and split them. Cell lines were maintained at 37°C in a 5% CO2 controlled atmosphere. HEK293T and HeLa cells were transfected with plasmid DNA using polyethylenimine (PEI, Polysciences) for 48 hours according to the manufacturer's instructions.

13.2.3 iPSC and Dopaminergic Neuron Differentiation

Human wild type and the isogenic DJ-1 mutant (A111L) induced pluripotent stem cells lines were generated in the lab of Dr. Mark Cookson (152). Flasks were coated with Matrigel hESC-Qualified Matrix, LDEV-free (Corning: 354277) for 30' at 37c° before cells seeding. Cell cultured were maintained in supplemented Stem Cell Basal Medium (GIBCO:

A3349401) at 37°C in a 5% CO2 controlled atmosphere. TrypLE™ Express Enzyme (GIBCO: 12605036) was employed to detach cells and generate subculture. The differentiation of iPSC into dopaminergic neurons was performed following an established protocol (dx.doi.org/10.17504/protocols.io.bsq5ndy6).

13.3 Behavioral Assays

13.3.1 Eclosion Rate

At 25C°, flies eclose after 10 days post eggs fertilization. After this period adult flies emerge from the larval stage. The number of larvae that did not develop to the adult life stage were counted and divided for the total number of larvae present in each vial.

13.3.2 Lifespan Assay

Adult males (1-3 day-old) were collected under brief CO 2 exposure and transferred into new tubes containing standard food (20 flies/vial). Flies were transferred to fresh food vials every 3-4 days and the number of dead flies was counted daily. The percentage of survival was calculated at the end of the experiments.

13.3.3 Negative Geotaxis (Climbing) Assay

Adult males (1-3 day-old) were collected and transferred to new tubes containing standard food. After 24 hours flies were placed in climbing vials. Negative geotaxis was evaluated by using a counter-current apparatus with 6 tubes in the lower frame and 5 in the upper frame. Flies were placed in the first plastic vial and gently tapped to the bottom. After 10 sec the upper frame was moved to the right, and the flies that passed in the upper tubes during this period were transferred to the next lower tubes by gently tapping. This procedure was repeated 5 times. The following formula was used to calculate the climbing index: CI = [(#F5x5)+(#F4x4)+(#F3x3)+(#F2x2)+(#F1x1)+(#F0x0)/(#FT)] .
Fn is the number of flies in the tube n (0= the initial tube and 5= the last tube) and #FT is the total number of flies.

13.4 Treatments

13.4.1 Drosophila melanogaster

To block autophagic activity adult fly males (1-3 days) were collected under brief CO2 exposure and transferred into tubes containing standard food (20 flies/vial) supplemented 10mM of Chloroquine (MedChemExpress: HY-17589A) for 24 hours before the experiment.

The scavenging of ROS was performed adding to standard food 1mM of N-acetyl-L-cysteine (NAC) (Sigma Aldrich: A7250). Adult fly males were collected and transferred to NAC-supplemented food vial for 7 days, during this period flies were transferred to fresh NAC-supplemented food vials every 2–3 days.

13.4.2 Cell Models

To induce autophagic activity cells were treated with 2,5 μM of the specific inhibitor of mTORC1 Torin1 (MedChemExpress: HY-13003) for 90 minutes (to observe TFEB nuclear translocation) or 3 hours (to induce autophagic activation). The blockage of the autophagic process was achieved by treating cells with 50 μM Chloroquine (MedChemExpress: HY-17589A) for 3 hours.

13.5 Plasmid

Eukaryotic expression of 3xFlag tagged PAK6 (wild type, K436M, S531N) constructs were generated as described previously (166). PAK6 S531N/S113A mutant construct was obtained from the 3xFlag PAK6 S531N using Quick-Change II site-directed mutagenesis kit (Stratagene) following the manufacturer's recommendations.

The following nucleotide oligomers were used:
for: CGGCGGGCACAGGCCCTGGGGCTGCTG;
rev: CAGCAGCCCCAGGGCCTGRGCCCGCCG.
eGFP-TFEB wild type was provided by prof. Diego Medina's lab (described in (192)). TFEB S467A was obtained through site-directed mutagenesis, using the following primers:

for: AGCAGCCGCCGGAGCGCTTTCAGCATGGAGGA.
rev: TCCTCCATGCTGAAAGCGCTCCGGCGGCTGCT

13.6 Transfections

HEK 293T and HeLa cells were transfected with plasmid DNA using polyethylenimine (PEI, Polysciences) for 48 hours according to the manufacturer's instructions. The DNA:PEI ratio used was 1:3.

13.7 Cell lysis and Protein Quantification

Flies and mice tissues or cells were lysed in home-made lysis buffer composed as follow:

50 mM Tris-HCl pH 7.5, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM EGTA, and 270 mM sucrose buffer. Protease inhibitors (Roche) were freshly added before cell lysis.

Lysates were incubated on ice for 30 minutes and cleared by centrifugation at 20000 x g for 30 minutes at 4°C. Supernatants were collected and stored at -20 C° until used.

13.8 SDS PAGE and Immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) allows the separation of proteins according to their size in polyacrylamide gels in the presence of SDS and applying an electric field.

30-50 µg of proteins samples were boiled for 10 minutes at 100 C° before loading on gels. According to the size of resolution required ExpressPlus™ PAGE 4–20% gels (GenScript), in MOPS running buffer or 7.5%, 10% Tris-glycine polyacrylamide gels in SDS/Tris-glycine running buffer were used. Precision Plus molecular weight markers (Biorad) were used for size estimation. The resolved proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad) or nitrocellulose membranes (Whatman), through a Trans-Blot® Turbo™ Transfer System (Bio-Rad).

Membranes were subsequently blocked in Tris-buffered saline plus 0.1% Tween (TBS-T) plus 5% non-fat milk for 1 h at room temperature (RT) and then incubated over-night at 4 °C with primary antibodies diluted in TBS-T plus 5% non-fat milk. Membranes were then washed in TBS-T (3x10 minutes) at RT and subsequently incubated for 1h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were then washed in TBS-

T (3x10 min) at RT and rinsed in TBS-T. Immunoreactive proteins were visualized using Immobilon® Forte Western HRP Substrate (Merck Millipore) at the Imager Chemi Premium (VWR). Densitometric analysis was carried out using the Image J software.

Antibodies utilized for western blot:

fly samples: mouse β-actin (Sigma Prestige); guinea pig p62 (Progen: GP62-C); mouse Ubiquitin (Santa Cruz Biotech: sc-8017), rabbit p-AMPK (Thr172) (Cell Signaling technology: 2535), rabbit p-S6K (Thr389)(Cell Signaling technology: 9205). rabbit LC3 (Thermo Fisher Scientific: PA1-16930); goat Anti PAK6 (Novus Biological: AF4265);mouse Hsp-70 (Stressgen Enzo Life science: SPA810).

Human and mouse samples: rabbit p62 (Abcam: ab109012), rabbit LC3 (Abcam: ab192890), mouse LAMP1 (Abcam: ab25630), rabbit TH (Pel-freez biological: P40101-150), rabbit AMPK- α (Cell Signaling technology: 2532), rabbit p-AMPK (Thr172) (Cell Signaling technology: 2535), rabbit S6K (Cell Signaling technology: 9202), rabbit p-S6K (Thr389)(Cell Signaling technology: 9205). rabbit anti-PARP (Cell Signalling Technology: 9542S); anti-Flag M2-Peroxidase (Sigma-Aldrich: A8592); anti GFP (Roche-Sigma: 11814150001); rabbit Anti-Phospho-PAK4/5/6 (pSer474) (Sigma-Aldrich: SAB4503964); rabbit Anti-Phospho-14-3-3 (pSer58) (Abcam: ab30554); Rabbit anti-TFEB (Bethyl Laboratories: A303-673A); mouse anti-LAMP1 (H4A3) (Santa Cruz Biotechnology: sc-20011); rabbit anti-p62 (Abcam:ab109012)

13.9 Pulldown Assay

Cells were harvested at 48 h post transfection and lysed in home-made buffer solution (20 mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 2,5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM sodium orthovanadate, 1% v/v Tween®, 20 and 1% of protease inhibitor cocktail (Roche)).

The resuspended cells were kept on ice for 30 minutes and then centrifuged for 30 minutes at maximum speed at 4°C. The supernatants were then incubated overnight with GFP-Trap® (Chromotek).

The GFP-tagged protein bound to resins were then washed 10 times with the following Washing Buffers (WB), two washes for each WB:

WB1 (20mM Tris-HCl, pH 7.5, 500mM NaCl, 1% v/v Tween® 20);
WB2 (20mM Tris-HCl, pH 7.5, 300mM NaCl, 0,5% v/v Tween® 20);
WB3 (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0,5% v/v Tween® 20);
WB4 (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% v/v Tween® 20);
WB5 (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.02% Tween® 20).

Immunoprecipitates were resuspended in sample buffer and stored at -20 C° until use.

Between 15 and 30 µg of proteins were resolved on 4-20% gels. For the following procedure see the "SDS PAGE and Immunoblotting" section.

13.10 Cell Fractionation

Cell fractionation of nuclear and cytoplasmic compartments was performed with the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo-Scientific) kit following the manufacturer's instructions. Cytoplasmatic and nuclear samples were resuspended in sample buffer and loaded in 10% Tris-glycine polyacrylamide gels as previously described.

13.11 Immunocytochemistry and Confocal Imaging

13.11.1 Immortalized Cell Models

HeLa or SH-SY5Y cells were cultured onto 12mm glass coverslips (Thermo-Scientific) coated with Poly-L-Lysine (Sigma- Aldrich). Cells were washed in PBS and fixed with 4% w/v Paraformaldehyde (PFA) for 20 minutes at RT. After cell permeabilization in PBS plus triton 0,1% for 20 minutes at RT and a blocking step performed in 3% BSA diluted in PBS for 30 minutes at RT, cells were stained with the appropriate primary antibody diluted in PBS plus 3% BSA for 1 hour at RT. Subsequently, cells were washed three times in PBS and incubated with the secondary antibody Alexa Fluor® 488 conjugated (Life Technologies) or Alexa Fluor® 568 conjugated (Life Technologies). Before mounting the coverslips on glass slides, cells were incubated with Hoechst 33258 (Invitrogen) diluted in PBS for 5 minutes.

Images were acquired with a LeicaSP5 confocal microscope (Leica Microsystems) and quantified using ImageJ.

The following antibodies were used: mouse anti-Flag F7425 (Sigma-Aldrich: F7425); rabbit anti-Phospho-PAK4/5/6 (pSer474) (Sigma-Aldrich: SAB4503964).

13.11.2 iPSC-Derived DA Neurons

IPS cells were cultured onto 12mm glass coverslips coated with 10 µg/ml Laminin (Sigma Aldrich: L2020) and 2 µg/ml Fibronectin (Corning: 356008). Cell staining was performed as previously described for HEK293T and HeLa cells.

Images were acquired using a Zeiss LSM 700 confocal microscope and quantified using ImageJ.

The following primary antibodies were used: sheep TH (Pel-freez biological: P60101-150), mouse LAMP1 (Abcam: ab25630), rabbit LC3 (Abcam: ab192890), rabbit p-AMPK (Thr172) (Cell Signaling technology: 2535).

The following secondary antibodies were used: Alexa Fluor® 488 conjugated (Life Technologies) or Alexa Fluor® 568 conjugated (Life Technologies), Alexa Fluor® 647 conjugated (Life Technologies).

13.12 Fly Brain Immunofluorescence and Confocal Imaging

Brains dissected from 1-3 days old male flies were labeled with anti-TH and anti-aggregated α-syn. In brief, brains were fixed in 0.4% paraformaldehyde (PFA) for 1h at RT. After permeabilization in 1% triton dissolved in PBS for 10 minutes and blocking in 1% BSA, 0,3% triton dissolved in PBS for 1 hour at RT. Then brains were stained overnight with primary antibodies diluted in PBS plus 0,3% triton, and 0,1% BSA at 4°C. Brains were then washed trice in PBS and incubated with the fluorophore-conjugated secondary antibodies (Alexa Fluor® 488 conjugated (Life Technologies) and Alexa Fluor® 568 conjugated (Life Technologies)) for 2 hours at RT. Finally, brains were mounted in Mowiol® 4-88 (Calbiochem, 475904), and analyzed.

Z-stack images were acquired using a LeicaSP5 confocal microscope (Leica Microsystems), the number of posterior dopaminergic neurons and synuclein-containing dopaminergic neurons were counted manually in each brain hemisphere.

The following antibodies were used for this experiment: rabbit anti-tyrosine hydroxylase (Merk millipore: AB152); mouse anti- aggregated-α-synuclein, clone 5G4 (Sigma Aldrich: MABN389).

13.13 TFEB Nuclear Translocation (Lab Medina)

Lipofectamine LTX protocol (Invitrogen, 15338) was followed for transfecting cells with PAK6 plasmids. 10k cells per well were plated in 96 well plates (PerkinElmer, 6055302) and transfected in suspension using Lipofectamine LTX (Invitrogen, 15338) according to manufacturer instructions. 24h after transfection, images were acquired with Opera Fenix (PerkinElmer). Analysis was performed with Harmony software (PerkinElmer).

Transfection-positive cells were first selected and then cytoplasmic and nucleus intensities were calculated. TFEB translocation is expressed as the ratio of nuclear intensity to cytoplasmic intensity.

13.14 *C. elegans* HLH-30 Nuclear Translocation (Lab Martinelli)

The cDNA corresponding to the wild-type or S531A human PAK6 alleles were subcloned into the pBy103 vector to be under the control of the unc-119 promoter, which drives pan-neuronal expression, and were injected at 30 ng/µl to generate multi-copy extrachromosomal arrays. The pJM371 plasmid (pelt- 2::NLS::tagRFP) (a kind gift from E. Di Schiavi, IBBR-CNR, Naples), which drives RFP expression in intestinal cell nuclei, was used as co-injection marker, and injected at 30 ng/ µl. Isogenic lines, that had lost the transgene, were cloned separately and used as controls.

The subcellular localization of HLH-30 prior to and following short-term (2 and 3 hours) or long-term (overnight) starvation was investigated by using an Eclipse Ti2-E microscope (Nikon Europe, Florence, Italy) equipped with DIC optics on live animals mounted on 2% agarose pads containing 10 mM sodium azide as anesthetic. Starvation was obtained by

moving young adult hermaphrodites to NGM plates without food for two or three hours, or overnight.

13.15 Lysotracker Staining

Adult male (1-3 day-old) brains were dissected in phosphate-buffered saline (PBS) followed by 15 minutes of incubation with 200 nM LysoTracker™ Red DND-99 Red probe (Thermofisher Scientific, L7528) in the dark. Then, brains were rinsed trice in PBS for 5 minutes, mounted in Mowiol ® 4-88 (Calbiochem, 475904), and immediately analyzed.

Z-stack images were acquired on a LeicaSP5 confocal microscope (Leica Microsystems). The area of the brain occupied by LysoTracker staining were quantified by ImageJ.

13.16 Autophagic Flux Assay (Atg8-mCherry-GFP reporter)

Adult male (1-3 days old) brains were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 1 h. Then, brains were washed thrice in PBS for five minutes and mounted in Mowiol® 4-88 (Calbiochem, 475,904). Samples were stored at 4 C° until analysis.

Z-stack images were acquired on a LeicaSP5 confocal microscope (Leica Microsystems). The number of mCherry-positive puncta were quantified by ImageJ.

13.17 DHE Assay

13.17.1 Drosophila melanogaster

1-3 days old male fly brains were dissected in Schneider's Drosophila medium (Biowest, L0207) and incubated for 5 minutes with 30 µM dihydroethidium (DHE) probe (Invitrogen, D11347) in the dark at RT. Subsequently, brains were washed thrice in Schneider's Drosophila medium for 5 min and mounted in Mowiol® 4-88 (Calbiochem, 475,904).

Samples were analyzed immediately after the treatment. Z-stack images were acquired with LeicaSP5 confocal microscope (Leica Microsystems). The fluorescence intensity of DHE staining was quantified by ImageJ.

13.17.2 iPSC-Derived DA Neurons

Cells were cultured onto an 8 well chambered coverslip for cell culture and live imaging (ibidi: 80806) coated with 10 µg/ml Laminin (Sigma Aldrich: L2020) and 2 µg/ml Fibronectin (Corning: 356008). Cells were incubated with 30 µM dihydroethidium (DHE) probe (Invitrogen, D11347) diluted in Hanks' balanced salt solution (HBSS) (Gibco: 14025092). The treatment lasted 30 minutes at 37 C° in the dark. Cells were then washed 3 times with HBSS and immediately analyzed.

Z-stack live images were acquired using a Zeiss LSM 700 confocal microscope. Quantification of the mean DHE fluorescence intensity was performed using ImageJ.

13.18 DQ-BSA Assay

13.18.1 Drosophila melanogaster

1-3 days old male fly brains were dissected in Schneider's Drosophila medium (Biowest, L0207) and incubated with 80 µg/ml, for 60 minutes at RT in the dark. Brains were then rinsed 3 times and chased for 2 hours at RT in Schneider's Drosophila medium before being mounted in Mowiol® 4-88 (Calbiochem, 475,904).

Z-Stack images were acquired immediately after the treatment with LeicaSP5 confocal microscope (Leica Microsystems). The mean intensity of the DQ-BSA positive structure were evaluated using ImageJ.

13.18.2 iPSC-Derived DA Neurons

IPS- derived DA-neuronal cells were cultured onto an 8 well chambered coverslip for cell culture and live imaging (ibidi: 80806) coated with 10 µg/ml Laminin (Sigma Aldrich: L2020) and 2 µg/ml Fibronectin (Corning: 356008).

Cells were incubated with 10 µg/ml of DQ-BSA probe (Thermofisher: D12051) diluted in PBS for 60 minutes in the dark at 37 C°. Cells were then washed 3 times with PBS. Live imaging and Z-stack images acquisition was performed using a Zeiss LSM 700 confocal microscope. DQ-BSA fluorescence intensity was quantified with ImageJ.

13.19 GCase Assay

GCase activity was measured using a protocol similar to the one described in (157). Briefly, flies tissue, mice tissues of cell lysates were obtained, and the total amount of proteins was quantified as described in “Lysis Buffer and protein quantification”.

The experiment was performed in 98-wells plate. 20 µl of lysate were diluted in 40 µl of a solution of citrate phosphate buffer (0.1 M citric acid, 0.2 M solution dibasic sodium phosphate), 0,2% Sodium taurodeoxycholate hydrate, and 3 mM 4-methylumbelliferyl β-D-glucopyranosidase (Sigma-Aldrich-M3633) and incubated at 37°C for 90 minutes in the dark. The reaction was stopped by adding ice-cold glycine buffer (0.2 M solution Glycine, 0.2 M NaOH) and fluorescence was detected on a multilabel plate reader Victor (Perkin Elmer) at excitation 360 nm and emission 440 nm.

Final GBA activity values were calculated normalizing the value obtained to the total amount of protein present in the sample.

13.20 RT qPCR

RNA was extracted from fly whole body or head tissues using the miRNeasy Mini Kit (Thermofisher: 217004) following manufacturer instuctions. RNA quality and concentration were assessed by Nanodrop. cDNA was synthesized from total RNA using PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara: RR037B). 5 ng of cDNA have been used for each qRT-PCR reaction, that was carried in triplicates on 98-well plate. RT-qPCR reaction was performed adding to the cDNA the KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich: KCQS02) and the DNA primers. Tubulin was used as housekeeping gene.

The following primers were used:

LAMP1

For: AATCTCAACGTCTTCGACAACAACT
Rev: TACAAGAGGTATTCCAAGCCCCAAT

Cathepsin D

For: AATTGCCGGTAATCAAGTTTGTGCT
Rev: ATGTCAAACTCGGTGTAGTATTT

Cathepsin B

For: TCCTTACGAGATTTCTCCCTGTGA
Rev: ATCTCCTCCTGAATTTCACGCACATT

AMPK

For: ATTCCCGAGTACCTCAACAAACAA
Rev: TAGGTGTCGATCACATTGGAGTCCT

Tubulin

Forward: ACAGATCAATGAACACATACTCGGTT
Reverse: AGACAAGATGGTTCAGGTCACCGTAT

Mitf

Forward: ATTCGGTGGTAGTTTTGTACGATGTGAT
Reverse: AAATAGGAGCTGAGGTAGACTGTAACGA

Gba1

Forward: ATATGCGGAGACAACGGAAATTTGTTT
Reverse: AGTTGTCACTCAACTTGATCCGAGATA

13.21 Statistics analysis

The data were expressed as the mean ± standard error of the mean (S.E.M). At least 3 biological replicates were analyzed in each experiment. Graphs and statistical analysis were performed using GraphPad PRISM 9.

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Review

Metformin Repurposing for Parkinson Disease Therapy: Opportunities and Challenges

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Abstract: Parkinson disease (PD) is a severe neurodegenerative disorder that affects around 2% of the population over 65 years old. It is characterized by the progressive loss of nigrostriatal dopaminergic neurons, resulting in motor disabilities of the patients. At present, only symptomatic cures are available, without suppressing disease progression. In this frame, the anti-diabetic drug metformin has been investigated as a potential disease modifier for PD, being a low-cost and generally well-tolerated medication, which has been successfully used for decades in the treatment of type 2 diabetes mellitus. Despite the precise mechanisms of action of metformin being not fully elucidated, the drug has been known to influence many cellular pathways that are associated with PD pathology. In this review, we present the evidence in the literature supporting the neuroprotective role of metformin, i.e., autophagy upregulation, degradation of pathological α-synuclein species, and regulation of mitochondrial functions. The epidemiological studies conducted in diabetic patients under metformin therapy aimed at evaluating the correlation between long-term metformin consumption and the risk of developing PD are also discussed. Finally, we provide an interpretation for the controversial results obtained both in experimental models and in clinical studies, thus providing a possible rationale for future investigations for the repositioning of metformin for PD therapy.

Keywords: Parkinson disease; metformin; neuroprotection; AMPK; epidemiology; bioavailability

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1. Introduction

Parkinson disease (PD) is the second most common and fastest-growing neurological disorder in the world [1]. It affects more than 6 million people worldwide and it is estimated to increase to more than 12 million by 2040 [1]. Motor symptoms, which represent the major clinical feature of PD, are associated with the preferential loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Another pathological hallmark of the disease is the presence in the surviving neurons of intracellular proteinaceous inclusions, referred to as Lewy bodies, mainly composed of α-synuclein (α-syn). Mutations in the *SNCA* gene, coding for the protein α-syn, have been associated with familial forms of PD [2], highlighting the role of the protein in the pathological mechanisms underlying the disease.

PD is still an incurable disorder and the currently available therapeutic approaches focus on stimulation of dopaminergic signaling, such as levodopa (L-DOPA), DOPA decarboxylase inhibitors, catechol-O-methyltransferase inhibitors, dopamine agonists, and inhibitors of the enzyme monoamine oxidase type B [3]. Unfortunately, all these treatments can only provide symptomatic relief but they do not hamper the clinical and pathological progression of the disorder. As a consequence, the development of new disease-modifying therapies is one of the major challenges for the treatment of PD. To design disease-modifying therapies is usually long, expensive, and highly risky, considering that it takes an average of 13–15 years with an estimated cost of bringing a new molecule to market of around

2.6 billion USD. Moreover, only ~10% of drugs entering clinical trials make it to the market [3,4]. In light of all these drawbacks, an alternative strategy is drug repurposing, also known as drug repositioning, which is the evaluation of existing drugs for new therapeutic purposes, outside their original clinical indication [5]. The advantage of this strategy is the possibility to bypass several preclinical and clinical phases, by the use of molecules whose pharmacokinetics, safety, and toxicology profiles have been already established.

Therefore, in the quest for new potential therapeutic compounds against PD progression, the knowledge of the pathological mechanisms involved in its etiopathogenesis and the comparative analysis for their potential roles in other disorders could help speed up drugs' repurposing. In this frame, we recently analyzed the literature data supporting a direct association between type 2 diabetes mellitus (T2DM) and an increased probability to develop PD, suggesting a potential molecular mechanism underlying such a correlation [6]. On the basis of this analysis, among the different marketed anti-diabetic drugs, metformin was proposed as a promising candidate for novel PD therapy. As recently reviewed, metformin is used as the first-line treatment of T2DM worldwide, being approved by the FDA in 1995 [7]. It is a low-cost medication, which is generally well tolerated with minimal side effects. Over time, the benefits of metformin have been described beyond diabetes and its potential application to treat various diseases has been proposed [8], making metformin one of the most promising drugs for repurposing.

In this review, we will analyze the therapeutic potentials of metformin repurposing for PD, considering the main mechanisms of action that would provide a neuroprotective effect. At the same time, we will present a critical appraisal of the use of metformin in both experimental PD models and clinical studies, highlighting the challenge in data interpretation and evaluation of metformin effectiveness as a disease modifier for PD.

2. Potential Neuroprotective Mechanisms of Action of Metformin

The antidiabetic action of dimethylbiguanide (metformin) is principally related to the inhibition of hepatic gluconeogenesis, the promotion of peripheral glucose uptake, the increased insulin sensitivity, and its anti-glycating action, which altogether stabilize patients' glycemia [9].

In recent years, metformin has gained increasing interest not only for its glucose-lowering capacity but also due to the possible beneficial effects in different pathological conditions, including neurodegenerative diseases. The impact of metformin on neuronal homeostasis has been increasingly studied in both in vitro and in vivo models (Tables 1 and 2), observing a general improvement in the lifespan of the animal PD models, a rescue of dopaminergic neuron loss, and motor phenotypes. However, the precise effect of the drug is still under debate and needs further characterization.

Table 1. Molecular read-outs of metformin administration to non-PD and PD cellular models.

Cellular Model	Concentration	Duration of Treatment	Read-Out	Ref.
Human neuronal stem cell	1 mM	48 h	– increased cell viability – increased mitochondrial functions – AMPK activation	[10]
Rat hepatocytes	0.05–0.1 mM	24–60 h	– inhibition of mitochondrial respiration – inhibition of complex I in isolated mitochondria	[11]
Rat hepatocytes	From 0.02 to 2 mM	1–7–39 h	– AMPK activation	[12]
Mitochondria isolated from frontal mouse brain	1 mM	15 min	– inhibition of complex I – reduced mitochondrial respiration	[13]
Mouse primary hepatocytes	0.5–1 mM	22 h	– reduced adenine nucleotides – reduced mitochondrial respiration	[14]
Mouse primary hepatocytes	0.075 mM	22 h	– increased mitochondrial respiration – increased mitochondrial fission – increased AMPK activity	[14]

Table 1. *Cont.*

Cellular Model	Concentration	Duration of Treatment	Read-Out	Ref.
In vitro PD cellular models				
SH-SY5Y overexpressing α-syn	0.5–1.0–2.5 mM	16–24 h	– PP2A activation and reduced α-syn pSer129 – AMPK activation and mTOR inhibition	[15]
SH-SY5Y neuroblastoma cells treated with rotenone	From 0.01 to 10 mM	2–3–6 h	– reduced cell death – reduced caspase 3/7 activity – AMPK activation – reduced mitochondrial ROS production – prevented antioxidant depletion and mitochondrial dysfunction – induced Nrf2 pathway via Akt	[16]
BV2 cells treated with LPS or IL-4	1 mM	3–12–24 h	– reduction of microglial activation – reduction of ROS – reduction of NADPH oxidase activity	[17]

Table 2. Molecular read-outs of metformin administration to non-PD and PD animal models.

Animal Model	Dosage	Duration	Administration Route	Read-Out	Ref.
C. elegans	50 mM		Oral	– increased lifespan – improved fitness – inhibition of TORC1 pathway and activation of the lysosomal pathway – activation of AMPK	[18]
C. elegans bcat-1 knock-down	0.05 mM	4 days	Oral	– complex I inhibition – rescue neuronal viability	[19]
D. melanogaster	From 1 to 100 mM	7 days	Oral	– AMPK activation – reduction of fat stores – disruption of intestinal fluid homeostasis	[20]
D. melanogaster	5 mM	7 days	Oral	– inhibition of age-related centrosome amplification in midgut stem cells – inhibition of Akt/TOR pathway	[21]
M. musculus	0.1–1% *w*/*w*		Oral	– increased lifespan and health span – mimicking of calorie-restriction transcriptome – Activation of AMPK – activation antioxidant response	[22]
M. musculus	200 mg/Kg		Intraperitoneal injection	– increased Ach levels – decreased choline levels	[13]
M. musculus	1–10 mM		Hypothalamus infusion	– increased Ach levels – decreased choline levels	[13]
M. musculus	From 6.25 to 50 mg/kg	Once a day for 12 weeks	Oral	– activation of complex I (50 mg/kg) – activation of AMPK	[14]
R. norvegicus	50–150 mg/ml	Once a day for 5 days	Oral intubation	– reduction of ATP/ADP ratio in the liver – change of glycolytic metabolite	[11]
In vivo PD animal models					
C. elegans treated with 50 mM 6-OHDA	5–10 mM	72 h	Oral	– increased lifespan – decreased degeneration of dopaminergic neurons – reduced α-syn aggregation – upregulation of dopamine synthetic gene cat-3 and antioxidant gene sod-3	[23]
M. musculus injected with MPTP (30 mg/kg/day) for 7 days	200 mg/kg/day	7 days (following MPTP)	Intraperitoneal injection	– recovery from motor dysfunction – increased TH expression in the striatum and restored dopamine levels – decreased caspase-3 and apoptosis inhibition – reduced astroglia activation – AMPK activation and mTOR inhibition – PP2A activation and reduced α-syn pSer129 – upregulation of neurotrophic factors (BDNF, GDNF) and activation of downstream signaling pathways (Akt, Erk1/2)	[24]

Table 2. *Cont.*

Animal Model	Dosage	Duration	Administration Route	Read-Out	Ref.
M. musculus injected with rotenone (2.5 mg/kg/day) for 10 days	300 mg/kg/day	10 days (co-admin.)	Intraperitoneal injection	– rescued dopaminergic neuron loss in SNpc – decreased caspase-3-mediated apoptosis – reduced α-syn accumulation – decreased levels of lipid peroxidation products (4-HNE, MDA) – no difference in motor behaviors	[25]
M. musculus treated with 15 µg 6-OHDA	100–200 mg/kg	Once a day for 4 weeks	Oral	– rescue of motor deficits – induced AMPK, AKT, BDNF, GSK3b, CREB pathway – reduced astrocyte activation	[26]
R. norvegicus injected with 2 µg of LPS	150 mg/Kg	Twice a day for 7 days	Oral	– reduction of the number of activated microglial cells – reduction of inflammatory mediators and microglial pro-inflammatory phenotypes – decreased activation of the inflammasome	[17]

Here, the most relevant cellular pathways that are modulated by metformin are summarized in Figure 1 and they will be discussed in the following paragraphs.

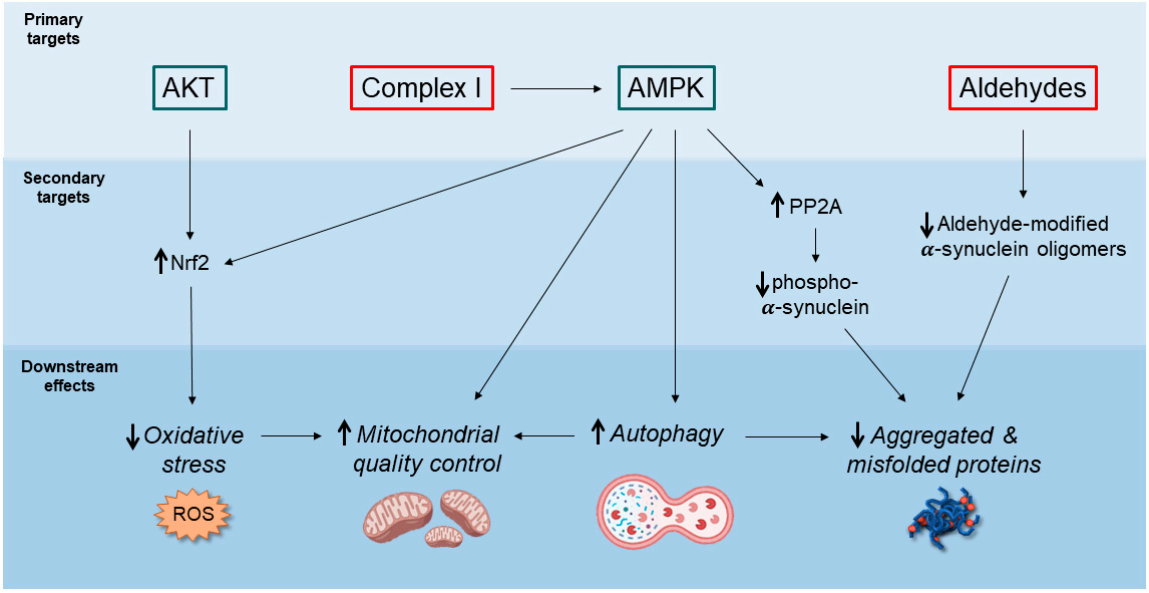

Figure 1. Potential neuroprotective mechanisms of action of metformin. This schematic diagram illustrates the most relevant molecular pathways and the cellular processes affected by metformin. In the upper section, the primary targets activated by metformin (AKT and AMPK) are highlighted in green. Metformin direct targets that are inhibited by the drug (Complex I and Aldehydes) are indicated in red. In the lower sections the secondary molecular targets are represented together with the downstream effects of metformin, which include the increase of autophagic activity, the reduction of aggregated or misfolded proteins, the decrease of ROS, and the improvement of mitochondrial functions. All these outcomes may account for the potential neuroprotective action of metformin.

2.1. AMPK-Mediated Autophagy Activation

One of the most studied and relevant effects of metformin is the modulation of the AMP-activated protein kinase (AMPK) activity. AMPK is a ubiquitously expressed kinase protein that is considered a sensor of cellular metabolic status and plays a crucial role in maintaining cellular energetic homeostasis [27,28]. AMPK function is affected by different stimuli, like changes in the intracellular calcium level or imbalance in the oxygen reactive species (ROS) concentration [27,29]. Moreover, the major stimulus that is known

to modulate AMPK activity is the variation of the ATP/AMP ratio, as the increase of AMP concentration results in its activation, due to the binding between AMPK and AMP. This interaction causes conformational changes in the protein that becomes more prone to be phosphorylated by the upstream activator kinases, in particular the Liver Kinase B1 (LKB1) [27]. In turn, AMPK can influence several important pathways, coordinating key cellular processes, such as autophagy, cell growth, and mitochondrial quality control [28]. Interestingly, some of the molecular mechanisms affected by AMPK function are crucial for neuronal cell survival and are known to be dysregulated in different neurodegenerative disorders, including PD. Thus, targeting AMPK to enhance its activity is considered a promising neuroprotective strategy [29,30]. Noteworthy, many studies demonstrated, both in vitro and in vivo, that metformin can activate AMPK [10] through a mechanism that is still under debate [31]. Importantly, this effect has been analyzed also in the neurodegeneration context, exploiting a cellular model of Alzheimer Disease (AD) characterized by the accumulation of amyloid-beta (Aβ) deposits. In this frame, the activation of AMPK mediated by metformin has been shown to increase cell viability and rescue mitochondrial defects by increasing mitochondrial mass and ameliorating mitochondrial function. These beneficial effects are associated with the activation of several AMPK downstream proteins including Bcl-1, CREB, and PGC1α [10].

Among the processes regulated by AMPK, the activation of autophagy is one of the best-studied. This mechanism has been analyzed in hippocampal rat neurons in which metformin was shown to activate AMPK, increasing neuroprotection through the mechanistic target of the rapamycin (mTORC1) pathway [32]. In this frame, the AMPK-mediated inhibition of mTORC1 has been described to be beneficial to maintain neuronal homeostasis by promoting autophagic activity and lysosomal biogenesis [18,33–35]. The AMPK-mTORC1 pathway has been proven to be activated by metformin also in vivo in *C. elegans* where the treatment with the drug increased lifespan through the induction of the lysosomal pathway [18].

Drosophila melanogaster has also been used as an in vivo model to evaluate the effect of metformin on the AMPK pathway. The oral administration of the drug was demonstrated to increase the levels of threonine 172 phosphorylation, which is necessary for the activation of the protein [20]. Although the use of metformin was not analyzed in fruit fly models for neurodegenerative disorders, increasing AMPK activity is beneficial in fly models of several diseases, suggesting the possibility to target this kinase with metformin as a therapeutic approach [36,37].

The metformin-mediated AMPK activation and the crucial role of AMPK activity have been widely investigated also in mammalian models, where it was shown that increasing AMPK activity may protect cells against different stress stimuli. For instance, in mice, metformin increases lifespan through the activation of AMPK [22].

Despite these positive observations, several questions about the activity of metformin are still unsolved and need further investigation in order to avoid possible negative effects. For example, the mechanism by which metformin modulates the activity of AMPK is still unclear. The hypothesis that metformin could directly activate AMPK was excluded long ago when the incubation of purified AMPK from rat liver with metformin failed to activate the kinase [12], indicating that the drug indirectly activates AMPK, likely affecting upstream regulators of the protein. Interestingly, metformin failed to activate AMPK in mice knockout for LKB1, an upstream activator of AMPK able to phosphorylate the protein at the T172 residue [38]. Therefore, it appears that the treatment can influence one or more upstream pathways.

2.2. Decreased Accumulation of α-Synuclein Pathological Species

Autophagy activation represents a good strategy to reduce the accumulation of toxic protein aggregates, which are detected at the histological level in different neurodegenerative diseases. In PD, the progressive accumulation of α-syn in neurons due to oxidative stress, certain post-translational modifications, or impairment of protein degradation

systems generates α-syn neurotoxic oligomers that are known to affect several cellular pathways [39]. Hence, the upregulation of the autophagic pathway by metformin might counteract α-syn pathology by rapidly disposing of the α-syn aggregates, as recently shown in a *C. elegans* PD model exposed to 6-hydroxydopamine (6-OHDA), where the treatment with metformin resulted in a reduction of both α-syn aggregation and dopaminergic neuron loss [23].

It has been also demonstrated that metformin can reduce the levels of phosphorylated α-syn at serine 129 (α-syn pSer129), which is usually considered a read-out of pathological and aggregated α-syn species. Specifically, both in vitro and in vivo, metformin administration resulted in time- and dose-dependent activation of protein phosphatase 2A (PP2A), which is known to mediate α-syn dephosphorylation [15]. According to the authors, PP2A activation by metformin can happen via both AMPK-dependent and -independent pathways. On the same line, metformin administration to mice previously injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) significantly reduced α-syn pSer129 levels through the activation of PP2A [24]. In this model, metformin-mediated decreased levels of pathological α-syn together with autophagy activation, upregulation of neurotrophic factors (BDNF, GDNF), and downstream signaling pathways (Akt, Erk1/2) resulted in restored dopamine concentration at the striatum and rescue of motor performance [24], thus underlying the multiple neuroprotective mechanisms of action of this drug.

On a different note, due to the ability of the guanidino group and the primary amine present in metformin structure to act as a scavenger of the aldehyde moiety of methylglyoxal (MGO), metformin has been used as an anti-glycating agent in T2DM therapy to prevent the accumulation of proteins modified by MGO and advanced glycating end products (AGEs) [40]. Interestingly, MGO has been demonstrated to covalently modify α-syn and trigger its oligomerization in several in vitro and in vivo models, exacerbating PD-like neurodegeneration [41]. In this frame, the administration of aminoguanidine, an analog of metformin, reduced α-syn aggregation and promoted its clearance via autophagy in MGO-treated cells and rescued the motor impairment observed in MGO-treated flies overexpressing the human α-syn [41]. Hence, we recently speculated that metformin could provide a similar scavenging activity towards MGO by preventing the accumulation of α-syn neurotoxic aggregates, but this could be extended to other aldehydes of neuropathological relevance in PD, i.e., aldehydes derived from oxidative stress and lipid peroxidation (4-hydroxynonenal (4-HNE), malondialdehyde (MDA)) or aldehydic molecules that accumulate from altered monoamine catabolic pathways (3,4-dihydroxyphenylacetaldehyde, 3,4-dihydroxyphenylglycoaldehyde, 5-hydroxyindole-3-acetaldehyde) [42]. Accordingly, a recent paper investigated the neuroprotective effect of metformin in a PD mouse model based on rotenone-induced dopaminergic neuron death [25]. The authors demonstrated that metformin co-administration with rotenone significantly reduced the nigral levels of 4-HNE and MDA, together with decreased α-syn accumulation and dopaminergic neuron degeneration in the SNpc [25]. Although the authors did not investigate it directly, the reduced α-syn buildup might derive, at least in part, from a scavenging activity of metformin towards lipid peroxidation products, preventing α-syn modification and oligomerization.

It is worth mentioning that the spreading of aggregated and phosphorylated α-syn toxic species has a crucial role in the progression of PD pathology [43–45]. Several mechanisms have been described to mediate the cell-to-cell transmission of pathological α-syn (trans-synaptic transmission, extra-cellular vesicles, and endocytosis) across diverse brain regions [46]. Moreover, according to the Braak hypothesis, the primary events of α-syn aggregation and spreading originate outside the brain, either in the olfactory bulb or the gastrointestinal tract, further propagating through the cranial nerves from the peripheral nervous system to the brainstem and the other vulnerable areas in the CNS (i.e., SNpc and the striatum) [47–49]. In this context, both the autophagic enhancing activity of metformin and the ability to degrade α-syn neurotoxic aggregates before propagation may be of great value to restrain α-syn spreading and to slow down the disease progression. Importantly,

the accumulation of α-syn aggregates is a feature shared by different synucleinopathies, which include, besides PD, Dementia with Lewy Bodies (DLB), and Multiple System Atrophy (MSA) [50]. Therefore, it is plausible to hypothesize that the treatment with metformin may be beneficial not only in the context of PD but also in other pathologies characterized by α-syn aggregation. These pathologies have been suggested to display structurally different α-syn fibrillary patterns or strains as well as different cell types, both neuronal and glial, in which the aggregates are found. All these issues may account for the heterogeneity of the clinical phenotypes associated with α-syn accumulation [51]. In this frame, it would be crucial to characterize in detail the action of metformin in the diverse brain regions and cellular subpopulations to assess whether the neuroprotective effect of the drug varies among the specific cellular target or α-syn conformation, thus resulting in a differential outcome according to the diverse synucleinopathy.

2.3. Inhibition of Mitochondrial Complex I and Regulation of Mitochondrial Dynamics

Metformin has been widely studied as a molecule able to influence mitochondrial functions. In this frame, more than 20 years ago it was observed that in rats treated with metformin, the molecule slowly penetrated across the inner mitochondrial membrane and accumulated inside the organelles, where it appeared to cause the inhibition of complex I activity possibly through a direct interaction [11]. Even though this effect is one of the most studied and established, the precise mechanism of metformin's function at the mitochondrial level is still under debate [52]. It is worth mentioning that while a mild inhibition of complex I may result in beneficial effects for cell homeostasis, the inhibition of the complex I may also be detrimental, in particular in the context of PD. In fact, the reduction of the mitochondrial respiratory chain function may determine a decrease in the ATP/ADP ratio, resulting in a relatively higher concentration of ADP that can bind AMPK and activate the protein [11]. However, in PD the decrease of complex I activity has been proposed to be one of the triggering factors for the disease [53]. Accordingly, some reports highlighted the possibility that the metformin-mediated inhibition of complex I may produce negative outcomes. For instance, a study performed in lipopolysaccharide (LPS)-induced rat model of PD showed that, despite some beneficial effects induced by metformin after the intranigral injection of LPS, such as the reduction of neuroinflammation, the drug not only did fail to rescue the dopaminergic neuronal loss induced by LPS but exacerbated it. The authors speculated that this result may be caused by the inhibition of complex I activity, affecting not only mitochondrial functionality but also cell homeostasis [17]. Along this line, metformin was shown to aggravate the dopaminergic neuronal loss in a PD mouse model based on the injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which inhibits mitochondrial complex I. In this case, the additive action of MPTP and metformin at the level of this complex and the consequent reduction of ATP may explain the detrimental effect of the drug [54]. Intriguingly, several PD models induced by the treatment with rotenone, which is another inhibitor of the complex I, showed opposite results. In fact, after rotenone injection in mice, metformin has been recently shown to reduce dopaminergic neuronal loss and rescue some PD behavioral phenotypes in mice [55]. Noteworthy, in a *C. elegans* model, it was observed that also mitochondrial hyperactivity may result in PD phenotypes, demonstrating that different alterations of mitochondrial homeostasis may be associated with PD pathology [19]. In this case, it is more intuitive to assume that the inhibitory effect mediated by metformin may be beneficial. Accordingly, metformin has been proven to rescue not only motor deficits but also the loss of dopaminergic neurons in *C. elegans* characterized by mitochondrial hyperactivation [19].

Besides the effects on the complex I activity, metformin has been also demonstrated to be important in maintaining mitochondrial quality control, improving mitochondrial fission, promoting respiration, and reducing oxidative stress [14,22]. For example, a recent work evaluated the effect of metformin in SH-SY5Y cells characterized by mitochondrial defects induced by the treatment with lead. In this model, the antidiabetic drug has been shown to reduce mitochondrial fragmentation and ameliorate mitochondrial mor-

phology. Moreover, in the same cellular model, metformin has been described to reduce the mitochondrial-derived ROS levels induced by the treatment with rotenone. This antioxidant property of metformin has been associated with the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), which is a transcription factor responsible for the transcriptional regulation of genes involved in the response against oxidative stress. Moreover, the metformin-mediated Nrf2 modulation has been shown to be determined by the activation of Akt, an upstream activator of Nrf2 [16]. Interestingly, also AMPK activity is known to influence the Nrf2 pathway [56,57], suggesting that metformin may induce the antioxidant response through Nrf2. However, it is worthwhile to report that Akt and AMPK have also been reported to exhibit antagonistic activity in the ROS regulation, modulating different molecular pathways [58]. For this reason, it is crucial to understand whether metformin activates preferentially AMPK rather than Akt in particular conditions or at certain drug concentrations.

Hence, the ability of metformin to influence mitochondrial functions may be therapeutically relevant in the context of PD, in which mitochondrial defects and excessive oxidative stress are known to be associated with the pathology. Nevertheless, the contradictory results presented here demonstrate that further characterization of the role of metformin at mitochondria needs to be further elucidated.

2.4. Anti-Inflammatory Action

Accumulating evidence highlights the important role of non-neuronal cells in the onset and progression of neurodegenerative disorders, and glial cells' activity has been widely studied in the PD context. These cells can regulate brain homeostasis and are essential for neuronal cell survival, modulating crucial processes, such as synaptic formation and maturation and response to stressful conditions [59,60]. Accordingly, several studies linked astrocyte dyshomeostasis to PD [61]. In particular, astrocytic activity has been evaluated in the case of neuroinflammation, one of the phenotypic traits associated with PD [62,63]. A study published in 2020 analyzed the effects of metformin on reactive astrocytes in a mouse 6-OHDA-based PD model, where the antidiabetic drug ameliorated astrocyte activation promoted by 6-OHDA and determined the downregulation of genes involved in astrocytes' reactivity. These effects may be mediated by the induction of important cellular pathways, such as Akt, GSK3b, CREB, and BDNF-mediated pathways [26].

The microglial activity in the inflammation processes has been evaluated too, both in vitro and in rats and mice PD models. Interestingly, it was shown that metformin reduced microglial activation in cells and rats after LPS treatment. More specifically, metformin promoted the decrease of the number of activated glial cells, reduced the expression of pro-inflammatory mediators, and lowered the activation of the inflammasome as well as the accumulation of ROS in microglia [17]. The same effects have been observed in a mouse model following the treatment with MPTP [54].

Taken together, these results seem to underly the beneficial effect of metformin in reducing neuroinflammation, highlighting the importance of increasing the efforts in this research field to understand more in detail how the drug exerts the anti-inflammatory activity. Noteworthy, also the effects on microglial cells might be related to the activation of AMPK, which has been linked to the anti-inflammatory pathway, suggesting that the activation of the AMPK pathway may account for many of the effects ascribed to metformin [17,54,64].

3. Metformin as a Disease Modifier for Parkinson Disease: How Effective Is It?

3.1. Evidence from Epidemiological Studies on T2DM Patients

Based on the molecular mechanisms described so far, metformin appears of great interest as a translational approach for PD. Several studies performed on both in vitro and in vivo models of neurodegenerative diseases promisingly suggested that the metformin-mediated AMPK activation may reduce the level of neuronal loss and alleviate several phenotypes associated with these disorders. In light of these considerations, some epi-

demiological studies investigated in cohorts of T2DM patients the correlation between metformin therapy and the risk of developing PD (Table 3).

Table 3. List of epidemiological studies on T2DM patients treated with metformin evaluating the association with the risk of PD.

Location	Study Period	Sample Size	Medication Users	Mean Age (Years)	HR [95% C.I.]	Follow-Up (Years)	Reference
Taiwan	1996–2007	11,730	1879	64.3 ± 9.6	0.95 [0.53–1.71]	11 or until PD onset	[65]
Norway	2004–2014	102,745	94,349	63.4 ± 11.1	1.39 [1.06–1.82]	6.95	[66]
South Korea	2009–2010	1,308,089	644,921	60.8 ± 10.0	1.22 [1.10–1.36]	6.3	[67]
USA	2004–2010	5530	2774	63.2 ± 10.9	0.19 [0.12–0.31]	5.2	[68]

The first study, conducted in a large cohort of T2DM patients in Taiwan, found a 2.2-fold increased risk to develop PD in T2DM patients [65]. Interestingly, the treatment with sulfonylurea, another anti-hyperglycemic agent, significantly increased the risk of PD (HR 1.57, 95% CI 1.15–2.13), but it was alleviated for those patients who received a co-therapy with metformin (HR 0.78, 95% CI 0.61–1.01). However, the administration of metformin alone did not prove to prevent the development of PD in diabetic patients (HR 0.95, 95% CI 0.53–1.71) [65]. Later on, other clinical studies in Australia (2013), Norway (2017), and South Korea (2020) investigated the correlation between metformin therapy and PD, revealing a positive correlation with dementia, cognitive decline, and Parkinson syndrome [66,67,69]. Conversely, a longitudinal study in the USA in 2019 analyzed a 5-year follow-up in more than 5500 veterans with T2DM (around 60 years old), pointing out that a metformin therapy for more than 4 years significantly decreased the risk of developing both PD and AD (HR 0.19, 95% CI 0.12–0.31) [68]. Despite this promising avenue, a recent systematic review and meta-analysis by Qin and coworkers indicated an overall lack of correlation between metformin therapy and PD development (HR 1.23, 95% CI 0.98–1.78) [70]. More importantly, the sole exclusion of the study in the USA cohort resulted in a significant increase in PD risk (HR 1.50, 95% CI 1.11–2.02) [70]. Here, it must be acknowledged that these conflicting results may derive from a high degree of heterogeneity among clinical studies, in terms of population, treatment regime, follow-up lengths, and adjusted factors. In these studies, the average age of the enrolled subjects was 60–65 years, which corresponds to the mean age of onset of PD, although the incidence of PD increases with age (1–2% over 65 years old and more than 5% over 80 years old). However, the follow-up usually took place for an additional 6 years on average, possibly underestimating the correlation between metformin consumption and PD.

This notwithstanding, these clinical studies appear less promising than the large body of evidence of metformin neuroprotective effect in PD pre-clinical models. On these premises, a few aspects of metformin pharmacokinetics and pharmacodynamics need further consideration to evaluate the possible drawbacks of metformin prolonged therapy, namely, the bioavailability of the molecule in the brain and potential molecular side effects.

3.2. Metformin Bioavailability in the Brain

Metformin bioavailability has been determined to be around 40–60% [71] with a half-life of about 6.2 h in the plasma and an elimination half-life of 17.6 h [72]. The molecule does not encounter hepatic metabolism and it is eliminated mainly through the urinary tract in its unmodified form [42]. One of the still-unsolved aspects regards the concentration and accumulation of the drug in the brain after administration and how metformin reaches the brain tissue. In this context, several works demonstrated that metformin can penetrate the

blood–brain barrier (BBB) in mammals, as indicated by the presence of the molecule in the cerebrospinal fluid (CSF) of rats after oral administration [73,74]. Along this line, another important factor to be assessed is the brain-to-plasma ratio of metformin, as this parameter indicates the ability of the molecule to pass through the BBB. This ratio has been evaluated independently in rats by two research groups that obtained different results. In the first publication, a rat model of inflammation induced by LPS showed that the brain-to-plasma ratio reached the maximum value 6 h after single oral administration. At this time point, the concentration of metformin in the blood plasma was comparable to the concentration in the brain, suggesting a high permeability of the drug across the BBB [73]. The same study also showed that the concentration of metformin was not the same in different brain regions. Moreover, acute and chronic administrations induced a different pattern of distribution with the highest concentration of the molecule in the cerebellum and the pituitary gland, respectively [73]. Conversely, the other study found in the CSF of rats only 4% of the plasma metformin concentration, suggesting a low ability to cross the BBB [74]. Even though these contradictory results may be explained by different methods used for the analysis or by variations in the method of administration, these data prompt the need to unequivocally estimate the percentage of metformin that penetrates the diverse brain districts.

Another critical factor that must be evaluated is the mechanism that governs the uptake of metformin within neuronal cells. Since metformin is positively charged it is improbable that it can easily pass the plasma membrane through simple diffusion [75]. Therefore, it is most likely that some membrane transporter plays a crucial role in the absorption and distribution of metformin in cells. In this frame, several studies confirmed that metformin is a substrate of numerous organic cation transporters (OCTs), including OCT1, OCT2, OCT3, MATE1, MATE2, PMAT, and OCTN1 [76]. These transporters are responsible for the recognition and transport of a broad variety of molecules and drugs, which are positively charged at physiological pH [77]. The pattern of expression of these transporters may highly influence the rate of metformin absorption in different tissues. For this reason, the analysis of the distribution of OCTs in the brain, with a specific focus on the expression of these transporters at the BBB level, as well as in different brain regions and different cell types, may be crucial to characterize the impact of metformin on brain physiology. An interesting work published in 2020 analyzed the expression of several OCTs in the BBB of rats, mice, and humans both in vivo and in vitro, using primary cell cultures [78]. The presence of the transporters was analyzed through the analysis of the OCTs' mRNA levels, and the authors performed also functional analysis evaluating the differences in permeability of known substrates through the membranes in the presence or absence of inhibitors of the transporters. This study revealed the lack or a negligible presence of OCT1, OCT2, OCT3, and MATE1 in the BBB of all organisms [78]. These results were quite unexpected considering the aforementioned ability of metformin to cross the BBB. This aspect may be partially explained by the fact that not all the OCTs responsible for metformin transport were evaluated in the study. Another possibility is that other unknown transporters in the BBB determine the passage of metformin. Further analyses are necessary to assess how metformin can reach the brain tissue. In contrast to the reported study, a previous work conducted in 2010 used confocal imaging and Western blot analysis to evaluate the presence of OCTs in the BBB. The authors found that both OCT1 and 2 are detectable in the BBB of rats, mice, and humans [79]. The discrepancies in the results of different scientific works are difficult to explain, but it is important to note that the different techniques used may have different levels of resolution. Moreover, the presence of the transporters detected in the latter study may also be determined by the signal contamination caused by other cells, such as neurons, in which the presence of OCTs seems to be more abundant. In fact, OCT2, OCT3, OCTN1, and, to a lesser extent, OCT1 expression has been demonstrated in human and rodent neurons and may account for the intake of metformin in neuronal cells [80,81].

To assess whether metformin can be therapeutically beneficial for neurodegenerative diseases it is also crucial to determine the concentration of the drug into the brain that is

necessary to promote protective effects and to understand the appropriate way to administer the molecule to gain the region of interest in the brain as well as inside neurons and glial cells. In this frame, the group of Kalyanaraman developed mitochondrial-targeted analogs of metformin (mito-metformin) by attaching a positively charged lipophilic triphenylphosphonium group to the molecule, thus increasing metformin bioavailability in the subcellular compartment of about 1000 times fold [82]. Despite being conceived to increase the anti-tumor potential of metformin [83], the administration of mito-metformin to the MitoPark transgenic mouse model of PD (knockout mouse for the mitochondrial transcription factor A in midbrain dopaminergic neurons) was able to promote mitophagy, restore striatal dopamine levels, and rescue both the motor and behavioral phenotype [84]. Moreover, the investigators tested the efficacy of mito-metformin in a cellular model of rotenone-induced mitochondrial dysfunction, revealing that the delivery of the drug by functionalized polyanhydride nanoparticles (NPs) provided a significant amelioration of cell viability at nanomolar concentrations of mito-metformin [85].

3.3. Side Effects Associated with Prolonged Metformin Consumption

Metformin is generally well tolerated with minimal side effects for the majority of medication users. The most frequent adverse effects are related to hypoglycemia and irritation of the gastrointestinal tract for which the subjects can experience bloating, flatus, diarrhea, nausea, and constipation. About 50% of T2DM patients under metformin therapy have been diagnosed with plasma acidosis [72]. This condition is defined by plasma pH < 7.3 and a lactate concentration >5.0 nmol/L, and, in the case of metformin, this is likely to derive from the alteration of glucose metabolism in hepatocytes, resulting in increased conversion of pyruvate to lactate, which is further released in the bloodstream. Although only a small percentage of cases of plasma acidosis is life-threatening due to complete kidney failure, reduced renal functions have been associated with prolonged consumption of metformin at high dosage (>2 g/day, when the average dose for T2DM patients is 500–1700 mg/day) for patients older than 65 years [72].

More importantly, a severe vitamin B12 (VitB12) deficiency has been found in metformin users [72]. VitB12 or cobalamin is an important cofactor for enzymes involved in the DNA synthesis and fatty acids' and amino acids' metabolism, as well as enzymes involved in neuroprotective functions, myelin synthesis, and blood cell maturation in the bone marrow. A decreased intestinal uptake of VitB12 results in peripheral neuropathy, axonal demyelination, and hematological abnormalities [72]. In a recent retrospective cohort study on patients with more than 1 year of metformin consumption, around 3.3% of the subjects displayed a significant VitB12 deficiency, with the highest correlation in people over 80 years old (63%) [86]. Although the precise mechanism of metformin inhibition of VitB12 uptake has not been fully elucidated, it might depend on an interference with the calcium-dependent binding of the gastric intrinsic factor-VitB12 complex with the cubilin receptors on enterocytes at the level of the ileum, a direct interaction between metformin and the cubilin receptor, which blocks VitB12 uptake, or a dysregulated intestinal microbiota outgrowth, which potentially affects cubilin receptor accessibility by VitB12 [87].

Interestingly, a case-control study carried out in Australia between 1998–2008 positively correlated the VitB12 deficiency concomitant to metformin consumption with increased risk of cognitive impairment and AD in T2DM patients over 65 years old (HR 1.71, 95% CI 1.12–2.60) [69]. The correction of the VitB12 levels in the plasma by calcium supplementation to promote VitB12 uptake was able to preserve the cognitive functions [69]. In addition, a recent study on idiopathic PD patients in Korea correlated VitB12 deficiency (<133 pg/mL) not only with cognitive impairment but also with the decreased motor performance of the individuals, while VitB12 supplementation was able to provide significant improvement of the motor symptoms [88]. On the same line, gastric cancer patients subjected to total gastrectomy (thus preventing the physiological VitB12 uptake) displayed a higher risk of developing PD (HR 1.55, 95% CI 1.03–2.32), whereas introducing VitB12 supplement significantly reduced the risk of PD of 60–70% (HR 0.36, 95% CI 0.17–0.76) [89].

Noteworthy, another recent cross-sectional analysis on PD patients from DATATOP [90] assessed the levels of VitB12 deficiency, indicating that 13% of patients presented VitB12 plasma concentration below the minimum threshold of 250 pg/mL [91]. This value decreased with aging between −17 and −47 pg/mL/year (which is more than the average VitB12 decrease of −5 pg/mL/year in the healthy elderly population) and was correlated with a progressive decrease in patients' motor performance (gait impairment). Moreover, at the time of diagnosis, PD patients presented the lowest VitB12 concentration as compared to other neurodegenerative disorders, i.e., AD, MSA, progressive supranuclear palsy, frontotemporal dementia, DLB, and mild cognitive impairment [91]. The authors discussed the possible causes of the decreased uptake of VitB12 at the gastrointestinal level in PD patients, suggesting, among others, the infection by *H. pylori*, a delayed gastric emptying, constipation, and bacterial outgrowth [91]. This is particularly relevant in light of the gut–brain axis involvement in PD, with the suggested possibility that the primary site of PD pathology could originate in the enteric system due to microbial dysbiosis and α-syn enteric aggregation and then propagate to the central nervous system through the vagal nerve [92]. Coincidently, it has been demonstrated that VitB12 negatively regulates α-syn fibrillation by direct binding to the protein and it reduces α-syn-induced cytotoxicity [93].

On these premises, the VitB12 deficiency and the gastro-intestinal alterations induced by metformin consumption should be taken into consideration during prolonged therapies with metformin. This is especially relevant when high dosages are required to ensure a proper bioavailability of the drug in the brain because they might exacerbate VitB12 depletion in elderly individuals who already have an increased risk of VitB12 deficiency at the age of PD clinical onset [87]. In addition, despite the putative role of metformin in counteracting α-syn spreading, it might have an indirect pro-aggregating action on the enteric α-syn, thus accelerating the early events of PD pathology.

3.4. Metformin Treatments in Experimental Models versus Human Subjects: Looking for a Key of Interpretation

An important aspect that still needs to be elucidated is whether metformin consumption in healthy individuals might be beneficial in targeting and delaying aging, as well as age-related disorders [94]. Indeed, potential metformin-dependent side effects displayed by T2DM patients might be absent in a diabetic-free background. However, at present, no clinical data are available aiming at assessing a decreased risk of PD other than for T2DM patients under the metformin regime.

Anyway, a few studies of in vivo experimental models investigated the role of metformin per sè in brain bioenergetic and behavioral response. Specifically, when 10-week-old mice were injected with 200 mg/kg/day of metformin, the activation of AMPK and the levels of GDNF and BDNF were significantly higher both in SNpc and striatum, resulting in TH upregulation and improved dopaminergic neuron health [95]. In another study, 4-month-old mice subjected to chronic administration of 180 mg/kg/day of metformin (which, according to the authors, should correspond to about 900 mg/day intake for humans) displayed an improved motor performance but cognitive impairment [96].

It is worth mentioning that, in both studies, the effects of metformin were tested in relatively young mice and in quantities that correspond, at least in theory, to the minimum clinical dosage. It has been estimated that in humans the plasma concentration of metformin ranges between 10 μM and 40 μM when assuming 1 g of drug per day [71]. However, the equivalence between the dosage in humans and the concentrations used in experimental models is still under debate, even though this would be extremely relevant to accurately correlate the molecular readouts detected in research models with the effects observed in humans. According to Lamoia and colleagues, the inhibition of mitochondrial complex I and AMPK activation detected in cellular models treated with millimolar concentrations of metformin correspond to supra-pharmacological concentrations, whereas the redox balance is stimulated at micromolar concentrations, defined as clinically relevant doses [71].

Another key issue is the inconsistency of dosage among the experimental studies present in the literature, together with an extremely high variability of effects according to the model system and the readouts considered. This is exacerbated when the metformin neuroprotective role is tested in both in vitro and in vivo PD models, where the neurodegenerative phenotype is induced either genetically (i.e., α-syn overexpression) or pharmacologically by LPS or neurotoxins such as 6-OHDA, rotenone, and MPTP. Indeed, since metformin has multiple direct and indirect targets (Figure 1), its mechanisms of action may significantly vary according to the animal model, the concentration, the administration route, and PD stimulus, thus challenging data interpretation and translation to humans. An example is represented by the inhibitory activity on mitochondrial complex I, which remains to be unresolved whether it is beneficial or not, in particular in the PD context. The controversial results present in the literature may depend on the amount and the duration of metformin treatment, as well as the PD model, as exemplified by the conundrum on the aggravating effect in the MPTP model versus the neuroprotective effect in the rotenone one [54,55]. Moreover, the different outcomes may be also due to different levels of complex I inhibition in different experimental models. A mild complex I inhibition might be advantageous as a reduced ATP/ADP ratio activates the AMPK signaling pathway, thus promoting autophagy-mediated degradation of misfolded proteins and dysfunctional organelles. Moreover, a partial complex inhibition could maintain under control the redox state of mitochondrial along with the dopaminergic neuronal projections that present higher rates of oxidative phosphorylation [97]. Conversely, an excessive inhibitory effect on complex I, comparable to the enzyme ablation, has been demonstrated to be sufficient in triggering a PD phenotype [53]. In addition, several contradictory results highlighted in some reports further complicate the analysis and the precise understanding of metformin activity at the mitochondria. For example, a recent paper demonstrated that low metformin concentrations may stimulate mitochondrial respiration and complex I activity in mice rather than inhibiting it [14]. Therefore, it is necessary to understand the precise metformin dosage necessary to alter the AMP/ATP ratio and activate AMPK, without the induction of detrimental effects.

4. Conclusions

Several works recently suggested that metformin may be used as a promising therapeutic strategy to counteract the progression of neurodegenerative disorders, including PD. However, despite intensive research, the precise mechanisms by which metformin exerts its activity are not completely understood. Therefore, to determine the potential therapeutic use of metformin as a disease modifier for PD, it is crucial to evaluate its main pharmacological properties, such as the bioavailability in the brain, the molecular pathways it affects, and the concentrations necessary to observe the beneficial effects. Unfortunately, the data available in the literature are often controversial and vary significantly among experimental models, prompting the necessity to outline a consensus in pre-clinical data interpretation.

At the same time, the prolonged consumption of metformin at a relatively high dosage might induce serious side effects that could worsen the risk of developing PD over time. In this frame, the epidemiological studies that investigated the association between metformin therapy and PD only assessed the effect of the drug in T2DM patients [65–69]. Additionally, to our knowledge, phase 1 clinical trials of metformin did not provide an appropriate follow-up on the enrolled subjects aiming at verifying a decreased incidence of PD with aging. Thus, it remains to be evaluated by ad hoc clinical studies whether metformin administration to non-diabetic subjects exerts a neuroprotective activity towards PD and other neurodegenerative disorders. In this case, the ability of metformin to effectively act as a disease modifier might depend on an early intervention in the prodromal phases of PD, when the neurodegenerative process is not irreversibly advanced. Hence, the development of new criteria for patients' stratification strategies should be a primary goal to identify those individuals who could benefit most from metformin therapy in the long run [42].

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The Regulation of MiTF/TFE Transcription Factors Across Model Organisms: from Brain Physiology to Implication for Neurodegeneration

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Abstract
The microphthalmia/transcription factor E (MiTF/TFE) transcription factors are responsible for the regulation of various key processes for the maintenance of brain function, including autophagy-lysosomal pathway, lipid catabolism, and mitochondrial homeostasis. Among them, autophagy is one of the most relevant pathways in this frame; it is evolutionary conserved and crucial for cellular homeostasis. The dysregulation of MiTF/TFE proteins was shown to be involved in the development and progression of neurodegenerative diseases. Thus, the characterization of their function is key in the understanding of the etiology of these diseases, with the potential to develop novel therapeutics targeted to MiTF/TFE proteins and to the autophagic process. The fact that these proteins are evolutionary conserved suggests that their function and dysfunction can be investigated in model organisms with a simpler nervous system than the mammalian one. Building not only on studies in mammalian models but also in complementary model organisms, in this review we discuss (1) the mechanistic regulation of MiTF/TFE transcription factors; (2) their roles in different regions of the central nervous system, in different cell types, and their involvement in the development of neurodegenerative diseases, including lysosomal storage disorders; (3) the overlap and the compensation that occur among the different members of the family; (4) the importance of the evolutionary conservation of these protein and the process they regulate, which allows their study in different model organisms; and (5) their possible role as therapeutic targets in neurodegeneration.

Keywords MiTF/TFE · TFEB · Autophagy · Neurodegeneration · Lysosomal storage disorders

Introduction

The transcription factors of the microphthalmia/transcription factor E (MiTF/TFE) family are crucial for the regulation of different cellular functions [1]. Among the four members of the mammalian MiTF/TFE family, the transcription factor EB (TFEB) is considered the master regulator of autophagy and lysosomal biogenesis because its nuclear translocation, which is controlled by different kinases and phosphatases, triggers the transcription of numerous genes involved in the regulation of this pathway. However, many aspects related not only to TFEB but also to the other MiTF/TFE transcription factors remain to be elucidated. For instance, the different functions of these proteins in different cellular types and tissues, such as the central nervous system (CNS), are still unclear. Indeed, how and to what extent defects in the regulation of the MiTF/TFE transcription factors contribute to the toxic events associated with neurodegenerative disorders are key questions in the understanding of the molecular mechanisms common to these pathologies [2–6].

Multiple other roles have been associated with the members of the MiTF/TFE family, including the regulation of mitophagy [7], lipid catabolism [8, 9], and mitochondrial

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biogenesis [10]. Although some of their activities may overlap, each homolog of the MiTF/TFE family seems to have a specific pattern of expression and individual functions, which will be addressed in the following sections. Despite the different cellular roles proposed, the most characterized process regulated by MiTF/TFE transcription factors remains to be autophagy.

Autophagy is a crucial process in cellular physiology and is responsible for the degradation of unnecessary and defective cellular components [11]. The autophagic machinery is known to be highly conserved throughout evolution. In fact, the orthologues of many of the genes necessary for this cellular mechanism are ubiquitously expressed in all eukaryotic organisms. Moreover, genetic, morphological, and sequence-based evidence for autophagy confirms the presence of this mechanism in metazoans, in plants, and also in Protista and fungi [12, 13].

Autophagy is a multistep process that determines the engulfment of cytoplasmic components, such as protein aggregates, damaged organelles, and cell debris in double membrane vesicles called autophagosomes (Fig. 1). These vesicles fuse with the lysosomes, membrane bound organelles characterized by an acidic lumen, and lead to the formation of autolysosomes, where the autophagic cargo is degraded [11]. The acidic pH of the lysosomes represents the ideal environment for the hydrolytic enzymes to exert their activity [14]. The products of the autophagic catabolic activity are then recycled back to the cytoplasm to sustain cell homeostasis [11].

Autophagy is ubiquitously performed at the basal level; what differs among cells and tissues is the regulation of the process and the speed of the autophagic flux, which measures the rate of autophagic degradation activity [15].

Autophagic flux is finely regulated by multiple signaling pathways, which are activated by different stimuli, including nutrients, reactive oxygen species and calcium, and by energy imbalance [11, 16]. Autophagy is particularly relevant in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and in lysosomal storage disorders (LSDs) [17]. All these diseases share a common pathological hallmark, which is the accumulation of aggregated proteins or dysfunctional organelles, such as mitochondria, which are not properly cleared because of defective degradative pathways.

As the MiTF/TFE family is conserved in many different organisms, ranging from mice to fruit flies, and from zebrafishes to worms, their study in these model organisms can provide a better perspective for the interpretation of different findings also in the field of neurodegeneration. The possibility of exploiting different model organisms to characterize the function of the MiTF/TFE family, the processes that they can regulate and their role in neurodegeneration, is also crucial for the identification of new targets for the development of novel therapeutic strategies against neurodegeneration. Finally, the activation of MiTF/TFE transcription factors, particularly of TFEB, can promote the clearance of intracellular waste in both LSDs and more common neurodegenerative diseases [18–20]. This may represent a novel therapeutic strategy to burst lysosomal and autophagic pathways in these disorders by targeting MiTF/TFE proteins.

The role of these transcription factors in autophagy and their link to neurodegeneration have been studied in different models. In this frame, the overexpression of the *Caenorhabditis elegans* orthologue of the MiTF/TFE proteins HLH-30 has been directly associated to autophagic induction and increased lifespan [21]. Coherently, a *Drosophila melanogaster* knockdown model for *Mitf*, the only fly orthologue of these

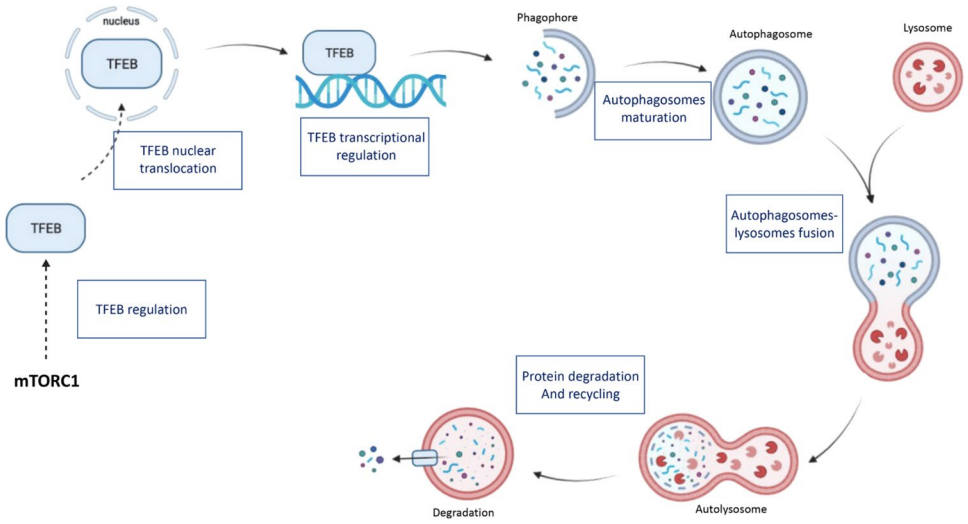

Fig. 1 Schematic representation of the different steps of the autophagic process, starting from the most characterized mechanism of TFEB regulation determined by mTORC1 activity, to the degradation of autophagic substrate

transcription factors, shows autophagic defects and accumulation of autophagic substrate [22]; the activity of Mitf has also been linked to the autophagosomal defects observed in a fly neurodegeneration model of amyotrophic lateral sclerosis (ALS) [23]. Moreover, overexpression of TFEB has been proved to be neuroprotective in a rat model of PD [24]. All these data, and others that will be discussed in detail in the following sections, clearly associate MiTF/TFE transcription factor activity with autophagy and suggest their important function in the field of neurodegeneration. Noteworthy, the knockout of *Tfeb* in mice leads to placental vascularization defects and embryonic death between 9.5 and 10.5 days [25], hampering the possibility to study the effects of *TFEB* knockout on the CNS in a mammalian model.

In this review, we will discuss the function and regulatory mechanisms of the MiTF/TFE family members by comparing their roles in different cell types and tissues, and in different model organisms. This will allow inferring their possible role in different brain cells and regions. We will also describe the role of MiTF/TFE family in neurodegenerative processes and the possibility of targeting these transcription factors to develop novel therapeutics.

By intersecting different aspects of this topic, ranging from function relevant to brain physiology to contribution to neurodegeneration, and by introducing an evolutionary perspective, we envision shedding light on different aspects of this scientific problem. This will also lead to discussing key issues and open questions with the aim of speculating on alternative research lines and on new experimental approaches within this research topic.

The MiTF/TFE Transcription Factor Family: an Overview

The microphthalmia/transcription factor E (MiTF/TFE) family is constituted in mammals by four members: (i) microphthalmia-associated transcription factor (MITF), (ii) transcription factor EB (TFEB), (iii) transcription factor E3 (TFE3), and (iv) transcription factor EC (TFEC) [4–6]. They share some common structural features: they all contain a basic domain, which is required for DNA binding, and a helix-loop-helix (HLH), and a leucine zipper (LZ) domain, which are critical for dimerization (Fig. 2). TFEB, TFE3, and MITF also contain a conserved transactivation domain, which is crucial for their transcriptional activation whereas TFEC, the most divergent member of the family, lacks this domain and appears to inhibit rather than activate transcription [5, 26, 27] (Fig. 2). The MiTF/TFE transcription factors bind the palindromic CACGTG E-box sequence, which conforms to the CANNTG motif that is recognized by other members of bHLH-zip family transcription factors. Flanking E-box sequences also influence the DNA binding specificity of the HLH/LZ family. The MiTF/TFE transcription factors have been described to prefer the GTCACGTGAC consensus region, named Coordinated Lysosomal Expression and Regulation (CLEAR) motif. Unlike other bHLH-zip transcription factors, the MiTF/TFE family members are also able to bind the asymmetric TCATGTG M-box sequence [28–30]. Sequencing of chromatin immunoprecipitate and mRNA analysis of HeLa cells overexpressing TFEB revealed that, through the binding with the CLEAR motif, TFEB enhances the expression of genes involved in lysosomal biogenesis, in lysosomal membrane formation, in acidification of lysosomes, in lysosomal hydrolases expression,

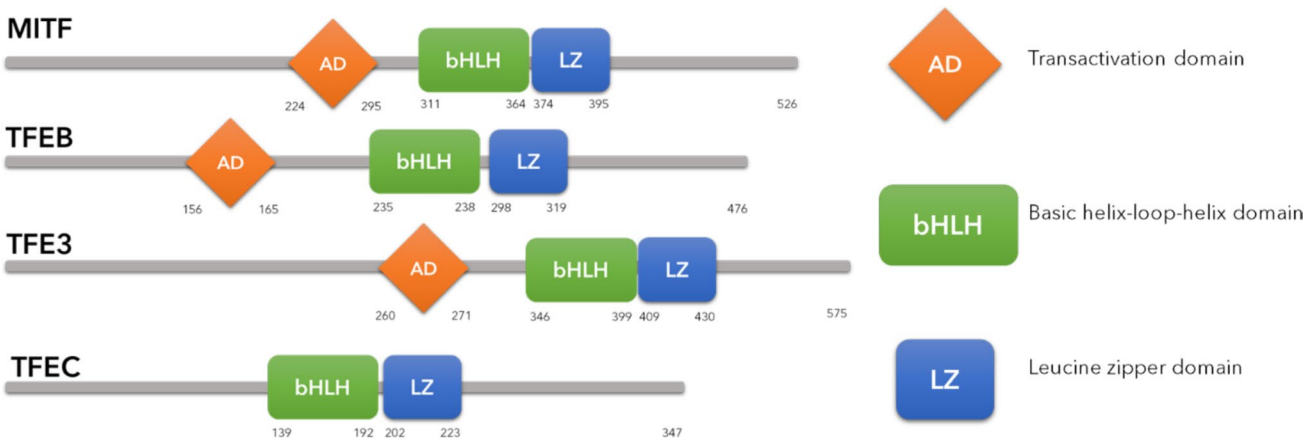

Fig. 2 Protein structure of MiT/TFE family members. MiT/TFE family members have high similarities in their sequences: they share basic-helix-loop-helix domain (bHLH) and a leucine zipper (LZ) domain. The activation domain is conserved in TFEB, MITF, and TFE3, but is missing in TFEC

and in the autophagic process [29, 31]. Interestingly, TFEB transcriptional activity is not only limited to the modulation of genes involved in lysosomal homeostasis, but also in the Golgi vesicle transport, protein transport, and mitochondrial homeostasis [31]. These data confirm the close link between TFEB and the other MiTF/TFE proteins and autophagy and underline the crucial role of TFEB in the overall cellular homeostasis.

The MiTF/TFE transcription factors have been shown to form, in vitro, both homodimers and heterodimers with any other family member, and the dimeric form is required for the binding to DNA and the transcriptional activation of target genes. However, they are unable to heterodimerize with other bHLH-zip transcription factors [4, 31]. The X-ray structure of MiTF was obtained using three fragments from the *Mus musculus Mitf* cDNA that were cloned in the pET-M11 vector and expressed in the *Escherichia coli* strain BL21 (DE3) RIL. This structural analysis revealed the presence of a three-residue shift within its ZIP domain, which generates an unusual leucine zipper kink, and is responsible for the specific dimerization of the MiTF/TFE members [31]. Multiple sequence alignment showed that this three-residue shift is conserved among all the MiTF/TFE members, while it is missing in the sequences of the canonical bHLH-zip transcription factors. However, the functional implication of the heterodimer formation has not been fully investigated, except for the MITF-TFE3 heterodimer, which does not appear to be essential for proper functioning, as suggested by MITF and TFE3 redundant roles at least in the development of osteoclasts [32].

The Regulation of MiTF/TFE Transcription Factors

The regulation of MiTF/TFE transcription factors can occur at different levels. Even though the activity of these proteins is mainly modulated through post-transcriptional modifications and highly depends on their subcellular localization, their regulation can also be performed at the transcriptional level. In this regard, most of the literature available is about TFEB. However, the high degree of homology among the MiTF/TFE members suggests that they may share common regulatory mechanisms. Several transcription factors are known to modulate the expression of *TFEB*: among them, androgen receptors, peroxisome-proliferator activated receptors-α (PPARα) [33], cAMP response element-binding protein (CREB) [34], and Krüppel-like factor 2(KLF2) [35], have been shown to enhance TFEB activity. Moreover, TFEB can also modulate its own regulation through a positive feedback loop [36]. The fact that TFEB is transcriptionally regulated by different transcription factors further suggests the importance of fine-tuning the expression of this protein and underlines that its level and activity are influenced by multiple stimuli. Another level of regulation is represented by alternative splicing. Tissue-specific expression of *MITF*, *TFEB*, and *TFEC* seems to be mediated, at least in part, by alternative transcription start sites, that also allows modulating the activity of the three proteins in different cells. *TFE3* is the only member of the MiTF/TFE family that does not present alternative first exons and is regulated by a single promoter [37].

At the post-transcriptional level, the necessary nuclear shuttling from the steady-state cytosolic localization, that activates TFEB-mediated transcriptional response, correlates with its phosphorylation status and it is positive regulated by de-phosphorylation in key serine residues [2]. The most known member of the family, TFEB, is characterized by several phosphorylation sites within its amino acid sequence. These sites are highly conserved throughout evolution and most of them are found in TFEB orthologues from invertebrates, as *C. elegans*, to humans. Moreover, the other mammalian MiTF/TFE transcription factors present the same phosphorylation sites: the most conserved are the residues crucial for the regulation, such as serine 141, serine 211, and serine 467 [38]. These data suggest that all the MiTF/TFE members may be regulated similarly to TFEB and that these mechanisms of modulation may be relevant for the activity of MiTF/TFE orthologues in different organisms. TFEB is the substrate of different kinase proteins, including extracellular signal-regulated kinase (ERK)2, protein kinase C (PKC)ß, and AKT, also known as protein kinase B (PKB). However, the most important protein involved in the regulation of TFEB is the mechanistic target of rapamycin (mTORC1), which phosphorylates the transcription factor at three serine residues, serine 122, serine 142, and serine 211 [39]. The mTORC1-mediated phosphorylation promotes the interaction between TFEB and 14–3-3 proteins, which sequester TFEB in an inactive state in the cytoplasm. It is important to note that through the binding to Rag (Ras-related GTP-binding) GTPases, both mTORC1 and TFEB are recruited at the lysosomal level, where the interaction occurs [40]. This mechanism is particularly important to respond to environmental cues and occurs in human, mouse, and *Drosophila melanogaster*-derived cells, providing evidence of a mechanism conserved both in mammals and in invertebrates [41, 42]. During normal nutrient conditions, a signaling pathway promoted by the availability of amino acids induces the binding between Rag GTPases and mTORC1 and its consequent activation at the lysosomal surface, where it phosphorylates TFEB [41]. In nutrient deprivation conditions, the calcium-sensitive phosphatase calcineurin dephosphorylates TFEB, thus promoting its nuclear translocation [2]. MITF and TFE3 have also been shown to be similarly regulated by

mTORC1, while much less is known about TFEC. Given the high degree of homology with the other members of the family, it is likely that also TFEC is regulated similarly [1].

mTORC1 being the hub of several important cellular processes [43], it is crucial to intensively study its activity and all its regulatory mechanisms to thoroughly understand how TFEB and the other MiTF/TFE proteins are modulated. Nutrient conditions are not the only parameter that affects these mTORC1 and, consequently, MiTF/TFE transcription factor activity. In fact, mTORC1 function may also depend on the activity of AMP-activated protein kinase (AMPK). AMPK is considered the sensor of cellular energetic status and is regulated by different upstream stimuli, such as oscillations in the intracellular calcium levels, alterations in oxygen reactive species, and changes in AMP/ATP ratio. AMPK activation inhibits mTORC1 promoting MiTF/TFE nuclear translocation [44]. Moreover, it has been recently reported that AMPK can directly activate TFEB and TFE3 by phosphorylating a cluster of serine residues in the C-terminus of these proteins [45]. Interestingly, it has been also shown that in mice AMPK activation does not enhance autophagy in neurons, further suggesting that these pathways need to be characterized specifically in different cell types and tissues [46].

These data reinforce the concept of MiTF/TFE transcription factors as crucial players in the maintenance of cellular energy balance and strengthen the hypothesis that their activity is particularly essential in highly energy-demanding tissues, like the brain. Noteworthy, the AMPK phosphorylation sites in TFEB are also present in MITF and TFEC and at least one of these residues is found in the MiTF/TFE orthologues of several organisms, both vertebrates and invertebrates, suggesting that this regulation mechanism may be highly conserved throughout evolution [38]. This data may open the possibility to characterize AMPK-mediated regulation of MiTF/TFE proteins not only in cellular models but also in vivo in different model organisms.

The final regulation mechanism is achieved through the degradation of the proteins. The available data for TFEB show that the transcription factor is degraded through the ubiquitin–proteasome pathway and that the half-life of the protein is about 13.5 h in neuronal-like cells, such as SH-SY5Y [47]. The relevance of the degradation process in the modulation of TFEB activity is confirmed by the fact that proteasome inhibition not only causes the accumulation of the protein, but also promotes its nuclear translocation and, in turn, the increased expression of TFEB target genes [47]. Interestingly, it has been shown that mTORC1 activation enhances the rate of TFEB proteasomal degradation, providing feedback mechanisms through which the mTORC1-mediated phosphorylation of TFEB inhibits its nuclear translocation and promotes also its degradation [48, 49].

Mammalian MiTF/TFE Transcription Factors

MITF, TFEB, TFE3, and TFEC are the four members of the mammalian MiTF/TFE family. Human and mouse MiTF/TFE proteins share a very high sequence identity (Table 1) and the information currently available on these factors mainly derives from data obtained in mouse models. In the following paragraphs, we will detail the pattern of expression and the main functions of the MiTF/TFE transcription factors. Special care, when possible, will be used in comparing the functions, the localization, and the regulation of the different MiTF/TFE family members.

MITF

In humans, the MITF locus is mapped in the short arm of chromosome 3 and spans 229 kbp, with the promoter region that is highly conserved with mice [26]. *MITF* transcription gives rise to several isoforms that are under the control of alternative promoters: *MITF-A* [50], *MITF-B* [51], *MITF-C* [52], *MITF-D* [53], *MITF-E* [54], *MITF-H* [55], *MITF-J* [56], *MITF-Mc* [57], and *MITF-M* [58] (Table 2). These isoforms share the same functional domains (transactivation domain, basic domain, bHLH domain, and LZ domain) but differ in the N-termini, as a result of alternative splicing of exon 1, and display a tissue-specific pattern of expression [37]. MITF is predominantly expressed in melanocytes, osteoclasts, mast cells, macrophages, NK cells, and B cells, and in the heart [59]. Moreover, it is expressed in the CNS. Immunohistochemical analysis of the mouse brain showed that MITF is especially expressed in the olfactory bulb (OB), and in tufted and mitral cells that receive signals from the olfactory neurons and transmit them to the olfactory cortex. MITF protein was not detected in other cell types of the OB, including granule cells or astrocytes [60], in contrast with a previous study based on an RT-PCR analysis showing the expression of *MITF* also in T98G and A-172 human glioblastoma cells [52]. One possible explanation for this discrepancy is that *MITF* expression is much higher in mitral and tufted cells of the OB, allowing the detection of the protein by immunohistochemical analysis only in these

Table 1 Identity and similarity values obtained by PROTEIN BLAST search using the amino acid sequence of human and mouse MiTF/TFE family members

MiTF/TFE transcription factor	Identity (%)	Similarity (%)
MITF	93%	95%
TFEB	93%	95%
TFE3	96%	97%
TFEC	70%	76%

Table 2 Pattern of expression of MiTF/TFE alternative transcripts

Protein name	Protein symbol	Transcripts	Expression	Referee
Microphthalmia-associated transcription factor	MITF	MITF-A	Ubiquitous	[50]
		MITF-B	N/A	
		MITF-C	Different cell types excluding melanocytes	[63]
		MITF-D	Preferentially in RPE cells, macrophages, osteoclasts, and mast cells	[53]
		MITF-E	Mast cells and osteoclasts	[54, 64]
		MITF-H	Ubiquitous	[50]
		MITF-J	Osteoclasts, RPE, and HeLa cells	[56]
		MITF-Mc	Mast cells	[57]
		MITF-M	Melanocytes, melanoma cells, and RPE cells	[65]
Transcription factor EB	TFEB	TFEB-A	Placenta, kidney, lung, and prostate Different tissues	
		TFEB-B	Different tissues excluding liver	
		TFEB-C	N/A	[37]
		TFEB-D TFEB-E	Brain	
		TFEB-F	Brain	
		TFEB-G	Spleen	
Transcription factor E3	TFE3	None	Ubiquitous with the highest expression levels in placenta, lung, and adrenal gland	[37]
Transcription factor EC	TFEC	TFEC-A	Testis, thymus, trachea, colon, and prostate	
		TFEC-B	Different tissues excluding heart and liver	[37]
		TFEC-C	Kidney and small intestine	

cell types [60]. Little is known about the function of MITF in the olfactory bulb. A recent study reported that mitral and tufted neurons from mutant mouse knockout for *Mitf* are characterized by a reduced A-type potassium current (IA) likely because of the decreased expression of the potassium channel subunit KCND3, leading thus to hyperexcitability. Moreover, *Mitf* mutant mice exhibit increased olfactory dishabituation, but reduced the ability to detect the odorant following long-term odor exposure. These findings suggest that MITF plays a key role in olfactory adaptation and intrinsic homeostatic plasticity through the regulation of *Kcnd3* expression. Accordingly, MITF signaling has been demonstrated to upregulate *Kcnd3* expression via an enhancer region located in an intron of *Kcnd3* [6]. It remains to be elucidated the possible importance of MITF in the regulation of KCND3, and therefore of potassium currents in other neuronal types. Worth mentioning is the fact that *Kcnd3* mutations were associated with the neurodegenerative disorder spinocerebellar ataxia type 19 [61, 62], suggesting that MITF impact on *Kcnd3* may also regulate neuronal function in the central and peripheral nervous system.

As already reviewed by Haq and Fisher in 2011 [66], MITF is required for many other cellular processes. It mediates the survival of melanoblasts and regulates the expression of genes encoding proteins implicated in the cell cycle in cell invasion by affecting actin cytoskeleton and in DNA damage repair and cell metabolism. In this frame, MITF modulates not only catabolic pathways, like autophagy, but also mitochondrial biogenesis and oxidative phosphorylation [67, 68]. MITF regulation of intracellular metabolism and of actin cytoskeleton is likely crucial not only for neurons, but also for other types of brain cells. In fact, specific types of neurons are known to have increased metabolic demands that make them specifically susceptible to neurodegeneration, suggesting that the MITF role may be particularly relevant in those cases. Similarly, MITF actin cytoskeleton remodeling is critical for neuronal shape and for the regulation of dendritic spine morphology [69]. Defects in neuronal metabolism and in the structure of dendritic spines were associated to different neurodegenerative diseases [70].

Through the binding of the CLEAR motif, MITF is also able to promote the expression of lysosomal and autophagy-related genes. Interestingly, in metastatic melanoma tumors, the lysosomal and autophagy genes under the control of MITF are different compared to the ones regulated by TFEB and TFE3, suggesting a distinct role of MITF [71]. These data support the idea that each member of the MiTF/TFE family may regulate the expression of its target genes in a cell/tissue-specific manner, making it crucial to investigate each of them separately not only in different neuronal types, but also in astrocytes and microglia, when studying their role in neurodegeneration.

In light of the numerous functions attributed to MITF, it is not surprising that mutations in the gene encoding the protein are associated with several pathological conditions. For instance, in the mouse models, MITF mutations induce

defects in neural crest-derived melanocyte and retinal pigment epithelium differentiation, osteoclastogenesis, mast cell differentiation, and notch signaling that manifest phenotypically as changes in coat color, small eyes, osteopetrosis, and a reduction in NK cell, B cell, and macrophage numbers [66]. In humans, different heterozygous and homozygous *MITF* mutations are associated with Waardenburg syndrome type 2A and type 4, respectively [72, 73]. Waardenburg syndrome is a neurogenic disorder characterized by the combinations of various degrees of sensorineural deafness and pigmentation abnormalities affecting the skin, hair, and eye [74]. Dominant-negative mutations in MITF are also associated with Tietz syndrome, which is characterized by profound deafness and generalized hypopigmentation [73]. Furthermore, biallelic *MITF* mutant alleles are associated with COMMAD syndrome characterized by coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism, and deafness [75].

TFEB

Among all members of the MiTF/TFE family, TFEB is the most studied and best characterized since it plays a pivotal role in the regulation of autophagy and lysosomal biogenesis. In contrast with the MITF pattern of expression, TFEB is ubiquitously expressed. Seven alternative 5' exons of the *TFEB* gene have been identified that originate from seven different transcripts, which encode different TFEB isoforms: *TFEB-A*, *TFEB-B*, *TFEB-C*, *TFEB-D*, *TFEB-E*, *TFEB-F*, and *TFEB-G*. Each transcript displays a different tissue distribution pattern, even though the existence of *TFEB-D* transcript still needs to be confirmed since its expression has not been detected in any of the tissues analyzed by RT-PCR. This could be due to very low expression levels.

TFEB is considered the master regulator of lysosomal biogenesis and autophagy signaling pathways because it induces the transcription of both autophagic/lysosomal-related genes through the binding with the CLEAR motif. This binding determines increased expression levels of the entire network of genes that contain this specific motif (the CLEAR network) [28]. The fact that autophagy is an essential cellular process and that TFEB promotes the expression of the CLEAR network can explain the ubiquitous expression of the protein. Moreover, as autophagic activity can vary between different tissues or cell types, the existence of alternative TFEB transcripts with different expression patterns may account for a very specific regulation of this evolutionarily conserved process. As already mentioned, it is well known that the autophagy-lysosomal pathway (ALP) is key not only for neuronal cells but also for other cell types in the brain, as astrocytes and microglia. In this regard, the contribution of non-neuronal cells to the maintaining of brain homeostasis and their role in neurodegeneration is gaining increasing attention. For example, astrocytes, that are the most abundant glial cells, are involved in the clearance of aggregated proteins and cell debris through the endo-lysosomal pathway. Therefore, the characterization of TFEB activity in non-neuronal brain cells is also very relevant. Increasing the astrocytic or microglial clearance capacity through TFEB upregulation may represent a promising therapeutic strategy for neurodegenerative diseases.

It is already known that TFEB plays an essential role in the tuning of several other basic cellular processes through the regulation of autophagy or lysosomal function in different tissues and cell types. For instance, TFEB-mediated lysosomal biogenesis in differentiated osteoclasts plays a crucial role in bone matrix resorption. Accordingly, mouse osteoclasts lacking TFEB show decreased expression of lysosomal genes, reduced number of lysosomes, and enhanced bone mass [76].

TFEB is also involved in the regulation of lipid metabolism, through a starvation-induced transactivation of *PPARα* and PPAR*α* co-activator 1α (*PGC1α*), which are two key regulators of lipid metabolism during TFEB-mediated starvation. This process has been deeply analyzed in a liver-specific *Tfeb* conditional knockout mouse that displays impaired lipid catabolism and a more severe metabolic imbalance in obese animals. Coherently, TFEB overexpression rescues obesity and associated metabolic syndrome in both diet- and genetically induced obese mice [77].

Through specific gain and loss-of-function approaches in mouse skeletal muscles, the role of TFEB in metabolic adaptation during physical activity has also been emphasized. As a result of exercise, TFEB is dephosphorylated via calcineurin and translocates into the nuclei of myofibers and directly controls glucose homeostasis by regulating the expression of glucose transporters and glycolytic enzymes. Moreover, TFEB modulates the expression of genes implicated in mitochondrial biogenesis, such as mitochondrial transcription factor A (TFAM) and nuclear respiratory factors 1 and 2 (NRF1 and NRF2). It is however unclear whether TFEB activation in response to the exercise depends or not on PGC1α [78, 79].

The overall metabolism of cells seems to be controlled by TFEB function. In neurons, glucose and lipid homeostasis, as well as mitochondrial ATP production, are key in the maintenance of neurons' capacity to meet the energy demands during neuronal activity. This suggests that if TFEB is defective, also these processes, together with the impairment of lysosomal function, may concur in damaging neuronal function.

The modulation of inflammatory and immune responses is another important function ascribed to TFEB. More specifically, depletion of *Tfeb* in murine macrophages results in a decreased expression and secretion of several pro-inflammatory cytokines, such as tumor necrosis factor (TNF),

interleukin-1β (Il-1β) and Il-6, and chemokines including (C–C motif)-ligand 2 (CCL2) and CCL5, after lipopolysaccharides (LPS) treatment [80]. Neuroinflammation is a well-established event that occurs in many neurodegenerative diseases, even if it is still unclear to which extent it contributes to the etiology of the disease and its progression. The findings on TFEB modulation of inflammation would suggest further investigating how TFEB impacts inflammatory mechanisms in brain-resident immune cells.

Overall, TFEB seems to transcriptionally regulate several pathways that are intertwined and that play a role in the maintenance of brain function, making it crucial not only to better understand each of them separately, but also to investigate how they cooperate.

TFE3

Like TFEB, TFE3 shows a ubiquitous pattern of expression with the highest levels observed in the placenta, lung, and adrenal gland. *TFE3* seems to be regulated by a single promoter since only one transcript for the *TFE3* gene has been identified [58]. Similar to TFEB, TFE3 binds CLEAR elements regulating the expression of genes related to lysosomal biogenesis and autophagy. However, the ability of TFE3 to control the transcription of lysosomal genes is TFEB-independent, suggesting that the relative abundance of TFEB or TFE3 and/or different regulatory mechanisms determine which of them prevails in activating the lysosomal response [81].

Coherently, many functions ascribed to TFE3 overlap with those associated with TFEB. For instance, like TFEB, also TFE3 is involved in the transcriptional regulation of the immune response. Both proteins orchestrate the cellular response to endoplasmic reticulum (ER) stress by upregulating the expression of the activating transcriptional factor 4 (ATF4) or other unfolded protein response (UPR) genes. While under prolonged ER-stress conditions, TFEB and TFE3 activation contributes to cell death by either a direct binding to pro-apoptotic factors promoters, such as C/EBP homologous protein (CHOP) and p53 upregulated modulator of apoptosis (PUMA), or, indirectly, through the regulation of ATF4, which is also involved in the control of CHOP and PUMA expression. These findings suggest that TFEB, TFE3, and ATF4 may play a dual role in cell fate depending on the severity of the stress [82]. Interestingly, TFE3 is also activated in response to Golgi stress and upregulates the transcription of Golgi-related genes [83].

Noteworthy, both ER and Golgi stress or dysfunction are frequently associated with neurodegeneration [84, 85]. Moreover, accumulating evidence links dyshomeostasis of these organelles with alterations in autophagy activity [86, 87], suggesting that it would be interesting to evaluate whether modulating TFE3 activity may affect the onset and progression of neurodegenerative disease, impacting not only ER and Golgi function but also autophagy.

TFEC

TFEC is the most divergent and least studied member of the MiTF/TFE family. Three alternative 5' exons of the *TFEC* gene have been identified: TFEC-A, TFEC-B, and TFEC-C, with the latter encoding for a shorter protein lacking exons 2 and 3. Mouse and rat *Tfec* lacks exon 5 that is found only in the human homolog. Human TFEC transcripts have a restricted and distinct pattern of expression [37]. In mice, TFEC expression is restricted to macrophages [88, 89] and mice lacking *Tfec* develop normally. They are viable and fertile, and normally pigmented, have normal eyes and mast cells, and show no osteopetrosis, thus indicating a redundant role of TFEC in myeloid cell development. *Tfec* expression at both the mRNA and protein levels is specifically induced in mouse macrophages by the Th2 cytokine IL-4. In macrophages lacking *TFEC* treated with IL-4, only few genes are affected by TFEC deficiency including G-CSFR (*Csf3r*) gene, which is upregulated to a lesser extent compared to IL-4 treated wild-type macrophages [90]. TFEC remains functionally uncharacterized and no information are available on its role in the nervous systems; thus, further studies to unravel its role in different human tissues/cells are needed.

TFEB and Autophagy in Neurodegeneration

The demand for basal autophagy differs among cells, and it appears to be particularly crucial in post-mitotic cells, like neurons, whose survival depends upon a strict regulation of cell homeostasis [91, 92]. Moreover, the role of glial cells is also crucial in the removal of extracellular waste and damaged neurons, which makes ALP important also in these cell types in the frame of neurodegenerative processes [93]. This suggests that autophagy and in particular TFEB and the other members of the MiTF/TFE family of transcription factor may be key not only in the development but also in the treatment of neurodegeneration. Further pieces of evidence supporting this idea are discussed in the following sections.

Autophagy and TFEB Impairment in Age-Related Neurodegenerative Diseases

It is established that autophagy impairments often occur in age-related or inherited neurodegenerative disorders and accumulating evidence suggests a primary involvement of this process in the pathogenesis of many of them, including PD, HD, and AD [94–96]. Several aggregation-prone proteins, such as huntingtin (HTT), α-synuclein (α-syn), amyloid beta (Aβ), and hyperphosphorylated-tau, are

eliminated through autophagy [46, 97–99]. Importantly, these proteins can also negatively impact the autophagic pathway, further contributing to their toxicity [100–103].

The link between autophagy and neurodegeneration is further supported by the fact that several genes whose mutations are associated with the familial forms of different neurodegenerative diseases have a role in the autophagic pathway and removal of key autophagy genes in the mouse brain leading to neurodegeneration [104, 105]. Interestingly, the dysregulation of autophagy in neurodegenerative diseases may also present as an increase in autophagy or TFEB activation, as discussed in Section ".TFEB and Autophagy in Neurodegeneration" This suggests that special care must be taken when designing possible therapeutic approaches impacting these mechanisms, in order to preserve autophagy homeostasis, rather than pushing the autophagic machinery without considering possible undesired effects.

Overall, these pieces of evidence suggest that basal clearance of cytosolic waste through autophagy is crucial for preventing the accumulation of cytoplasmic inclusion in neurons, and in astrocytes and microglia, which are involved in the clearance of brain waste via phagocytosis [93]. Therefore, the upregulation of autophagy may have beneficial effects and many research efforts in the field are aimed at finding molecular modulators of this process, possibly in a cell-type specific manner [6, 7].

TFEB, which is by far the most investigated protein in the MiTF/TFE family, shows impaired activity and regulation in many age-related neurodegenerative diseases, further supporting the importance of its role in the maintenance of cellular homeostasis (Fig. 3) [106].

For instance, analysis of postmortem PD midbrains revealed a selective loss of nuclear TFEB. In this frame, TFEB colocalizes with α-syn in Lewy bodies contained in surviving nigral neurons in PD human brains [107]. α-Syn shares structural homology with several regions of 14–3-3 proteins, and 14–3-3 proteins are well-established binding partners of the phosphorylated form of TFEB and prevent its translocation to the nucleus. This homology could lead to the binding between α-syn and TFEB that, consequently, is maintained inactive in the cytoplasm. Indeed, impaired α-syn degradation due to defective autophagy could initiate a vicious cycle leading to non-physiological α-syn-TFEB interaction, which may further amplify ALP dysfunction [107, 108].

The expression of *TFEB* and its target genes was found to be reduced also in the striatum of a mouse model of HD [16]. A reduction of TFEB expression levels and its nuclear localization was also observed in postmortem AD brains as well as in monocytes and lymphocytes from patients with AD [16, 109]. Mouse embryonic fibroblasts from double KO of the AD-associated proteins presenilin 1 and 2 and human AD neurons display higher levels of TFEB phosphorylation compared to the controls, which correlates with TFEB cytoplasmic retention and a decreased CLEAR gene network activity [110].

TFEB nuclear translocation appeared to be negatively affected in a dose-dependent manner in primary microglial cells treated with different concentrations of Aβ [111]. All

Autophagic process	TFEB regulation	TFEB nuclear translocation	TFEB transcriptional regulation	Autophagosomes maturation	Autophagosomes-lysosomes fusion	Protein degradation and recycling
Defects in neurodegenerative diseases	TFEB regulation and protein level ↓ LSD ↓ HD ↓ AD	TFEB activation and nuclear translocation ↓ PD ↓/↑ AD ↑ LSD	TFEB transcriptional activity ↓ PD ↓ HD ↓ /↑ AD ↑ LSD	Cargo accumulation and autophagosome maturation ↓ LSD ↓ PD	Lysosomal activity and fusion with autophagosome ↓ LSD ↓ PD	Autophagic vesicles and autophagic substrates accumulation ↑ PD ↑ AD ↑ HD ↑ LSD
Possible TFEB-targeted therapeutic strategies	TFEB overexpression AD PD LSD HD	TFEB activation AD PD HD LSD				

Fig. 3 Schematic recapitulation of the defects that characterize different neurodegenerative diseases in every step of the autophagic process (as described in Fig. 1). In the third line of the table are highlighted the possible therapeutic strategies to counteract the progression of these pathologies, like TFEB overexpression and TFEB activation. In blue and red are reported the diseases in which the modulation of TFEB activity has been demonstrated to be beneficial or detrimental, respectively. In magenta the pathology in which the modification of TFEB function may have divergent effects

these data support the presence of an inverse correlation between TFEB nuclear levels and the pathological state, but the picture is more complex and presents also some contrasting results. For example, increased TFEB expression levels were detected in patient-derived fibroblasts carrying the AD-associated presenilin 1 A246E mutation [112]. Another study reported an upregulation of genes within the CLEAR network in brains of presenilin 1, 2 double knockout mice with no changes in TFEB expression level [113]. Analysis of hippocampal CA1 neurons of AD patients microaspirated by laser capture microdissection revealed increased expression levels and nuclear translocation of TFE3, together with an elevated expression of its target genes, while no changes in TFEB levels were detected in the same neurons. Increased TFEB expression levels and nuclear translocation were observed in glia from AD hippocampal tissues, suggesting that TFEB, in this cellular type, may play a crucial role in scavenging aggregated proteins and neuronal debris [114]. Furthermore, these data suggest that TFEB could be upregulated as a compensatory mechanism in certain conditions: if this has a positive effect on the degradation of intracellular waste or contributes to further clump the system is still controversial.

A decline in TFEB levels with age in human immune cells has been recently reported [115], suggesting that it could negatively affect the ALP. This mechanism could contribute to the accumulation of toxic aggregation prone proteins at the early stages of the development of neurodegenerative diseases.

The opposite findings of TFEB being up- or downregulated during neurodegeneration suggest that the extent and length of the neurodegenerative process may impact differently TFEB expression, with an increase in the early phases and a reduction in the late stages of the disease. The same could be hypothesized for TFEB nuclear or cytoplasmic localization. A further degree of complexity is represented by cell type and tissue specificity, which are still understudied, and by the role of the different MiTF/TFE family members in neurodegeneration. The investigation of these aspects in both familial and sporadic forms of these diseases in suitable models may provide interesting clues about the role of these transcription factors in their etiology and further help in the identification of novel therapeutic targets for these disorders.

Autophagy and TFEB Impairment in Lysosomal Storage Disorders

Another group of diseases in which TFEB and the other transcription factors of the MiTF/TFE family seem to play an important role are lysosomal storage disorders (LSDs). They comprise more than sixty diseases caused by mutations in genes involved in lysosomal function, such as lysosomal hydrolases or lysosomal membrane proteins. About two-thirds of LSDs determine neurological symptoms and are counted among the neurodegenerative disorders [116, 117].

Although these pathologies are characterized by lysosomal defects, in the majority of the cases, they can affect various stages of the autophagic process causing impairments in autophagosome maturation and in autophagosome-lysosome fusion and, eventually, accumulation of undigested material in cells [117]. Because of its role in the regulation of ALP, TFEB has been extensively investigated in the context of LSDs, as a factor that can contribute to the progression of the pathology and as a possible therapeutic target.

As expected, both the activity of TFEB and its nuclear localization together with the autophagic process are affected in several lysosomal disorders (Fig. 3).

Gaucher disease (GD) is the most common LSD and in the most severe cases it determines neurological defects. The pathology is caused by mutation in the *GBA1* gene, which encodes the lysosomal enzyme glucocerebrosidase (GCase), and is characterized by a decrease in the degradation of autophagosome content after its fusion with lysosomes [118]. Lysosomal GCase is responsible for the hydrolysis of the lipid glucosylceramide into glucose and ceramide. When GCase is mutated, accumulation of the substrate and ALP impairment occur in many cell types [118].

Decreased levels of TFEB have been observed in induced pluripotent stem cell (iPSC)-derived neurons from GD patients, probably due to an increased proteasomal degradation of the transcription factor. In the same iPSC-derived model, the instability of TFEB was linked to the hyperactivation of mTORC1 [119]. mTORC1-mediated phosphorylation of TFEB has been shown not only to inhibit its nuclear translocation, but also to promote the targeting of the protein to the proteasomal degradation machinery [48]. Surprisingly, even though mTORC1 activity is increased in GD-derived cells, TFEB was shown to be predominantly localized in the nuclei compared to control cells [118, 119]. These data suggest that another mechanism of TFEB regulation, besides the mTORC1-mediated one, may act in GD cells to stimulate the nuclear translocation and, in turn, the activity of the transcription factor to compensate for the lysosomal defects.

In another study, Sardiello and colleagues investigated the subcellular localization of TFEB in embryonic fibroblast from mouse models of three LSDs: mucopolysaccharidoses types II and III (MPSII, MPSIII) and multiple sulfatase deficiency (MSD). The first two diseases belong to a group of metabolic disorders caused by impairment of lysosomal enzymes required for the degradation of glycosaminoglycans, while the latter is caused by the deficiency in the formylglycine-generating enzyme [120]. As in the case of GD, TFEB was predominantly observed in the nuclei, further supporting the idea that the activation of TFEB is

promoted in this type of diseases as a cellular response to enhance lysosomal activity [28].

Despite the nuclear translocation of TFEB observed in several LSDs, this compensatory mechanism does not seem to be enough to counteract the progression of the disease and TFEB activity is not sufficient to properly remove the intracellularly accumulated debris. As observed in GD, it is possible that the stability and accumulation of TFEB are affected by increased proteasomal degradation, resulting in a decreased total amount of the protein. This hypothesis is supported by an experiment performed on myotubes in a mouse model of Pompe disease, another LSD caused by mutation in the *GAA* gene. This disease is characterized by deficiency of the lysosomal enzyme acid α-glucosidase and leads to the accumulation of lysosomal glycogen [121, 122]. While cells treated with the mTOR inhibitor Torin1, which induces a downstream activation of TFEB, failed to rescue the lysosomal phenotype in this model, the overexpression of TFEB in the same model was able to induce cellular clearance, suggesting that in these pathological conditions the amount of endogenous TFEB is not enough to support lysosomal function [121]. Similarly, the overexpression of TFEB in both cellular and mouse models of MSD and MPSIII-A diseases promotes clearance and ameliorates phenotypic hallmarks of these diseases [18].

As previously discussed, also the other members of the MiTF/TFE family are involved in the regulation of the autophagy and lysosomal activity. However, as in the case of the neurodegenerative disorders previously discussed, little is known about the involvement of TFEC and MITF in the onset and progression of LSDs. Recently, it has been demonstrated that the overexpression of TFE3 can induce lysosomal exocytosis and cellular clearance in a model of Pompe disease, suggesting that also this homologue of TFEB can play an important function in the regulation of cell fate in these disorders [123].

TFEB as a Possible Therapeutic Target in Neurodegeneration

Although the contribution of TFEB to the pathogenesis of neurodegenerative disorders is still under debate, several studies have evaluated the effects induced by the exogenous TFEB overexpression (Fig. 3). In a mouse model of tauopathy, the adenovirus-mediated overexpression of TFEB drastically reduces the levels of the disease marker phospho-Tau 16 weeks post-injection. In this model, TFEB has been shown to participate in the selective elimination of misfolded and hyperphosphorylated tau by promoting the expression of the phosphatase and tensin homolog (PTEN) protein, attenuating neurofibrillary tangles pathology. Moreover, injected mice displayed increased neuronal survival and brain weight, associated with a rescue of behavioral and synaptic deficits [19].

Another study reported decreased levels of tau aggregates in the hippocampus and cortex upon neuron-targeted TFEB overexpression [124], together with attenuated learning and memory skill deficits, in a different mouse model of tauopathy [124].

Extracellular tau is considered to be responsible for the spreading of tau pathology and represents the primary target for tau immunotherapy [125]. Interestingly, TFEB loss of function in PS19 mice, a transgenic mouse line expressing P301S mutant tau, causes a reduction of intestinal fluid tau. The authors proposed a model in which TFEB plays an active role in the secretion of mutant tau via lysosomal exocytosis mediated by TFEB and Transient Receptor Potential Mucolipin 1 (TRPML1) signaling [125]. Accordingly, astrocyte-specific TFEB overexpression in the hippocampus of PS19 mice was able to reduce tau spreading from the ipsilateral to the contralateral hippocampus [126]. A recent study reported that TFEB overexpression in another AD mouse model is responsible for a reduction of the levels of the β-secretase-derived β-amyloid precursor protein fragment C99, which is a precursor of the toxic Aβ peptide. Coherently, the overexpression of TFEB in hippocampal astrocytes contributed to the reduction of Aβ levels in the brain interstitial fluid and of the hippocampal amyloid plaque load [127, 128]. A recent study reported that intracerebral injection of TFEB in the *substantia nigra pars compacta* of a PD rat model overexpressing the human disease-associated A53T α-syn mutant reduced the accumulation and the aggregation of α-syn as well as astrogliosis and prevented the behavioral deficits typical of this PD model [24]. Furthermore, TFEB injection in the striatum of $HD^{Q175/Q7}$ mice reduced the levels of mutant HTT (mHTT) while preserving the levels of wild-type HTT. However, in this model, TFEB overexpression was also accompanied by ER stress and reactive gliosis [129].

In contrast with the later work, another study reported that the co-injection of human TFEB and mHTT in the mouse striatum has no impact on the level of mHTT aggregates even though autophagy appears to be activated. In this case, the accumulation of late autophagic structures seems to impair the global process [130].

Overall, these results seem to indicate that TFEB might represent a promising therapeutic target for the treatment of neurodegenerative disorders. However, it is important to notice that autophagy must be strictly regulated to guarantee the correct homeostasis in each type of cell. Therefore, while a regulated induction of autophagic flux may have positive effects in neurons, the overactivation of this process may be deleterious in other cells. Thus, deciphering the physiological and pathological role of TFEB in the different cell types that constitute the central nervous system will be necessary

to develop efficient and safe therapeutic strategies for neurodegenerative disorders.

The possibility of modulating the activity of the MiTF/TFE factors, and in particular of TFEB, has also generated a great interest as a possible therapeutic strategy for LSDs. The role of TFEB as a potential target for the treatment of several LSDs has been largely investigated, and the overexpression of TFEB or its activation through the inhibition of mTORC1 has been reported to be beneficial in the rescue of the lysosome-associated pathological phenotype.

Despite the promising observations in cellular models of LSDs and the first proof of concept in vivo, additional data on the effects of chronic activation of TFEB in animal models is required. Thus, the uncontrolled expression of MiTF/TFE factors is linked to various human rare genetic cancers [131]. In this regard, the most logical approach to promote clearance is the pharmacological activation of TFEB. Using small molecules could allow the modulation of the amplitude and duration of TFEB activity in vivo. Also, this approach could be combined to other therapeutic approaches such as enzyme replacement therapy (ERT) or gene therapy.

To evaluate the effect of TFEB modulation in vivo, it would be very useful to take advantage of the different transgenic animal models already used to study LSDs, which are quite reliable compared to the transgenic mouse models for some age-related neurodegenerative diseases. Animal models can provide the opportunity to assess the effects of the constitutive long-term activation of TFEB, for example by evaluating in which way TFEB activation affects the lifespan, the homeostasis, and the function of neurons and of other CNS cell types. They could also help to understand to what extent the modulation of TFEB may be beneficial in the context of LSDs, and how to prevent possible negative effects.

MiTF/TFE Family Transcription Factors: Putative Therapeutic Targets in Neurodegenerative Diseases and Lysosomal Storage Disorders?

In recent years, several compounds able to modulate TFEB activity have been found to enhance autophagy and lysosomal biogenesis and might have therapeutic potential for the treatment of neurodegenerative diseases and LSD (Table 3). Very recently, a repurposing approach to identify drugs able to ameliorate two subtypes of Batten disease, the most frequent of rare neurodegenerative disorders in children, resulted in the identification of tamoxifen [20]. Tamoxifen ameliorates the phenotype of disease relevant cellular models of CLN3 and CLN7 disease, including neuronal progenitor cells (NPCs) from CLN7 patient-derived induced pluripotent stem cells (iPSC). Also, the treatment with tamoxifen was able to ameliorate the phenotype of a mouse model of CLN7 disease. Interestingly, tamoxifen exerts its action through a mechanism that involves activation of the transcription factor EB (TFEB) [20].

The idea of enhancing autophagy to counteract neurodegeneration has been explored by many [152] and in most cases the preferred molecular target for the proposed therapeutic strategies is mTOR, particularly using specific mTOR inhibitors. Despite their efficacy in certain models, they have also proved to have limited capacity of impacting neurodegenerative diseases in certain clinical trials. We suggest that this may be associated to the fact that mTOR regulates not only TFEB and its downstream pathways but also many other targets, and this could be an issue when proposing a therapeutic approach for a chronic progressive disease. Nevertheless, TFEB is a non-canonical substrate of mTOR and can be activated by inhibitors that impact Rags pathways but not on canonical mTOR substrate, such as the ribosomal protein S6 kinase (S6K). For example, in a recently published paper, the mTOR inhibitor fluoxetine was identified as a possible corrector of neurodegeneration in MPS-IIIA via TFEB activation in a Rag-dependent manner [153]. This suggests that careful evaluation of mTOR inhibitors should be performed before moving them towards tests in pre-clinical models for neurodegenerative diseases or to clinical trials. Or, even better, specific therapeutic strategies targeting TFEB should be identified and tested.

Another interesting aspect to evaluate is the fact that research is mainly focused on TFEB among all MiTF/TFE family members, but even though MiTF/TFE transcription factors display some functional overlap, it remains to be established to which extent they have common functions, whether they are complementary or differ, and which factors orchestrate their interplay. Therefore, despite the promising perspective to fight neurodegenerative diseases and LSDs by enhancing autophagy/lysosomal biogenesis via TFEB modulation, a better understanding of the factors that regulate TFEB activity as well as the interplay between TFEB and the MiTF/TFE transcription factors is strongly required. This would ensure a safe development of targeted therapies for the treatment of these diseases. In fact, enhancing autophagy and lysosomal activity may be beneficial for the treatment of neurodegenerative diseases and LSDs but could have adverse effects. For example, it is quite well established that altered regulation of MiTF/TFE proteins can be linked to cancer development. *MITF* gene amplification was found in in 20% of melanomas. Translocations and rearrangements of *TFE3* and *TFEB* are associated with a rare subtype of kidney cancer termed translocation-renal cell carcinoma (tRCC) and alveolar soft part sarcomas (ASPS), a rare lung cancer variant [154]. A recent study reports that TFEC is expressed at higher levels in ovarian cancer tissues, compared to normal tissues, and correlates with malignant progression and

Table 3 Modulators of TFEB activity

Name compound	Mechanism of action	Effect	Reference
2-Hydroxypropyl-β-cyclodextrin (2-HPβCD)	Nuclear translocation and consequent activation of TFEB upon treatment	Increased clearance of ceroid lipopigment in late infantile neuronal ceroid lipofuscinosis (LINCL) fibroblasts	[132]
		Increased autophagic clearance of aggregated α-syn in H4 cells stably transfected for the expression of α-syn-EmGFP	[133]
Aspirin (acetylsalicylic acid)	Upregulation of TFEB and increased lysosomal biogenesis via PPARα	Enhanced uptake and degradation of Aβ in primary astrocytes. Reduced intraneuronal Aβ accumulation. Decreased amyloid plaque pathology in 5XFAD mice	[134]
Cerium oxide nanoparticles (nanoceria) coated with N-acetylglucosamine, polyethylene glycol, and polyvinylpyrrolidone	Nuclear translocation and consequent activation of TFEB upon treatment	Promoted clearance of ceroid lipopigment in fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL)	[135]
Chlorogenic acid (CGA)	Upregulation of cathepsin D, protein expression induced by the mTOR/TFEB signaling pathway	Promoted lysosomal activity in APP/PS1 mice and Aβ25-35-exposed SHSY5Y cells. Improved spatial memory and attenuated neuron damage in APP/PS1 mice	[136]
Cinnamic acid	Upregulation of TFEB via PPARα	Enhanced lysosomal biogenesis in mouse primary brain cells. Decreased amyloid plaque pathology and improved memory in 5XFAD mice	[137]
Curcumin analog C1	Activation of TFEB and promotion of autophagy and lysosome biogenesis in a mTOR-independent manner	Reduced APP, CTF-β/α, β-amyloid peptides, and Tau aggregates accompanied by improved synaptic and cognitive function in mouse models of beta-amyloidosis, tauopathy, and combined amyloidosis-tauopathy	[138]
		Rescue of cell death in 6-OHDA-induced PD models (SH-SY5Y cells, iPSC-derived DA neurons and mice nigral DA neurons)	[139]
Curcumin derivative (E4)	Activation of TFEB by AKT-MTORC1 inhibition and promotion of autophagy and lysosome biogenesis	Decreased level of overexpressed α–syn in In Neuro2a (N2a) cells transfected with A53T α–syn and reduced cell death in PC12 cells treated with MPP^+	[140]
Dynasore	Blocking of mTORC1 activity by repressing the lysosomal localization of mTOR, which induces nuclear translocation of TFE3 and TFEB	Enhanced autophagy promotes the clearance of protein aggregates formed by mutant huntingtin	[141]
Fisetin (3,7,3′,4′ -tetrahydroxyflavone)	Activation of TFEB via mTORC1 inhibition	Decreased level of phosphorylated tau in cortical cells or primary neurons	[142]
Flubendazole	Induced TFEB nuclear translocation via mTOR deactivation caused by disruption of dynamic microtubules	Reduction of p-tau in N2a cells	[143]
Genistein (5,7-dihydroxy-3 (4-hydroxyphenyl)-4H-1 benzopyran-4-one)	Impairment of glycosaminoglycans (GAGs) synthesis and enhancement of their degradation. It also alters the expression of genes involved in lysosomal metabolism via TFEB nuclear translocation	Genistein might have beneficial effects for the treatment of lysosomal storage disorders such as mucopolysaccharidoses caused by mutations leading to impaired degradation of GAGs	[144]
GSK3 inhibitor VIII	Activation of TFEB by GSK3 inhibition	Lysosomal clearance of APP in N2a cells stably transfected with the APP-695 Swedish mutation and of its CTF in CHO cells inducibly expressing the APP-CTF	[145]

Table 3 (continued)

Name compound	Mechanism of action	Effect	Reference
Gypenoside XVII (GP-17)	TFEB activation by releasing TFEB from TFEB/14–3-3 complexes	Elimination of AβPP, Aβ40, and Aβ42 in APP695swe cells. Formation of Aβ plaques in the hippocampus and cortex of APP/PS1 mice is prevented and spatial learning and memory are restored	[146]
Ibudilast	Enhanced TFEB nuclear translocation by inhibiting mTORC1 activity	Increased clearance of disease-linked TAR DNA binding protein (TDP-43) and superoxide dismutase 1 (SOD1) protein aggregates in cells transfected with corresponding mutated forms. Protective effect of TDP-43-induced cytotoxicity in motor neuron-like NSC-34 cells	[147]
Ouabain	Activation of TFEB via inhibition of the mTOR pathway	Reduced accumulation of p-tau in GFP-TauP301S-overexpressing SH-SY5Y, in primary cortical neurons, in a *Drosophila melanogaster* tau model and in TauP301L mice. Ameliorated memory defects in in TauP301L mice	[148]
Pseudoginsenoside-F11 (PF11)	Induced TFEB nuclear translocation by suppressing mTORC1 activity	Increased degradation of oligomeric Aβ in cultured microglia	[149]
Threalose	Inhibition of Akt which in turn activates TFEB independently of mTORC1	Enhanced clearance of lipopigments and reduced neuropathology in a mouse model of Batten disease. Promoted cellular clearance in fibroblast derived from patient with Batten disease	[150]
	Rapid enlargement and transient permeabilization of lysosomes leading to calcineurin activation and subsequent TFEB dephosphorylation and nuclear translocation	Promoted clearance in mouse motoneuron-like hybrid cell line (NSC34) of polyQ-containing androgen receptor, TDP-43, and SOD1 mutated forms	[151]

poor survival for ovarian cancer patients [155]. Moreover, increased TFEB expression is found in glioblastoma patients and contributes to the glioblastoma resistance to chemotherapy. In fact, drug-mediated inhibition of TFEB expression and oligomerization can enhance glioblastoma cell sensitivity to conventional chemotherapeutic agents [156]. All these observations deserve attention, and therefore a comprehensive characterization of the potential deleterious effects of uncontrolled expression of MiTF/TFE3 factors in vivo. We must consider that alterations to the regulation of MiTF/TFE transcription factors are accompanied by the hyperactivation of other key pathways involved in tumorigenesis and cell proliferation [131, 157]. Therefore, we can expect that controlled pharmacological activation of MiTF/TFE proteins will recapitulate the pathological features of MiTF/TFE-driven cancer.

Overall, given that (1) autophagy and TFEB translocation may already by overactivated in certain neurodegenerative diseases or LSDs to compensate for the defective mechanisms already in place and to remove undigested cellular waste, (2) uncontrolled boosting of autophagy may impact the overall cellular homeostasis, and (3) overactivation of TFEB may trigger downstream pathways other than ALP, the final goal of novel therapeutic strategies would be to restore the homeostatic regulation of these processes and the homeostasis of the MiTF/TFE transcription factors, rather than promoting their uncontrolled activation.

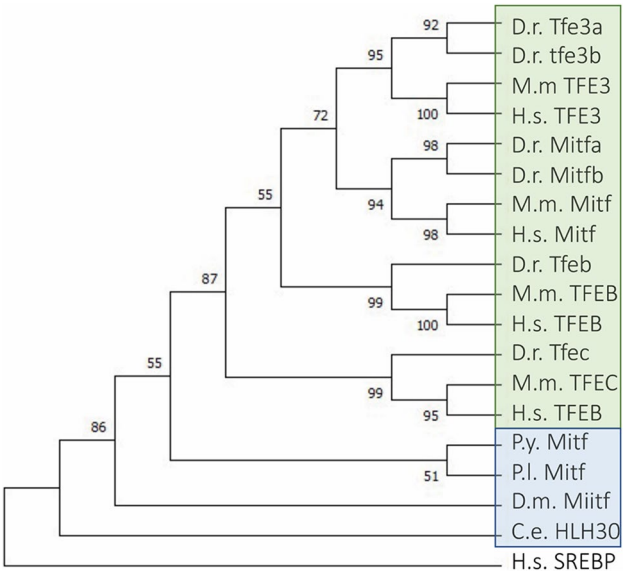

Fig. 4 Phylogenetic tree of the MiTF/TFE family of transcription factors. At every node, the bootstrap values are shown. In green and in blue are highlighted vertebrate and invertebrate organisms respectively. *Homo sapiens* Sterol Regulatory Binding Protein (SREBP) has been used as an outgroup protein to root the tree. H.s, *Homo sapiens*; M.m, *Mus musculus*; D.r, *Danio rerio*; P.y, *Patinopecten yessoens*; P.l, *Paracentrotus lividus*; D.m, *Drosophila melanogaster*; C.e, *Caenorhabditis elegans*

What Can We Learn from the Study of MiTF/TFE Family in Non-mammalian Organisms?

Non-mammalian model organisms can have a role in the implementation of our current knowledge on MiTF/TFE transcription factors. All MiTF/TFE family members are conserved in vertebrates, while invertebrates have only a single MiTF orthologue (Fig. 4). Considering the high degree of conservation of the entire autophagic machinery, also organisms that are phylogenetically distant from humans can be exploited for the in vivo characterization of these proteins and these pathways (Fig. 5). These animal models can give information on unclear or uncharacterized aspects that would otherwise be difficult to study in humans or mice. Every animal model is characterized by specific features that provide unique advantageous tools to understand in detail the activity and the complex mechanisms of regulation of MiTF/TFE transcription factors. For example, the transparent body of worms and zebrafish larvae allows exploiting these organisms for the in vivo visualization of these proteins to study their intracellular localization and movements across the cell compartments. The complex behavioral features of fruit flies may be of great interest for the evaluation of phenotypes associated to the modulation of MiTF/TFE transcription factors activity to study short/long-term collateral effects (if any) and to perform rescue experiments. In addition, exploiting invertebrate models may allow the analysis of a high number of individuals that could be highly valuable for the screening of drugs or compounds that modulate MiTF/TFE protein activity. These simpler organisms are usually easier to be genetically manipulated; they allow the possibility to generate animals carrying specific mutations or useful constructs for imaging that help the understanding of the role of these transcription factors. These are only few of the reasons why the use of non-mammal animal models in the research may be worthwhile. The choice for the proper organism should be based on the questions the researchers want to assess. Moreover, the exploitation of different models to answer the same biological question may be crucial to get more informative results, and to increase the soundness of the data and the value of the research.

The following paragraphs will describe the current knowledge about MiTF/TFE transcription factor in model organisms other than rodents, by focusing, when possible, on phylogenetic and mechanistic aspects.

Caenorhabditis elegans

The nematode *Caenorhabditis elegans* is one of the most used model organisms in biology [158]. Several features of

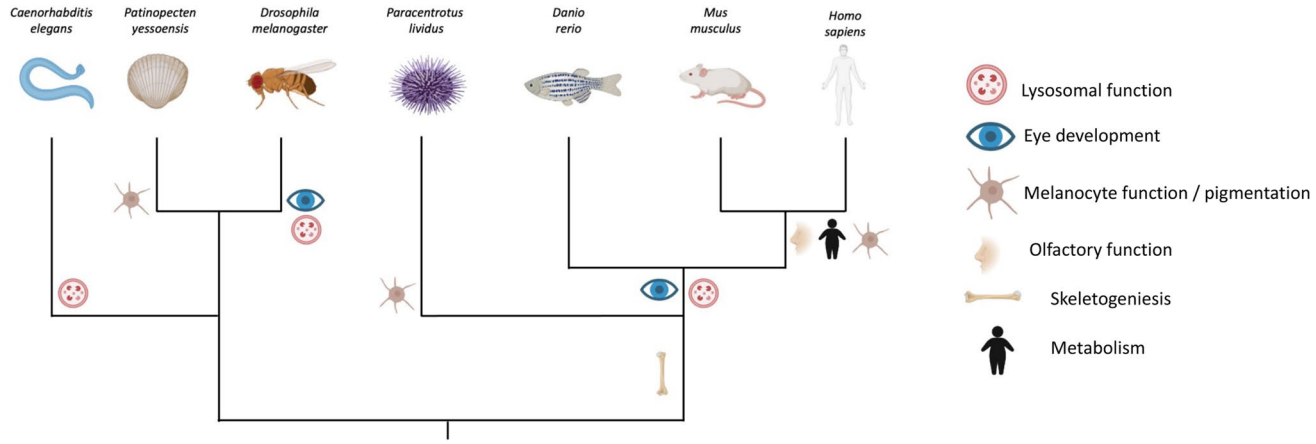

Fig. 5 The most important functions ascribed to the MITF/TFE transcription factors are reported in the phylogenetic tree of the species. The position of a specific function in the tree shows when it appeared during evolution. Autophagy and lysosomal activities are the most conserved functions: sea urchins and molluscs are the only organisms in the tree in which this function has not been reported; however, it is highly probable that this function is common to all the organisms and is present in the progenitor of all these animals. Mitf/TFE transcription factors have been associated also to pigmentation and eye development in different organisms. Skeletogenesis seems to be a common function to all deuterostomes. Some peculiar functions, such as the role in the olfaction and the control of metabolism, have been described only in mammals so far

C. elegans make this animal an advantageous model for biology research. First, it is relatively easy to produce transgenic worm lines that overexpress or lack a gene of interest; it is possible to limit the expression of genes to specific cell types and to study protein activity by tagging them with fluorescent probes. Several studies characterized TFEB activity in worm lines lacking the *C. elegans* TFEB orthologue gene or studied its subcellular localization by expressing the protein tagged with a GFP [159–161]. It is also possible to assess autophagic activity in worms through the expression of specific construct for the analysis of autophagic flux [162]. Second, the small size of this organism and the possibility of analyzing many individuals allow performing large-scale screens. While its nervous system is relatively simple compared to that of other model organisms, several cellular and molecular pathways are well conserved. Moreover, about 70% of genes linked to human diseases have orthologues in worm genome. For these reasons, this animal is considered a good model for the neurodegeneration research [163].

A functional orthologue gene of human *TFEB* has been found in *C. elegans*, called *hlh-30*, which shares high homology to the human protein in both the DNA-binding and activation domain. The protein HLH-30 is the only member of the MiTF/TFE family, which is present in *C. elegans* and, like the human orthologue, it modulates autophagy and lysosomal function [164].

Interestingly, also the regulation of HLH-30 protein seems to be conserved, with the worm transcription factor that is modulated via post-transcriptional modifications, in a similar manner to its mammalian orthologues. In fact, the silencing of mTOR has been demonstrated to enhance the HLH-30 nuclear localization and to increase the expression of several genes that are the Nematoda orthologues of the human TFEB targets, including autophagy-related genes [159].

Moreover, the *C. elegans* HLH-30 downstream genes are characterized by the presence in their promoter region of an E-box sequence that overlaps with that of the CLEAR motif, further confirming the conservation between the HLH-30 and its mammalian orthologues. This region in *C. elegans* is crucial for the specificity of HLH-30 binding to DNA [159, 164].

Drosophila melanogaster

Drosophila melanogaster, commonly known as fruit fly, is one of the most used model organism in biology and in biomedical research [165]. In particular, it is a powerful organism to model the physiology and pathology of the brain, mainly because it can be genetically manipulated and because many genes and molecular pathways are conserved between humans and flies [166]. Synapse formation, membrane trafficking, and neuronal communication are a few examples of processes that are similar in flies and in more complex organisms [167]. In addition, *D. melanogaster* brain is developed enough to promote elaborated behavioral features, but it is still small enough and relatively simple, thus allowing the detailed analysis of its structure and functions.

The genome of *Drosophila melanogaster* contains a single *Mitf*, which shares several features and functions with its mammalian orthologues, providing evidence of high

conservation of this gene throughout the evolution. Interestingly, *Mitf* is not phylogenetically closer to the mammalian *MITF* than to *TFEB*, suggesting that the ancestral gene underwent multiple duplication events after the separation of the invertebrate to the vertebrate lineages [22]. The presence of only one gene in flies and the high degree of conservation with the four mammalian MiTF/TFE family members make the fruit fly an advantageous organism to study its physiological role in a simplified model.

Like the mammalian transcription factors of the MiTF/TFE family, *Drosophila melanogaster* Mitf can regulate gene expression through the binding to the DNA. The conservation of the DNA binding region in the protein suggests that the fly transcription factor recognizes the same target domain of its mammalian orthologues. This DNA region is represented by the already described CLEAR motif, which is crucial for the binding specificity to the DNA. The conservation of the CLEAR motif in *Drosophila* has been confirmed in the promoter region of genes whose expression resulted significantly upregulated by overexpression of Mitf protein [22, 23, 168].

In fruit flies, Mitf has been shown to play a role in the transcriptional regulation of lysosomal biogenesis, in autophagy, and in the catabolism of lipids, further supporting a functional similarity with its mammalian orthologues TFEB and TFE3 [22]. Moreover, a reduction in Mitf activity leads to an impairment of autophagic flux with accumulation of autophagy substrates, such as polyubiquitinated proteins and dysfunctional mitochondria. Mitf determines the cellular response to starvation, which is well known to activate autophagy. Upon starvation, Mitf upregulates lysosomal biogenesis and several autophagy-related genes, especially those involved in the formation and maturation of autophagosomes [22, 168]. In this frame, mTOR has been shown to negatively modulate Mitf activity, and also in flies the treatment with Torin1, a specific inhibitor of mTOR, is able to induce the activation and the nuclear translocation of Mitf [22]. Interestingly, the similarity goes further at the structural level. It is well established that Ser142 and Ser211 are the residues of human TFEB that are phosphorylated by mTORC1, inhibiting TFEB activity. Corresponding serine residues, Ser240 and Ser346, respectively, are also present in the fly orthologue.

Besides its role in autophagy, *D. melanogaster* Mitf is involved in eye development, a function that is performed by MITF in mammals. More specifically, Mitf is expressed in the eye-antennal imaginal disc during the second and third larval stages of the fruit fly and the expression of a dominant negative *Mitf* mutant impairs the correct development of the eye [168].

Overall, the data concerning *D. melanogaster* Mitf suggest that the protein function is highly conserved and that the distinct roles described for the mammalian proteins might coexist in the unique fly orthologue. This makes *D. melanogaster* a perfect asset to test drugs able to modulate Mitf activity in a cost-effective but valuable model organism, and before moving towards more complex organisms.

The presence of a unique *Mitf* orthologue both in *C. elegans* and *D. melanogaster* points at them as good organisms to study this protein in a simple model in which the presence of only one protein of the MiTF/TFE family removes the potential uncertainty derived from the redundancy. On the other side, this hinders the possibility of studying the interplay among the different MiTF/TFE family members, which may be crucial for the understanding of the molecular mechanisms that regulate the downstream pathways of these transcription factors. Under these circumstances, it may be worthwhile to exploit more complex model organisms.

Danio rerio

Among the vertebrates, *Danio rerio*, commonly known as zebrafish, is one of the most studied model organisms [169]. As a vertebrate, it is evolutionary closer to humans than invertebrate models and present several advantages over other vertebrate organisms, such as the high rate of fecundity, the external fertilization, and the fact that in the first developmental stages the organism is transparent and allows to visualize internal structures and tissues by in vivo imaging [169]. Furthermore, in the neurodegenerative field, zebrafish is largely used because its brain organization shows high similarities with human brain, with specific brain regions of zebrafish that are highly conserved and can be related to mammal brain [169]. Genomic analyses have demonstrated that zebrafish and other teleost species underwent an event of gene duplication that may have occurred at least 100 million years ago and determined the presence of approximately 20–30% of an extra complement of genes in their genome. This is probably the reason for the presence in zebrafish of six genes belonging to the MiTF/TFE family. Besides *tfeb* and *tfec*, it also presents two orthologues of the mammalian *MITF* (*Mitfa* and *Mitfb*) and two orthologues of the mammalian *TFE3* (*Tfe3a* and *Tfe3b*). Since often-duplicated genes are characterized by tissue-specific expression or by the activation in precise developmental stages, the research may take advantage of the duplication of the genome to study in detail the role of the protein of interest in different cell types or at different times. This feature seems to apply to the proteins Mitfa and Mitfb that share high homology in their sequence, with the differences mostly located in their amino and carboxy termini. Interestingly, Mitfa seems to correspond to the mammal melanocytic "M" isoform, whereas Mitfb shares the highest homology with the mammalian "A" isoform. Moreover, the zebrafish *Mitf* genes have restricted expression profiles that approximate the localized expression of their mammalian orthologue [170].

Regarding the role of tfeb, the major functions of this transcription factor are conserved. In zebrafish, as in mammals, it controls the network of genes involved in lysosomal biogenesis and autophagy. Moreover, similarly to its mammalian orthologue, tfeb activation is regulated by mTORC1 and Rag-GTPases. Like in mouse, tfeb can repress in zebrafish the process of myelination during the development of the CNS. More specifically, tfeb has been shown to upregulate several of its target genes in the oligodentrocytes, leading the authors to speculate that the activity of tfeb may impair the trafficking of endo-lysosomal organelles to the membrane and the synthesis of lipids, two crucial processes for the membranous myelin sheath. As the activity of tfeb might disrupt the process of myelination, it appears to be specifically repressed by mTORC1 and other inhibitory kinases during myelination [171]. This function of tfeb, although poorly investigated, may be crucial for neuronal physiology and could be very relevant in the context of neurodegeneration. Thus, it would be very worthwhile to investigate whether this activity of tfeb is conserved in other models and in mammal and understand how it impacts brain homeostasis.

In comparison to *Mitf* and *Tfeb*, which are the most studied and characterized genes among the members of the MiTF/TFE family in zebrafish, much less is known about the two *tfe3* genes. *Tfe3a* has been described to encode a protein of 539 amino acids that shares about 50% of homology with the human *TFE3*. As observed for the other members of the family, the most conserved region of *Tfe3a* is the helix-loop-helix/leucine zipper region. Like the human orthologue that has been shown to regulate immunoglobulin expression, *tfe3a* in zebrafish is present in the ventral mesoderm, which gives origin to blood cells, suggesting a possible functional conservation of this gene between different organisms [170].

Tfe3 genes seem to be co-expressed with *mitf* genes in several tissues. In fibroblast cell cultures, the two genes share comparable activities, suggesting a possible redundant role. Nevertheless, in mitf knockout zebrafish models, Tfe3 has been demonstrated to support very inefficiently the role of Mitf [170], indicating that, in vivo, the different members of the MiTF/TFE family exert different roles being only partially redundant at the functional level [170].

In zebrafish, *Tfec* is the less characterized member of the Mitf family. The protein has been proposed to be a key regulator of zebrafish embryonic hematopoiesis, the process responsible for the formation of all types of blood cells from hematopoietic stem cells (HSCs) [172]. How and if this has a role in the nervous system, at least in this model, remain to be elucidated.

Other Organisms

Beside the most common and studied animal models, orthologues of the MiTF/TFE family of transcription factors have been found in other organisms. Even though these organisms are considered irrelevant in biomedical research, they may inspire the study of alternative pathways or regulatory mechanisms in more conventional models.

Among the organisms in which an orthologue of Mitf has been described, *Paracentrotus lividus* is a sea urchin that has an important phylogenetic position because it belongs to the phylum of Deuterostome, like vertebrates. Given the conservation of many important molecular pathways, the study of Mitf in *Paracentrotus lividus* may provide interesting information about the role and the signalling pathways of this transcription factor.

Pl-Mitf protein is characterized by all the functional domains of the MiTF/TFE protein family, including the DNA binding domain and the bHLHzip domain. However, in this domain, only four out of five canonical leucine residues are observed and two of them are conservative substitutions. This imperfect leucine zipper has also been found in *D. melanogaster*. Among the phosphorylation sites of Pl-Mitf, some of them are conserved, further providing evidence of a possible common pattern of regulation of this protein. Mitf has been found in the pigment cells of the sea urchin, coherently to the role of the mammalian orthologue in melanocyte. Moreover, while MITF in mammals is an important transcription factor in osteoclasts, Pl-Mitf is expressed in the presumptive mesenchymal cells (PMC) that are progenitor cells of the sea urchin larval skeleton. Some studies have highlighted similar features between PMC and osteoclasts: both cell types are involved in the skeleton development, have migratory capability, and can form multinucleated syncytia. These data may suggest an unknown role of Pl-Mitf in the skeletogenesis of sea urchin [173].

Given the well-established role of MITF in the pigment cells, another group of organisms may be interesting to study the function of this gene. In fact, molluscs are characterized by a vast pattern of colors, mainly in shells. The yesso scallop, *Patinopecten yessoensis*, is a large group of molluscs that live in the bottom of the northwestern Pacific Ocean. The genome and the transcriptome of Yesso scallop have been widely studied and a unique orthologue of *MITF* (*Py-Mitf*) has been found in this organism. It shares less homology with vertebrate organisms, highlighting the fact that the evolution of this gene is consistent with the species taxonomy. This gene is formed by eight exons, in contrast with the mammalian MITF that is organized into nine exons. As observed in other organisms, the most conserved region of the *Py-Mitf* gene is the bHLH-LZ motif [174]. Shell color is determined in these organisms by the presence of biological pigments, like melanin, carotenoids, and tetrapyrroles,

but the mechanisms that underline these features are poorly understood. Melanin biosynthesis is initiated with tyrosine oxidation and tyrosine in mammals is known to be positively regulated by MITF, which has been reported to be a master regulator of melanogenesis. Interestingly, in yesso scallop, the expression of *Py-Mitf* has been shown to correlate with the shell color, further confirming the high degree of conservation of these genes among different organisms. Moreover, the higher level of Py-Mitf mRNA was detected in the mantel, the organ involved in shell color formation. Notably, even though the two valves of the same organism usually are characterized by different colors, no difference in the level of Py-Mitf expression was detected between the right and the left mantels. This result may indicate that Py-Mitf is strictly regulated, and a different regulation process may modulate the shell color in the same animal. Reports about Mitf involvement in autophagic regulation in these organisms are lacking; however, given the high conservation of autophagy throughout evolution and the similarities between mammalian MITF and the invertebrate orthologue, it is highly probable that this transcription factor may control ALP also in these invertebrates.

The high degree of conservation of the MiTF/TFE transcription factors across evolution should be exploited for the research of modulators of these proteins. The possibility of being inspired not only of many classical model organisms but also of several non-canonical animal models may be very relevant to implement the in vivo characterization of these factors and represent very good tools to analyze in detail different aspects of the MiTF/TFE transcription factors that are still unclear.

Conclusions and Future Directions

Autophagy dysfunction has been described in different neurodegenerative disorders. All the members of the MiTF/TFE family have been shown to participate in the regulation of autophagy, and in other processes that are relevant for brain physiology. However, many aspects related to the basic biology of these transcription factors remain unknown. For instance, MiTF/TFE transcription factors can form both homodimers and heterodimers with any other family member, but little is known about the functional difference between homodimers and heterodimers. Furthermore, with the exception of TFE3, all family members have alternative transcripts which display different tissue distribution patterns, and it remains to be determined the functional importance of these transcripts and whether this may result in cell-type–specific regulatory networks. As MiTF/TFE transcription factors are conserved across species, comparing MiTF/TFE protein function and regulation in different and appropriate animal models may provide a better understanding of their physiological function in the CNS. Moreover, the use of different model organisms may provide a valuable tool for understanding the roles of these transcription factors common to all forms of eukaryotic life and how their impairment may be implicated in neurodegeneration. Thus, it appears fundamental to decipher the factors that are responsible for MiTF/TFE transcription factor regulation and their interplay. This would allow modulating autophagy and other relevant pathways for brain cells via MiTF/TFE family members in a tissue/cell-specific manner thus avoiding negative side effects.

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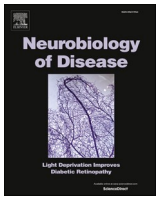

DJ-1 promotes energy balance by regulating both mitochondrial and autophagic homeostasis

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ABSTRACT

The protein DJ-1 is mutated in rare familial forms of recessive Parkinson's disease and in parkinsonism accompanied by amyotrophic lateral sclerosis symptoms and dementia. DJ-1 is considered a multitasking protein able to confer protection under various conditions of stress. However, the precise cellular function still remains elusive. In the present work, we evaluated fruit flies lacking the expression of the DJ-1 homolog dj-1β as compared to control aged-matched individuals. Behavioral evaluations included lifespan, locomotion in an open field arena, sensitivity to oxidative insults, and resistance to starvation. Molecular analyses were carried out by analyzing the mitochondrial morphology and functionality, and the autophagic response. We demonstrated that *dj-1β* null mutant flies are hypoactive and display higher sensitivity to oxidative insults and food deprivation. Analysis of mitochondrial homeostasis revealed that loss of dj-1β leads to larger and more circular mitochondria, characterized by impaired complex-I-linked respiration while preserving ATP production capacity. Additionally, *dj-1β* null mutant flies present an impaired autophagic response, which is suppressed by treatment with the antioxidant molecule N-Acetyl-L-Cysteine. Overall, our data point to a mechanism whereby DJ-1 plays a critical role in the maintenance of energy homeostasis, by sustaining mitochondrial homeostasis and affecting the autophagic flux through the maintenance of the cellular redox state. In light of the involvement of DJ-1 in neurodegenerative diseases and considering that neurons are highly energy-demanding cells, particularly sensitive to redox stress, our study sheds light on a key role of DJ-1 in the maintenance of cellular homeostasis.

List of abbreviations

ALS	Amyotrophic Lateral Sclerosis
AMPK	5'-AMP-Activated Protein Kinase
BN-PAGE	Blue Native-Polyacrylamide Gel Electrophoresis
DHE	Dihydroethidium

(*continued on next column*)

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ETC	Electron Transport Chain
HPLC	High Pressure Liquid Chromatography
KO	Knockout
LC3	Microtubule-associated protein 1A/1B-light chain 3
NAC	N-Acetyl-L-Cysteine

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(*continued*)

OCR	Oxygen Consumption Rate
p-AMPK	Phosphorylated AMPK
PBS	Phosphate-Buffered Saline
PD	Parkinson's Disease
ROS	Reactive Oxygen Species
TEM	Transmission Electron Microscopy

1. Introduction

Alterations in the function of the protein DJ-1 have been associated with different neurodegenerative disorders. Mutations in the DJ-1 encoding gene (*PARK7*) have been originally implicated in familial forms of autosomal recessive Parkinson's disease (PD) in 2003. Bonifati et al. discovered that a 14 Kbp deletion and a missense mutation (L166P) in the *PARK7* locus were associated with parkinsonian symptoms (Bonifati, 2003; Bonifati et al., 2003; Hague et al., 2003). Since then, a growing number of mutations have been reported, confirming the association of the protein with a form of parkinsonism with early-onset motor symptoms and slow progression, followed by non-motor manifestations, such as cognitive decline and depression (Bonifati, 2003; Abou-Sleiman et al., 2003). Subsequent studies reported broader participation of DJ-1 in forms of parkinsonism accompanied by amyotrophic lateral sclerosis (ALS) symptoms and dementia (Annesi et al., 2005; Hanagasi et al., 2016; Rizzu et al., 2004), suggesting the involvement of DJ-1 in a more complex pathological scenario. DJ-1 is a 20 kDa protein expressed in most body tissues, with abundant expression in the brain (Olzmann et al., 2004). At the subcellular level, DJ-1 is mainly found in the cytoplasm, although a fraction of the protein has also been detected in the nucleus and mitochondria, especially under conditions of stress (Canet-Avilés et al., 2004; Junn et al., 2009; Kim et al., 2012; Zhang et al., 2005). Indeed, mounting evidence suggests that the protein confers cellular protection and coordinates a number of survival pathways, such as oxidative stress response (Oh and Mouradian, 2017). So far, different mechanisms of action have been proposed, including modulation of signal transduction and transcription, redox sensing, maintenance of mitochondrial homeostasis, and energy balance (Biosa et al., 2017; Dolgacheva et al., 2019; Mencke et al., 2021). However, a general consensus on its specific cellular activity is still missing (Biosa et al., 2017). Here, we took advantage of using *Drosophila melanogaster* as an *in vivo* model to gain insights into the physiological role of DJ-1. Our data show that loss of DJ-1 in *Drosophila* does not result in overt phenotypic abnormalities, confirming previous studies, but instead confers subtle bioenergetic impairments, which are exacerbated under conditions of stress. Moreover, we provide a novel characterization of *DJ-1* null mutant flies, highlighting both behavioral and molecular aspects associated with loss of function of the protein.

2. Materials and methods

2.1. Fly strains and husbandry

Drosophila w^{1118} (BDSC_5905), dj-1$\beta^{\Delta 93}$ (BDSC_33601), daughterless (da)-Gal4 (BDSC_8641), UAS-dj-1β (BDSC_33604), and UAS-GFP-mCherry-Atg8a (BDSC_37749) fly lines were obtained from the Bloomington *Drosophila* Stock Center. All strains were reared on common cornmeal food in a humidified, temperature-controlled incubator at 25 °C on a 12 h light/dark cycle. Unless otherwise stated, for each experiment, 1-to-3-day-old male flies were collected under brief CO_2 exposure and transferred into new tubes containing standard food (20–25 flies/vial). The individuals were left aging for 7 days and then analyzed.

2.2. Longevity

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred into new tubes containing standard food (20–25 flies/vial). Flies were transferred to fresh food vials every 3–4 days and the number of dead flies was counted daily. The percentage of survival was calculated at the end of the experiments.

2.3. NAC treatment

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred into new tubes containing standard food (20 flies/vial) added with 1 mM N-Acetyl-L-Cysteine (NAC) (Sigma-Aldrich: A8199). Treatment lasted for a period of 7 days and flies were transferred to fresh NAC-added food vials every 2–3 days.

2.4. Starvation resistance

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred into new tubes containing standard food (20 flies/vial). After 7 days, flies were transferred to vials containing 1.5% agar and the number of dead individuals counted till all flies died.

2.5. Bodyweight measurements

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (20 flies/vial). After 7 days, flies were kept on standard food or starved for 24 h and then weighed as a pool of 10 flies.

2.6. DHE assay

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (20 flies/vial). After 7 days, reactive oxygen species (ROS) measurement was performed according to Owusu-Ansah et al. protocol (Owusu-Ansah and Yavari, 2008). Briefly, fly brains were dissected in Schneider's *Drosophila* medium (Biowest, L0207) followed by 5 min of incubation with 30 μM dihydroethidium (DHE) probe (Invitrogen, D11347) in the dark. Subsequently, brains were washed thrice in Schneider's Drosophila medium for 5 min and mounted in Vectashield medium (Vector laboratories, H-1000). Images were acquired utilizing LeicaSP5 confocal microscope (Leica Microsystems) equipped with a 20 × 0.5 NA objective by using a 514 nm laser. The intensity of DHE staining was quantified by ImageJ.

2.7. Locomotion in open field arena

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (20 flies/vial). After 7 days, locomotor behavior was analyzed. Fly movements were investigated in an open field arena of 10 cm diameter custom designed in order to confine flies in a two-dimension space. Flies were loaded into the arena by means of a mouth aspirator and then left to acclimatize in complete darkness for 5 min. Recording started at light on and lasted 10 min (15 frames/s; video resolution: 1296 × 964 pixel). All experiments were conducted between zeitgeber time 2 and 4 at a room with controlled temperature. The videos obtained were analyzed by the CTRAX software (California Institute of Technology Fly Tracker) for fly position tracking (Qin et al., 2020). Subsequently, eventual errors made by the tracking process were manually corrected with a MATLAB Toolbox (FixErrors), provided by the developers of CTRAX. Locomotor parameters were finally computed with another MATLAB script (Behavioral Microarray) also belonging to CTRAX package. Data were then imported into the R software (R Development Core Team, 2017) environment for data analysis and statistical computing by means of custom scripts. The locomotor parameters evaluated were: velocity (maximal speed of the fly measured in millimeter/s), length (distance in millimeter walked by flies without interruptions after each walking step

with a velocity > 1 mm/s), number of starts (number of times that the fly stops walking), and sharp turns (number of times that the fly changes direction with a velocity > 60 degree/s).

2.8. Climbing

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (20 flies/vial). After 7 days, flies were assessed for their climbing ability through a counter-current device, composed of 6 testing vials. Briefly, 20 males were put into the first vial, tapped to the bottom, and let climb vertically for 10 s. This practice was repeated five times. At the end of the entire procedure, the number of individuals that have remained in each vial was counted. The climbing index was calculated by normalizing the weighted performance of each group of flies to the maximum possible score (Greene et al., 2003).

2.9. Respiration

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (20 flies/vial). After 7 days, mitochondrial respiration was monitored at 30 °C using an Oxygraph-2 k high-resolution respirometer (OROBOROS Instruments) using a chamber volume set to 2 ml. Calibration with air-saturated medium was performed daily. Five male flies per genotype (equal weight) were homogenized in respiration buffer (120 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 4 mM KH_2PO_4, 2 mM $MgCl_2$, and 1 mM EGTA, 1 g/l fatty acid-free BSA, pH 7.2). For coupled (state 3) assays, complex I-linked respiration was measured at saturating concentrations of malate (2 mM), glutamate (10 mM) and ADP (2.5 mM) Complex II-linked respiration was assayed in respiration buffer supplemented with 0.15 μM rotenone, 10 mM succinate and 2.5 mM ADP. Obtained values were normalized for total protein levels.

2.10. Mitochondrial isolation

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food (200 flies/vial). After 7 days, approximately 200 males were homogenized in 7 ml of BSA-added Mannitol-Sucrose buffer pH 7.4 (225 mM D-Mannitol, 75 mM Sucrose, 1 mM Hepes, 5 mM EGTA pH 8, 1% BSA) with 15 strokes at 700 rpm. The fly homogenate was centrifuged at 1000 x*g* for 10 min at 4 °C and the supernatant was subsequently filtered through a fine cotton mesh and centrifuged at 6000 x*g* for 10 min at 4 °C. Pellet was then washed with 10 ml of BSA-added Mannitol-Sucrose buffer and centrifuged at 6000 x*g* for 10 min at 4 °C. This step was repeated using Mannitol-Sucrose Buffer without BSA addition and centrifuged at 7000 x*g* for 10 min at 4 °C. The obtained mitochondrial pellet was resuspended in 500 μl of Mannitol-Sucrose Buffer without BSA and quantified with BCA kit to estimate the mitochondrial protein concentration.

2.11. Blue Native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was performed to evaluate abnormalities in complexes abundance. Briefly, mitochondria-enriched fractions previously prepared were subjected to membrane solubilization to extract the mitochondrial complexes. The mitochondrial pellet was resuspended using Solubilization Buffer (1.5 M aminocaproic acid, 50 mM Bis-Tris/HCl pH 7.0) to obtain a final concentration of 10 mg of mitochondrial protein/ml and solubilized with 4 mg digitonin per mg of protein. After 5 min of incubation on ice, mitochondria were then centrifuged at 20000 x*g* at 4 °C for 30 min. Supernatants were transferred into clean tubes and mixed with 10 μl of Blue-Native Sample Buffer (0.75 M aminocaproic acid, 50 mM Bis-Tris/HCl pH 7.0, 0.5 mM EDTA, 5% Serva Blue G). The equivalent volume of 50 μg was subsequently run in NativePAGE™ 3–12% Bis-Tris Protein Gels (Thermo Fisher Scientific). The gel was subsequently stained with Staining Solution (0.2% Coomassie Blue G250, 50% methanol, 7% acetic acid) to reveal mitochondrial complexes. After destaining (20% methanol and 7% acetic acid), the gel was scanned.

2.12. Complex I in-gel activity

Complex I in-gel activity was performed after complexes separation by BN-PAGE as previously described. Briefly, the gel was rinsed quickly with ultrapure water and incubated with Complex I Reaction Solution (5 mM Tris–HCl pH 7.4, 0.14 mg/ml NADH, 1 mg/ml Nitro Blue Tetrazolium) for about 10 min in a rocking shaker at room temperature. After the band signal has reached the desired intensity, the gel was washed with ultrapure water to stop the reaction and scanned for quantification analysis by ImageJ.

2.13. ATP and ADP quantification by High-Pressure Liquid Chromatography (HPLC)

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food. After 7 days, flies were kept in standard conditions or starved for 24 h. Subsequently, a pool of 10 flies was weighed, homogenized in 400 μl 0.6 M perchloric acid and neutralized with 71.5 μl of 2 M potassium carbonate. Samples were centrifuged at 14,000 x*g* for 10 min at 4 °C and the supernatant was immediately subjected to HPLC analysis according to Menegollo et al. 2019 (Menegollo et al., 2019). ATP and ADP were separated on a Waters 2695 Separation Module (Milfold, MA, USA) with a Waters 996 Photodiode Array Detector equipped with a Phenomenex Jupiter C18 column (particles diameter 5 μm, porosity 300 Å, 250 × 4.6 mm) and guard column (3 μm, 4,6 × 3 mm). The mobile phase consisted of ammonium dihydrogen phosphate pH 6.0, 1% methanol. Nucleotides were monitored by UV detection ($\lambda = 254$ nm) and the peaks were analyzed with Origin software (8.5.1). To determine ATP and ADP concentrations of the fly extract, standard curves of the adenosine nucleotides were used as reference. The values obtained were then normalized to the total weight of the fly pool.

2.14. Western Blot

Fly bodies were mechanically lysed in 50 mM Tris-HCl pH 7.5, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM EGTA, and 270 mM sucrose buffer added with protease inhibitors (Roche) and incubated on ice for 30 min. Subsequently, lysates were cleared by centrifugation at 20000 x*g* for 30 min at 4 °C. Supernatants were used to quantify protein by using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). For each sample, 50 μg of protein were loaded in 13% Tris-glycine polyacrylamide gels in SDS/Tris-glycine running buffer or ExpressPlus™ PAGE 4–20% gels (GenScript), in MOPS running buffer, according to the size-resolution required. The resolved proteins were transferred to polyvinylidenedifluoride membranes (Bio-Rad), through a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were subsequently blocked in Tris-buffered saline plus 0.1% Tween (TBS-T) and 5% non-fat milk for 1 h at 4 °C. The membranes were then washed in TBS-T and subsequently incubated overnight at 4 °C with primary antibodies in TBS-T plus 5% non-fat milk. Membranes were then washed in TBS-T and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated α-mouse or α-rabbit IgG. Blots were then washed in TBS-T (3 × 10 min) at room temperature and rinsed in TBS-T, and immunoreactive proteins were visualized using Immobilon® Forte Western HRP Substrate (Merck Millipore). Densitometric analysis was carried out using ImageJ software. The antibodies used for Western Blot are as follows: mouse β-actin (Sigma Prestige 1:2000), mouse ATP5-α (Abcam: ab14748, 1:10000), rabbit GABARAP/Atg8a (Abcam: ab109364, 1:1000), rabbit p-AMPK

(Thr172) (Cell Signalling: 2535, 40H9), rabbit dj-1β (a generous gift from Prof. Yuzuru Imai, University of Juntendo, 1:5000).

2.15. LysoTracker staining

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food. After 7 days, flies were kept in standard conditions or starved for 24 h. To evaluate the lysosomal content, fly brains were dissected in phosphate-buffered saline (PBS) followed by 15 min of incubation with 200 nM LysoTracker™ Red DND-99 probe (Thermofisher Scientific, L7528) in the dark. Subsequently, brains were washed thrice in PBS for 5 min, mounted in Mowiol® 4–88 (Calbiochem, 475,904), and immediately analyzed. Images were acquired utilizing either Zeiss LSM770 confocal microscope equipped with Plan Apochromat 40 × 1.3 NA objective by using a 561 nm laser (fed condition) or LeicaSP5 confocal microscope (Leica Microsystems) equipped with a 40 × 0.5 NA objective by using a 543 nm laser (starvation). The area of the brain occupied by LysoTracker staining and the number of acidic compartments was quantified by ImageJ.

2.16. Quantification of autolysosomes by using the GFP-mCherry-Atg8a autophagy reporter

Adult males (1–3 day-old) were collected under brief CO_2 exposure and transferred to new tubes containing standard food. After 7 days, fly brains were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 1 h. Subsequently, brains were washed thrice in PBS for 5 min, mounted in Mowiol® 4–88 (Calbiochem, 475,904), and analyzed. Images were acquired utilizing LeicaSP5 confocal microscope (Leica Microsystems) equipped with a 40 × 0.5 NA objective by using a 543 nm laser. The number of mCherry-positive puncta were quantified by ImageJ.

2.17. Transmission Electron Microscopy (TEM)

To assess the mitochondrial morphology, flies were fixed for 2 h in 0.1 M sodium cacodylate (pH 7.4), containing 2.5% glutaraldehyde and 2% paraformaldehyde, and then dissected to isolate thoraces. Briefly, the head, legs, and wings were removed with forceps, taking care of removing the gut as well. The dissected thoraces were then fixed in the same fixative solution overnight at 4 °C. Dissected thoraces of flies grown under basal conditions were fixed in 0.1 M sodium cacodylate (pH 7.4), containing 2.5% glutaraldehyde and 2% paraformaldehyde, overnight at 4 °C. Subsequently, samples were incubated with a solution of 1% of tannic acid for 1 h at room temperature and then post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. After three water washes, samples were dehydrated in a graded ethanol series and embedded in an epoxy resin (Sigma-Aldrich). Ultrathin sections (60–70 nm) were obtained with an Ultrotome V (LKB) ultramicrotome, counterstained with uranyl acetate and lead citrate and viewed using a Tecnai G2 (FEI) transmission electron microscope, operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera.

2.18. Analysis of mitochondrial morphology

Mitochondrial morphology and electron density were evaluated by analyzing the TEM images with Fiji. Mitochondrial boundaries were manually annotated, saved as different regions of interest (ROI), and relative morphology was assessed by measuring area (μm^2) and circularity (0–1) as shape descriptors. Data were then plotted as frequency distributions and fitted by using the function sum of Gaussians. Fitting was performed by using OriginLab software (version 2019). Mitochondrial electron density was calculated by using the function "Histogram" which results in the pixel distribution of the selected ROI over a 0–255 Gy range. Obtained values were normalized for the mitochondria area (total number of pixels counted per organelle) and for the picture contrast by dividing for the mean gray value of the muscular fibers. Values were then converted into the percentage pixel distribution over the 0–255 pixel range. Electron density values were also divided into three subgroups, referred to as high (0–85), intermediate (86–170), and low (171–255) electron density, and the percentage of mitochondria belonging to each group was determined for both genotypes and plotted.

2.19. Statistical analysis

Graphs and statistical analyses were performed by using GraphPad Prism 7. Generally, data are represented as box plots showing median, 25th and 75th percentiles, minimum and maximum values. Non-linear fitting of mitochondrial morphology parameters was carried out by utilizing OriginLab software (version 2019). Statistical difference among mean values was assessed by Mann-Whitney test (two groups) and by the Kruskal-Wallis test, with the Dunn's multiple comparisons test or One-Way ANOVA test, with Bonferroni's multiple comparisons test (three groups). For grouped analysis two-way ANOVA test, with Bonferroni's multiple comparison test, was used. Statistical significance was defined for p-value <0.05 (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Additional details are reported in the legend of each figure.

3. Results

3.1. Loss of dj-1β impairs the fly free locomotor ability and redox balance

The fly genome encodes two *DJ-1* homologs genes, referred to as *dj-1α* and *dj-1β*. While expression of the former is mainly restricted to testes, the latter is widely expressed in all tissues, similar to the human protein (Meulener et al., 2005). Moreover, only dj-1β has been shown to protect against oxidative insults, supporting the hypothesis that this homolog better recapitulates the function of human DJ-1 (Meulener et al., 2005; Meulener et al., 2006). To get insights into the role of DJ-1 in *Drosophila*, we exploited *dj-1β*$^{\Delta 93}$ strain, a null mutant strain carrying a 1960 bp deletion in the gene encoding the fly protein dj-1β, which was previously generated (Meulener et al., 2005). Under basal conditions, adult flies lacking the expression of *dj-1β* (Fig. 1a) do not present overt abnormalities, displaying a lifespan (Fig. 1b) and climbing performance (Fig. 1c) comparable to that of age-matched controls (w^{1118}). To investigate fine behavioral aspects, we recorded the free locomotion of flies in an open field arena. Differently from the climbing apparatus, which only assesses provoked negative geotaxis, with this set-up, fruit flies are free to move in a two-dimension space without external stimulus, allowing the evaluation of fine locomotor parameters. More specifically, within a set time, we analyzed the length walked, the maximal velocity, the number of times that flies start moving, and changes in the walking direction. Representative images of the fly traces showed that while control individuals tend to uniformly move around the arena, *dj-1β* knockout (KO) mutant flies display a reduced exploratory activity and spend most of their time near the arena edge, suggesting mild locomotor impairments (Fig. 1d). Moreover, our data indicated that *dj-1β* null mutant flies move less and with lower velocity, when compared to controls (Fig. 1e-f). The lack of *dj-1β* is also associated with a higher number of changes in direction and path stops (Fig. 1g-h), indicative of uncoordinated and discontinuous locomotion, reminiscent of the disease condition. Therefore, the absence of *dj-1β* impairs the free locomotor behavior, suggesting a role played by the protein in movement coordination.

As DJ-1 has been recurrently involved in redox homeostasis, in parallel to the locomotion analysis, we evaluated whether loss of dj-1β impairs the fly redox state by staining *Drosophila* brains with the reactive oxygen species (ROS) probe dihydroethidium (DHE), which emits fluorescence upon reaction with superoxide anions. In agreement with

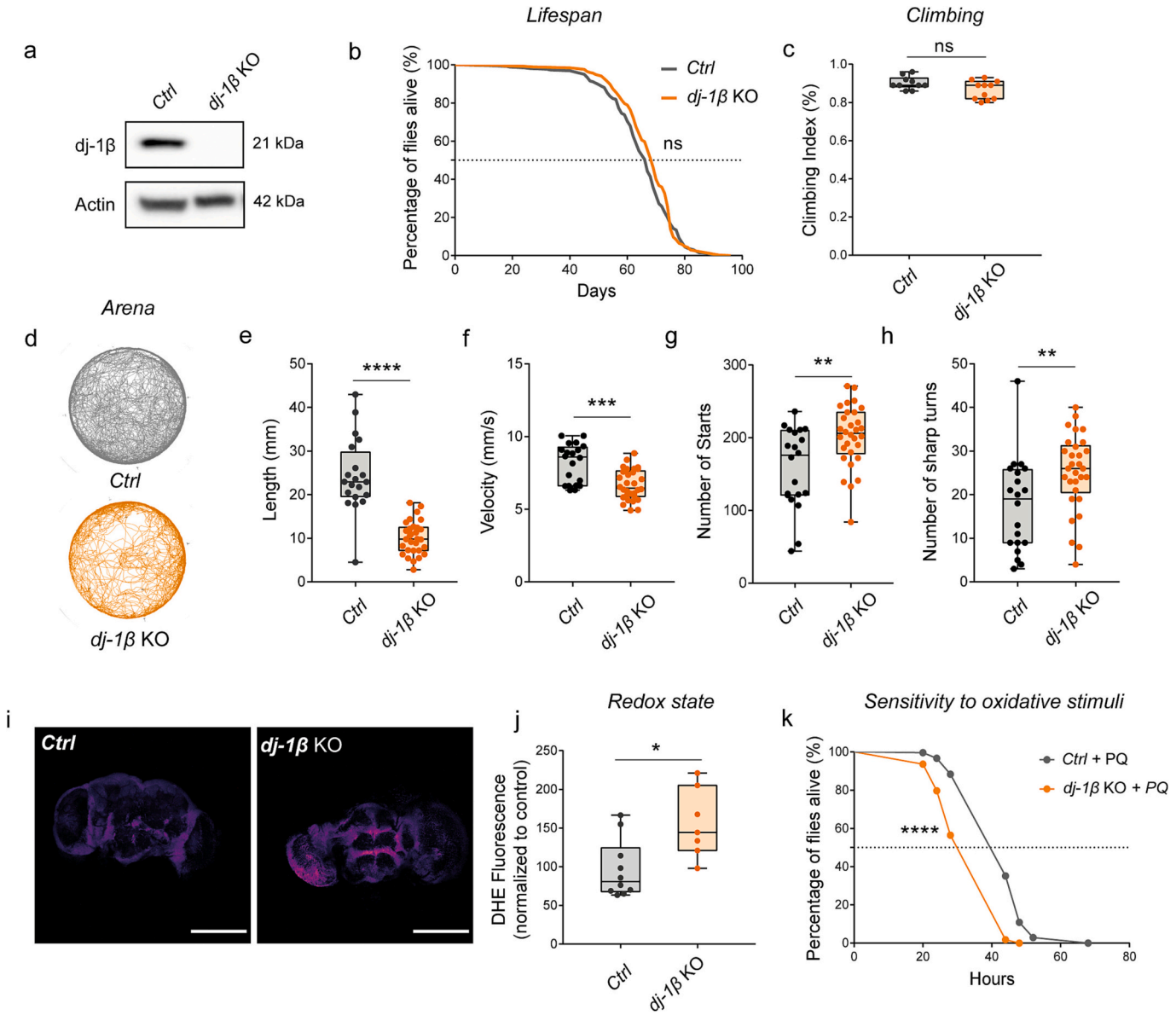

Fig. 1. Phenotypic characterization of *dj-1β* null *Drosophila*.
(a) Western blot showing the lack of *dj-1β* expression in KO flies. Actin was used as loading control. (b) Lifespan of *dj-1β* null flies as compared to control individuals (*Ctrl*). Analysis has been performed by using 260 *Ctrl* and 370 *dj-1β* KO flies. Log-rank (Mantel-Cox) test, ns (non significant). (c) Climbing ability of *dj-1β* null flies as compared to controls, $n = 10$ *Ctrl* and $n = 11$ *dj-1β* KO flies, each dot represents a pool of 20 flies. Results have been analyzed by unpaired *t*-test, ns (non significant). (d-h) Fly locomotor behavior in open field arena. (d) Representative locomotor traces of control (upper chart) and *dj-1β* deficient flies (lower chart) in arena. (e) Length walked by each fly expressed in millimeter (mm), (f) maximal velocity reached by each fly expressed in millimeter/ s (mm/s), (g) number of start (number of times that each fly started to move), (h) number of sharp turns (number of times that each fly changed direction). For each genotype, 20 flies were analyzed. Data are presented as box and whisker and have been analyzed by Mann-Whitney test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (i) Pseudocolor representative images (scale bar: 200 μm) and (j) relative quantification of adult *Drosophila* brains stained with DHE ROS reporter. Data are presented as box and whisker and have been analyzed by Mann-Whitney test, * $p < 0.05$. $n = 10$ *Ctrl* and $n = 7$ *dj-1β* null flies, each dot represents a fly brain. (k) Sensitivity to pro-oxidant stimuli. Survival rate of control and *dj-1β* null flies exposed to 20 mM paraquat (PQ). Analysis has been performed by using 239 *Ctrl* and 280 *dj-1β* KO flies. Statistical significance was tested by Log-rank (Mantel-Cox) test, **** $p < 0.0001$.

other studies performed on different *dj-1β* KO strains (Casani et al., 2013; Lavara-Culebras et al., 2010; Stefanatos et al., 2012), *dj-1β* null mutant flies show increased ROS levels as compared to control individuals (Fig. 1i-j). Additionally, loss of *dj-1β* affects the fly resistance to oxidative stressors, such as the pro-oxidant molecule paraquat, which generates superoxide radicals upon redox cycling (Cochemé and Murphy, 2008) (Fig. 1k). Collectively, these data indicate that the *Drosophila* DJ-1 homolog *dj-1β* plays a role in the regulation of the fine locomotor behavior and corroborates the involvement of the protein in maintaining the redox balance.

3.2. Loss of dj-1β causes morphological changes at the mitochondrial level

The observed alterations in the locomotor behavior and redox homeostasis might be attributable to bioenergetic deficits. As DJ-1 has been associated with mitochondrial homeostasis (Dolgacheva et al., 2019), we next investigated organelle integrity in *dj-1β* KO flies. To this end, we imaged by electron microscopy the fly thoracic muscles, in which a high abundance of mitochondria are regularly distributed, enabling a fine morphological evaluation of the organelle sections. We also searched for possible anatomical abnormalities in the muscle fibers

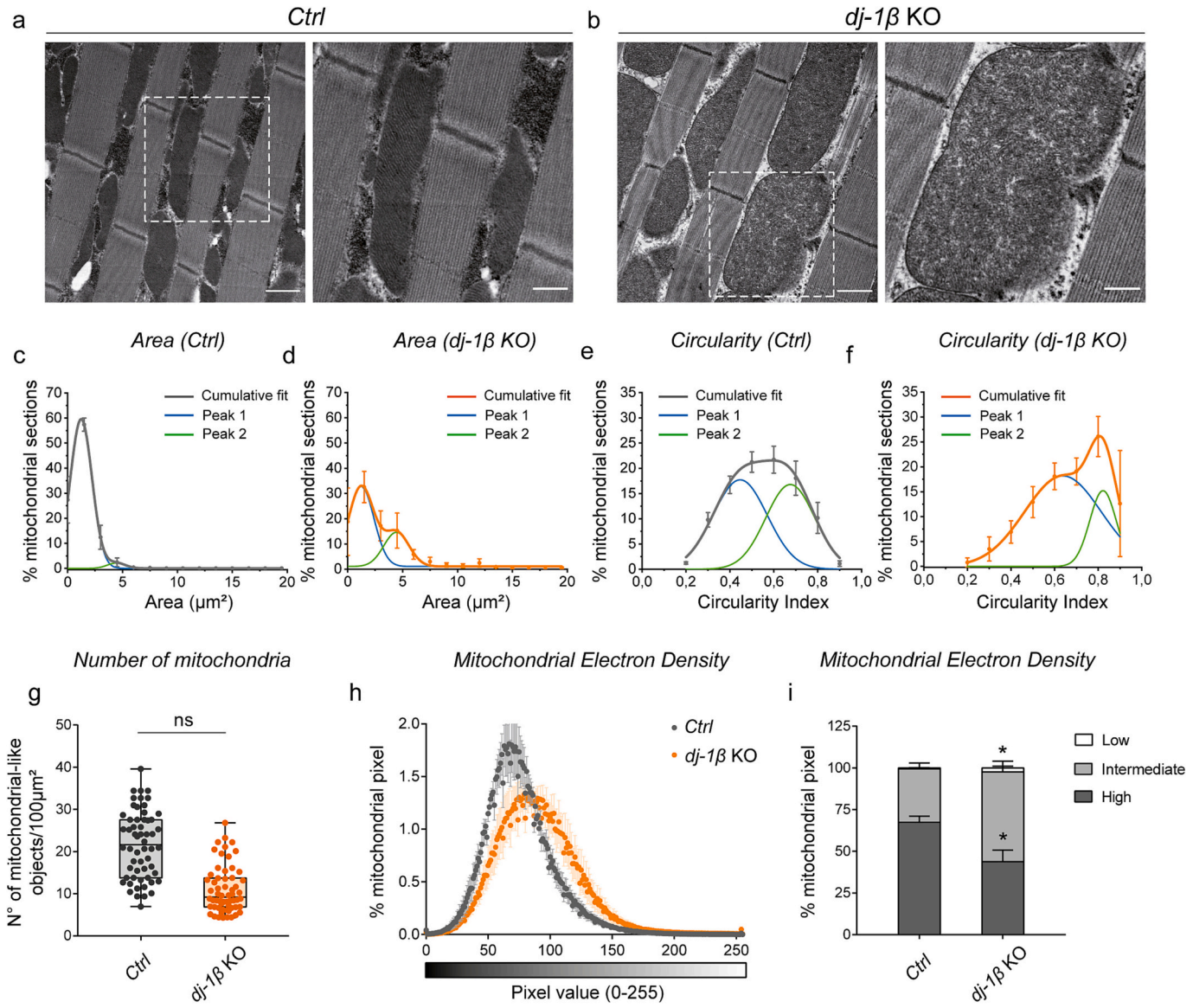

Fig. 2. The loss of function of dj-1β affects the mitochondrial morphology and ultrastructure.
(a,b) Representative electron microscopy images of thoracic muscle mitochondria (left panel, scale bar: 1 μm) and relative magnified pictures (right panel, scale bar: 500 nm) of (a) control and (b) *dj-1β* KO flies. $n = 3$ and $n = 4$ fly thoraxes were analyzed respectively for *Ctrl* and *dj-1β* null individuals. (c,d,e,f) Morphological analysis of the mitochondrial shape. Frequency distribution of mitochondrial (c,d) area and (e,f) circularity of (c,e) control and (d,f) *dj-1β* null flies. Data are presented as mean ± SEM of independent biological replicates and have been fitted by using sum of gaussian function. Blue (peak 1) and green (peak 2) curves represent single gaussian fitting, while gray and orange curves indicate cumulative fitting, respectively for control and *dj-1β* null flies. (g) Number of mitochondrial-like objects per 100 μm^2. Results are presented as box and whisker, showing pooled data, and have been analyzed by Mann-Whitney test, ns (non significant). At least 12 fields of view have been analyzed per fly. (h,i) Analysis of the mitochondrial electron density. (h) Percentage distribution of mitochondrial pixel in the 0–255 pixel range, where 0 indicate black pixels (more electron dense organelles) and 255 white pixels (less electron dense organelles). (i) Classification of mitochondrial pixels according to relative electron density into three subgroups: high (0–85), intermediate (86–170), and low (171–255) electron density. Data are presented as mean ± SEM of three (*Ctrl*) or four (*dj-1β* KO) independent replicates. Statistical significance was tested by Two-way ANOVA with Bonferroni's post-hoc test, * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that could explain the observed locomotion defects. As reported in Fig. 2a-b, *dj-1β* null mutant individuals do not present evident muscular abnormalities, indicating that the absence of *dj-1β* does not seem to affect the muscular integrity and that the altered locomotor behavior is not caused by muscular degeneration. However, in contrast to control individuals which display tightly packed mitochondria between the muscular fibers and with densely organized cristae, *dj-1β* KO flies exhibit an altered mitochondrial morphology. A more detailed quantitative analysis of the mitochondrial area revealed that two major mitochondrial subpopulations are present, one centered at 1.2 μm^2 (peak 1, blue curve) and another one at 4.4 μm^2 (peak 2, green curve). While control flies present almost exclusively smaller mitochondrial sections (~97%) and only a minority of larger structures (~3%), in *dj-1β* null mutant flies the percentage of large sections is increased (~30%) (Fig. 2c-d). Additionally, the evaluation of the circularity index showed that the absence of dj-1β causes an increased number of spherical mitochondrial structures (circularity index >0.8), differently from controls that have a more elongated shape (circularity index <0.7) (Fig. 2e-f). Collectively, the lack of dj-1β induces larger and more circular mitochondrial sections, with a trend towards a reduced number of structures per unit of area (Fig. 2g). Moreover, we observed that *dj-1β* null mutant flies display a mild reduction in the mitochondrial electron density. Therefore, to

investigate mitochondrial ultrastructure, we also assessed the mitochondrial electron density, as a parameter reflecting the internal mitochondrial organization (Chakraborty et al., 2018). The analysis of the distribution of pixel intensities revealed that while control flies present a high percentage of electrodense (darker) organelles, *dj-1β* KO *Drosophila* show a slight reduced cristae density (paler organelles), indicating that dj-1β may also have a role in maintaining the mitochondrial ultrastructure (Fig. 2g-i). Taken together, our data indicate that the loss of dj-1β influences mitochondria shape and density, inducing a general rearrangement of the organelle architecture.

3.3. Loss of dj-1β does not affect the ATP levels

Changes in mitochondrial morphology and ultrastructure were reported to impact the organization of the respiratory complexes with consequent effects on mitochondrial functionality (Cogliati et al., 2016). Based on the observed morphological differences, we wondered whether *dj-1β* KO flies may display some respiratory deficits. To this end, we first separated the mitochondrial complexes by Blue Native electrophoresis to evaluate possible impairments in the electron transport chain (ETC) organization, but this revealed no obvious alteration in abundance of the respiratory chain complexes in *dj-1β* null mutant flies (Fig. 3a). However, respirometry analysis showed a decreased complex-I linked oxygen consumption rate (OCR), while complex-II linked respiration was unaltered (Fig. 3b). In light of previous *in vitro* studies supporting a stimulatory role of DJ-1 towards complex I activity (Hayashi et al., 2009; Zhang et al., 2018) and considering our respirometry data, we next wondered whether dj-1β deficiency causes impairments in the activity of complex I. As shown in Fig. 3c, we did not find any significant alteration in complex activity, suggesting that dj-1β may not be involved in the regulation of the complex functionality *per se* but it may play a role in regulating the electron flux through complex I or its coordination into supercomplexes. To investigate the bioenergetic status of *dj-1β* KO flies, we next quantified ATP and ADP levels by high-pressure liquid chromatography (HPLC) (Fig. 3d). However, we could not observe differences in ATP and ADP pools in *dj-1β* null mutant flies (Fig. 3e). Although mitochondria account for most of the ATP production, we cannot rule out that other pathways may be involved, such as glycolysis. Therefore, we measured ADP and ATP levels after 24 h of starvation, to reduce the glycolytic contribution and boost the mitochondrial-derived energy production. Also in this case, we did not reveal evident differences in the nucleotide pools between control and KO flies (Fig. 3e-f), implying that the loss of the protein does not alter ATP levels. Overall, our data demonstrate that dj-1β influences complex I-linked OCR, without interfering with the respiratory chain stability and ATP levels.

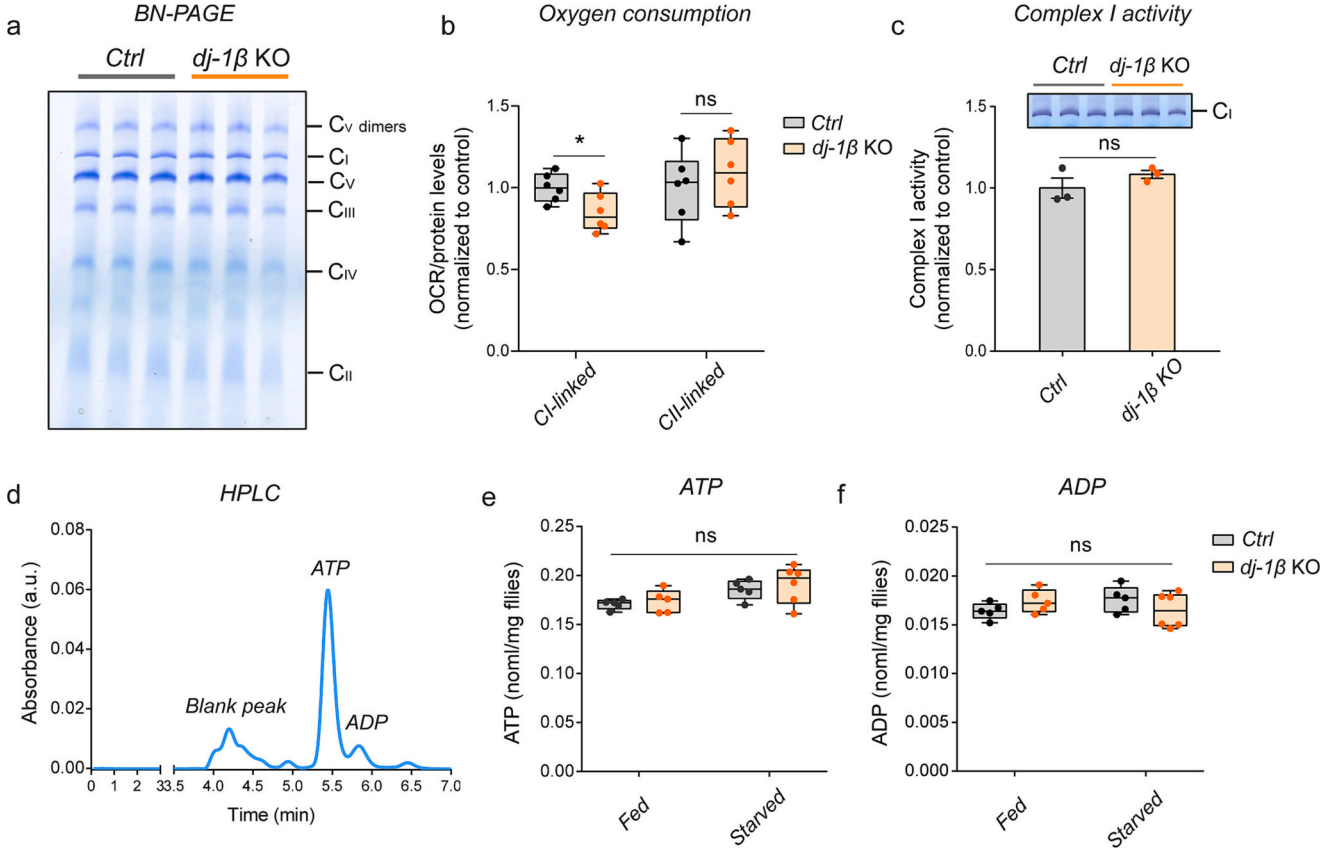

Fig. 3. The absence of dj-1β mildly impacts on the mitochondrial function.
(a) Analysis of the mitochondria complexes organization by Blue-Native Page (BN-PAGE) in control and *dj-1β* KO flies. Mitochondria were extracted from 250 flies and 50 μg of mitochondrial extract was loaded in gel. Each lane indicates an independent experiment, n = 3 for both genotypes. (b) Oxygen consumption rate (OCR) of complex I ($n = 6$) and complex II ($n = 5$). Each measurement derived from a pool of 5 flies. Data are presented as box and whisker of OCR values normalized to protein levels. Statistical significance was measured by unpaired t-test, ns (non significant), * $p < 0.05$. (c) Complex I in gel activity. Data are presented as mean ± SEM of three independent replicates. Statistical significance was test by Mann-Whitney test, ns (non significant). (d,e,f) High pressure liquid chromatography (HPLC) measurements of ATP and ADP in fly lysate. (d) Representative chromatogram of a fly lysate, showing separation of ATP and ADP pools. Quantification of (e) ATP and (f) ADP levels in fly lysates under fed and starved (24 h) conditions, expressed as nanomoles per mg of flies (nmol/mg flies). Data are presented as box and whisker derived from at least five independent replicates and have been analyzed by Two-way ANOVA with Bonferroni's post-hoc test, ns (non significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

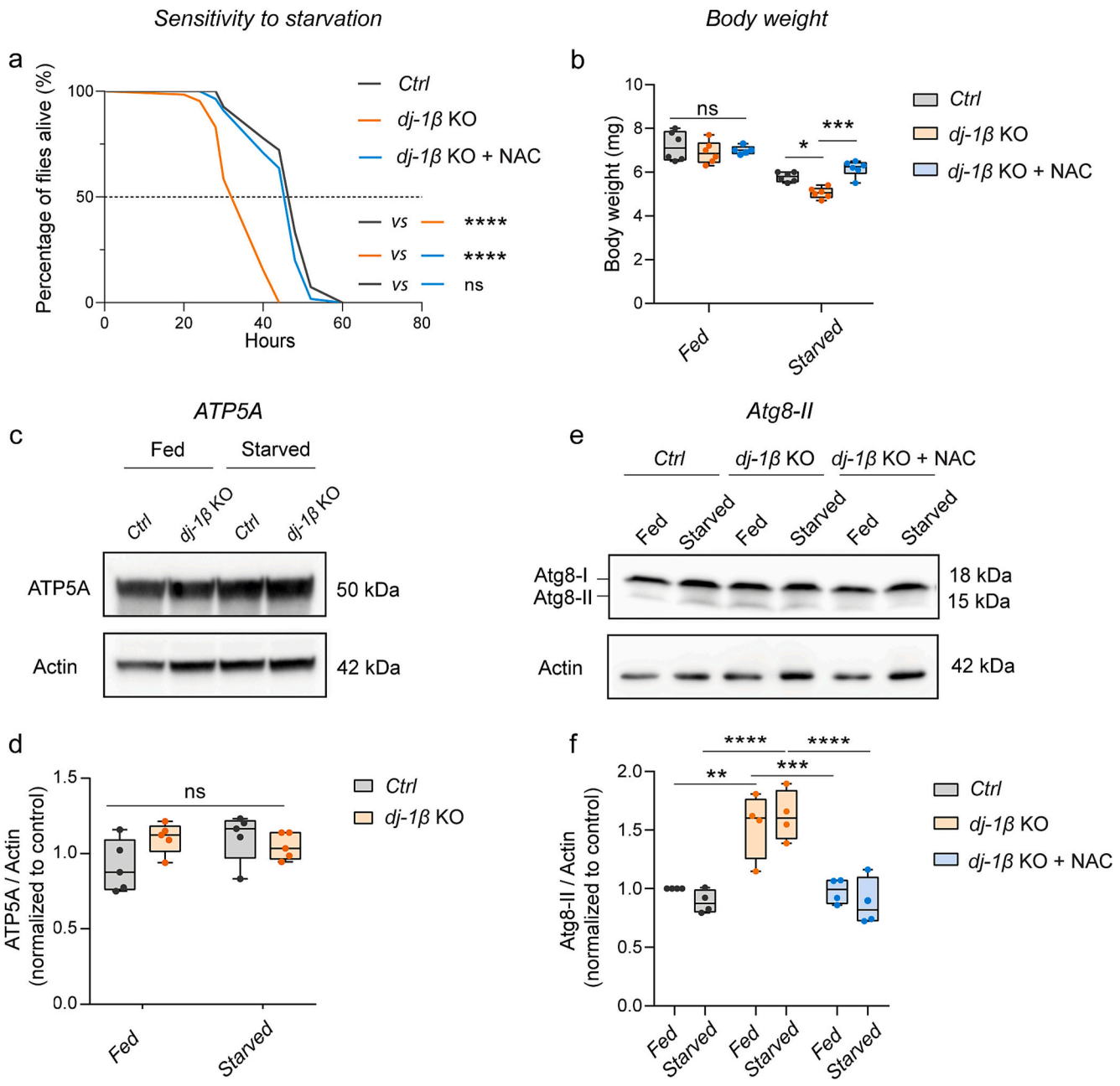

Fig. 4. The loss of dj-1β impairs the fly response to starvation by altering the redox balance.
(a) Survival rate of *Ctrl*, *dj-1β* KO, and *dj-1β* KO flies treated with NAC (*dj-1β* KO + NAC) flies under starvation. Groups of twenty flies, raised in standard food, have been transferred into vials with 1.5% agar to evaluate resistance to starvation. Analysis has been performed by using 54 *Ctrl* flies, 65 *dj-1β* KO and 55 *dj-1β* KO + NAC flies. Statistical significance has been measured by Log-rank (Mantel–Cox) test, **** $p < 0.0001$. (b) Body weight measurements of *Ctrl*, *dj-1β* KO, and *dj-1β* KO + NAC flies under fed and starved conditions. Body weight was measured on pools of ten flies kept on standard food or starved for 24 h. Data are presented as box and whisker derived from at least five independent replicates. Statistical significance was tested by using Two-way ANOVA with Bonferroni's post-hoc test, ns (non significant), * $p < 0.05$, *** $p < 0.0001$. Western blot analysis and relative quantification of (c-d) ATP5A and (e-f) Atg8-II (LC3-II) levels in *Ctrl*, *dj-1β* KO, and *dj-1β* KO + NAC flies under fed and starved conditions. Actin was used as loading control. Data are presented as box and whisker derived from at least four independent replicates and have been analyzed by Two-way ANOVA with Bonferroni's post-hoc test, ns (non significant), ** $p < 0.01$, *** $p < 0.0001$, **** $p < 0.0001$.

3.4. dj-1β null mutant flies are sensitive to nutrient deprivation and display autophagic impairments

Despite the lack of impairments in ATP production, when performing the experiments described above, we noticed that *dj-1β* null mutant individuals were more susceptible to food deprivation. As represented in Fig. 4a, *dj-1β* KO flies have a higher mortality rate as compared to control individuals, showing first death events after 20–24 h of starvation. Moreover, *dj-1β* null mutant flies lose more weight than age-matched controls under food deprivation (Fig. 4b), suggesting some dysregulations at the catabolic level. Importantly, these phenotypes, along with the altered redox state, are rescued by re-expressing dj-1β, confirming that this effect is specifically due to a role of dj-1β in response to food deprivation (Supplementary Fig. 1a-c). As starvation represents a major trigger for autophagy, we sought to investigate the autophagic response, starting from the evaluation of the mitochondrial mass, as a readout of mitochondrial autophagy (mitophagy). Specifically, to investigate mitochondrial content, we assessed the levels of the protein

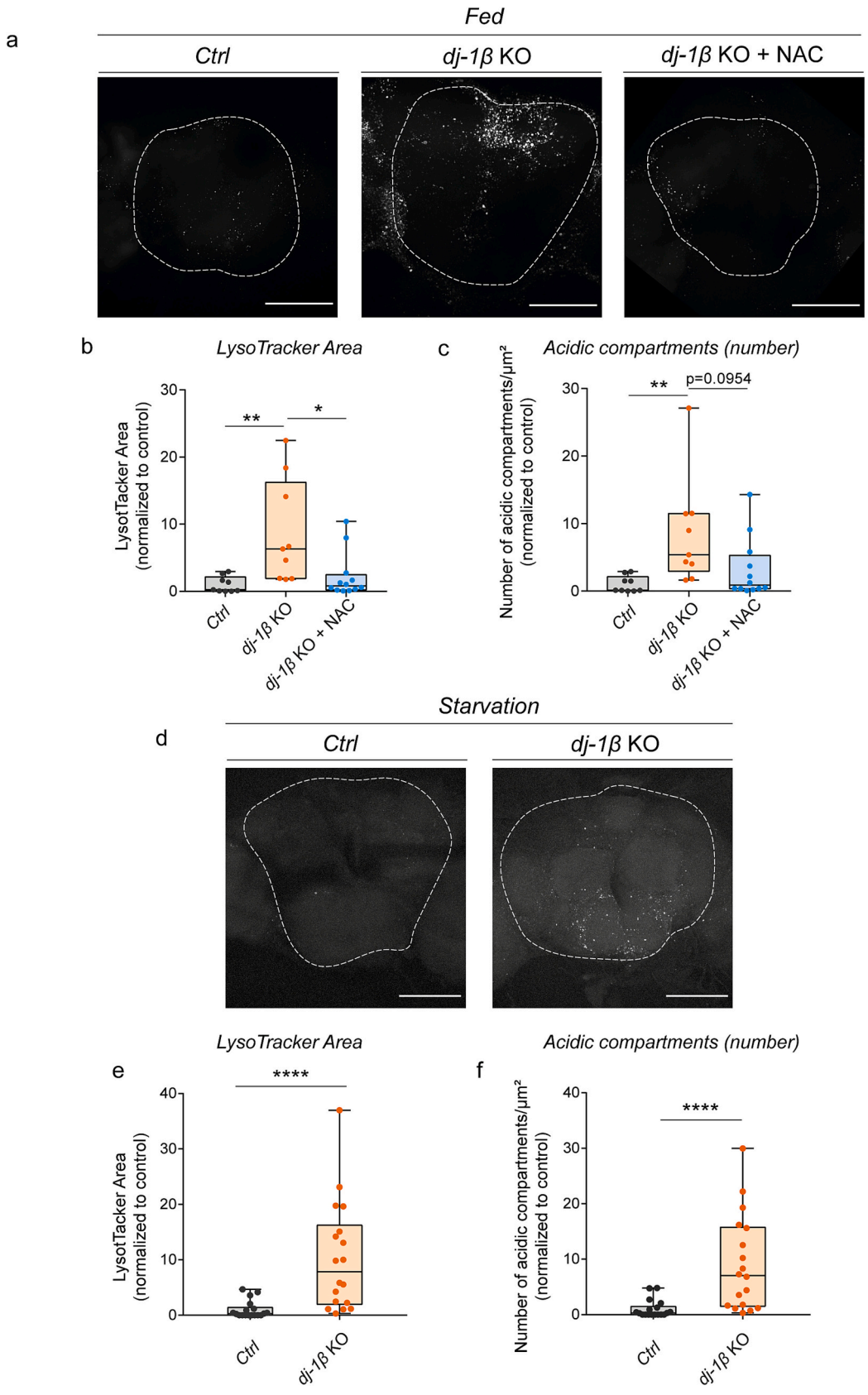

Fig. 5. The loss of dj-1β alters the autophagic-lysosomal pathway by increasing the number of acidic compartments in a ROS-dependent manner.
(a,d) Pseudocolor representative images (scale bar: 100 µm) and (b-c, e-f) relative quantification of adult *Drosophila* brains stained with LysoTracker Red probe under (a-c) fed conditions and after (d-f) 24 h of starvation. NAC treatment was evaluated under fed condition. Data are presented as box and whisker and have been analyzed by (b-c) Kruskal-Wallis test, with Dunn's multiple comparison * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ or (e-f) Mann-Whitney test, **** $p < 0.001$. Each dot represents a fly brain. For each genotype, at least 9 fly brains were analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

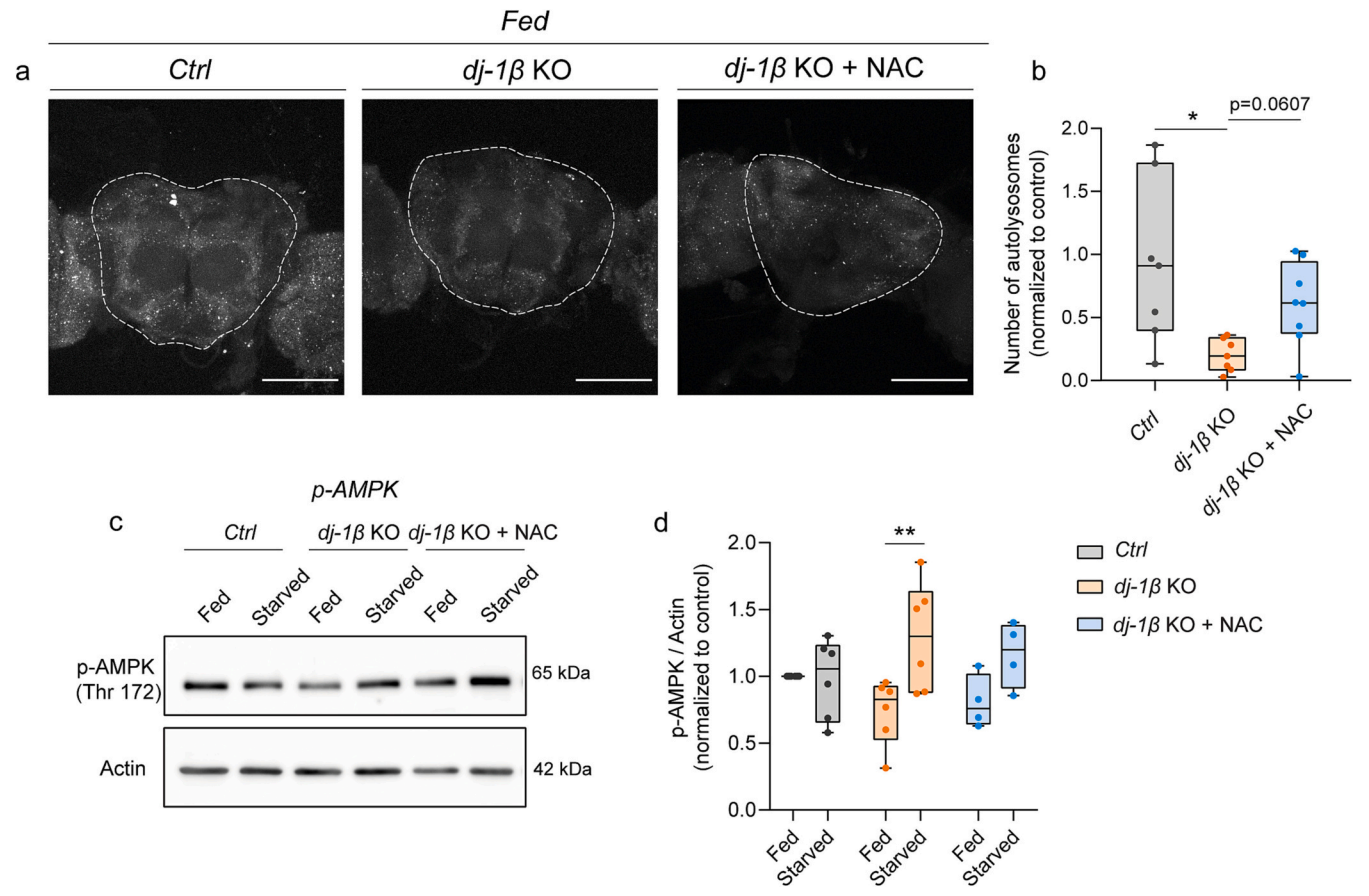

Fig. 6. In the absence of dj-1β the autophagosome-lysosome fusion rate is impaired in a ROS-associated mechanism, while the hyperactivation of p-AMPK, observed under dj-1β deficiency, is ROS-independent.
(a) Pseudocolor representative images (scale bar: 100 μm) of adult *Drosophila* brains expressing GFP-mCherry-Atg8a autophagy reporter and (b) relative quantification. Images show mCherry signal, as an indication of the autolysosomal content. Data are presented as box and whisker and have been analyzed by Kruskal-Wallis test, with Dunn's multiple comparison, * $p < 0.05$. For each genotype, at least 7 fly brains were analyzed. (c-d) Western blot analysis of phosphorylated AMPK (Thr 172) levels in *Ctrl, dj-1β* KO, and *dj-1β* KO + NAC under fed and starved conditions. Actin was used as loading control. Data are presented as box and whisker derived from four independent replicates and have been analyzed by Two-way ANOVA with Bonferroni's post-hoc test, ns (non significant), ** $p < 0.01$.

ATP5A, which represents a catalytic subunit of the complex V. We found that the absence of dj-1β does not alter the mitochondrial mass, not even under starvation (Fig. 4c). Since the mitochondrial content is not affected, we then evaluated the levels of the protein microtubule-associated protein 1 light chain 3 (LC3), as a general readout of autophagy. In conditions of active autophagy, the cytosolic form of the protein, referred to as LC3-I, is converted into the lipidated form LC3-II, through phosphatidylethanolamine conjugation, and targeted to the autophagosome (Yoshii and Mizushima, 2017). Interestingly, we observed that *dj-1β* null mutant flies present higher levels of Atg8-II (the fly homolog of LC3-II) under both fed and starved conditions. (Fig. 4d). Overall, these results indicate that *dj-1β* null individuals may accumulate autophagosomes, suggesting some autophagic alterations. As we have reported that *dj-1β* null flies present an altered redox state (Fig. 1i-j), we successively sought to evaluate whether the phenotypes observed are ROS-mediated. Therefore, flies were treated with the antioxidant molecule N-Acetyl-L-Cysteine (NAC). As expected, NAC treatment rescues ROS levels in *dj-1β* null flies (Supplementary Fig. 2c-d) but, more importantly, we observed that it ameliorates the sensitivity of *dj-1β* null flies to starvation, body weight loss, and Atg8-II levels (Fig. 4a, b and d). Of note, NAC did not show effects on control flies starvation sensitivity or body weight. Thus, these results suggest that the increased susceptibility of *dj-1β* null individuals to food deprivation could be primarily caused by impaired redox homeostasis.

3.5. *The absence of dj-1β alters the autophagic-lysosomal response and stimulates the activation of AMPK under starvation*

During autophagy, newly formed autophagosomes, containing dysfunctional or old cellular components, fuse with lysosomes, which are acidic organelles enriched in hydrolytic enzymes (Galluzzi et al., 2017). To explore the possible accumulation of autophagic organelles, we analyzed lysosomal-like compartments in the fly brain by confocal microscopy. To this end, we stained the brains of *dj-1β* KO and control flies with LysoTracker, an acidotropic probe that labels acidic cellular compartments, namely lysosomes and autolysosomes (DeVorkin and Gorski, 2014). We observed that in fed *dj-1β* null mutant flies, the area occupied by and the number of acidic compartments is significantly increased as compared to control brains (Fig. 5a-c) and that these compartments are even more numerous upon starvation (Fig. 5d-f). Of note, this effect is reversed by re-expressing dj-1β, further supporting the role of the protein in autolysosomes/lysosomes regulation (Supplementary Fig. 1d-f). To further dissect the autophagic process, we then took advantage of the GFP-mCherry-Atg8a flux reporter, whose signal is influenced by the pH. Specifically, due to the low lysosomal pH, the GFP signal is rapidly quenched after autophagosome-lysosome fusion, thus autophagosomes are both GFP and mCherry positive, while autolysosomes are only positive for mCherry (Lőrincz et al., 2017). Although we could not identify both GFP- and mCherry-positive puncta (autophagosomes), we quantified mCherry-positive signal (autolysosomes) and

found that the absence of dj-1β significantly decreases the autolysosomal number (Fig. 6a-b). This finding may indicate that there is a reduced autophagosomal-lysosomal fusion in the absence of dj-1β, consistent with causing an accumulation of autophagosomes (Atg8-II levels). While there was a trend towards amelioration by NAC treatment, this did not reach significance (p-value = 0.0607). Finally, we evaluated the levels of the phosphorylated form of AMP-activated kinase (AMPK), which is a major inducer of the autophagic pathway. Conditions of stress, such as starvation, mitochondrial impairments, or excessive ROS production, are known to induce AMPK activation, mainly through phosphorylation of a threonine residue localized at position 172. The active form of the kinase then stimulates the activation of catabolic processes, such as autophagy, while inhibiting anabolic pathways, thus restoring energy balance (Li and Chen, 2019). While phosphorylated AMPK (p-AMPK) levels are similar between control and KO flies under basal conditions, we noticed that the level of p-AMPK significantly increases in starved *dj-1β* null mutant flies as compared to starved controls (Fig. 6c-d). We reasoned that the activation of p-AMPK may represent a compensatory response to promote the autophagic pathway. However, NAC treatment was not sufficient to significantly affect p-AMPK activation in these conditions. Together, these results implicate that loss of dj-1β alters the cell redox state, eventually affecting autophagy, and that cells may stimulate feedback compensatory mechanisms to sustain cargo degradation, though p-AMPK activation may rely on ROS-independent stimuli.

4. Discussion

Despite more than two decades of intense research on DJ-1, no consensus has been found on its precise cellular role. So far, DJ-1 has been described as a multifaceted protein placed at the crossroad of different mechanisms implicated in cellular protection, though the precise protective role still remains elusive. Here, we carried out a wide characterization of DJ-1 null *Drosophila*, aiming at better defining the physiological role of the protein from the neurodegenerative perspective. *Drosophila melanogaster* has for a long time contributed to untangle diverse aspects of human pathologies, especially in the field of neurodegenerative diseases (Bolus et al., 2020; Lu and Vogel, 2009). Indeed, the fly genome shares about 60% of homology with the human one, bearing numerous disease-associated genes. Moreover, fruit flies show a complex nervous system, allowing the investigation of neuronal populations and circuits relevant in human neurodegeneration, including evaluation of locomotor impairments, neuronal loss, changes in redox homeostasis, and autophagic alterations (Bolus et al., 2020; Lu and Vogel, 2009). Therefore, with our study, we exploited *Drosophila* to evaluate behavioral aspects and molecular pathways that could lie behind the physiological function of DJ-1 and its involvement in neurodegeneration.

A defective locomotor performance is a typical symptom of different neurodegenerative pathologies, such as PD and ALS (Hardiman et al., 2017; Moustafa et al., 2016). In flies, classical approaches to study motor defects commonly rely on the evaluation of the startle-induced negative geotactic ability, namely climbing, where fruit flies are assessed for their capacity to climb vertically, following an obligated path (Madabattula et al., 2015). In this study, we also took advantage of a more sophisticated setup to explore the flies' free locomotion in an open field arena. Interestingly, we found that *dj-1β* null mutant flies display normal startle-induced negative geotaxis, while they present an altered free-walking behavior, characterized by discontinuous and uncoordinated locomotion. Multiple reasons could be responsible for this phenotype, such as neurodegeneration, muscular defects, or impaired bioenergetics. Since *dj-1β* KO flies have not been associated with dopaminergic cell loss (Meulener et al., 2005; Hwang et al., 2013; Menzies et al., 2005; Park et al., 2005), in agreement with *DJ-1* null mice (Chandran et al., 2008), we excluded dopaminergic neurodegeneration as a possible contributive factor. Moreover, the preserved climbing ability is consistent with intact and functional muscular tissue, as we confirmed by TEM analysis of the fly musculature. In light of these observations, and considering the altered redox state reported in the absence of dj-1β, we reasoned that behind the behavioral phenotype may instead lie defective mitochondrial homeostasis, being this organelle primarily responsible for the cellular energic state and ROS balance.

Longstanding evidence has supported the possible role of DJ-1 at the mitochondrial level. Indeed, DJ-1 has been proposed to translocate inside the organelle under oxidative conditions (Junn et al., 2009; Zhang et al., 2005; Calì et al., 2015; Maita et al., 2013), where it appears to preserve the organelle functionality, for example, by sustaining the activity of complex I (Hayashi et al., 2009; Heo et al., 2012) and complex V (Chen et al., 2019). Moreover, the protein has been involved in mitochondrial dynamics (Irrcher et al., 2010; Krebiehl et al., 2010), mitophagy (Hao et al., 2010; McCoy and Cookson, 2011; Ozawa et al., 2020), and mitochondria-endoplasmic reticulum contact sites (Liu et al., 2019; Ottolini et al., 2013). However, a final consensus on the role of DJ-1 at the mitochondrial level has not been reached. Additionally, most of these investigations have been performed *in vitro*, lacking *in vivo* relevance. In this study, we evaluated morphological and functional aspects of the organelle, to obtain a more complete picture. TEM analysis of the fly thorax showed that *dj-1β* null mutant flies present a trend towards a reduced number of mitochondria, characterized by increased area and roundness. Moreover, western blotting analysis of the mitochondrial marker ATP5A did not reveal alterations in the mitochondrial mass, supporting the notion that in the absence of dj-1β, mitochondria rearrange their shape without altering the total organelle content. Notably, an increase in the total mitochondrial area could represent a compensatory mechanism to sustain organelle functionality in conditions of stress, such as ROS imbalance or metabolic changes/insufficiencies. The evidence of an altered mitochondrial ultrastructure led us to hypothesize possible functional impairments in the respiratory chain. The analysis revealed that *dj-1β* null mutant flies display a stable respiratory chain, where complex I activity appeared preserved, despite showing a reduced respiratory rate. As complex I activity has been measured in the isolated complex (in gel activity), while OCR has been evaluated in an intact respiratory chain, these data suggest that dj-1β does not affect complex I activity *per se*, while it influences the electron flux through this complex. Of note, despite reduced complex I-linked respiration, we did not reveal significant variations in ATP and ADP levels, not even under starvation. However, *dj-1β* KO flies exposed to starvation showed premature mortality as compared to controls. This observation may indicate that some compensatory mechanisms, such as glycolysis, gluconeogenesis, and/or fatty acid oxidation, are activated to preserve ATP levels, in fed *dj-1β* KO flies, but they fail to sustain this state for long under starvation. In the presence of mild mitochondrial functional impairment and/or under food deprivation, alternative energetic reservoirs are mobilized, principally involving glycogen or protein breakdown and lipolysis. In this regard, the significant weight loss of *dj-1β* null mutant flies under starvation could result from alterations in these catabolic pathways. Interestingly, DJ-1 deficiency has been reported to cause reduced adipogenesis and body weight accompanied by low glucose levels and insulin resistance in mice (Kim et al., 2015; Wu et al., 2017; Xu et al., 2018) and increased glycolytic rate in *Drosophila* and human cells (Solana-Manrique et al., 2020; Solana-Manrique et al., 2022), supporting that DJ-1 indeed affects the energetic metabolism.

Autophagy plays a crucial role in the preservation of energy balance and is intimately connected to metabolism. Indeed, besides participating in the renewal of old and dysfunctional cellular components, autophagy has been also shown to contribute to the mobilization of energetic stores, *via* lysosomal degradation (Lahiri et al., 2019). In light of the overt sensitivity of *dj-1β* null mutant flies to starvation, we then sought to investigate whether the loss of dj-1β could affect the autophagic response. We found that, under basal and starved conditions, *dj-1β* KO flies accumulate higher levels of the autophagic marker Atg8-II with respect to controls. Atg8-II levels are considered a marker of

autophagosome quantity, as the subsequent fusion of autophagosomes with lysosomes leads to decreased Atg8-II protein levels due to the degradation of the luminal cargo together with the inner autolysosomal membrane (Yoshii and Mizushima, 2017). As we observed an increased number of autophagosomes under both fed and starved conditions, we reasoned that autophagy flux may be impaired in the absence of *dj-1β*. Interestingly, the analysis with the autophagy flux reporter GFP-mCherry-Atg8a showed that the number of autolysosomes is reduced in *dj-1β* KO fly brains (Fig. 6a-c), suggesting an alteration in the autophagosomal-lysosomal fusion, which may explain the accumulation of the autophagosomal marker Atg8-II (Fig. 4d). Interestingly, LysoTracker staining revealed that *dj-1β* KO flies display an increase in the lysosomal content while control flies rarely show signal, even under starvation. This result could be caused by the reduced autophagosomal-lysosomal fusion occurring in *dj-1β* KO individuals, which eventually may lead to the accumulation of the lysosomal content. Indeed, an increase in the LysoTracker signal has been already observed in other fly models of lysosomal dysfunction (Kinghorn et al., 2016; Mao et al., 2019). Thus, our *in vivo* data highlight the role of DJ-1 in modulating autophagy, in agreement with previous investigations mostly performed employing *in vitro* systems (Krebiehl et al., 2010; González-Polo et al., 2009; Nash et al., 2017; Qin et al., 2020; Ren et al., 2010; Thomas et al., 2011; Vasseur et al., 2009; Xue et al., 2017). Additionally, we also found that starved *dj-1β* KO flies present an increased stimulation of the metabolic player AMPK. In addition to promoting both glucose utilization and the mobilization of lipid stores, AMPK is also a recognized autophagic inducer. Our results suggest that, in response to the loss of dj-1β, AMPK phosphorylation is more enhanced in *dj-1β* KO flies than in controls, under starvation. The overactivation of pAMPK in the absence of *dj-1β* could represent an alternative mechanism through which the cell tries to compensate for the autophagic impairment, as also reported in the literature (Jia et al., 2020). Of interest, autophagy markers are rescued by NAC administration, suggesting that the effects associated with the loss of *dj-1β* KO are due to an impaired redox state. However, pAMPK levels were unaffected by NAC, indicating that ROS may not be the principal signal regulating its activation. Indeed, besides AMP and ROS, calcium, glucose, and fatty acids could regulate AMPK phosphorylation (Li and Chen, 2019; Jeon, 2016). Therefore, further analysis is required to untangle this aspect in *dj-1β* null background.

Collectively, our findings point to DJ-1 as a regulator of energy balance at the crossroad between mitochondrial and autophagic pathways. Indeed, the protein appears to sustain mitochondrial homeostasis, while concomitantly participating in autophagy modulation. Importantly, our data showed that the modulatory role of DJ-1 on autophagy mostly depends on the cellular redox state. Thus, DJ-1 could help to sustain the energy balance by preserving the redox homeostasis. This function has high relevance for the neurodegenerative context, as neurons are highly energy-consuming cells, which are particularly sensitive to redox stress and require a fine regulation of energy metabolism to sustain their activity. Therefore, our data highlight the possible role of DJ-1 in the maintenance of neuronal health, placing the protein at the crossroad of crucial redox-associated bioenergetic pathways.

Drosophila melanogaster represents a valuable model for studying molecular and cellular processes relevant to neurodegenerative disorders, as stated above. However, the evolutionary distance between humans and flies should call for caution when using insects in translational research. In the present work, we cannot exclude that the function of DJ-1 may require interaction with a panel of proteins, some of which could not be conserved between humans and flies. Moreover, though *Drosophila* possesses astroglia-like cells and other glial populations that share functional similarity with human microglia, the glia to neuron ratio differs from humans. Moreover, they do not present oligodendrocytes (Yildirim et al., 2019; Kremer et al., 2017). Since these cells participate in the regulation of neuronal metabolism, such differences could affect the metabolic impairment derived from alterations in DJ-1 functionality. In addition, a major technical limitation of *Drosophila*-based studies is the general lack of specific antibodies, which complicates a full characterization of the underlying molecular mechanisms. Thus, future studies should complement and expand these findings in a human cellular environment using brain cells and organoids derived from pluripotent stem cells induced from patients carrying loss-of-function *DJ-1* alleles.

5. Conclusions

With our study, we shed light on different aspects of DJ-1 physiology *in vivo*, through a fine characterization of the *DJ-1* null fly model. Loss of DJ-1 leads to hypoactivity and enhances susceptibility to food deprivation, supporting the existence of possible metabolic impairments. Our findings suggest that DJ-1 contributes to mitochondrial homeostasis, by sustaining the organelle morphology and complex I respiration, and participates in autophagy regulation through the preservation of the cellular redox state. Therefore, we unveiled a critical role of DJ-1 in the maintenance of energy balance in highly consuming tissues. Considering that neurons are extremely vulnerable to a defective energy supply, this study could conceivably explain the involvement of the protein in a complex neuropathological scenario.

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Ethics approval

All *Drosophila* experiments were performed in line with local guidelines and codes of practice.

Availability of data and material

Data generated and analyzed during this study are included in this published article. Datasets generated and analyzed during the current study are available from corresponding authors on reasonable request.

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CRediT authorship contribution statement

Federica De Lazzari: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Francesco Agostini:** Methodology, Investigation, Formal analysis. **Nicoletta Plotegher:** Methodology, Formal analysis, Writing – review & editing. **Michele Sandre:** Methodology, Investigation. **Elisa Greggio:** Resources, Writing – review & editing. **Aram Megighian:** Methodology, Resources, Writing – review & editing. **Luigi Bubacco:** Resources, Writing – review & editing. **Federica Sandrelli:** Resources, Writing – review & editing. **Alexander J. Whitworth:** Resources, Writing – review & editing, Project administration, Funding acquisition, Supervision. **Marco Bisaglia:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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DJ-1 and SOD1 Act Independently in the Protection against Anoxia in *Drosophila melanogaster*

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Abstract: Redox homeostasis is a vital process the maintenance of which is assured by the presence of numerous antioxidant small molecules and enzymes and the alteration of which is involved in many pathologies, including several neurodegenerative disorders. Among the different enzymes involved in the antioxidant response, SOD1 and DJ-1 have both been associated with the pathogenesis of amyotrophic lateral sclerosis and Parkinson's disease, suggesting a possible interplay in their mechanism of action. Copper deficiency in the SOD1-active site has been proposed as a central determinant in SOD1-related neurodegeneration. SOD1 maturation mainly relies on the presence of the protein copper chaperone for SOD1 (CCS), but a CCS-independent alternative pathway also exists and functions under anaerobic conditions. To explore the possible involvement of DJ-1 in such a pathway in vivo, we exposed *Drosophila melanogaster* to anoxia and evaluated the effect of DJ-1 on fly survival and SOD1 levels, in the presence or absence of CCS. Loss of DJ-1 negatively affects the fly response to the anoxic treatment, but our data indicate that the protective activity of DJ-1 is independent of SOD1 in *Drosophila*, indicating that the two proteins may act in different pathways.

Keywords: amyotrophic lateral sclerosis; DJ-1; *Drosophila melanogaster*; Parkinson's disease; SOD1

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1. Introduction

Superoxide dismutase 1 (SOD1) is one of the most important enzymes involved in the control of cellular redox homeostasis, owing to its ability to catalyze the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen. SOD1 is a 32 kDa homodimeric metalloenzyme, which is mainly found in the cytosol, although it is also present in the nucleus and mitochondrial intermembrane space [1,2]. Each subunit is built upon an eight-stranded β-barrel and comprises two functionally important loops, called the electrostatic loop and the metal-binding loop, respectively, which play roles in protein folding and activity [1,2]. Each monomer incorporates one copper and one zinc ion in close enough proximity to share an imidazole ligand [1,2]. The zinc ion exerts a structural role, whereas the copper ion is the core of the enzymatic activity of the protein. The metal ions both contribute to the stability of the mature enzyme, which is further enhanced by an intramolecular disulfide bridge between the residues Cys57 and Cys146 [1].

After the discovery, in 1993 that mutations in the *SOD1* gene are involved in familial forms of amyotrophic lateral sclerosis (ALS), research concerning the physiopathology of the protein strongly increased. Since then, approximately 200 *SOD1* gene modifications have been described [1,2]. Pathogenic mutations are distributed throughout the entire sequence of the protein and all of them accentuate structural instability of the metal-free SOD1 (apo-SOD1) and promote the accumulation of disordered immature SOD1

conformers, which leads to the formation of intracellular aggregates [1,2]. Interestingly, data obtained in ALS mouse models, incorporating the pathological G37R or G93A SOD1 mutations, suggested that the copper content rather than the amino-acid mutations per se isi a greater determinant in motor neuron death and the ALS-like phenotype [3–5].

In addition to a prominent role exerted by SOD1 in the pathogenesis of ALS, a recent work reported an accumulation of abnormal deposits of SOD1 also in idiopathic Parkinson's disease (PD) brains, strictly mirroring the pattern of neuronal loss observed in the disease [6]. Based on their results, the investigators proposed a model in which copper deficiency was associated with a reduction in the metal loading into the active site. As a consequence, apo-SOD1 becomes less stable, accumulates within aggregates, and loses its ability to protect neurons from oxidative damage [6–8].

Although both ALS and PD are complex pathologies, caused by the interplay of multiple genetic and environmental factors, the "metalation" status of SOD1 might be one of the key pathological determinants in both SOD1-related familial forms of ALS and sporadic PD. The copper acquisition has been shown to rely on a dedicated protein, referred to as copper chaperone for SOD1 (CCS) [9], through an oxygen-dependent pathway [10]. CCS is composed of three distinct domains, i.e., D1, D2, and D3. The first domain contains the copper-binding motif "MxCxxC", which seems to be responsible for the acquisition of the metal ion from the plasma membrane copper transporter 1 (Ctr1) and for its delivery to SOD1. The D2 domain shares high homology with SOD1 and is involved in heterodimer formation. The last domain contains a CxC copper-binding motif that has been proposed to deliver copper into the SOD1 active site in an alternative way with respect to domain D1 [11].

Although SOD1 maturation mainly occurs via the CCS-dependent pathway, an alternative mechanism for copper insertion is known to exist and accounts for around 15% of SOD1 activity [12]. Moreover, the CCS-independent pathway is able to activate SOD1 even under anaerobic conditions [10]. In this frame, we and others demonstrated, in vitro, the ability of the protein DJ-1 to bind copper [13–15] and to interact with and activate SOD1, through copper transfer [15,16], suggesting a possible involvement of DJ-1 in the CCS-independent SOD1 maturation pathway.

DJ-1 is a multifunctional protein implicated in oxidative stress responses, even though its specific role is still controversial [17–19]. Several different *DJ-1* gene mutations have been associated with familial autosomal recessive forms of PD [19,20]. Moreover, two independent studies have associated mutations in the *DJ-1* gene with ALS [21,22] and altered DJ-1 protein levels have been detected in cerebrospinal fluid, spinal cord, and motor cortex sections of ALS-affected patients [23,24].

Given the involvement of both SOD1 and DJ-1 in ALS and PD, and considering the purported participation of DJ-1 in the SOD1 maturation pathway, this work aimed to evaluate in vivo, using *Drosophila melanogaster* as an animal model, whether the antioxidant properties of DJ-1 were dependent on the presence of SOD1.

2. Materials and Methods

Drosophila Strains and Culture Maintenance—Flies were raised on agar, cornmeal, and yeast food, at 25 °C, under 70% relative humidity in 12-hour light/dark cycles. Only male flies were used in all experiments. The following strains were obtained from the Bloomington *Drosophila* Stock Center: w^{1118} (#5905), $dj\text{-}1\beta^{\Delta 93}$ (#33601), Ccs^{n29E}/Cyo (#24755), $Sod1^{x39}$ (#24490), $Sod1^{n1}$ (#24492), *daughterless-Gal4* (#8641, da*Gal4*), and UAS-*dj-1β* (#33604). The w^{1118} strain was used as a control line when analyzing *dj-1β*, *Ccs*, and *Sod1* mutant flies.

Western blot analysis—To extract proteins, fly bodies were homogenized with a pestle in lysis buffer (50 mM Tris-HCl pH 7.5, 1% *v/v* Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM p- glycerophosphate, 5 mM sodium pyrophosphate, and 270 mM sucrose), incubated on ice for 30 min, and then centrifuged at maximum speed for 30 min at 4 °C. Proteins were separated in 13% polyacrylamide SDS gels, and then transferred into nitrocellulose membranes (Bio-Rad), through a Trans-Blot® Turbo™ TranSystem (Bio-Rad, Hercules, CA, USA). Membranes were blocked in Tris-buffered

saline solution containing 0.1% Tween (TBS-T) and 5% w/v skimmed milk for 1 h at room temperature. After this step, membranes were incubated overnight at 4 °C with primary antibodies dissolved in 5% w/v skimmed milk in TBS-T. After incubation, membranes were washed 3 times for 10 min with TBS-T, and subsequently incubated with secondary antibodies conjugated with horseradish-peroxidase (HRP), diluted in 5% w/v skimmed milk in TBS-T for 1 h at room temperature. Finally, membranes were washed in TBS-T 3 times for 10 min. Proteins detection was performed using an ECL-Plus detection kit (GE Healthxare, Chicago, IL, USA), and images were acquired using a VWR® CHEMI Premium analyzer. Protein levels were quantified by densitometry using the ImageJ software (US National Institutes of Health). The primary antibodies were rabbit α-SOD1 (HPA001401, Sigma Prestige, 1:1000) and mouse α-actin (MAB1501, EDM Millipore 1:5000).

Survival experiments under anoxia—Adult males (1–3 days old) were collected under brief CO_2 exposure and placed in fresh food vials 1 day before the experiment. On the day of the experiment, flies were incubated in an anaerobic glove box (MBRAUN MB-200B). Flies were kept under anoxia for up to 6 h, and, subsequently reintroduced into the normoxic atmosphere to monitor fly lethality.

Survival experiments under paraquat treatment—Groups of 20 flies (1–3 days old) were collected under brief CO_2 exposure and placed in fresh food vials containing 1 mM paraquat. Paraquat sensitivity was determined by counting death events every day for 4 days.

Locomotion Assay—Groups of 20 flies (1–3 days old) were collected under brief CO_2 exposure and placed in fresh food vials and the locomotion behavior was assessed the following day. When experiments were performed in the presence of paraquat, flies were transferred into new tubes every 2 days and the locomotion assays were performed after 7 days of treatment. The mobility of flies from each treatment group was assessed through a negative geotaxis climbing assay using a counter-current apparatus with 6 tubes in the lower frame and 5 in the upper frame. Flies were placed in the first plastic vial (1.5 cm diameter and 10 cm height) and gently tapped to the bottom. After 10 sec, the upper frame was moved to the right, and the flies that passed in the upper tubes during this period were transferred to the next lower tubes by gently tapping. This procedure was repeated 5 times. For each genotype, the climbing index was calculated using the following formula:

$$CI = [(\#F5 \times 5) + (\#F4 \times 4) + (\#F3 \times 3) + (\#F2 \times 2) + (\#F1 \times 1) + (\#F0 \times 0)/(\#FT)] \quad (1)$$

where #Fn is the number of flies in the tube n (being 0 in the initial tube and 5 in the last tube) and #FT is the total number of flies.

Transmission electron microscopy *(TEM)*—To assess the mitochondrial morphology under basal conditions, adult males (1–3 days old) were fixed for 2 h in 0.1 M sodium cacodylate (pH 7.4), containing 2.5% glutaraldehyde and 2% paraformaldehyde, and then dissected to isolate thoraces. Briefly, the head, legs, and wings were removed with forceps, taking care to remove the gut as well. Subsequently, samples were incubated with a solution of 1% tannic acid for 1 h at room temperature, and then post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. After three water washes, samples were dehydrated in a graded ethanol series and embedded in an epoxy resin (Sigma-Aldrich). Ultrathin sections (60–70 nm) were obtained with an Ultrotome V (LKB) ultramicrotome, counterstained with uranyl acetate and lead citrate, and viewed using a Tecnai G2 (FEI) transmission electron microscope, operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System, Muenster, Germany) digital camera.

Statistical analysis—Data were collected from at least three independent experiments. Graphs were produced and statistical analyses were performed by using GraphPad Prism 9 software. One- or two-way ANOVA, followed by Tukey's multiple comparisons post hoc test, was used for grouped comparisons. Survival analysis was performed by Mantel–Cox log-rank test. p-values <0.05 were considered to be significant.

3. Results

3.1. Ccs-Dependent and Ccs-Independent Sod1 Maturation Modulate D. melanogaster Life Expectancy, and Capability to Cope with Oxidative Stress Conditions

The *D. melanogaster* genome encodes homologs of the human proteins SOD1 and CCS, referred to as Sod1 and Ccs, respectively, and the CCS-dependent SOD1 maturation pathway has been described to be conserved in humans and flies [25]. More specifically, the physiological role of Ccs has been investigated through the generation of a *Ccs* null line (Ccs^{n29E}), carrying a 1907 bp deletion at the *Ccs* locus level, which has been characterized long ago [25]. The strain shows a reduced lifespan and high sensitivity to oxidative conditions. Moreover, these effects appear to be derived from a reduced amount of functional Sod1, as the loss of Ccs affects both the levels and activity of Sod1. In this work, we confirmed some of the previously described effects induced by the depletion of Ccs, and we further characterized the Ccs^{n29E} strain, by comparing it with the control line w^{1118} and with a *Sod1* mutant. To this aim, we first tested the $Sod1^{x39}$ strain, characterized by a 395 bp deletion in the *Sod1* gene [26]. However, as the *Sod1* deletion was lethal in homozygosis, we moved to the $Sod1^{n1}$ strain bearing the G49S mutation that affects the formation of hydrogen bonds at the dimer interface. The G49S substitution makes the protein much more unstable as compared with the wild-type Sod1 form [26], but allows the development of alive homozygous adults, most probably because of a very small amount of active protein that is still present in these flies.

In agreement with previous results [25], when we evaluated the lifespan of *Ccs* null individuals, we observed premature mortality with respect to controls, with the median survival time ($t_{1/2}$)that decreases from 67 to 38 days, although they lived significantly longer than *Sod1* mutants, which were characterized by a $t_{1/2}$ of only 8 days. (Figure 1A).

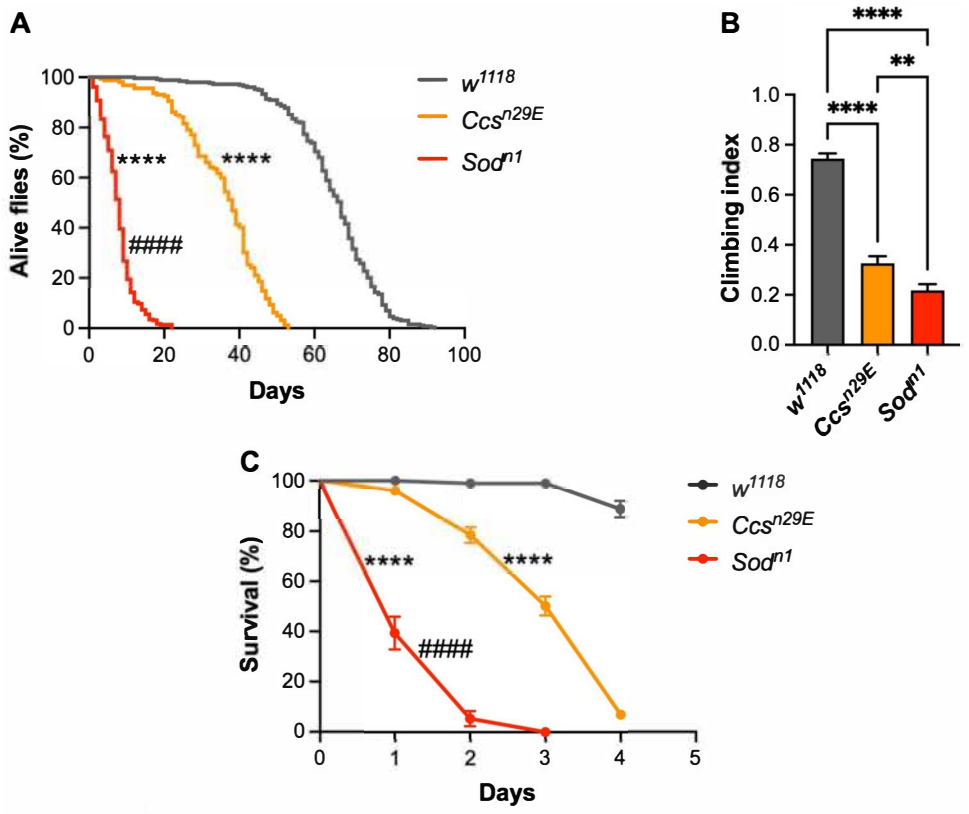

Figure 1. *Cont.*

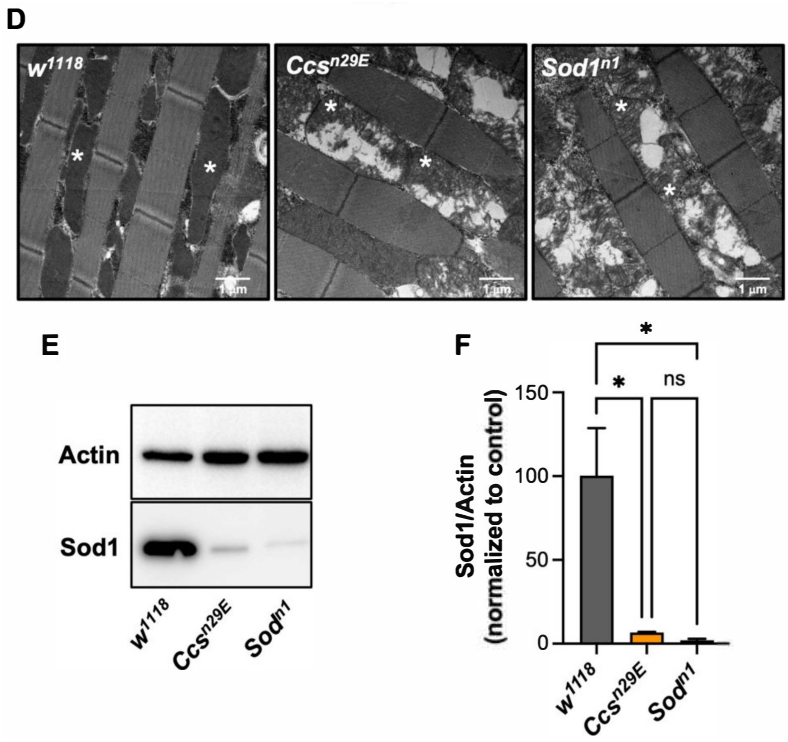

Figure 1. Ccs^{n29E} null flies show milder phenotypes than $Sod1^{n1}$ mutants: (**A**) Survival analysis. Ccs^{n29E} mutants lived significantly less than w^{1118} controls but longer than $Sod1^{n1}$ flies (Mantel–Cox log-rank test: w^{1118} vs. Ccs^{n29E} (****), w^{1118} vs. $Sod1^{n1}$ (****), Ccs^{n29E} vs. $Sod1^{n1}$ (####), $p < 0.0001$ for all comparisons. N: 162 Ccs^{n29E}, 288 $Sod1^{n1}$, and 260 w^{1118}); (**B**) climbing activity (mean ± SEM). Analysis of variance and post-hoc tests indicate significant locomotor impairments in Ccs^{n29E} and $Sod1^{n1}$ flies as compared with w^{1118} controls, with $Sod1^{n1}$ mutants showing the strongest phenotype (one-way ANOVA $F_{2, 516} = 123.7$ $p < 0.0001$; Tukey's multiple comparisons test: w^{1118} vs. Ccs^{n29E} (****) and w^{1118} vs. $Sod1^{n1}$ (****), $p < 0.001$; Ccs^{n29E} vs. $Sod1^{n1}$ (**), $p = 0.0075$. N: 178 Ccs^{n29E}, 164 $Sod1^{n1}$, and 177 w^{1118}); (**C**) survival analysis under mild oxidative stress conditions (1 mM paraquat). Ccs^{n29E} flies were significantly more and less sensitive as compared with w^{1118} and $Sod1^{n1}$ flies, respectively (Mantel–Cox log-rank test: w^{1118} vs. Ccs^{n29E} (****), w^{1118} vs. $Sod1^{n1}$ (****), Ccs^{n29E} vs. $Sod1^{n1}$ (####), $p < 0.0001$ for all comparisons. N: 172 Ccs^{n29E}, 56 $Sod1^{n1}$, and 99 w^{1118}); (**D**) representative TEM images of the mitochondrial morphology of Ccs^{n29E}, $Sod1^{n1}$ mutants, and w^{1118} controls. Asterisks in the pictures indicate representative mitochondria; (**E**) representative Western blot; (**F**) relative quantification of Sod1 levels (mean ± SEM) in Ccs^{n29E}, $Sod1^{n1,}$, and w^{1118} flies; in (**F**), Sod1 levels are reported as the Sod1/Actin ratio, with Actin signal used as a loading control. Ccs^{n29E} and $Sod1^{n1}$ flies showed a significantly lower Sod1 amount with respect to controls (one-way ANOVA: $F_{2, 6} = 11.51$, $p = 0.0088$; Tukey's multiple comparisons test: w^{1118} vs. Ccs^{n29E} (*) and w^{1118} vs. $Sod1^{n1}$ (*) $p < 0.05$; Ccs^{n29E} vs. $Sod1^{n1}$ (ns, non-significant) $p = 0.97$. N = 3).

Then, we compared the locomotor behavior of flies through a negative geotaxis-based climbing assay, observing that, with respect to the controls, both Ccs^{n29E} and $Sod1^{n1}$ lines showed a strong locomotor impairment, with slightly more pronounced effects in the case of *Sod1* mutants (Figure 1B). As a further characterization, we analyzed the effects derived by increasing oxidative stress conditions through the addition of paraquat into the food. Paraquat is an herbicide whose toxicity is generally ascribed to the generation of oxidative stress conditions through the production of free radical species, including superoxide and hydroxyl radicals, both at the cytosolic and mitochondrial levels [27–29]. Since it was previously shown that in the presence of 2 mM paraquat, Ccs^{n29E} flies display the same extreme hypersensitivity to the redox cycling agent as exhibited by the $Sod1^{n1}$ line, with less than 5% of survivors after 24 h [25], here, we used a lower concentration of paraquat and measured fly survival for a longer period (4 days). As expected, in the presence of

1 mM paraquat, the absence of Ccs induced a high sensitivity to oxidative conditions in contrast to control flies, whose survival was almost unaffected throughout the time course of the experiment. However, as for the lifespan and the climbing ability, the lack of Ccs produced milder effects than the loss of the Sod1 protein itself (Figure 1C). Given that oxidative stress and mitochondrial damage are often correlated, to further evaluate the consequences of the loss of Ccs, we evaluated the mitochondrial morphology of both Ccs^{n29E} and $Sod1^{n1}$ mutants and negative controls. In contrast to w^{1118} control flies and similarly to $Sod1^{n1}$ mutants, the absence of Ccs in Ccs^{n29E} individuals led to swollen and vacuolized mitochondria characterized by an altered ultrastructure. (Figure 1D). Therefore, the loss of either Ccs or Sod1 largely impacts mitochondrial homeostasis. Since it has been reported that upon Ccs depletion, the *Drosophila* Sod1 protein becomes unstable and its levels dramatically drop [25], we finally evaluated the amount of Sod1 in *Ccs* null flies, by immunoblotting. Consistent with the previous report, the loss of Ccs highly impairs Sod1 protein levels (Figure 1E), confirming that Sod1 becomes unstable in the absence of its copper chaperone. Nevertheless, we detected a higher residual amount of Sod1 in *Ccs* null flies with respect to $Sod1^{n1}$ mutants (Sod1 levels (mean ± SEM): 6.6 ± 0.5 and 2.1 ± 0.9 in Ccs^{n29E} and $Sod1^{n1}$, respectively) (Figure 1F), although the difference was not statistically significant, which might explain the less robust phenotypic effects in Ccs^{n29E} as compared with $Sod1^{n1}$ flies. Overall, our results indicate that the absence of Ccs strongly affects the capability of flies to cope with oxidative stress conditions. Furthermore, taken together, our data confirm that also, in the *D. melanogaster* model, Sod1 maturation can be achieved through an alternative Ccs-independent pathway.

3.2. Drosophila dj-1β Participates in the Protection against Oxygen Deprivation without Affecting Sod1 Expression

In light of the results presented above, we were interested in assessing whether DJ-1 could be implicated in the CCS-independent SOD1 maturation. The *D. melanogaster* genome encodes two DJ-1 homologs, referred to as dj-1α and dj-1β, with a different pattern of expression, being dj-1α expression mainly restricted to the testis while dj-1β shows a ubiquitous expression profile. Double *dj-1α*; *dj-1β* knock-out animals display enhanced sensitivity to oxidative conditions [30]. Interestingly, this sensitivity has been demonstrated to depend on the loss of dj-1β, since single *dj-1β* null flies ($dj1\beta^{\Delta 93}$) are as sensitive to oxidative stressors as double *dj-1α* and *dj-1β* knock-out flies, while *dj-1α* single knock-out individuals behave as control flies [30]. Moreover, it has been previously demonstrated that human DJ-1 can rescue *dj-1β* knock-out phenotypes when flies were treated with paraquat, underscoring the similarity between the human and fly proteins [31]. Consequentially, we focused on the protective effects of the dj-1β isoform. As the CCS-independent SOD1 maturation process does not require the presence of molecular oxygen, to investigate the possible involvement of dj-1β in this pathway, we first tested how the modulation of dj-1β expression levels affects fly survival by comparing *dj-1β* knock-out with w^{1118} and Ccs^{n29E} flies under anoxic conditions. With respect to mammals, fruit flies represent a valuable in vivo model for this kind of experiment as they are very resistant to anoxia for up to a few hours [32].

When flies were exposed to anoxia, they rapidly stopped moving and fell into a coma-like condition within a few minutes. Since the rescue of locomotor activities after the reintroduction into a normoxic atmosphere required several hours to be completed, we measured fly survival one day after treatment as a readout of the anoxic effect. Figure 2A shows the survival profiles of $dj1\beta^{\Delta 93}$, Ccs^{n29E}, and control flies kept under anoxic conditions for different periods (3, 4, 5, and 6 h). For each genotype, the percentage of surviving flies decreases with increasing time of treatment, indicating a direct correlation between the extent of the anoxia period and lethality. Moreover, while the percentages of survivors in $dj1\beta^{\Delta 93}$ and Ccs^{n29E} strains were similar to control individuals after 3 h of anoxic treatment, 4 and 5 h of anoxic exposure induced significantly higher mortality in both $dj1\beta^{\Delta 93}$ and Ccs^{n29E} as compared with the controls (Figure 2A). The difference among genotypes disappeared after 6 h of treatment, which determined the death of most of the flies in

both *dj1β*$^{\Delta 93}$ and *Ccs*^{n29E}, as well as in control samples (Figure 2A), indicating that 6 h of anoxic exposure was too drastic to detect statistically significant differences among the three *Drosophila* genotypes. Interestingly, the effects observed under oxygen deprivation did not significantly differ between *dj1β*$^{\Delta 93}$ and *Ccs*^{n29E} strains (Figure 2A).

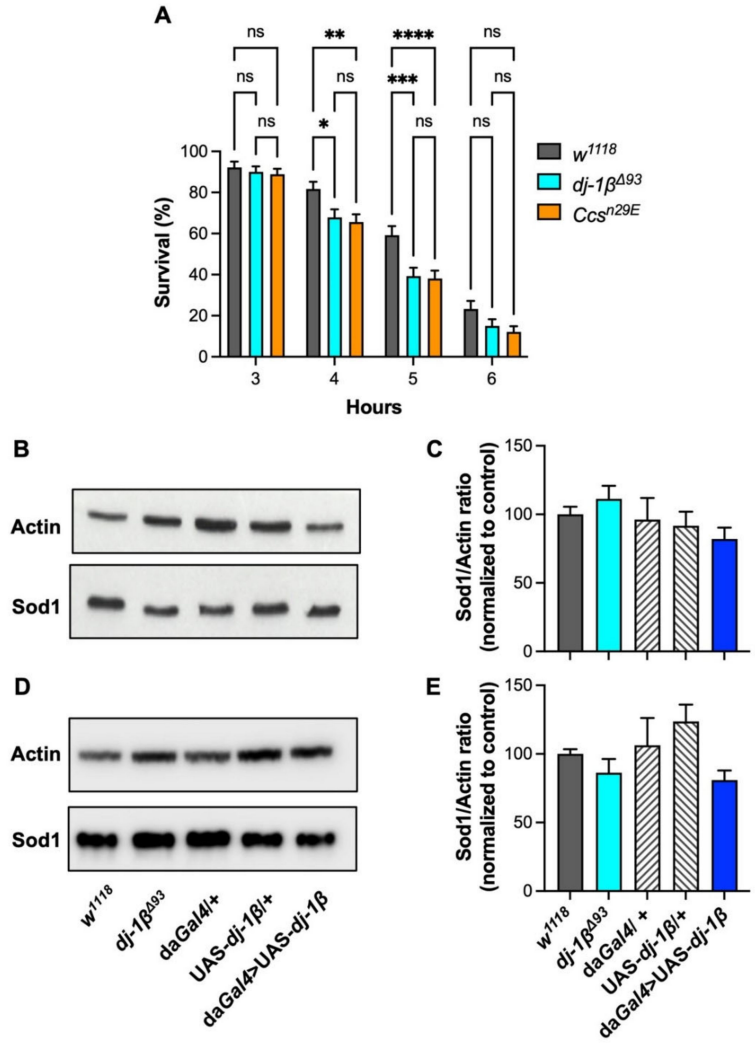

Figure 2. *dj-1β* protects against oxygen deprivation without affecting Sod1 expression: (**A**) Percentage of survivors (mean ± SEM) in *dj-1β*$^{\Delta 93}$, *Ccs*^{n29E}, and *w*^{1118} control flies, after 3, 4, 5, and 6 h of anoxic treatment. *dj-1β*$^{\Delta 93}$ and *Ccs*^{n29E} flies showed significantly higher mortality as compared with *w*^{1118} controls after 4 and 5 h of anoxia. Anoxia induced similar effects in both *dj-1β*$^{\Delta 93}$ and *Ccs*^{n29E} flies, at all time points (two-way ANOVA (time of treatment X genotype) $F_{6, 1578} = 1.28$, $p = 0.263$; time of treatment effect: $F_{3, 1578} = 222.4$, $p < 0.0001$; genotype effect: $F_{2, 1578} = 13.83$, $p < 0.0001$; in the graph ****, ***, **, *, and ns indicate $p < 0.0001$, < 0.001, < 0.01, < 0.05, and non-significant, in Tukey's multiple comparisons post-hoc tests, see text for details); (**B**) representative Western blot and (**C**) relative quantification of Sod1 protein levels (mean ± SEM) under basal conditions, in *dj-1β* KO and *dj-1β*-overexpressing (da*Gal4* > UAS-*dj-1β*) flies as compared with controls (*w*^{1118}, da*Gal4*/+ and UAS-*dj-1β*/+); in (**C**), Sod1 levels are reported as the Sod1/Actin ratio, with Actin signal used as a loading control. No significant differences in Sod1 protein signals were detected among genotypes (one-way ANOVA $F_{4, 23} = 0.90$, $p = 0.483$, N > 4 per genotype); (**D**) representative Western blot and (**E**) relative quantification of Sod1 protein levels (mean ± SEM) in *dj-1β* KO and *dj-1β*-overexpressing (da*Gal4* > UAS-*dj-1β*) flies as compared with controls (*w*^{1118}, da*Gal4*/+, and UAS-*dj-1β*/+), after 7 days of exposure to 1 mM paraquat; in (**E**), Sod1 protein amounts are reported as the Sod1/Actin ratio, with Actin signal used as a loading control. No significant differences in Sod1 protein amounts were detected among genotypes (one-way ANOVA $F_{4, 17} = 2.29$ $p = 0.101$; N > 3 per genotype).

In mammals, DJ-1 has been reported to stimulate the nuclear translocation of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), which phosphorylate ets like-1 protein (Elk1), a transcription factor involved in the expression of various antioxidant genes, including SOD1 [33]. Therefore, to assess whether the protective role of dj-1β observed during anoxic treatments was associated with its capability to promote the expression of Sod1, we measured Sod1 protein levels in *dj-1β* knock-out and control flies. As represented in Figure 2B,C, the absence of dj-1β does not affect the amount of protein. Additionally, Sod1 protein levels resulted similar in dj-1β overexpressing flies (da*Gal4* > UAS-*dj-1β*) as compared with their appropriate negative controls (da*Gal4*/+ and UAS-*dj-1β*/+) (Figure 2B,C), further ruling out a direct involvement of dj-1β in the modulation of Sod1 expression under our experimental conditions.

Since the most corroborated function of DJ-1 deals with its protective role against oxidative stress, we wondered whether the participation of dj-1β in the modulation of Sod1 expression could become relevant under oxidative conditions. However, simple discrimination between dead and alive individuals immediately after the anoxic incubation period was impossible, because of the coma-like state of alive flies. Moreover, during the long-time interval required by flies to completely recover after the anoxic treatment, protein levels could change. For these reasons, the analysis of Sod1 expression under anoxia was not informative and we adopted an alternative approach: we measured Sod1 protein levels after treatment with a sub-lethal concentration of paraquat (1 mM). Our data indicate that, also under oxidative conditions, dj-1β is unable to modulate Sod1 expression (Figure 2D,E), suggesting that the protective effects mediated by dj-1β do not depend on *Sod1* transcriptional activation.

3.3. The Overexpression of dj-1β Does Not Rescue the Effects Induced by Ccs Depletion

After having characterized several experimental readouts related to the loss of the Ccs protein, we investigated the potential complementarity between dj-1β and Sod1 in the antioxidant response, by either using *dj-1β* knock-out flies or by ubiquitously overexpressing dj-1β in a *Ccs* null background. First, through a series of standard crosses, we produced a Ccs^{n29E}/Cyo; $dj\text{-}1\beta^{\Delta 93}/dj\text{-}1\beta^{\Delta 93}$ line with the aim of characterizing the *Ccs*; *dj-1β* double knockout individuals. Interestingly, the genetic deletion of both *Ccs* and *dj-1β* was lethal, as homozygous flies did not eclose, coherently with the initial hypothesis that both proteins could participate in Sod1 maturation in two independent ways. To evaluate this indication, we assessed the effects of dj-1β overexpression in the absence of Ccs, using Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* flies, and relative controls (Ccs^{n29E}; da*Gal4*/+ and Ccs^{n29E}; UAS-*dj-1β*/+). Considering the involvement of dj-1β in the antioxidant response and the existence of a Ccs-independent Sod1 maturation pathway which does not rely on the presence of oxygen, we carried out survival experiments after anoxic treatments or in paraquat-induced oxidative conditions. Unexpectedly, in both cases, the results did not support a complementary role for dj-1β in the transfer of copper into the Sod1 active site. In fact, under oxygen deprivation, the survival of dj-1β overexpressing flies was comparable to those of both parental lines at each time point considered (Figure 3A).

Moreover, the effects induced by the presence of paraquat were very similar between controls and dj-1β-overexpressing flies (Figure 3B). Since, as described above, upon Ccs depletion, *Drosophila* Sod1 becomes unstable and its levels drop dramatically, we used Sod1 protein levels as an experimental readout to further evaluate the possible participation of dj-1β in the Ccs-independent Sod1 maturation pathway. As represented in Figure 3C, the expression levels of Sod1 in each strain presenting a *Ccs* null background were very low as comparison with w^{1118} flies, even in the presence of dj-1β overexpression, excluding, once again, the involvement of dj-1β in the process that leads to the accumulation of the mature form of Sod1.

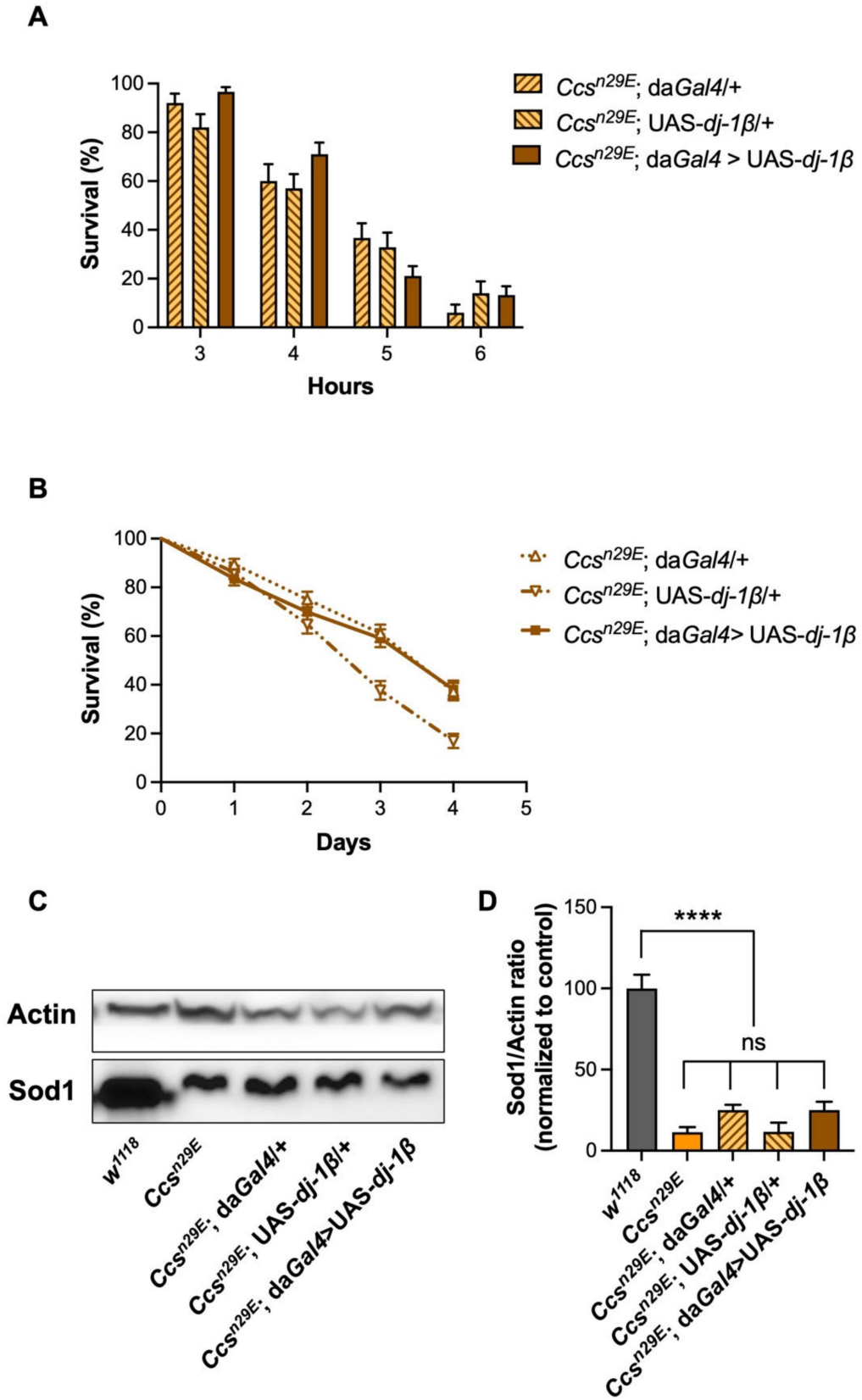

Figure 3. *dj-1β* overexpression in a Ccs^{n29E} background does not rescue the effects induced by a Ccs

depletion: (**A**) Percentage of survivors (mean ± SEM) in Ccs^{n29E}, da*Gal4* > UAS-*dj-1β*, and relative controls (Ccs^{n29E}, da*Gal4*/+ and Ccs^{n29E}, UAS-*dj-1β*/+), after 3, 4, 5, and 6 h of anoxic treatment. Time of anoxic treatment significantly affected survival in all fly strains, with no differences among genotypes (two-way ANOVA (time of treatment X genotype) $F_{6, 798} = 2.78$, $p = 0.011$; time of treatment effect: $F_{3, 798} = 144.1$, $p < 0.0001$; genotype effect: $F_{2, 798} = 0.76$, $p = 0.47$, non-significant). For each time of treatment, 50–90 males per genotype were analyzed; (**B**) survival analysis under mild oxidative stress conditions (1 mM paraquat) in Ccs^{n29E}; da*Gal4* > UAS-*dj-1β*, and relative controls (Ccs^{n29E}, da*Gal4*/+ and Ccs^{n29E}, UAS-*dj-1β*/+). The Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* survival profile was intermediate between the two controls, indicating the differences were not due to a *dj-1β* overexpression in a *Ccs* null background (Mantel–Cox log-rank test: Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* vs. Ccs^{n29E}; da*Gal4*/+: $p = 0.8$; Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* vs. Ccs^{n29E}; UAS-*dj-1β*/+: $p = <0.0001$; Ccs^{n29E}; da*Gal4*/+ vs. Ccs^{n29E}; UAS-*dj-1β*/+: $p < 0.0001$); (**C**) Representative Western blot and (**D**) relative quantification of Sod1 levels (mean ± SEM) in Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* and control flies; in (**D**), Sod1 levels are reported as the Sod1/Actin ratio, with Actin signal used as a loading control. Sod1 levels in Ccs^{n29E}; da*Gal4* > UAS-*dj-1β* flies were significantly lower from that of w^{1118} controls but similar to those of Ccs^{n29E} null flies and Ccs^{n29E}; da*Gal4*/+ or Ccs^{n29E}; UAS-*dj-1β*/+ controls (one-way ANOVA: $F_{4, 10} = 46.1$, $p < 0.0001$; Tukey's multiple comparisons test: w^{1118} vs. all other genotypes $p < 0.0001$ (****), Ccs^{n29E}, all other comparisons, $p \geq 0.4$, ns. N = 3).

4. Discussion

Currently, reactive oxygen species (ROS) have been recognized to play important functions as endogenous mediators in several signaling pathways. However, ROS are extremely reactive molecules and, if their concentration rises above physiological levels, they can exert deleterious effects. Accordingly, numerous neurodegenerative disorders, including ALS and PD, are characterized by ROS-associated oxidative damage. It follows that the fine-tuning between ROS generation and their elimination is essential for cell survival. Among the different enzymes involved in the antioxidant response, in this work, we focused on SOD1 and DJ-1 and their possible interplay, since both have been associated with the pathogenesis of ALS and PD.

While the enzymatic function of SOD1 is very well characterized, being the enzyme involved in the dismutation of superoxide radicals, much less is known about the precise mechanism through which DJ-1 exerts its antioxidant function. Interestingly, independent lines of research have suggested a possible involvement of DJ-1 in binding copper ions and protecting against copper-induced cytotoxicity [13–15], although contrasting results have also been reported [34]. Moreover, based on in vitro and cellular indications, we and others have proposed the potential participation of DJ-1 in the SOD1 activation [15,16,35,36], suggesting the possibility that the antioxidant activity of DJ-1 is mediated by SOD1.

In this work, this hypothesis has been explored using *D. melanogaster* as an in vivo model. Fruit flies possess orthologs of the human protein objects of this study that share similar functional properties with their human counterparts. Consistent with previously published data [25], our results suggest that the presence of Sod1 plays a protective role for adult flies, especially in the presence of clear oxidative insults, since the amount of protein is directly correlated with both lifespan and fly survival in the presence of paraquat. More importantly, the phenotypes observed in *Ccs* null mutants support the presence, as in humans, of a Ccs-independent Sod1 maturation pathway.

As the Ccs-independent pathway does not rely on the presence of oxygen, then, we decided to carry out experiments under anaerobic conditions. First, we demonstrated that the lack of Ccs makes flies more sensitive to oxygen depletion. This observation can be explained considering that, in the absence of Ccs, the Sod1 protein does not accumulate in its active form. Unexpectedly, however, in the *Ccs* null background, the overexpression of dj-1β was unable to provide protection under anoxia and similar results were also observed in the presence of paraquat, a herbicide that has been demonstrated to increase the cellular production of ROS. Several hypotheses might explain the discrepancy between

the results presented in this work and previous studies from our and other laboratories, which suggested a role for DJ-1 in the CCS-independent SOD1 maturation pathway.

One possible explanation could be linked to the fact that, in *Drosophila*, the absence of Ccs makes Sod1 highly unstable, actually subtracting Sod1 to the action of dj-1β. Elevated Sod1 instability in *Ccs* null mutant flies has been reported in a previous study [25] and validated in our work. Moreover, such behavior has been confirmed by expressing *Drosophila* Sod1 in a yeast strain depleted of the endogenous CCS but was not observed with yeast and human SOD1 [25]. The authors also demonstrated that Sod1 stabilization was dependent on copper transfer and/or disulfide oxidation and proposed that, in *Drosophila*, Ccs afforded stability to Sod1 by activating the enzyme through copper insertion and/or disulfide oxidation [25]. In this frame, it is plausible that DJ-1 requires the presence of SOD1 in a folded state to transfer the copper ion and activate the enzyme.

However, considering that DJ-1 seems to possess redundant cellular functions and that only mild phenotypes are observed in DJ-1 knock-out animal models, a further possibility to explain our results is that the protective role of dj-1β could become particularly important only under mildly stressful situations. In other words, the strong phenotypes caused by the loss of Ccs could be too strong to allow the detection of the limited protective effects mediated by dj-1β. In agreement with this hypothesis, it has recently been shown that while the loss of DJ-1 makes cells more sensitive to methylglyoxal-associated glycation, its overexpression does not improve cellular viability against the toxicity of exogenously added methylglyoxal [37].

On the contrary, we consider it unlikely that the discrepancy between our in vivo results and previously published in vitro and cellular data is due to an evolutionary functional divergence between *Drosophila* dj-1β and human DJ-1. This hypothesis could find support in the fact that in human DJ-1, two cysteine residues, namely Cys53 and Cys106, have been described as fundamental in forming two different copper-binding sites [14,15], while *Drosophila* dj-1β lacks the cysteine residue corresponding to human DJ-1 Cys53. However, dj-1β possesses the Cys104 residue, corresponding to human DJ-1 Cys106, which has been shown to be a key site allowing the transfer of the metal ion to SOD1 in vitro [15]. Moreover, the Cys53 residue is also absent in the *Arabidopsis thaliana* DJ-1 homolog, whose role in SOD1 maturation was described for the first time [16].

Finally, we also tend to exclude that our in vivo data are an indication that, in *Drosophila*, dj-1β works upstream from Ccs in the same cellular pathway, in agreement with the hypothesis that a still unknown protein is responsible for the transfer of intracellular copper from glutathione to CCS [38]. In fact, while both *Ccs* and *dj-1β* single mutants reach the adult stage, the *Ccs*; *dj-1β* double knock-out is lethal, therefore, suggesting that Ccs and dj-1β proteins exert their role in distinct protective pathways.

Notably, our results indicate that the absence of dj-1β affects adult fly survival under oxygen depletion, in line with a purported protective role of DJ-1 against hypoxia injury [18]. Remarkably, the effects observed in *dj-1β* knock-out mutants do not seem to rely on the Ccs-dependent Sod1 maturation pathway, as *dj-1β* knock-out flies showed an accumulation of Sod1 similar to controls. Additionally, the overexpression of dj-1β did not alter Sod1 expression, either at the RNA or protein levels, again indicating that the protective function of dj-1β was independent of Sod1.

In conclusion, as summarized in Figure 4, the picture arising from the data presented here is that dj-1β and Sod1 exert their antioxidant activity through two distinct mechanisms, and further research is still required to unmask the precise molecular mechanisms underlying the protective role of DJ-1.

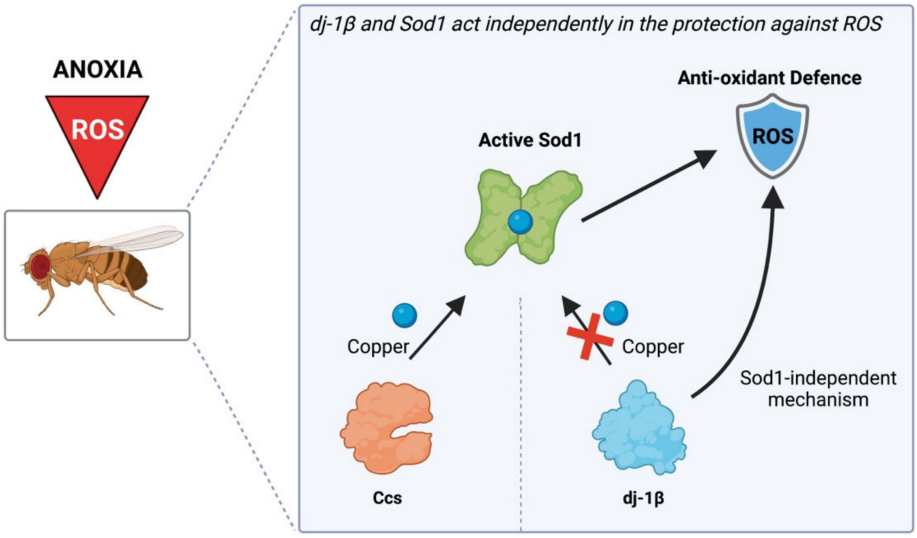

Figure 4. Proposed model describing the independent actions of Sod1 and dj-1β in the protection against anoxia. In *Drosophila melanogaster*, Ccs is responsible for copper loading into the Sod1 active site through the so-called Ccs-dependent Sod1 maturation pathway. The loss of Ccs increases the susceptibility of flies to oxidative stress. dj-1β does not participate in the Ccs-independent Sod1 maturation pathway, as in a *Ccs* null genetic background, dj-1β overexpression does not induce any protection. The loss of dj-1β increases the susceptibility to oxidative stress without affecting the Ccs-related Sod1 maturation pathway, as Sod1 levels in *dj-1β* knock-out flies are similar to controls. Moreover, dj-1β protection appears independent from Sod1 as the modulation of dj-1β expression does not affect Sod1 levels either under basal conditions or in the presence of oxidative stress (created with BioRender.com, accessed on 7 July 2022).

Author Contributions: Conceptualization, M.A.Z., L.B., F.S. and M.B.; methodology, F.D.L., F.A., D.D. and S.M.; formal analysis, F.D.L., F.S. and M.B.; writing—original draft preparation, F.S., and M.B.; writing—review and editing, F.D.L., S.M., M.A.Z. and L.B.; supervision, P.C., F.S. and M.B.; funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

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