UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department of Biology

Ph.D. COURSE IN: Biosciences
CURRICULUM: Cell Biology and Physiology
SERIES XXXII

Exploring the physiological functions of the protein DJ-1 in redox homeostasis

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Acknowledgements

These years as a PhD student have been a life-changing experience for me, during which I grew both scientifically and personally. This achievement would not have been possible without the support received from many people.

Firstly, I would like to express my gratitude to my supervisor Prof. Marco Bisaglia, who believed in me and supported me constantly in this journey. His assistance, guidance, and patience have been important for my scientific growth. A well-deserved thank you to Prof. Federica Sandrelli, whose expertise has profoundly contributed to enhanced my knowledge regarding the use of *Drosophila* as a model organism. Special appreciation goes to Prof. Luigi Bubacco for his invaluable advice on research as well as on my career. I am also grateful to Dr Sandro Malacrida, Dr Simona Mrakic-Sposta, and Dr Alessandra Vezzoli for the productive collaboration. I would like to also thank all my colleagues for their support and the funny moments.

In addition, I would genuinely like to thank Dr Alex Whitworth for mentoring me during my stay in his lab at the Mitochondrial Biology Unit (Cambridge,UK). I am very thankful to Alvaro for sharing his expertise with me and for his critical help on the electron microscopy. A special thank you to Leonor, with whom it has been a pleasure discussing about science and DJ-1. Furthermore, I thank Simonetta for her kind support and Tom for his useful tips on the Oroboros. Of course, I am grateful to Erika for her precious help with my experiments on the mitochondrial fucntionality. Finally, I thank all the lab members for their collaborative spirit and kindness.

I would additionally like to express my sincere gratitude to Prof. Mike Murphy and all his lab team, especially Anja and Hiran, whom I had the pleasure to collaborate with. Moreover, I take this opportunity to thank Prof. Mike Murphy and Dr. Stefania Girotto for their valuable comments on my thesis as reviewers of this manuscript. Also, I would like to thank my PhD evaluators Prof. Luca Scorrano and Dr. Tito Calì for their important suggestions and insights on my research project.

Last but not least, a heartfelt thank you to my family who supported my choices and encouraged me continuously in this path since the very beginning.

“The whole history of science has been the gradual realization that events do not happen in an arbitrary manner but that they reflect a certain underlying order.”

Stephen Hawking

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Abstract

Reactive oxygen and nitrogen species, collectively called RONS, are crucial molecules involved in multiple beneficial functions at the physiological level. However, RONS can assume detrimental roles at higher concentrations.

Among the different cellular sources of RONS, mitochondria play a central role, in particular, when they are not properly functioning. In fact, a defective mitochondrial homeostasis has been recurrently associated with many pathological states, such as neurodegenerative diseases. In this scenario, the anti-oxidant defence exerts a pivotal role in counteracting the damaging effects of RONS. Among the different anti-oxidant molecules, superoxide dismutases (SODs) are often considered as the first line of defence due to their ability to eliminate superoxide anions, from which more harmful species can arise. In recent years, mounting evidence is highlighting the protective role of the Parkinson's disease-associated protein DJ-1 against redox alterations. In fact, various reports have reported the participation of the protein in the anti-oxidant protection under different oxidative conditions, including exposure to exogenous pro-oxidants and ischemia-reperfusion injury. Nonetheless, the exact mechanism of action of DJ-1 has not been completely clarified yet.

In light of the aforementioned considerations, our project focused on the elucidation of the physiological functions of the protein *in vivo*. To this aim, we exploited *Drosophila melanogaster* as a model organism, using fruit flies lacking the expression of the fly DJ-1 homologue.

The loss of DJ-1 does not affect lifespan but results in mild locomotor dysfunctions. Moreover, the absence of the protein appears to influence the cristae morphology, supporting that the protein could play a role at the mitochondrial level. Therefore, we then explored the consequences of DJ-1 loss of function in the mitochondrial homeostasis, under basal and oxidative conditions. Our study showed that the absence of DJ-1 impairs mitochondrial functionality and morphology, especially, under oxidative stimuli. Furthermore, although not affecting the total levels of ATP, DJ-1 null flies are more sensitive to starvation than controls, suggesting a dysregulation in the mobilisation of the energetic storages.

We also set up the experimental conditions to investigate the fly metabolic response to anoxia, through the evaluation of succinate accumulation and ATP depletion, laying the ground for future experiments focused on the fly metabolic response to anoxia and on the role of DJ-1 in this pathway.

A second part of the project was dedicated to the exploration of the involvement of DJ-1 in the SOD1 maturation pathway, which is normally accomplished by a dedicated copper chaperone, named CCS. Nevertheless, a residual activity has been described in the absence of CCS, supporting the existence of a CCS-independent pathway. Our group has found that human DJ-1 can bind and transfer copper to SOD1, rendering the enzyme active *in vitro*, suggesting a role of DJ-1 in the alternative activation of SOD1. To investigate this pathway *in vivo*, we overexpressed or silenced the expression of DJ-1, under the absence of the fly CCS homologue.

Our results evidenced that DJ-1 is essential under the absence of CCS and that the ubiquitous overexpression of DJ-1 in *CCS* null background seems to rescue SOD1 protein levels. Since fly SOD1 is unstable in the absence of CCS, this data may suggest a possible participation of DJ-1 in the maturation of the enzyme, though further confirmation is required.

Overall, with this project, we contributed to add some pieces of information concerning the anti-oxidant role of DJ-1 *in vivo*. In accordance with the multifaceted nature of the protein, our data indicate that DJ-1 may exert its protective activity acting at different levels, ranging from the maintenance of the mitochondrial homeostasis to the possible activation of the anti-oxidant enzyme SOD1.

Chapter 1

Introduction

Reactive oxygen and nitrogen species and the anti-oxidant defence

1. Introduction

1.1 ROS and RNS species

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), collectively called reactive oxygen nitrogen species (RONS, see Figure 1), consist of radical and non-radical molecules formed by the reduction of oxygen (Weidinger and Kozlov, 2015). These species are the result of the normal cellular metabolism and play an important role in redox signalling at the physiological level. Contrary to other cellular signalling pathways based on protein-protein interaction or ligand-receptor binding, redox signalling employs ROS and RNS as effectors, which transfer the signal through protein modification (Schieber and Chandel, 2014). Nonetheless, when RONS production overwhelms the anti-oxidant capacity of the cell, these species assume a deleterious role, with detrimental effects on the cellular physiology (Valko et al., 2007). The principal cellular ROS are the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). Superoxide anion is not highly reactive toward cellular macromolecules and it has been shown to change proteins redox state without damaging their structures (Weidinger and Kozlov, 2015). This ROS species often is referred to as a "primary ROS", since its toxic potential principally relies on the ability to generate more reactive "secondary" ROS (Valko et al., 2006). Even though hydrogen peroxide is considered an inert molecule, it can combine with $O_2^{\bullet -}$ and iron through the Haber-Weiss reaction, generating the harmful hydroxyl radical. This species is currently recognised as the most reactive, because it possesses a highly damaging potential towards all macromolecules and it is associated with lipid oxidation, resulting in alterations in the membrane fluidity and integrity (Halliwell and Gutteridge, 1984; Lushchak, 2014).

Oxygen also participates in the production of RNS, a class of reactive nitrogen molecules, which includes nitric oxide radical ($NO^{\bullet}$), peroxynitrite ($ONOO^-$), and nitrogen dioxide radical ($NO_2^{\bullet}$) (Dhawan, 2014). Nitric oxide is an uncharged lipophilic radical, which can easily diffuse in the cytoplasm as well as permeate biological membranes. This molecule possesses a half-life of few seconds in aqueous solution. However, under low oxygen concentrations, its half-life is

extended up to 15 seconds (Valko et al., 2006). $NO^{\bullet}$ is normally generated during L-arginine breakdown by nitric oxide synthase (NOS) enzyme. Albeit involved in numerous physiological functions, under oxidative conditions, $NO^{\bullet}$ can react with $O_2^{\bullet-}$, favouring the generation of peroxynitrite ($ONOO^-$), a highly oxidizing molecule, which leads to protein nitration (Valko et al., 2006). After its synthesis, $ONOO^-$ can be rapidly decomposed into $OH^{\bullet}$ and $NO_2^{\bullet}$, further supporting oxyradicals production. Similarly to the superoxide anion, nitric oxide acts as a "primary" RNS, which is responsible for the formation of more dangerous "secondary" reactive molecules. Therefore, the principal defence mechanism against "secondary" RONS can be accomplished through their avoidance, a condition that can be reached by tightly regulating the concentration of "primary" RONS. Considering that most of RONS derive from the superoxide radical, a strict control over its synthesis or removal represents a pivotal mechanism to maintain the cellular integrity.

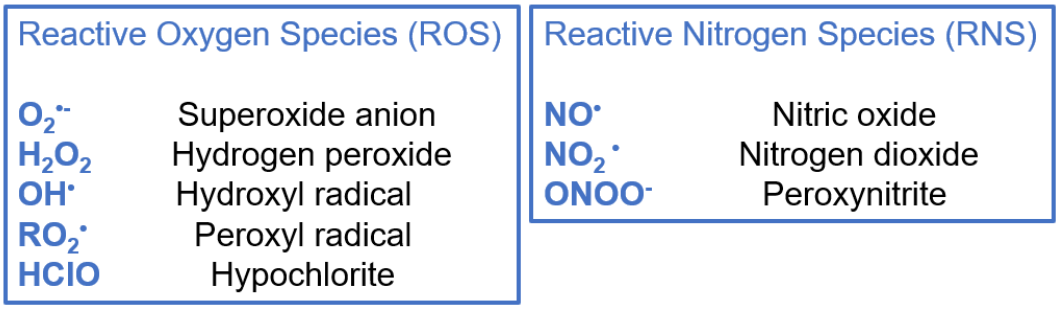


Figure 1. Principal reactive oxygen (ROS) and nitrogen species (RNS).

1.2 Cellular sources of RONS

Within the cell, multiple mechanisms contribute to RONS generation, with mitochondria being the main source, followed by other cellular sites that act as additional sources. In the following sections, general mechanisms of RONS production will be presented, focusing, in particular, on mitochondria-derived species.

1.2.1 Mitochondria

Dealing with oxygen, mitochondria represent the major contributors to ROS production. Indeed, even under optimal conditions, the redox reactions occurring during oxidative phosphorylation render each electron carrier in the electron transport chain (ETC) prone to react with molecular oxygen, eventually producing ROS. Superoxide anion and hydrogen peroxide represent the major ROS species produced during oxidative phosphorylation, and their generation is substantially increased when ETC is impaired in dysfunctional mitochondria. Complex I and complex III are considered the major sites of ROS production (Figure 2).

Complex I is the first component of the respiratory chain, where electrons are initially accepted from NADH by flavin mononucleotide (FMN) and released to Coenzyme Q (CoQ). Superoxide anion production is observed upon interaction of molecular oxygen with reduced FMN, under high $NADH/NAD^+$ ratio in the mitochondrial matrix (Murphy, 2009). Under physiological conditions, the FMN redox state mainly depends on the $NADH^+/NAD$ ratio, condition that is determined by the rate of mitochondrial respiration. When mitochondria are properly respiring, the $NADH^+/NAD$ ratio is maintained at low levels, and $O_2^{\bullet-}$ production is limited. Differently, under low ATP requirement or when mitochondria are damaged, NADH can accumulate, sustaining FMN reduction, and, therefore, driving $O_2^{\bullet-}$ generation (Murphy, 2009).

Alternatively, complex I has been shown to produce abundant ROS by the so-called reverse electron transport (RET), which is associated with a reversion of the electron flux. Indeed, when the CoQ pool is reduced ($CoQH_2$) and in the presence of a high proton driving force, electrons can be induced to go back to the FMN site, driving $O_2^{\bullet-}$ formation (Robb et al., 2018). Despite having been considered an *in vitro* phenomenon for long, it is becoming evident that RET occurs also *in vivo*, under different conditions (Scialò et al., 2017). For example, RET has been shown to participate in the redox signalling activated during the inflammatory response (Mills et al., 2016), to play a role in the reorganisation of the respiratory complexes under metabolic variations (Guarás et al., 2016), as well as to contribute to the adaptation of the carotid body to changing oxygen levels (Fernández-Agüera et al., 2015).

Furthermore, RET has gained attention as a primary mechanism leading to the production of superoxide anions upon reperfusion, due to the oxidation of the succinate accumulated during ischemia (Chouchani et al., 2016). In addition, RET has been described to promote lifespan extension in *Drosophila* (Scialò et al., 2016). In conclusion, complex I can promote ROS generation both during the forward and reverse electron flux. Importantly, the superoxide radical produced by complex I is released in the mitochondrial matrix, where it is held due to its charged state. In this compartment, then, it will be rapidly detoxified by the superoxide dismutase enzyme SOD2 (Valko et al., 2007).

Through the "Q-cycle", complex III has been described as the second site of ROS production within the respiratory chain (Cadenas et al., 1977; Turrens et al., 1985). The physiological function of this complex is to receive reducing equivalents generated by complex I and II through ubiquinol, eventually transferring electrons to cytochrome c. The functional elements of this complex are two cytochrome b subunits that constitute a hydrophobic pocket into which CoQ diffuses. In this process, the ubiquinol delivers its electrons to the Rieske iron-sulphur protein, becoming an unstable ubisemiquinone, which acts as electron donor for cytochrome b units. Through this step, ubisemiquinone is oxidised to ubiquinone. Under physiological conditions, the semiquinone radical is rapidly oxidised to ubiquinone, reducing the probability of electron transfer to molecular O_2 (Zorov et al., 2014). However, when this cycle is disrupted, for example by antimycin A, oxygen can interact with ubisemiquinone leading to $O_2^{\bullet-}$ generation (Murphy, 2009; Zorov et al., 2014). Differently from complex I, complex III-derived $O_2^{\bullet-}$ is reversed both within the matrix and in the intermembrane space (Kudin et al., 2004; Muller et al., 2004; St-Pierre et al., 2002). In this last compartment, SOD1 is principally responsible for $O_2^{\bullet-}$ clearance with concomitant H_2O_2 production (Kawamata and Manfredi, 2010). Nonetheless, $O_2^{\bullet-}$ has been shown to cross the outer membrane via the voltage-dependent anion channels (VDAC), accumulating in the cytosol (Han et al., 2003). It is noteworthy that the contribution of complex III to mitochondrial ROS production under physiological conditions remains still ambiguous, since it gives rise to $O_2^{\bullet-}$ production only under antimycin A treatment. However, its participation in ROS generation may become significant under conditions affecting the stability of the

ubisemiquinone radical, such as the CoQ and cytochrome c redox state, variations in the proton driving force, and cytochrome c depletion (Murphy, 2009).

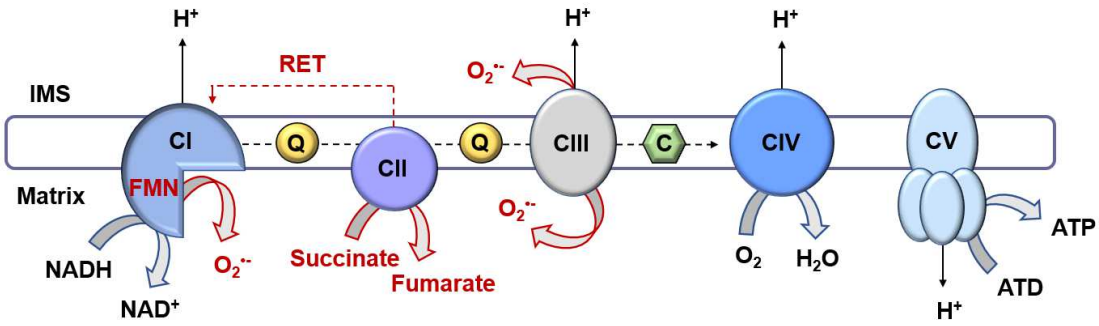


Figure 2. Mechanisms of production of superoxide anions within the respiratory chain. CI is the entry point, where electrons are accepted from NADH by flavin mononucleotide (FMN) and delivered to Co-enzyme Q (Q). Q accepts electrons from CI and CII and delivers them to CIII. From CIII electrons are transferred through cytochrome c (C) to CIV, which forms water from oxygen. Finally, the CV produces ATP exploiting the proton gradient generated by CI, CIII, and CIV. In this process, CI and CIII are the major sites for ROS formation. CI generates ROS when FMN interacts with molecular oxygen generating $O_2^{\bullet-}$. Alternatively, CI produces ROS through reverse electron transport (RET), e.g. upon ischemia-reperfusion (succinate accumulation). CI-derived $O_2^{\bullet-}$ is reversed in the matrix. CIII participates in ROS generation upon disruption of the Q cycle. CIII-derived $O_2^{\bullet-}$ is reversed both in the matrix and in the intermembrane space (IMS). Sites and mechanisms of ROS generation are highlighted in red. Abbreviations: complex I (CI), complex II (CII), complex III (CIII), complex IV (CIV), and complex V (CV).

Besides complex I and III, other mitochondrial site are plausible sources for ROS generation. One source is associated with NADH pool in the mitochondrial matrix (Murphy, 2009; Zorov et al., 2014). In this compartment, there are two NADH-dependent enzymes, known as α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, which include dihydrolipoamide dehydrogenase in their complex. This component is a flavin-containing enzyme that can produce ROS when NAD^+ concentrations are restricted (Bunik and Sievers, 2002; Starkov, 2004; Tretter, 2004).

Another possible mitochondrial ROS source relies on CoQ-interacting sites, causing CoQ reduction (Murphy, 2009). In the mitochondrial matrix, β-fatty acid oxidation

has been associated with $O_2^{\bullet-}$ and H_2O_2 generation (Quijano et al., 2016). In particular, the reduction of CoQ from electron transfer flavoprotein (ETF) by ETF-CoQ oxidoreductase has been found as potential $O_2^{\bullet-}$ production site (St-Pierre et al., 2002). Similarly, on the outer side of the inner mitochondrial membrane, the glycerol-3-phosphate dehydrogenase enzyme, which participates in lipid metabolism, transfers electrons from glycerol-3-phosphate to CoQ, with consequent ROS generation (Drahota et al., 2002; Tretter et al., 2007). In the same compartment, the enzyme dihydro-orotate dehydrogenase, which participates in pyrimidine nucleotide biosynthesis, has been reported to produce $O_2^{\bullet-}$, by transporting electrons from dihydro-oratate to CoQ (Forman and Kennedy, 1976). Hence, albeit the contribution of these additional sources remain to be clarified, it is plausible that complex I and III are not the only players in mitochondrial ROS generation and that, under specific conditions, these alternative sites could become relevant (Murphy, 2009).

It is worth mentioning that mitochondrial-derived ROS have been mostly evaluated using isolated organelles, representing a more artificial condition. Indeed, *in vitro* analyses have estimated that approximately 0.1-2% of O_2 is converted into H_2O_2, however, under *in vivo* conditions, ROS production could be much lower (Murphy, 2009). In this regard, more recently, *in vivo* and *ex-vivo* techniques have been introduced, which could help to better estimate the rate of mitochondrial ROS production under more physiological conditions (Dikalov and Harrison, 2014).

1.2.2 Extra-mitochondrial sources

In addition to mitochondria, there are other cellular sources contributing to RONS generation. For example, the membrane-bound NADH-oxidase (NOX) produces superoxide anion as a host-defence mechanism (S. Hernandes and R.G. Britto, 2012). Similarly, the activation of protein myeloperoxidase (MPO), which is involved in pathogen defence mechanisms, accounts for the generation of hypochlorite (HClO), a “secondary” ROS involved in lipid peroxidation (Lefkowitz and Lefkowitz, 2008). Another class of ROS producing enzymes are cyclooxygenases and lipoxygenases, which indirectly promote the formation of peroxyl radicals. These enzymes are involved in the conversion of arachidonic acid to different eicosanoids,

which participate in blood pressure regulation, platelet aggregation, and inflammation (Dennis and Norris, 2015). In addition, the metalloflavoprotein xanthine oxidase also cooperates in ROS generation, synthesising $O_2^{\bullet-}$ via nucleic acids degradation (Dhawan, 2014). Regarding RNS, the primary source is represented by NOS enzymes, which produce nitric oxide. There are three isoforms of NOS, two of which are constitutively expressed in neuronal (nNOS) and endothelial (eNOS) cells, both activated by calcium flux. Their activity guarantees the formation of $NO^{\bullet}$ required for signals transmission and to maintain a basal vasodilator tone. Macrophages express an inducible NOS isoform (iNOS), which is regulated by inflammatory mediators, principally involved in pathogen defence (Dhawan, 2014). Although NOS plays an important physiological role, it is worth mentioning that when the enzyme is not properly assembled, condition known as uncoupling, these enzymes can produce $O_2^{\bullet-}$ rather than $NO^{\bullet}$, exacerbating RONS production (Pou et al., 1992; Stuehr et al., 2001).

1.3 Physiological functions of RONS

As aforementioned, under low concentrations, RONS act as messengers in redox signalling, mediating important physiological processes. Usually, redox signalling exploits H_2O_2 as a mediator to modify protein redox state through cysteine oxidation. Thiol groups of cysteines exist in multiple redox states, which can promote different post-translational modifications, eventually influencing protein structure, activity, and localisation (Wang et al., 2018). Moreover, being cysteines principally located in the active site of proteins, this modification eventually impacts on the enzymatic activity, determining protein activation or inhibition according to the target involved (Schieber and Chandel, 2014). In addition to cysteines, also methionines participate in signal transmission, via redox modifications. Indeed, this amino acid bears sulphur atoms that can undergo reversible oxidation (Drazic and Winter, 2014).
An important aspect to be considered in the determination of RONS effects is their subcellular compartmentalisation. Targets of redox signalling usually localise near the site of RONS production to be readily oxidised when required (Schieber and Chandel, 2014). In this way, RONS participate in the modulation of signalling cascades involved in the fine regulation of cell cycle and cell death. Numerous

pathways are sensitive to RONS concentrations through redox modifications. For example, during ischemia, H_2O_2 is required for the activation of the hypoxia-inducible factor HIF1-α, an oxygen-sensitive transcription factor involved in angiogenesis. Indeed, mitochondrial-derived ROS have been reported to stabilise HIF1-α under hypoxia (Guzy et al., 2005; Schroedl et al., 2002). Similarly, the activation of the nuclear transcription factor-erythroid 2-related factor 2 (Nrf2), a master transcription factor for anti-oxidant responses, relies on oxidative modifications. Under physiological levels of RONS, Nrf2 is normally sequestered in the cytosol, bound to its inhibitor named kelch-like ECH-associated protein (Keap1). Nonetheless, upon oxidative stimuli, Keap1 can be readily oxidised, removing its inhibitory activity towards Nrf2 and allowing its nuclear translocation (Ma, 2013). Furthermore, the immune system utilises RONS to limit pathogen infection. For example, $O_2^{\bullet-}$ has been proposed to modulate inflammation through inflammasome activation and cytokines release (Zhou et al., 2011, p. 3). Interestingly, RONS have also been implied in muscle regeneration after injury. In fact, RONS production, in particular, H_2O_2, is important to regulate staminal muscle cells proliferation and differentiation through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) (Lee et al., 2011). Moreover, NOS activity has been observed to increase during myogenic differentiation, supporting a role of $NO^{\bullet}$ in this process (Lee et al., 1997). Besides muscle regeneration, $NO^{\bullet}$ seems to participate in multiple physiological processes, such as inhibition of platelet aggregation, vasodilatation, neuronal firing, and synaptic plasticity (Kim et al., 2004; Wang et al., 1998; Zhong et al., 2013).

Among the positive roles of RONS, it is also worth mentioning the concept of *mitohormesis*. According to this hypothesis, mild mitochondrial dysfunctions and consequent RONS production can promote longevity (Ristow and Zarse, 2010). Multiple studies performed in fruit flies and worms have shown the positive effects of ETC dysfunctions in life extension (Sanz, 2016). A proposed explanation is that a slight increase in RONS levels triggers the anti-oxidant defence compensating for the initial boost of reactive species and their damage (Ristow and Zarse, 2010).

Considering these findings, the beneficial role of RONS species in several biological processes is well accepted in literature. Nonetheless, this function is ensured only under low RONS levels and seems to be mainly restricted to $O_2^{\bullet-}$, H_2O_2, and $NO^{\bullet}$ molecules, while other "secondary" RONS are usually considered harmful species. Therefore, to define a positive or negative effect of a reactive species, the mechanism of its synthesis or removal, its concentrations, and subcellular localisation are all features that should be considered.

1.4 Altered RONS homeostasis and pathological consequences

Chronic excessive levels of pro-oxidant species are considered the cornerstone of many pathologies (Figure 3). As previously discussed, when RONS production overwhelms the anti-oxidant capacity of the cell, the entire cellular homeostasis is heavily affected. Indeed, when "primary" RONS are not tightly regulated, they can assume detrimental functions rather than being redox messengers. For example, $NO^{\bullet}$ was reported to bind to the iron-sulphur cluster of cytochrome c oxidoreductase, inducing a reversible inhibition of the enzyme (Cleeter et al., 1994). Moreover, nitric oxide has been shown to affect complex III function, exerting an antimycin-A like activity (Iglesias et al., 2015). In addition, excessive cysteine oxidation by H_2O_2 can inactivate redox-sensitive proteins, impairing multiple signalling cascades (Friguet, 2006). More importantly, the persistent production of $O_2^{\bullet-}$, H_2O_2, and $NO^{\bullet}$ molecules favours the formation of "secondary" species characterised by higher reactivity. Among them, $HO^{\bullet}$ is one of the most reactive, lacking a dedicated detoxification system. Having an *in vivo* half-life of 10^{-9} seconds, $HO^{\bullet}$ acts near the site of its formation, affecting all biological macromolecules. Moreover, $NO^{\bullet}$ can react with $O_2^{\bullet-}$ generating $ONOO^-$, a strong oxidising agent able to induce DNA fragmentation and lipid oxidation (Valko et al., 2007). Therefore, under these conditions, nucleic acids, proteins, and lipids become irreversibly damaged, eventually promoting cytotoxicity. This redox unbalance is frequently observed in many pathologies, and, in particular, neurodegenerative diseases have been strictly associated with an impaired RONS homeostasis (Liu et al., 2017).

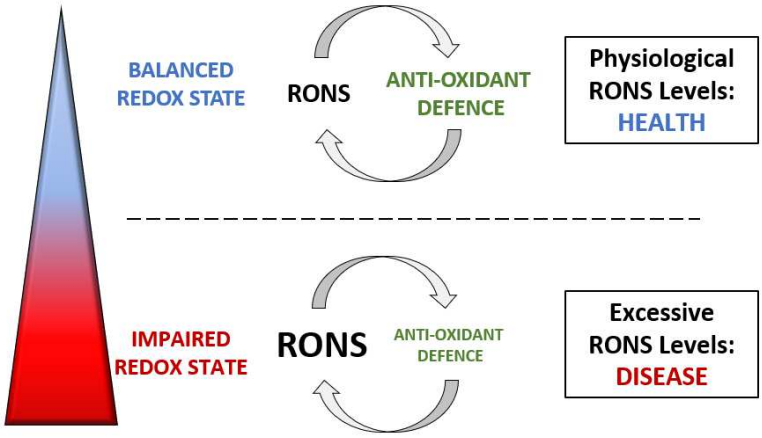


Figure 3. Redox homeostasis in health and disease. Under physiological conditions, the production of RONS is counterbalanced by an appropriate anti-oxidant defence (health). However, when this equilibrium is impaired, due to an excessive RONS generation or to a defective anti-oxidant defence, the uncontrolled RONS levels can lead to pathological conditions (disease).

From the metabolic point of view, the brain is one of the most active organs, characterised by the highest oxygen demand, consuming about 20% of total oxygen for aerobic metabolism (Halliwell and Gutteridge, 1984). Therefore, neurons are strictly dependent on mitochondrial ATP production to sustain their synaptic activity and to maintain ionic gradients (Cobley et al., 2018). Due to reduced antioxidant buffering, high content in transition metals, and low regenerative capacity, brain neurons are highly vulnerable to uncontrolled RONS concentrations. Indeed, mounting evidence demonstrated that neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are all characterised by high levels of oxidised lipids, proteins and nucleic acids (Ramalingam and Kim, 2012). Multiple factors seem to contribute to this state, including mitochondrial dysfunction, neuroinflammation, and ischemic episodes, which altogether boost RONS production (Liu et al., 2017; Snyder et al., 2017). In the following paragraphs, the sources of RONS will be discussed specifically in the context of PD, focusing, in particular, on the mitochondrial role in the pathology (summarised in Figure 6).

1.5 Parkinson's disease

PD is a chronic and progressive movement disorder that affects approximately 1-2% of the population over the age of 65 years. Clinically, the main symptoms are tremor, rigidity, bradykinesia and postural instability. Nonetheless, nowadays PD is considered a more complex illness encompassing both motor and non-motor symptoms (Przedborski, 2017). From the neuropathological point of view, PD is characterized by a selective degeneration of dopaminergic neurons within Substantia Nigra pars compacta (SNpc) and by the formation of intracellular aggregates, called Lewy bodies (LBs), in the surviving neurons (Dickson, 2012) (Figure 4). Although the disease is principally of sporadic origin, the identification of genetic forms of the pathology has helped to partially understand the cellular mechanisms involved in the syndrome. Familial cases account for 5-10% of the disease and are classified into dominant and recessive forms according to the pattern of inheritance. Among the causative genes, *SNCA* and *LRRK2* are associated with dominant PD cases, while *Parkin*, *PINK1*, *DJ-1* contribute to recessive forms of the disease (Kalia and Lang, 2015). α-Synuclein is the major component of LBs also in sporadic PD manifestations, while LRRK2 is a kinase implicated in different cellular functions, among which vesicle trafficking, synaptic morphogenesis, and neurite outgrowth. Differently, Parkin, PINK1, DJ-1 are all proteins involved in mitochondrial homeostasis. Parkin, an E3 ubiquitin ligase, and PINK1, a serine-threonine protein kinase, cooperate to maintain mitochondrial quality control driving the disposal of damaged or old mitochondria, while DJ-1 is a multitasking protein principally implicated in the activation of anti-oxidant responses (Kalia and Lang, 2015). Besides these genes, numerous other *loci* have been associated with the pathology, further supporting the involvement of genetics in the disease onset (Deng et al., 2018). Although the precise PD aetiology is still unclear, there is a growing body of evidence suggesting that oxidative and nitrosative stress are important players in the disease development (Tsang and Chung, 2009).

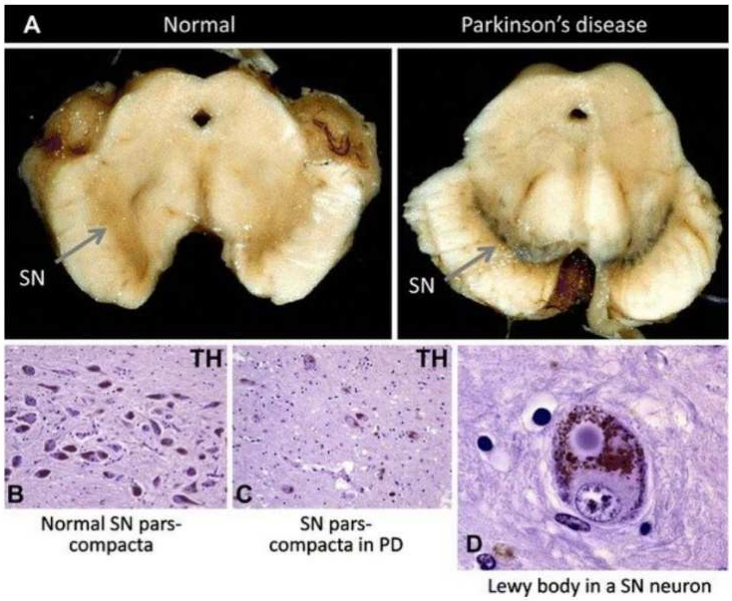


Figure 4. Histopathological hallmarks of PD. (A) PD is characterised by the loss of SN dopaminergic neurons, which results in the SN depigmentation (B) and loss of tyrosine hydroxylase (TH, an enzyme responsible for dopamine synthesis) positive neurons. (C) Another PD hallmark is the formation of proteinaceous aggregates, called Lewy bodies (LBs). (Mandel et al., 2010).

1.5.1 RONS sources in Parkinson's disease

1.5.1.1 Mitochondrial dysfunctions

Mitochondria have received special interest in PD etiopathology, especially considering that numerous PD-related genes and neurotoxins have shown to affect mitochondrial functionality (Haddad and Nakamura, 2015). The first evidence suggesting the involvement of mitochondria in the disease came when MPTP (1methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a contaminant of a synthetic opiate, was shown to induce parkinsonism. In fact, its metabolite 1-methyl-4-phenylpyridinium (MPP+) was reported to primarily accumulate in the dopaminergic mitochondria, interfering with complex I activity and consequently leading to ROS production (Javitch et al., 1985). Successively, other complex I inhibitors, among which rotenone, pyridaben, and fenpyroximate, have been identified as risk factors of the disease (Bose and Beal, 2016). In addition, the weed-killer paraquat was found to increase PD-risk probably acting at mitochondrial level (Castello et al., 2007; Tanner et al., 2011; A. Wang et al., 2011). Although its toxicity seems to rely on multiple mechanisms, including neuroinflammation (Purisai et al., 2007; Wu et al., 2005), the compound was also observed to cause mitochondrial oxidative damage through the generation of $O_2^{\bullet -}$ by redox-cycling, via complex I-dependent reduction (Cochemé and Murphy, 2008). Altogether these findings have

corroborated the involvement of complex I dysfunctions in the pathology. In accordance, *post-mortem* studies on PD patients' brain have demonstrated that mitochondrial complex I activity is frequently impaired (Hattingen et al., 2009; Hattori et al., 1991; Schapira et al., 1990). Moreover, further analyses highlighted that also complex II and complex IV are affected in the SN neurons of PD patients (Bender et al., 2006; Grünewald et al., 2016; Kraytsberg et al., 2006). A recent study has suggested that alterations in the respiratory functionality might be driven by an impaired protein import machinery into mitochondria (Franco-Iborra et al., 2018). Indeed, respiratory complexes are composed of both nuclear- and mitochondrial-encoded proteins, therefore, a defective import of the nuclear-encoded subunits can affect the stability and functionality of the ETC complexes. In this regard, authors have found that PD patients' SN presented a downregulation in the mitochondrial import machinery associated with a defective assembly of complex I (Franco-Iborra et al., 2018). Besides an impaired protein import, mitochondrial functionality might be also impaired by a defective mitochondrial DNA (mtDNA) homeostasis. In fact, PD SN has been associated with a higher mutational rate at the mtDNA level, also affecting genes encoding proteins of the respiratory chain (Coxhead et al., 2016). Moreover, recent papers have found that PD patients tend to display a reduced mtDNA copy number than healthy individuals and that this deregulation could contribute to drive the mitochondrial dysfunction observed in the disease (Dölle et al., 2016; Grünewald et al., 2016).

A proper mitochondrial functionality relies also on the maintenance of the mitochondrial homeostasis. In this context, an important role is played by mitophagy, which is a physiological process responsible for the disposal of old or damaged mitochondria. Remarkably, defects in this pathway have been reported in PD (Liu et al., 2019). In fact, Parkin and PINK1 are well-recognised players in this pathway and their loss of function has been shown to largely impair the mitochondrial homeostasis with detrimental cellular consequences (Liu et al., 2019). Furthermore, Parkin and PINK1 have been found to regulate the ubiquitination of mitofusin 1 and 2 (Gegg et al., 2010; Glauser et al., 2011), proteins involved in the process of mitochondrial fusion, supporting a role of mitochondria dynamics in PD (Burté et al., 2015). Additionally, loss of function mutations in *DJ-1* gene have been associated

with mitochondrial fragmentation and altered respiration (Wang et al., 2012). Furthermore, mutations in OPA1 protein, a key regulator of mitochondrial dynamics, have been linked to parkinsonism, further supporting the role of mitochondrial dynamics in the pathology (Carelli et al., 2015). Indeed, fission and fusion are pivotal processes for the proper maintenance of synaptic functions and their impairment could represent a further factor contributing to dopaminergic neurons vulnerability (Burté et al., 2015). Altogether, these alterations in the mitochondrial function, turnover, and dynamics eventually lead to dysfunctional mitochondria, which can act as a major source of dangerous reactive species.

1.5.1.2 Ischemia-reperfusion injury

Besides these alterations, mitochondrial can further contribute to ROS generation in the pathology, through ischemia-driven RET mechanism. Indeed, hypoxic brain injury has been suggested as a potential condition contributing to PD development (Karunasinghe and Lipski, 2013; Lin et al., 2010; Rodriguez-Grande et al., 2013; Swaminath et al., 2006). Notably, PD patients frequently experience respiratory deficiency and sleep apnoea with pneumonia representing a primary cause of death (Shafqat et al., 2017; Shill and Stacy, 2002). Moreover, PD patients have been reported to display a reduced ability to respond to hypoxia, accompanied by a blunted perception of dyspnoea, even at early stages of the disease (Onodera et al., 2000). Although still poorly explored, the relationship between oxygen supply deficiency and PD is now emerging, representing an additional cause of neurodegeneration.

The detrimental effects of hypoxia depend mainly on the adaptive metabolic changes that occur under oxygen depletion. When perceiving low oxygen levels, cells slow down the metabolic rate and cellular activity (Kalogeris et al., 2012). Due to the lack of oxygen, the mitochondrial respiration is inhibited, with consequent ATP depletion. Since ATP is required as the regulator of different ATP-dependent ionic exchangers, involving Na^+, Ca^{2+}, K^+ , and H^+ ions, the ischemic period leads to ionic imbalance and pH changes (Kalogeris et al., 2012). Moreover, under this state, there is a conspicuous accumulation of succinate, which is a key metabolite normally produced by the tricarboxylic acid (TCA) cycle and used as substrate by

complex II. However, under ischemia, complex II has been shown to reverse its activity, generating succinate from fumarate (Chouchani et al., 2014). Upon reoxygenation, the accumulated succinate is rapidly oxidised driving a massive ROS production, via RET (Chouchani et al., 2016) (Figure 5). In addition, NOXs and xanthine oxidases have been suggested as additional mechanisms participating in RONS production in this condition, although their contribution seems negligible compared to complex I (Braunersreuther et al., 2013; Jassem et al., 2002). The consequence of this initial burst of RONS is the following activation of cell death signalling cascades, which mainly involve the RONS-dependent opening of the mitochondrial permeability transition pore (mPTP) (Kim et al., 2006). mPTP opening is rapidly triggered upon reoxygenation and causes a loss of permeability in the inner mitochondrial membrane, favouring uncoupling of the oxidative phosphorylation and mitochondrial swelling, with consequent activation of cell death pathways (Kim et al., 2006; Seidlmayer et al., 2015). Therefore, considering the established role of ischemia-reperfusion in ROS generation, it could represent an additional ROS source in the disease.

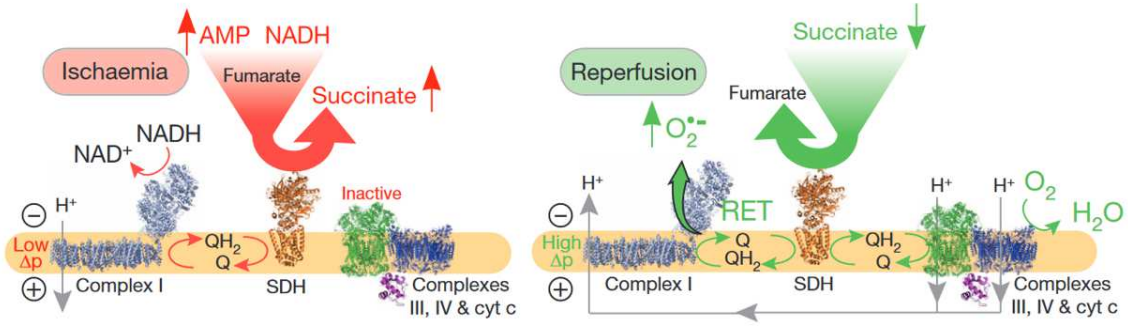


Figure 5. Proposed mechanism of ROS production by ischemia-reperfusion. During ischemia, the respiratory chain is inhibited due to the lack of oxygen, leading to NADH accumulation, reduction of the CoQ pool ($CoQH_2$), low protonmotive force (Δp), and ATP depletion. Under this condition, complex II seems to reverse its activity, producing succinate from fumarate, which accepts electrons from $CoQH_2$. When oxygen is reintroduced, succinate is rapidly oxidised by complex II. This phenomenon induces a high Δp, driving the reverse electron transport (RET) back to the FMN site of complex I, with concomitant conspicuous production of superoxide anions. Figure taken from Chouchani et al., 2014.

1.5.1.3 Neuroinflammation

Another important source that has been reported to contribute to RONS accumulation in PD is neuroinflammation. This stressful condition is determined by hyperactivation of microglia, the resident immune cells of the brain (Kettenmann et al., 2011). Under physiological conditions, microglial cells have a quiescent phenotype and regulate brain homeostasis, by releasing neurotrophic and anti-inflammatory molecules. Upon activation, microglia trigger the immune response to eventually restore the tissue homeostasis. Usually, this protective response is concluded after that the eliciting stress factor has been eradicated. Nonetheless, if the stimulus is not properly removed, chronic microglia activation promotes the establishment of a permanent inflammatory state that leads to the accumulation of toxic RONS species (Kettenmann et al., 2011). The major species released are $O_2^{\bullet-}$ and $NO^{\bullet}$, generated respectively by NOX and NOS enzymes (Valko et al., 2007). The role of neuroinflammation in PD pathology has been firstly recognised by the finding of reactive microglia in PD patient's brain (McGeer et al., 1988). Several factors have been proposed as microglia triggers in the disease. One of them is neuromelanin, a pigment derived mainly from the co-polymerization of dopamine and peptide moieties, which is responsible for the dark colour of SN dopaminergic neurons. Albeit its involvement remains uncertain, it has been reported that neuromelanin released from dying dopaminergic neurons functions as a microglial stimulus, through NOX activation (Zhang et al., 2011). α-Synuclein and LRRK2 have also been proposed as modulators of neuroinflammation (Schapansky et al., 2015) In fact, the release of extracellular α-synuclein aggregates by dying neurons has been shown to favour NOX activation, upon internalisation by microglia cells (Gao et al., 2011; W. Zhang et al., 2005). Differently, LRRK2 appears to positively regulate inflammation, since its down-regulation has been associated with a reduced microglia activation (Moehle et al., 2012; Russo et al., 2015). In conclusion, although it is still uncertain whether neuroinflammation represents a cause or consequence of the disease, its participation in RONS generation and in PD progression is currently well recognised.

1.5.1.4 Dopamine metabolism

An additional source of RONS in PD pathology arises from dopamine metabolism (Bisaglia et al., 2014, 2013). Because of its chemical nature, dopamine is a molecule prone to oxidation at its electron-rich catechol moiety. Although, normally stable inside synaptic vesicles, due to the acidic environment, when exposed to the cytosolic pH it becomes unstable and can auto-oxidised, leading to the production of $O_2^{\bullet-}$ and H_2O_2 (Bisaglia et al., 2014). Moreover, this process is accompanied by the formation of highly reactive dopamine-derived quinones (DAQs) that are involved in nucleophilic addition reactions, affecting macromolecules stability (Bisaglia et al., 2014; Monzani et al., 2019). One major target of DAQs are cysteines, being among the strongest cellular nucleophiles (Bisaglia et al., 2013; Hastings et al., 1996). Furthermore, DAQs can also interact with guanine bases of DNA, forming DNA adducts, which are cause of genomic alterations and mutations (Stokes et al., 1999). Therefore, besides directly producing reactive species via oxidation, dopamine can induce cellular damages by DAQs formation, exacerbating the general state of oxidative stress.

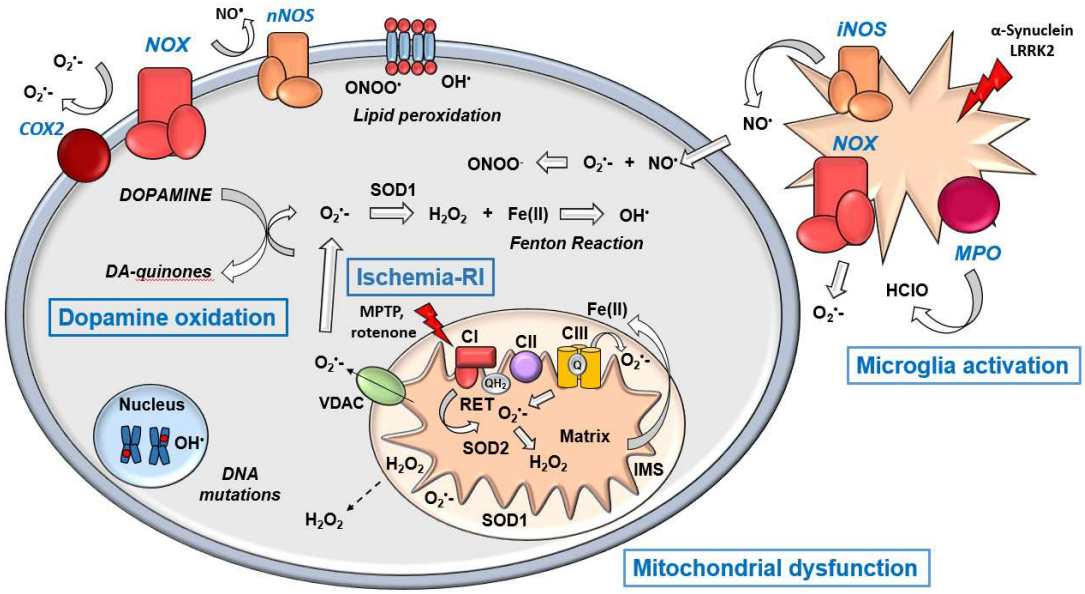


Figure 6. Sources of oxidative stress in PD. Mitochondrial dysfunction, ischemia-reperfusion injury, dopamine metabolism and neuroinflammation co-participate in promoting oxidative damage in dopaminergic neurons. Complex I and complex III are the

major sources of $O_2^{\bullet-}$ inside the cells. The PD-associated toxins MPTP and rotenone act as complex I inhibitors. Complex I produces $O_2^{\bullet-}$ via by forward electron flux and by reverse electron transport (RET), upon ischemia-reperfusion. Chronic activation of microglia further contributes to the generation of $O_2^{\bullet-}$ and $NO^{\bullet}$ radicals by the action of NADPH-oxidase (NOX) and inducible nitric oxide synthase (iNOS), respectively. In addition, activated microglia produces HClO via myeloperoxidase (MPO). The PD-related proteins α-synuclein and LRRK2 stimulate microglia activation. In dopaminergic neurons, the cytosolic oxidation of dopamine to dopamine-quinones induces the production of the $O_2^{\bullet-}$. Cytosolic $O_2^{\bullet-}$ is converted by superoxide dismutase 1 (SOD1) in H_2O_2, which can react with iron leading to the generation of the highly toxic radical HO• through the Fenton reaction. In the mitochondrial matrix, $O_2^{\bullet-}$ is detoxified by SOD2. Differently, $O_2^{\bullet-}$ can react with NO• to form the harmful molecule ONOO-. (IMS: intermembrane space; nNOS: neuronal nitric oxide synthase; VDAC: voltage-dependent anion channels). Figure adapted from De Lazzari et al., 2018.

1.6 Counteracting RONS: the role of the antioxidant defence

Considering the numerous sources of RONS and their pathological effects, the antioxidant response plays a pivotal role in counteracting this stressful condition to preserve the cellular physiology. As previously described, RONS class includes multiple species with variable reactivity. However, most of them, if not all, derive from superoxide anion, recognised as the “primary” ROS. Therefore, the principal cellular anti-oxidant defence relies on the presence of superoxide dismutases (SODs), which are enzymes devoted to the detoxification of superoxide anion. For this reason, SODs are considered the first line of defence against RONS, because their activity prevents the formation of highly damaging “secondary” species. Cells present three SOD isoforms with different spatial distribution. SOD1 is predominantly found in the cytosol but is also found in the nucleus and in the intermembrane space (Gertz et al., 2012; Kawamata and Manfredi, 2010). SOD2 detoxifies the mitochondrial $O_2^{\bullet-}$ within the matrix, while SOD3 acts at the extracellular level (Wang et al., 2018). All three isoforms convert superoxide anion into hydrogen peroxide and molecular oxygen, but they use different ions for their catalytic activity. In particular, both SOD1 and SOD3 rely on copper and zinc, while SOD2 utilises manganese (Wang et al., 2018). Considering their intracellular

localisations, special interest has been given to SOD1 and SOD2 enzymes. SOD2 exerts a pivotal role in detoxifying the respiratory chain-derived $O_2^{\bullet-}$, especially considering that complex-I-derived $O_2^{\bullet-}$ is exclusively released into the matrix (Valko et al., 2007). In addition, SOD1 plays a role in mitochondrial $O_2^{\bullet-}$ scavenging as well, being distributed also in the intermembrane space (Kawamata and Manfredi, 2010). In this sub-compartment multiple mitochondrial processes take place, including protein import, oxidative folding, transport of metabolites, lipids and metals ions, assembly of the respiratory complexes, and redox signalling (Herrmann and Riemer, 2010). Consequently, alterations in this space impact on the entire mitochondrion and its homeostasis. Therefore, thanks to its broad expression, SOD1 preserves the physiological redox state of the entire cell, mainly buffering cytosolic $O_2^{\bullet-}$, while limiting the propagation of the mitochondrial-derived $O_2^{\bullet-}$ from the intermembrane space of mitochondria.

The detoxifying activity of SODs enzymes results in the production of H_2O_2, which is detoxified by multiple mechanisms. Hydrogen peroxide is eliminated by glutathione peroxidases, thioredoxins, peroxiredoxins, and glutaredoxins. In addition, H_2O_2 clearance is also accomplished by catalase, enzyme principally found within peroxisomes (Ighodaro and Akinloye, 2018; Wood et al., 2003).
Besides the presence of the anti-oxidant enzymes, cells display an additional line of defence based on scavenging molecules, such as glutathione, vitamin C and E, and carotenoids, which convert radicals into less harmful species via electron donation (Ighodaro and Akinloye, 2018). Nonetheless, the enzymatic system represents the principal defence against oxidative insults, where SODs are primarily involved (De Lazzari et al., 2018).

1.6.1 The enzyme SOD1: features and maturation pathways

As previously discussed, SOD1 is often considered the primary defence against ROS, being expressed in different cellular compartments (Furukawa and O'halloran, 2006). In its fully mature state, SOD1 is a homodimeric metalloenzyme (32 kDa), where each subunit presents an immunoglobulin-like fold containing an active site with one zinc and one copper ion, which participate in protein stability and catalysis,

respectively (Furukawa and O'halloran, 2006). The mature enzyme is stabilised by an intramolecular disulphide bridge involving Cys57 and Cys146. Interestingly, this disulphide bond is a remarkable characteristic of the SOD1 enzyme, being maintained under the reducing cellular environment (Furukawa and O'halloran, 2006). While the pathway leading to zinc insertion is still obscure, copper acquisition and disulphide bridge formation have been shown to mainly depend on a dedicated protein, named Copper Chaperone for SOD1 (CCS) (Culotta et al., 2006) (Figure 7).

1.6.1.1 The CCS-dependent maturation pathway

Human CCS is a ubiquitous dimeric protein of 28 kDa, principally localised in the cytosol. From the structural point of view, CCS is characterised by three distinct domains, referred to as D1, D2, and D3 (Furukawa and O'halloran, 2006). Domain D1 includes the N-terminal region of the protein and harbours a "MXCXXC" copper binding motif, which appears to be essential for copper delivery, *in vivo*. Due to its high homology to the SOD1 sequence, domain D2 is involved in CCS-SOD1 recognition step, favouring their interaction during the maturation process. Domain D3 is a small unstructured peptide localised at the C-terminal region and it is essential for completing SOD1 activation. This domain seems to be primarily involved in the formation of SOD1 disulphide bridge through its cysteine residues at positions 244 and 246 (Furukawa and O'halloran, 2006). Bearing a "CXC" motif, this domain has been hypothesised to play a role in copper delivery. Nonetheless, its low copper binding affinity compared to domain D1 seems to exclude this possibility (Allen et al., 2012; Banci et al., 2012). CCS binds the reduced form of copper (Cu^{+}) and has been described to retrieve the ion from the copper transporter 1 (Ctr1) localised in the plasma membrane (Maryon et al., 2013). According to Banci and colleagues, SOD1 activation through CCS appears to follow a multi-step pathway. Initially, zinc-loaded SOD1 interacts with CCS forming a heterodimer via D2 domain, allowing domain D1 to transfer copper to SOD1. Successively, a CCS intramolecular disulphide bond is converted into a transient intermolecular disulphide bridge between CCS and SOD1, which eventually evolves in an intrasubunit disulphide

bond between SOD1 Cys57 and Cys146. After this step, SOD1 dimerises, accomplishing its fully maturation and activation (Banci et al., 2012).

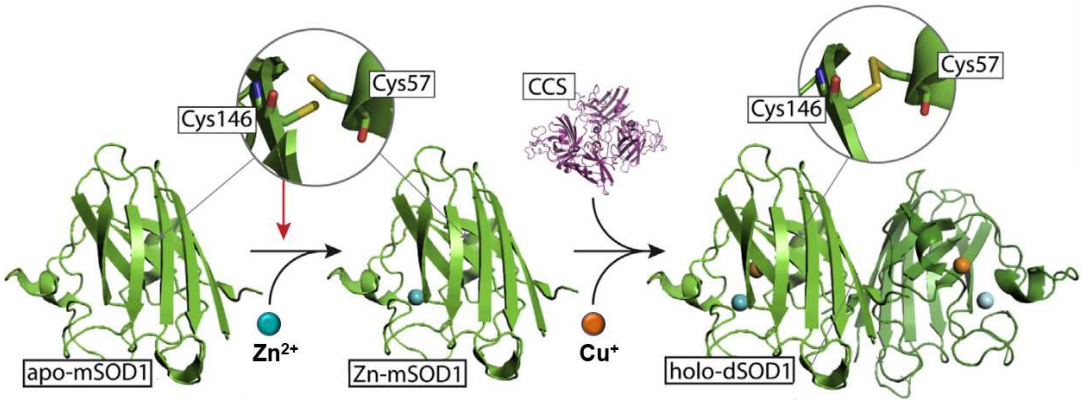


Figure 7. Pathway of maturation of SOD1 through CCS chaperone. During the process of activation, the apo-SOD1 firstly acquires zinc from an unknown source and, successively, copper is inserted by CCS, with the concomitant formation of a disulphide bridge between Cys57 and Cys146. In this holo-form, the enzyme is fully mature and active. Figure adapted from Trist et al., 2018.

1.6.1.2 The CCS-independent maturation pathway

Most of the initial studies regarding SOD1 maturation were performed in yeast, where CCS represents the only way to activate SOD1. Subsequently, additional studies involving superior organisms evidenced that SOD1 can still preserve a residual activity under the absence of CCS. Indeed, analyses performed in mouse, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* showed that SOD1 still retained a partial activity under the absence of CCS (Carroll et al., 2004; Chu, 2005; Kirby et al., 2008; Wong et al., 2000). Moreover, human SOD1 was found to be active when expressed in yeast lacking CCS expression, suggesting the existence of an alternative pathway (Carroll et al., 2004). Although displaying a good degree of sequence homology, this different behaviour between human and yeast SOD1 has been revealed to be dependent on a key residue positioned at the C-terminal region of the protein (Leitch et al., 2009a). While the yeast SOD1 bears a proline residue located at position 144, the human protein

harbours a leucine. Interestingly, the replacement of Pro144 by a leucine in yeast SOD1 abolishes CCS requirement and, vice versa, the introduction of Pro144 in human SOD1 precludes its activation in the absence of CCS, confirming that this residue dictates SOD1 maturation pathway (Leitch et al., 2009a). Notably, an extended analysis of SOD1 sequences among different organisms, including mammals, avians, fish, insects, fungi, and plants, has revealed that this proline is mainly restricted to Ascomycota fungi, while, in the other eukaryotes this residue is frequently substituted by leucine, isoleucine, valine, and alanine. Therefore, this analysis has evidenced that most organisms do not strictly rely on CCS chaperone to activate SOD1, supporting the existence of a CCS-independent activation (Carroll et al., 2004; Leitch et al., 2009b). Indeed, while this protein is generally well conserved, not all organisms display a CCS homologous. For instance, the *C. elegans* genome does not encode CCS so that SOD1 is activated only through an independent mechanism (Jensen and Culotta, 2005). This feature is not unique to *C. elegans*, since nematodes, platyhelminthes, molluscs, and arachnids genomes do not seem to encode a CCS chaperone to activate SOD1 (Leitch et al., 2009b). In the attempt to better comprehend the CCS-independent pathway, Leitch *et al*. have compared *S. cerevisiae* SOD1 activation, which exclusively relies on CCS, to *C. elegans* SOD1 that is completely CCS-independent and to human SOD1, which exploits both mechanisms. This analysis has revealed an important difference regarding the SOD1 intramolecular disulphide bond formation (Leitch et al., 2009a). While *S. cerevisiae* SOD1 disulphide bridge formation depends solely on copper-loaded CCS, *C. elegans* SOD1 disulphide is normally completely oxidised, and human SOD1 disulphide is approximately 50% oxidised in the absence of copper and CCS (Furukawa et al., 2004; Leitch et al., 2009a). This study has revealed that SOD1 molecules, which can become active regardless of the presence of CCS, show a higher tendency towards disulphide oxidation in the reducing cellular environment. Moreover, the substitution of proline 144 with leucine, serine, or glutamine in yeast conferred a CCS-independent activation, also increasing the formation of the disulphide bond (Leitch et al., 2009a). As this amino acid is positioned 5.5 Å away from Cys146, which is one of the residues involved in the SOD1 disulphide bridge formation, it could impose some constraints in

conformational dynamics and/or affect the pKa of the cysteines involved in disulphide bridge formation. Under this scenario, the presence of CCS may be required to overcome these restrictions (Leitch et al., 2009b). Besides disulphide stability, another major difference that emerged between the CCS-dependent and -independent pathway resides in the molecular oxygen demand. In fact, the CCS-dependent maturation pathway requires the presence of oxygen to be accomplished (Furukawa et al., 2004), while the alternative pathway can function in limiting oxygen conditions and even under anoxia (Leitch et al., 2009a) (Figure 8). As Leitch *et al*. have proposed, the evolution of a dual mechanism for SOD1 activation in superior organisms may reflect different conditions under which SOD1 activity is required. Accordingly, normally SOD1 promptly detoxifies superoxide anions and its maturation is accomplished by CCS chaperone. Nonetheless, under limiting oxygen concentrations, the alternative pathway guarantees a residual SOD1 activation, which may be relevant for redox signalling (Leitch et al., 2009b). Considering the essential role of SOD1, it is not surprising that organisms have evolved different mechanisms to preserve its protective activity.

Although SOD1 plays a pivotal role against oxidative insults, as aforementioned, cells rely on numerous mechanisms of defence, which all together confer a higher level of protection against RONS and their detrimental effects. In this sophisticated machinery, the Parkinson-disease associated protein DJ-1 is receiving increasing interest due to its validated role in the anti-oxidant response. Indeed, consistent evidence has demonstrated that the protein exerts cytoprotection against multiple oxidative insults (Raninga et al., 2017). In the following sections, DJ-1 protein will be thoroughly described, emphasising its involvement in the anti-oxidant defence.

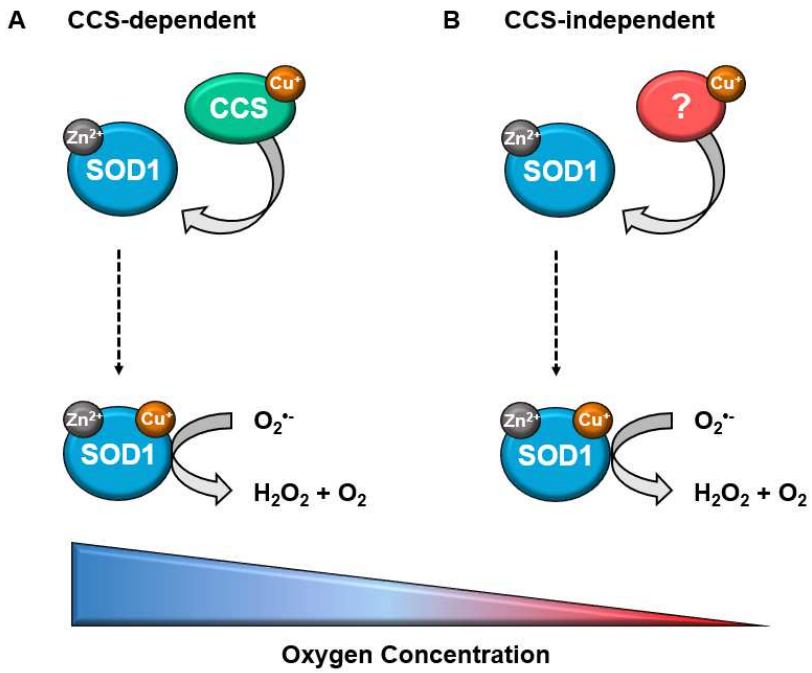


Figure 8. The pathways of activation of the enzyme SOD1. (A) The CCS-dependent pathway: under normal oxygen concentration, SOD1 maturation is accomplished by the dedicated copper chaperone CCS, which inserts copper, rendering the enzyme active. (B) The CCS-independent activation pathway: under hypoxia, SOD1 becomes mature through an unknown player that inserts copper, conferring a residual activity to the enzyme.

1.7 The protein DJ-1: discovery and association with diseases

DJ-1 was firstly identified in 1997 by Nagakubo and colleagues, who defined DJ-1 as a novel oncoprotein able to transform mouse cells in cooperation with H-Ras (Nagakubo et al., 1997a). This finding led to further investigation of the oncogenic properties of the protein, showing that DJ-1 upregulation is frequently correlated to many cancer types, with a high level of malignancy (Cao et al., 2017). Successively, Bonifati *et al*. reported a 14 kbp deletion and T497C transition (L166P) in the DJ-1 encoding gene (referred to as *PARK7*) causing early-onset forms of PD, characterised by recessive inheritance (Bonifati, 2003). Since then, multiple studies revealed a growing number of mutations, confirming the association of the protein with the pathology (see the Parkinson Disease Mutation Database and relative references therein http://www.molgen.ua.ac.be/PDmutDB/default.cfm?MT=0-

&ML=0&Page=Home). Although the involvement of the protein in PD and cancer are the most studied research fields, DJ-1 has been also implicated in male fertility, diabetes, stroke, chronic obstructive pulmonary disease (COPD), and familial adenomatous polyposis (FAP) (Ariga et al., 2013).

1.7.1 Pattern of expression

The participation of DJ-1 in several diseases reflects its wide pattern of expression, being found in most body tissues (Nagakubo et al., 1997a). At subcellular level, DJ-1 localises principally in the cytoplasm, though a fraction of the protein translocates into the nucleus and mitochondria, under stress conditions (Canet-Avilés et al., 2004a; Junn et al., 2009a; S.-J. Kim et al., 2012; L. Zhang et al., 2005). Albeit not bearing a DNA binding domain (Yamaguchi et al., 2012), the protein has been shown to move inside the nucleus under the S phase upon growth factors supplementation, however, the specific roles exerted in these compartment remains to be completely elucidated (Nagakubo et al., 1997b). Similarly, the function played within mitochondria is still unclear. Moreover, the specific mitochondrial localisation has not been well characterised, since DJ-1 has been reported in the matrix, in the intermembrane space (L. Zhang et al., 2005) and in the outer mitochondrial membrane (Canet-Avilés et al., 2004a; Junn et al., 2009a). In addition, DJ-1 seems also to be secreted in the extracellular space, since it has been detected in the plasma or serum of patients affected by melanoma, breast cancer, and stroke (Allard, 2005; Le Naour et al., 2001; Pardo et al., 2006). Although the role in this compartment is still uncertain, some reports have suggested a participation in the autocrine/paracrine signalling cascade (J.-M. Kim et al., 2012; Pantcheva et al., 2014).

1.7.2 Protein features

DJ-1 is 189 amino-acid long protein and exists as a homodimer of 20 kDa, where the two monomers interact mainly through hydrophobic bonds (Wilson et al., 2003) (Figure 9). Sequence analysis has revealed that the protein belongs to the DJ-1/ThiJ/PfpI superfamily, which is highly conserved among phyla (Lucas and Marin, 2006). In bacteria ThiJ participates in thiamine biosynthesis, generating thiamine derivatives that are thought to protect cells against ROS, while PfpI acts as a protease (Halio et al., 1996; Mizote et al., 1996). Although not presenting similar activities, DJ-1 shares with this family a highly conserved cysteine residue, localised at position 106, which seems to regulate several functions attributed to the protein (Tao and Tong, 2003). This residue has a low thiol pKa value of ~5 and exists almost exclusively as a reactive thiolate anion at physiological pH (Witt et al., 2008). This residue has been reported to be particularly sensitive to oxidation and to determine the activation of the protein. In fact, under mild oxidative conditions, the thiolate anion undergoes oxidation to sulfinic acid (-SO_2H), which is considered the active form of the protein, while, further oxidation promotes the formation of sulfonic acid (-SO_3H), which appears to render the protein inactive (Kiss et al., 2017; Wilson, 2011). Interestingly, the oxidised forms of the protein have been detected in idiopathic PD patients' brain and urine, suggesting that oxidised DJ-1 may be exploited as biomarkers for PD diagnosis (Choi et al., 2006; Jang et al., 2018).

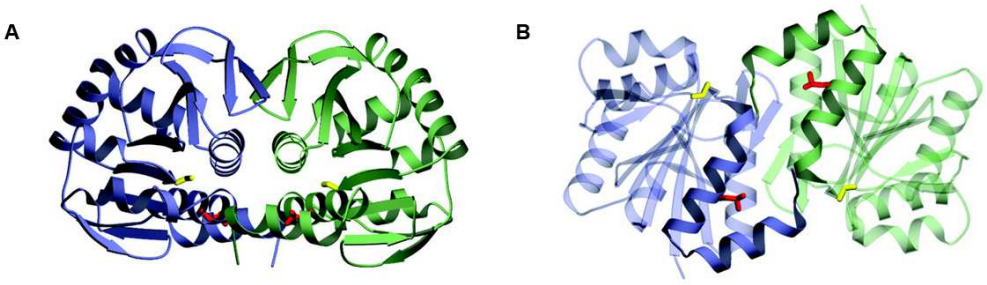


Figure 9. Crystal structure of human DJ-1. (A) and (B) Dimeric structure of human DJ-1, with the two monomers coloured in blue and green. In (B) the view is rotated of 90° with respect to the view in (A). In both pictures, the highly conserved Cys106 and Leu166, which is mutated in PD, are highlighted in yellow and red, respectively. Figure adapted from Wilson et al., 2003.

1.7.3 Proposed functions

Albeit the structural data relative to DJ-1 are known since 2003, the physiological function is only partially understood. To date, several functions have been ascribed to DJ-1, including tumorigenesis, modulation of signalling cascades, maintenance of ROS homeostasis, regulation of transcription, maintenance of glucose levels, and protection upon stroke (Ariga et al., 2013; Raninga et al., 2017). Nonetheless, the most widely accepted function is its protective role against excessive ROS levels, a condition under which the protein seems to orchestrate specific responses to promote cell survival (Cookson, 2010; Raninga et al., 2017) (Figure 10).

1.7.3.1 Modulation of signalling pathways and gene transcription

DJ-1 has been shown to indirectly affect transcription by interacting with transcription factors and their regulators, eventually regulating gene expression (Yamaguchi et al., 2012). Interestingly, DJ-1 appears to favour the activation of pro-survival and proliferative pathways, while simultaneously inhibiting cell death signalling cascades. For example, under stressful conditions, DJ-1 has been shown to promote the nuclear translocation of the extracellular signal-regulated protein kinases 1 and 2 (ERK), where it phosphorylates Elk, the transcription factor involved in the expression of different anti-oxidant genes, including *SOD1*. (Z. Wang et al., 2011). In a similar manner, DJ-1 seems to play a role in the activation of the Nrf2 pathway, through a direct interaction with the inhibitor Keap1, and therefore, releasing Nrf2 transcription factor (Clements et al., 2006).

This mechanism of action of DJ-1 is also found in the activation of the Akt pathway, a pivotal signalling cascade involved in cell growth regulation. Indeed, in the absence of survival signals, a key player in this pathway is the Phosphatase and TENsin homologue deleted from chromosome 10 (PTEN), which inhibits phosphoinositide 3-kinase (PIK3) kinase activity, hence preventing Akt activation (Manning and Toker, 2017). In this scenario, under stressful situations, DJ-1 has been shown to bind directly to PTEN, blocking its inhibitory function, and, therefore, inducing cell survival (Kim et al., 2005, 2009). If on the one hand the protein is primarily active in the stimulation of survival mechanism, on the other it has been

reported to mediate the repression of apoptotic cascades. In this frame, DJ-1 has been implicated in the inhibition of the pro-apoptotic transcription factor p53, interacting with the p53-DNA binding domain (Kato et al., 2013). In addition, DJ-1 has been reported to exert its anti-apoptotic activity by precluding the activation of the apoptosis signal-regulating kinase 1 (ASK1) pathway, inhibiting ASK1 and death domain associated protein (Daxx) interaction, which is a key event in the activation of cell death mechanisms (Junn et al., 2005; Klawitter et al., 2013; Waak et al., 2009).

1.7.3.2 Maintenance of the mitochondrial homeostasis

Under increasing ROS levels, a fraction of DJ-1 has been demonstrated to located at the mitochondrial level (Junn et al., 2009a; L. Zhang et al., 2005). Although the precise mechanism of action in this district is still elusive, DJ-1 seems to participate in the maintenance of mitochondrial homeostasis, sustaining respiration and preventing mitochondrial-derived ROS damages. Indeed, different reports have pointed out that DJ-1 loss of function is associated with mitochondrial fragmentation and impaired respiration (Irrcher et al., 2010; Krebiehl et al., 2010; Wang et al., 2012). In this frame, DJ-1 null dopaminergic neurons have been shown to consume less oxygen and to present an altered membrane potential (Heo et al., 2012). Albeit still under investigation, it has been proposed that the protein may exert its protective role by acting at different levels. Hayashi and colleagues firstly identified DJ-1 as an interactor of the complex I subunit $NDUFA_4$ in NIH3T3 and HEK293 cell lines by two-hybrid assay (Hayashi et al., 2009). Accordingly, through blue native-polyacrylamide gel electrophoresis and 2-dimensional gel analysis, Heo *et al.* have found that the protein contributed to complex I stability and that DJ-1 null cells presented reduced complex I activity and oxygen consumption (Heo et al., 2012). Interestingly, it has been demonstrated that this ability to bind and sustain complex I function is also a mechanism of protection exerted by the protein against ischemia-reperfusion injury (Ding et al., 2018; Zhang et al., 2018). Besides binding complex I, it has been recently shown that DJ-1 is able to interact with the F_1F_O ATP synthase β subunit, decreasing mitochondrial uncoupling and enhancing ATP production in dopaminergic neurons (Chen et al., 2019). In accordance, Calì *et al.* have found that

mitochondrially-driven DJ-1 sustains the energetic metabolism, favouring respiration, super-complexes assembly, and ATP levels (Calì et al., 2015).

In addition to the purported role on mitochondrial complexes, it is worth to mention that DJ-1 has also been suggested to exert its mitochondrial function through regulation of signalling pathways. Zhang and colleagues have observed that the mitochondrial dysfunctions evidenced under DJ-1 deficiency were partially caused by a reduced activation of both Akt and Nrf2 pathways in a DJ-1-dependent manner (Zhang et al., 2019). Importantly, these signalling cascades have been implicated in the maintenance of mitochondrial activity, supporting respiration, membrane potential and ATP production (Bijur and Jope, 2003; Dinkova-Kostova and Abramov, 2015; Goo et al., 2012). Furthermore, authors reported that DJ-1 knock-down in dopaminergic cells induced ROS-derived mitochondrial dysfunction, with consequent activation of the pro-apoptotic protein C-Jun N-terminal kinase (JNK) (Ren et al., 2010; Zhang et al., 2019). Considering these findings, DJ-1 appears to preserve mitochondrial functionality both directly, by modulating the activity of the respiratory complexes, and indirectly, by orchestrating mitochondrial-associated signalling cascades.

Additionally, DJ-1 has been partially associated with mitophagy, which is normally regulated by the coordinated action of the proteins Parkin and PINK1 (Joselin et al., 2012; Strobbe et al., 2018; Thomas et al., 2011). When mitochondria are damaged, PINK1 is stabilised on the mitochondrial outer membrane, where it recruits and phosphorylates Parkin, promoting its E3 ligase activity and labelling mitochondria for degradation (Pickles et al., 2018). Although DJ-1 does not appear to be primarily involved in mitophagy, some findings reported its possible participation. Indeed, Cookson's lab has reported that *DJ-1* upregulation protected *PINK1*-null cells under rotenone treatment, although not reversing mitochondrial fragmentation in *Parkin* or *PINK1* mutant mammalian cell lines. Moreover, they observed that *PINK1* or *parkin* overexpression rescued mitochondrial fragmentation in *DJ-1*-deficient cells and that *DJ-1* deficiency induced an increased in the microtubule associated protein 1A/1B-light chain 3 (LC3) puncta, which are markers of enhanced autophagy, closely associated with mitochondria (Thomas et al., 2011). Furthermore, DJ-1 has been implicated in the mitochondrial morphology and dynamics. In fact, the loss of DJ-1

has been associated with increased fragmentation, supporting a role in mitochondrial fusion and fission (Irrcher et al., 2010; Wang et al., 2012). Finally, the protein has been suggested to be involved in the modulation of the mitochondrial Ca^{2+} transients, by induction of the mitochondrial-endoplasmatic reticulum tethering, antagonising p53 activity (Ottolini et al., 2013). Collectively, from these results, it emerges that DJ-1 regulates mitochondrial functionality performing multiple activities to preserve the homeostasis of the organelle, especially under oxidative insults.

1.7.3.3 Protection against ischemia-reperfusion injury

In accordance with its anti-oxidant role, DJ-1 has also been proved to participate in the hypoxia-reoxygenation response, conferring protection at both cerebral and cardiac level. DJ-1 has been found to act on two levels: firstly, by sustaining the ischemic response under oxygen depletion, and then by protecting against reperfusion damages. During limiting oxygen concentrations, DJ-1 seems to stabilise HIF1-α (Parsanejad et al., 2014; Vasseur et al., 2009). In fact, Vasseur and colleagues have found that *DJ-1* silencing in U2OS cells reduced HIF-1α levels of 30% under hypoxic treatment (Vasseur et al., 2009). DJ-1 has been suggested to stabilise HIF-1α through negative regulation of Von Hippel-Lindau (VHL), an E3-ubiquitin ligase that normally labels HIF-1α for degradation under physiological oxygen concentrations (Parsanejad et al., 2014). In this way, DJ-1 sustains the HIF-1α-related response pathway, favouring cellular adaptation to the ischemic period. Successively, upon oxygen reintroduction, DJ-1 has been shown to mainly protect against reoxygenation-derived ROS damages. In fact, *in vivo* studies have reported that the absence or down-regulation of DJ-1 in mice results in higher sensitivity to brain ischemia, increased infarct size, and major locomotor dysfunctions (Molcho et al., 2018; Peng et al., 2019). On the contrary, *DJ-1* upregulation has been observed to elicit neuroprotection, *in vivo*, as its overexpression was reported to rescue post-ischemic cerebral damages (Molcho et al., 2018; Yanagisawa et al., 2008). Similarly, administration of sodium phenylbutyrate, a histone deacetylase inhibitor that upregulates *DJ-1* expression, has shown beneficial effects upon brain reperfusion (R.-X. Yang et al., 2017). Although the protective activity exerted by the

protein is becoming more and more evident, the mechanism of action remains to be completely clarified. In this regard, different *in vitro* studies have found that the protein could act at the mitochondrial level. Accordingly, upon ischemic insults, DJ-1 was observed to translocate into mitochondria of rat primary neural cells (Kaneko et al., 2014). In this compartment, the protein has been reported to reduce mitochondrial fragmentation and to sustain complex I activity in cardiac cells (Ding et al., 2018; Dongworth et al., 2014; Shimizu et al., 2016; Zhang et al., 2018). Apart from the mitochondrial related effects, other studies have showed that DJ-1 protection could rely on the expression of anti-oxidant genes. For example, DJ-1 was shown to induced Nrf2-dependent gene expression in rat heart cells, such as *SOD2*, *catalase*, and *glutathione peroxidase* genes (Yan et al., 2015; Yu et al., 2013). Moreover, DJ-1 has been described as a mediator of resveratrol protective effects against cerebral ischemia via the modulation of PI3K/Akt pathway (Abdel-Aleem et al., 2016). Overall, DJ-1 appears to participate in ischemia-reperfusion responses acting at multiple levels. If during ischemia, the protein stabilises HIF-1α transcription factor, upon reoxygenation, it ensures mitochondrial protection and activates anti-oxidant pathways to guarantee survival.

1.7.3.4 Participation in the SOD1 maturation pathway

As thoroughly discussed in the previous paragraphs, DJ-1-mediated protection seems to derive from its ability to mediate numerous protective mechanisms. One important function is the upregulation of the anti-oxidant defence. In this frame, independent lines of research have suggested that DJ-1 may be involved in the SOD1 maturation process. Björkblom and colleagues firstly reported the ability of DJ-1 to bind copper in both its reduced (Cu^{+}) and oxidised (Cu^{2+}) states and to protect against copper toxicity *in vitro* (Björkblom et al., 2013). According to this study, DJ-1 binds Cu^{+} in a 1:1 ratio through a bis-cysteinate site positioned at the interface of the two subunits, which involves Cys53 (Puno et al., 2013). This binding site was then confirmed by Girotto *et al*. who have also identified another site capable of binding copper. In fact, DJ-1 has been described to bind Cu^{2+} with a binding affinity of 3.47×10^{-4} M via Cys106. This second binding site coordinates copper through the residues Cys106, Glu18, and a water molecule, while the amino

acids Gly75 and His126 seem to stabilise the binding, probably through hydrogen bonds (Girotto et al., 2014). More importantly, the authors have demonstrated that Cu^{2+}-loaded DJ-1 can transfer the metal to SOD1 resulting in an active enzyme, *in vitro*. As Cys53 mutation does not seem to affect DJ-1-dependent SOD1 maturation, Cys106 seems to be the primary involved (Girotto et al., 2014). Consistent with these results, another group has proposed the involvement of DJ-1 in SOD1 activation, working with the *A. thaliana* homologue AtDJ-1a and human DJ-1 (Xu et al., 2010). Through bimolecular fluorescence complementation (BiFC) assays and isothermal calorimetric (ITC) assays, Xu and co-workers showed that AtDJ-1a interacts with *A. thaliana* copper superoxide dismutase 1 (CSD1) and stimulates its activity. In a similar manner, they reported that human DJ-1 interacts with SOD1 in CHO-S cells, promoting SOD1 function. An indirect evidence of the ability of DJ-1 to transfer copper to SOD1 derived from a recent paper showing that DJ-1 and SOD1 interaction is enhanced upon treatment with $Cu^{(II)}$ATSM, a hypoxia sensitive positron emission tomography imaging agent, with anti-oxidant properties (Srivastava et al., 2016). According to these promising results, DJ-1 seems to possess the required characteristics to be an additional player in the SOD1 maturation pathway, although further *in vivo* validation is particularly awaited to confirm this role.

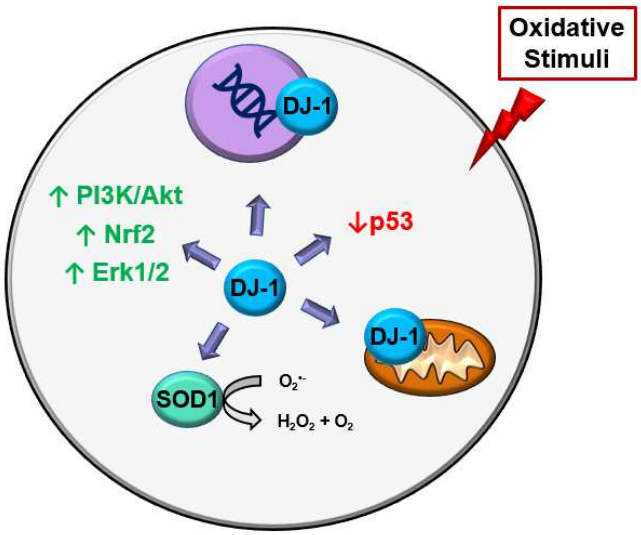


Figure 10. Some of the functions attributed to DJ-1. Under oxidative insults, the protein orchestrates different protective mechanisms. In the cytosol, the protein sustains the activation of pro-survival cascades, such as the Nrf2, PI3K/Akt, and Erk1/2 pathways, while it inhibits pro-apoptotic signalling cascades, like p53. In addition, it may play a role in the activation of the protective enzyme SOD1, which is responsible for the detoxification of the superoxide anion. Under oxidative stimuli, a fraction of the protein also translocates within the nucleus and the mitochondrion, where its functions are less elucidated.

1.8 Aim of the Project

The maintenance of an adequate redox homeostasis is a prerequisite of a healthy state. When the concentration of reactive oxygen and nitrogen species, collectively called RONS, overwhelms the anti-oxidant capacity of the cell, they can acquire damaging functions (Weidinger and Kozlov, 2015). Mitochondria are the principal contributors to RONS generation, particularly, when not properly working, a condition frequently observed in several neuropathological states (Stefanatos and Sanz, 2018). To protect cells from RONS-related damages a complex anti-oxidant system exists, which includes multiple players able to target different RONS species, conferring, in such a way, a broad anti-oxidant protection. Superoxide dismutases (SODs) play a primary role in the detoxification of the superoxide anion, from which more harmful species can originate (Wang et al., 2018). Besides SODs, the anti-oxidant machinery includes multiple additional players, among which it is emerging the protective role of the Parkinson's disease-associated protein DJ-1 (Raninga et al., 2017). Though not being directly implicated in RONS scavenging, the protein seems to orchestrate multiple responses to preserve the cellular redox balance (Raninga et al., 2017). To date, a growing number of activities have been attributed to DJ-1, nonetheless, a consensus on its actual role is still missing. Considering this open question, with this project, we aimed at exploring the physiological functions of DJ-1 in the redox homeostasis, using *Drosophila melanogaster* as a model organism.

A first part of the thesis will be focused on the involvement of DJ-1 in the mitochondrial homeostasis. Interestingly, DJ-1 has been reported to translocate in this compartment under oxidative stimuli, though its precise function in this organelle remains partially understood (Canet-Avilés et al., 2004b; Junn et al., 2009a; L. Zhang et al., 2005). In this regard, we will describe how the absence of the protein affects the mitochondrial homeostasis, investigating the mitochondrial functionality, morphology, and metabolism under basal condition and under oxidative insults.

A second part of the project will be dedicated to the exploration of the possible participation of DJ-1 in the maturation pathway of the enzyme SOD1. This anti-oxidant enzyme normally undergoes a process a maturation during which it is assisted by a dedicated copper chaperone, called CCS. In this process, SOD1

acquires copper and a disulphide bridge, becoming fully mature and active (Banci et al., 2012). Nevertheless, SOD1 has been shown to present a residual activity also in the absence of CCS, suggesting the existence of an alternative pathway of maturation (Leitch et al., 2009b, 2009a). In this frame our and other groups have reported that DJ-1 may play a role in this mechanism, as it has been shown to have the ability to bind and transfer copper to SOD1 *in vitro* (Girotto et al., 2014; Xu et al., 2010). Hence, we will investigate this mechanism *in vivo*, assessing the effects of the absence and overexpression of the protein in the maturation of the enzyme SOD1. Overall, with this research project, we explored different protective roles in which the protein may be implied, trying to contribute to answer to the still open question concerning its physiological role.

Chapter 2

Experimental Model

Drosophila melanogaster as a model organism in Parkinson's disease

2. Introduction

The history of *Drosophila melanogaster* as a model organism in science started more than 100 years ago by Thomas Morgan, who elucidated the theory of inheritance, previously demonstrated by Mendel. Since then, the model has contributed to the dissection of a wide spectrum of biological processes, as well as to the investigation of different forms of human pathologies. Indeed, its versatility and biological features have rendered the fruit fly an advantageous model in research (Allocca et al., 2018a). The following paragraphs will be dedicated to present *Drosophila* organism and its utilisation in research to model human diseases.

2.1 *Drosophila* life cycle and reproduction

Fruit flies are characterised by a rapid life cycle, developing in about 10 days at 25°C. Moreover, a single fertile female can lay hundreds of eggs (Hales et al., 2015). After fertilisation, embryogenesis is completed in 24 hours, establishing the larval body plan, through initial expression of maternally-derived mRNAs. Successively, the embryo undergoes three larval stages, known as L1, L2, and L3 which altogether take about 4-5 days. During larval morphogenesis, many cell types and tissues, including the larval brain, are already differentiated and functional. After encapsulation of the L3 larva, the pupal stage begins to orchestrate the organisation of the adult body (Figure 11). In particular, most of the larval tissue degenerates, while adult head, wings, legs, thorax and reproductive apparatus originate from the imaginal disk, i.e. tissue-specific progenitor cells residual of the larval period. Once eclosed, the adult individual becomes sexually mature within 10 hours and lives for about 2-3 months (Hales et al., 2015).

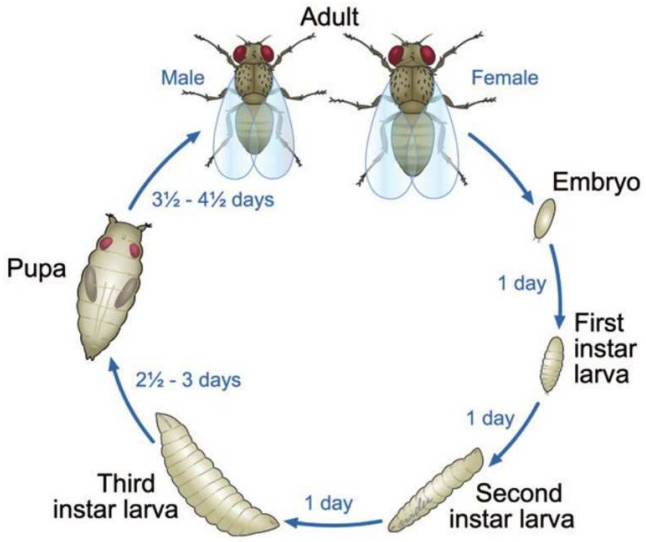


Figure 11. *Drosophila melanogaster* life cycle. The fruit fly life cycle lasts approximately 10 days at 25°C. Embryos develop into adult flies undergoing three larval stages (L1, L2, and L3) and a pupal phase from which flies emerge. Females and males present sexual dimorphism, with males being smaller than females (Ong et al., 2015).

2.2 *Drosophila* genome and genetic tools

The *Drosophila* genome has been extensively studied and it has been completely sequenced in 2000 (Adams, 2000). It encompasses 4 pairs of chromosomes. The first pair includes the sex chromosomes, with an X chromosome and a small Y chromosome, which is principally composed of heterochromatin. The second and third autosomes include most of the encoding genome, while the fourth pair, called the dot chromosome, is a small autosome. The characterisation of the *Drosophila* genome has led to the development of a wide array of genetic techniques to manipulate genes of interest. Indeed, the sophisticated toolkit allows to knock-down, mutate, overexpress, and tag a gene. Most of these methods rely on the insertion of an exogenous DNA sequence in the fly genome, generating a transgene. The transgene can be a complete genic sequence, a mutated region, a regulatory fragment, or any type of genomic sequence of interest (Allocca et al., 2018a). The most utilised genetic tools to manipulate gene expression in *Drosophila* will be briefly described below.

2.2.1 Inducing mutations: P-element insertion and EMS

To promote gene deletions a common tool exploits the insertion of P-elements, which are mobile genetic elements naturally occurring in the *Drosophila* genome. When these elements are induced to transpose, they can disrupt a gene sequence, eventually inducing its loss of function. Moreover, they can be associated with a tag, which helps in identifying the presence of the P-element in the genome. However, some genomic regions are more/less prone to allow P-elements insertion (known as hot/cold spots), thus, representing a limitation of the technique (Allocca et al., 2018a; Bellen et al., 2011).

In addition to P-elements mobilisation, another mechanism to induce mutations is the ethyl methanesulfonate (EMS)-based technique. EMS generates random mutations, mainly point mutations, with high frequency, being more efficient than P-element in mutagenesis. This tool is based on the production of O^6-methylguanine, an altered guanine which incorrectly pairs with thymine during DNA replication, favouring GC to AT transitions. This aberration can alter codon frame or destroy splice sites, potentially destroying the gene product. The generated mutation can lead to an evident phenotype or be undetectable, requiring extensive screenings to identify which DNA alteration is linked to the observed phenotype (Allocca et al., 2018a; St Johnston, 2002).

2.2.2 Manipulating gene expression level: the GAL4/UAS system

A commonly used tool to manipulate the expression of a gene of interest is the so-called GAL4-UAS system (Brand and Perrimon, 1993). For this system, the yeast transcription factor GAL4 is inserted into a P-element vector, carrying in frame a specific promoter. Separately, a second line, which has a P-element construct containing a gene of interest in frame with the upstream activating sequence (UAS), to which GAL4 will bind, is generated. When the two parental lines are genetically crossed, their progeny will overexpress the transgene in a controlled way (Brand and Perrimon, 1993) (Figure 12). GAL4 expression can be spatially regulated through a tissue-specific promoter or temporally controlled using the temperature-sensitive transcription factor $GAL80^{ts}$. Indeed, $GAL80^{ts}$ can bind to GAL4 repressing

its transcriptional activity at the permissive temperature of 18°C, while its inhibitory function is removed at higher temperatures, allowing GAL4 binding to UAS (Zeidler et al., 2004). A refinement of the technique includes also the split GAL4, which improves the temporal control of gene expression. In this system, the transcriptional activation domain of GAL4 and its DNA binding domain are cloned in separated promoters. Hence, only when the two domains bind each other, GAL4 is reconstituted and functional (Luan et al., 2006). The number of GAL4 lines available is increasing over time, allowing multiple combinations. Moreover, the UAS construct can be in frame with either a transgene or an RNA silencing sequence to overexpress or knock down a specific gene (Allocca et al., 2018a).

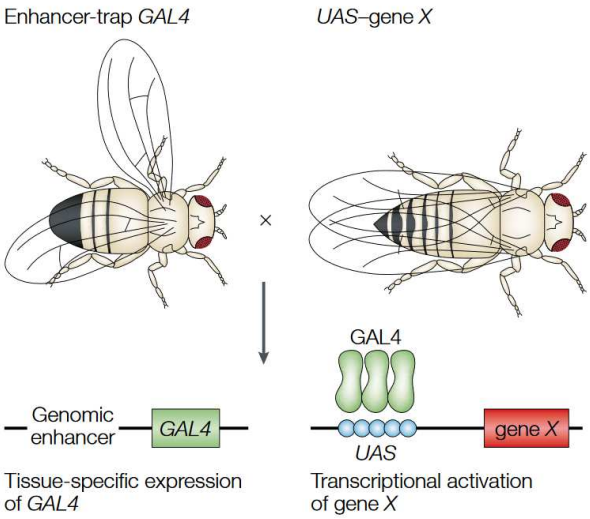


Figure 12. Basic GAL/UAS system in *Drosophila*. A *Drosophila* line bears the yeast transcriptional activator GAL4 in frame with a tissue-specific promoter, while a second fly line carries the upstream activating sequence (UAS) in frame with a gene of interest. When the two parental lines are crossed, the progeny will express the gene of interest in a specific tissue. Figure adapted from St Johnston, 2002.

2.2.3 Balancer chromosomes

Among the fly genetic tools, it is worth to mention the use of balancer chromosomes, which are genetically engineered chromosomes used for multiple purposes in fruit fly genetics. A balancer chromosome normally carries recessive deleterious mutations and inversion breakpoints, which confer lethality in homozygosity. Moreover, they frequently present a dominant marker that renders easy to follow their inheritance in the mating schemes. Thanks to these features, balancers are used to maintain sterile or lethal mutations in stable stocks, to avoid crossing over during homologous recombination, or to generate complex genotypes. Balancers are available for all the fly chromosomes, apart from the fourth and the Y chromosomes. Having a wide range of applications, balancers are considered a fundamental part of the fly genetic toolbox for *Drosophila* researchers (Kaufman, 2017).

2.3 *Drosophila* as a model to investigate redox alterations and pathological consequences

As aforementioned, fruit flies have significantly contributed to the comprehension of multiple physiological processes, with relevance also for human pathologies. In particular, considering the relevance of redox biology to multiple pathological states, *Drosophila* has been extensively used in this field. Interestingly, albeit the evolutionary distance, fruit flies and mammals share many genes, cellular mechanisms, signalling pathways, and neuronal processes. Additionally, flies conserve the same biological mechanisms of RONS production. Indeed, *in vitro* studies have reported that *Drosophila* mitochondria function as the mammalian organelles, with complex I being the primary $O_2^{\bullet-}$ source, by both forward and reverse electron flux (Miwa et al., 2003; Sanz et al., 2010b). Moreover, at extra-mitochondrial level, *Drosophila* displays homologues proteins to NOX enzymes, xanthine oxidases, and mieloperoxidases (Aguirre and Lambeth, 2010; Edwards et al., 1977; Nelson et al., 1994). Importantly, anti-oxidant systems are conserved as well, showing protein counterpart for SODs, catalase, glutathione peroxidase, and DJ-1 (Meulener et al., 2005; Missirlis et al., 2001). Thanks to this high degree of

conservation and its versatility, *Drosophila* has become an attractive model to explore the *in vivo* responses to reactive species, giving insights to the role of redox homeostasis in health and disease. Fruit flies have been exposed to multiple stressors, evaluating the response to different reactive species, concentrations, and biological sources. Major analyses have been focused on the evaluation of ROS effects on life expectancy, aiming at elucidating how reactive species are correlated to the ageing process and ageing-related diseases. Although oxidative stress has been largely considered a hallmark of ageing, there are confounding results regarding the exact role of reactive species on longevity. For example, the removal of the cytosolic or mitochondrial *SOD* homologues in fruit flies, referred to as *Sod1* and *Sod2* respectively, has been reported to shorten remarkably lifespan (Duttaroy et al., 2003; Kirby et al., 2002; Martin et al., 2009; Melov, 2000; Wicks et al., 2009). On the contrary, their overexpression has mainly been associated with longer lifespan (Curtis et al., 2007, p.; Sun et al., 2002; Sun and Tower, 1999) Moreover, paraquat and H_2O_2 exposure or severe mitochondrial impairments have been associated to premature mortality. Nonetheless, some studies have evidenced that manipulating specific ROS species can have beneficial effects on *Drosophila* lifespan. For example, RNA interference of the respiratory complexes or selective knock-down of complex I, and RET have been reported to prolong lifespan in fruit flies (Copeland et al., 2009; Owusu-Ansah et al., 2013; Scialò et al., 2016). Moreover, it is worth mentioning that some studies have found that *Sod1*, *Sod2*, and *catalase* overexpression in long-lived fruit flies did not further improve lifespan (Mockett et al., 2010; Orr et al., 2003), suggesting that long-lived animals may not benefit from an enhanced anti-oxidant defence under physiological conditions. Overall, these observations have highlighted that the use of *Drosophila* can help in shedding some light on the complexity of *in vivo* responses to ROS manipulation (Sanz, 2016).

Besides longevity studies, *Drosophila* has also been used to investigate the contribution of redox unbalance to pathological states, including neurodegeneration. To this end, the utilisation of redox-sensitive probes has been very useful in the elucidation of *in vivo* redox changes occurring in normal physiology and under pathological states (Albrecht et al., 2011; Liu et al., 2012). Moreover, thanks to the

remarkable conservation of many human disease genes, fruit flies have been widely used to dissect critical redox pathways involved in the neurodegenerative process (Lessing and Bonini, 2009). For example, a recent research carried out in *Drosophila* has revealed that neuronal changes in GSH redox state correlated with AD onset and progression, through the concomitant enhancement of the JNK signalling cascade (Stapper and Jahn, 2018). Similarly, the oxidative stress-dependent activation of the JNK pathway has been found to drive the aggregation of Tau protein in an AD fly model (Dias-Santagata et al., 2007). Moreover, the stimulation of the Nrf2 anti-oxidant pathway, in fly dopaminergic neurons, has been shown to attenuate α-synuclein-driven neurodegeneration (Wang et al., 2015). In addition to genetic manipulations of redox pathways, fruit flies have also been exploited to screen anti-oxidant compounds with therapeutic potential in neurodegenerative disease. In fact, these molecules can be delivered in the standard fly food and their effects evaluated through behavioural assays (Deshpande et al., 2019; Maitra and Ciesla, 2019; Sanz et al., 2017). For example, the administration of the Mn-SOD mimetic M40403 in fruit flies has been shown to protect against paraquat toxicity in *Sod1* and *Sod2* null flies, recovering early lethality and locomotor defects (Filograna et al., 2016). Altogether, these findings demonstrate that *Drosophila* represents a valuable model to dissect redox-related neurodegenerative mechanisms and to explore possible protective anti-oxidant systems (Hales et al., 2015).

2.3.1 Modelling PD neurodegeneration in *Drosophila*

The fly central nervous system is a bilaterally symmetrical brain composed of both neurons and glial cells. Notwithstanding its simplicity, *Drosophila* brain coordinates complex behaviours, such as circadian rhythms, sleep, memory, locomotion, and learning. Moreover, *Drosophila* has been found to respond to drugs acting on the central nervous system similarly to mammals (Andretic et al., 2008; Rothenfluh and Heberlein, 2002; Satta et al., 2003). The fruit fly brain includes a well-characterised set of dopaminergic (DA) cells. DA neurons are subdivided into multiple clusters, which are symmetrically distributed and easily recognisable in the fly brain (Mao, 2009; Whitworth et al., 2006). Similarly to mammals, the DA system is involved in

the control of locomotion and other complex behaviours, including olfaction, memory, learning, and sleep (Yamamoto and Seto, 2014). This remarkable degree of conservation combined with the possibility to expose flies to PD-linked toxins has led to the investigation of PD-related phenotypes, including DA neurodegeneration and dysfunctional locomotion. DA neuronal loss is normally investigated by counting the number of neurons positive to tyrosine hydroxylase (TH) immunostaining, the rate-limiting enzyme in dopamine synthesis (Mao, 2009). Locomotor alterations are usually assessed via evaluation of a startle-induced negative geotaxis, a typical *Drosophila* behaviour, known also as climbing. Once inside the experimental tubes, flies display the spontaneous tendency to climb following a light stimulus (Nichols et al., 2012). Besides the availability of these tools, the advantage of using *Drosophila* to study the disease derives also from the conservation of many PD-genes. In fact, *Parkin*, *PINK1*, *LRRK2*, and *DJ-1* mutant strains have been generated, to investigate the mechanisms leading to the disease. Interestingly, *Parkin* and *PINK1* mutants are characterised by reduced lifespan, mitochondrial pathology, apoptotic muscle degeneration, and DA neurons degeneration (Park et al., 2006; Pickrell and Youle, 2015). Differently, *DJ-1* loss of function confers milder phenotypes (Meulener et al., 2005), as thoroughly described in the following section. Regarding dominant *loci*, *LRRK2* null animals have been shown to present inconsistent phenotypes. Indeed, according to some reports, deficient flies showed DA cell loss and locomotor deficits (Lee et al., 2007), while other studies did not find evidence of degeneration (Wang et al., 2008). Interestingly, the expression of human *LRRK2* or the *Drosophila* homologue confers age-dependent DA cell death and locomotor alterations (Liu et al., 2008; Ng et al., 2009; Venderova et al., 2009). Differently from the other PD-genes, the fly genome does not bear an *α-synuclein* encoding gene. Hence, *α-synuclein* fly models are uniquely based on the overexpression of the human gene, both in mutated and wild-type form. Interestingly, transgenic flies overexpressing the protein develop Lewy body–like inclusions, DA cells degeneration, and locomotor defects (Feany and Bender, 2000). Furthermore, *Drosophila* shares high homology with the mammalian innate immune response of the brain, allowing the investigation of the PD-related neuroinflammatory mechanisms (Lye and Chtarbanova, 2018). Therefore, considering these findings, the fruit fly represents a

valuable system to model PD-linked genes and to dissect the related mechanisms of neurodegeneration (Figure 13).

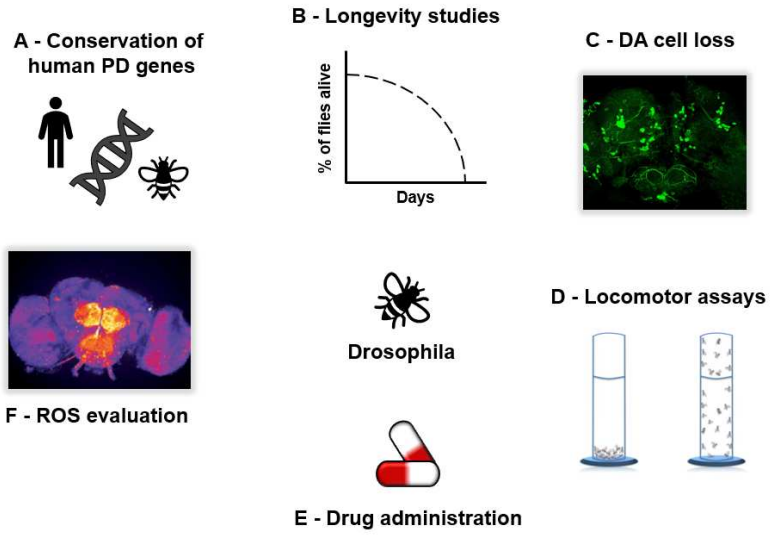


Figure 13. Advantages of *Drosophila* as a model organism to investigate PD. *Drosophila* offers numerous advantages as a model organism to study PD-related features. (A) Fruit flies shared many of the human genes involved in PD, allowing the generation of PD-mutant organisms, which can be assessed through different approaches: (B) longevity, (C) evaluation of DA cell loss, (D) locomotor defects, e.g. by climbing, (E) response to PD-toxins and therapeutic compounds, and (F) ROS levels. The picture representing ROS evaluation in the fly brain derives from Biosa et al., 2018, while the figure representing the climbing device derives from Madabattula et al., 2015. The picture showing the DA system was produced in our lab, generating flies that overexpress the green fluorescent protein (GFP) in DA neurons via TH-driver.

2.3.2 *Drosophila* as a model for hypoxia/anoxia studies

As previously mentioned, several neurodegenerative diseases have been associated with an impaired oxygen supply, highlighting a role of hypoxia/anoxia in their development and/or progression. Thanks to its versatility, *Drosophila* has also been exploited in the investigation of the hypoxic/anoxic responses, mainly using whole body organisms (Armstrong et al., 2011; Ma and Haddad, 1997). As many other insects, *Drosophila* displays great resistance to severe hypoxia and even

anoxia for short exposure time. Under limiting oxygen concentrations, adult flies become motionless within 1 minute, due to interruption of the electrical responses in fly muscles (Krishnan et al., 1997). Recovery time increases proportionally to the treatment duration, but flies cannot survive more than 12 hours (Van Voorhies, 2009). Although it is still completely undefined which mechanisms trigger *Drosophila* resistance to such extreme conditions, some peculiarities have been discovered. For example, fly neurons have been found to become hyperpolarised under anoxia, reducing excitability, probably as a mechanism to preserve energy (Gu and Haddad, 1999). Moreover, *Drosophila* appears to upregulate chaperone proteins, such as Hsp70 and Hsp23, as a response mechanism to avoid protein unfolding upon prolong periods of oxygen depletion. Accordingly, the overexpression of Hsp chaperones has been shown to increase the survival rates compared to controls (Azad et al., 2009). In addition, fruit flies have been reported to accumulate trehalose, which exerts protection in multiple types of stress, including heat, dehydration, hyperosmotic shock, and oxidant damage (Becker et al., 1996; Chen et al., 2002). Therefore, its accumulation under anoxia has been shown to reduce protein aggregation (Chen et al., 2003). Moreover, a role has been attributed to the gene *hypnos-2* encoding a *Drosophila* pre-mRNA adenosine deaminase (dADAR), which regulates in RNA editing of ion channel proteins in the central nervous system (Palladino et al., 2000). Indeed, its disruption has been reported to delay the anoxic coma recovery (Ma and Haddad, 1997), supporting a role for the modulation of ion channels in the anoxic response. From the metabolic point of view, *Drosophila* has been reported to present an atypical behaviour. In most anoxic-tolerant organisms, this adaptation has been supposed to depend on their ability to balance ATP supply and demand, favouring anaerobic metabolism to preserve the ATP pool (Boutilier and St-Pierre, 2000). Surprisingly, albeit its high resistance to extreme oxygen condition, *Drosophila* does not seem to present this feature, displaying a mild ATP decrease as that reported in mammals (Campbell et al., 2018). Moreover, fruit flies have been reported to accumulate acetate, lactate, and alanine under anoxia, instead of succinate as known for mammals (Feala et al., 2009, 2007). Notwithstanding these differences, *Drosophila* shares similar response pathways to those known in mammals. In fact, low oxygen availability determines the

stabilisation of HIF1-α homologous protein, referred to as Similar (Sima) in flies (Bacon et al., 1998). As HIF1-α, Sima belongs to the family of basic helix-loop-helix (bHLH)/PAS transcription factors and guides gene expression. Moreover, similarly to HIF1-α which regulates vascularisation in mammals (Semenza, 2009), Sima controls tracheal sprouting towards oxygen-deprived tissues in flies during hypoxia (Centanin et al., 2010). Considering the high degree of conservation of this molecular response pathway and the notable *Drosophila* endurance to limiting oxygen conditions, fruit flies have emerged as an interesting model to explore the molecular mechanisms involved in hypoxia, anoxia and reoxygenation processes. Indeed, determining the basis of this resistance may reveal interesting protective mechanisms, which might have a useful impact on human oxygen-related pathophysiology.

2.4 The *Drosophila* anti-oxidant system: focus on SOD1 and DJ-1

As previously mentioned, the *Drosophila* genome encodes an anti-oxidant machinery similar to mammals. *Drosophila* presents a first line of defence based on Sod enzymes and a second line of defence, based on catalase, glutathione peroxidases, thioredoxines, and peroxiredoxins (Missirlis et al., 2001). *Drosophila* also shares multiple redox-sensitive pathways, including Nrf2, NfKβ, and PI3K/Akt, which counteract ROS-damages, enhancing the anti-oxidant defence and promoting survival (Hetru and Hoffmann, 2009; Pitoniak and Bohmann, 2015, p. 2; Santabárbara-Ruiz et al., 2018). In addition, the fruit fly anti-oxidant machinery also includes homologues of the multitasking protein DJ-1 (Meulener et al., 2005). Considering the established role of the cytosolic SOD1 in the cellular anti-oxidant defence, the emerging role of DJ-1 in this field, and the proposed interplay between the two proteins, the following paragraphs will be dedicated to the description of SOD1 and DJ-1 proteins in *Drosophila*.

2.4.1 *Drosophila* SOD1: features and mechanism of activation

Drosophila Sod1 protein displays a ubiquitous pattern of expression, being principally localised at the cytosolic level, as the human protein. The protein sequence shares large homology to human SOD1, and, as most eukaryotes, it does not bear Pro144, which is instead substituted by isoleucine. Therefore, fruit fly Sod1 can be activated also in the absence of its copper chaperone, referred to as Ccs, which has been reported to have peculiar features. Indeed, albeit conserving the D2 and D3 domains, involved in Ccs-Sod1 docking and disulphide bridge formation, respectively, fruit fly Ccs does not harbour the MXCXXC motif in the D1 domain (Kirby et al., 2008). Therefore, *Drosophila* Ccs must deliver copper to Sod1 through D3 domain. In addition, *Drosophila* Sod1 is also unique for being largely unstable in the absence of its chaperone Ccs. As shown by Kirby and colleagues, upon Ccs depletion, Sod1 protein levels are dramatically reduced, as well as its dismutating activity, which is almost undetectable (Kirby et al., 2008). Nevertheless, while *Sod1* mutants properly develop till the larval stage, late pupae and adults are characterised by premature lethality (half-life of about 10 days), infertility and hypersensitivity to excessive ROS levels (Phillips et al., 1989). *Ccs* null individuals show a similar sensitivity to oxidative stress, but live longer than *Sod1* mutants, with a half-life of 40 days. These findings have suggested that under non stressful conditions, the residual levels of Sod1, activated by an alternative mechanism, are enough to extend lifespan (Kirby et al., 2008). The players of this alternative pathway are still under investigation, although some studies have proposed potential copper-donor candidates (Carroll et al., 2004; Girotto et al., 2014). In this frame, using the human proteins, our and other groups have observed that DJ-1 may have a role in SOD1 maturation *in vitro*, acting as copper chaperone (Girotto et al., 2014; Srivastava et al., 2016; Xu et al., 2010), laying the background for the *in vivo* investigation of this mechanism.

2.4.2 *Drosophila* DJ-1

Drosophila genome encodes two DJ-1 protein homologous, referred to as dj-1α and dj-1β, which are defined by a different spatial pattern of expression. dj-1α protein is mainly restricted to male testes, with minor expression in the brain, while dj-1β presents an ubiquitous expression profile, as the human protein (Aradska et al., 2015; Meulener et al., 2005). The two fly homologues share a similar identity with human DJ-1 (56% identity, 70% similarity for dj-1α and 52% identity, 69% similarity for dj-1β). Double knocked-out animals are viable and fertile but display enhanced sensitivity to high amounts of ROS (Meulener et al., 2005). Interestingly, this sensitivity seems to depend on dj-1β, since single *dj-1β* null flies are as sensitive as double knocked-out flies to oxidative stressors, while *dj-1α* single knocked-out individuals behave as control flies. This has suggested that dj-1β is the main factor involved in protection under uncontrolled ROS concentration, while dj-1α may have a different role, due to its specific expression pattern (Meulener et al., 2005). Interestingly both proteins bear the highly conserved Cys104, which has been shown to be essential for dj-1β-mediated protection against ROS. Indeed, as observed for the human protein, also dj-1β undergoes an acidic shift upon paraquat exposure via Cys104 oxidation to sulfinic and sulfonic acid (Meulener et al., 2006). Crystallographic analysis has revealed that dj-1β protein is structurally similar to the human counterpart, with the exception of the intrinsically mobile solvent-exposed regions (Lin et al., 2012).

Although being involved in PD pathology in humans, dj-1 null flies do not show evidence of gross dopaminergic neurons death, not even during ageing (Menzies et al., 2005; Meulener et al., 2005). To date, only two reports have shown that *dj-1β* single null flies display DA cell loss under oxidative insults (Hwang et al., 2013; J. Yang et al., 2017), and a research group has reported that *dj-1α* silencing causes DA cells degeneration (Faust et al., 2009; Yang et al., 2005). Notwithstanding the lack of evident neurodegeneration, double knocked-out and *dj-1β* single null flies have been shown to have locomotor deficits (Hao et al., 2010; Hwang et al., 2013; Park et al., 2005; J. Yang et al., 2017). Concerning the function, *Drosophila* dj-1 homologues have been reported to have a conserved role in modulating signalling pathways, especially under stressful conditions to promote survival. For instance,

dj-1β has been observed to negatively regulate the Daxx homologue through dFOXO (Hwang et al., 2013) and to promote Akt cascade through PTEN inhibition (Kim et al., 2005). Moreover, the protein has been found to induce the expression of the mitochondrial isocitrate dehydrogenase enzyme, by the Nrf2 pathway under oxidative stress (J. Yang et al., 2017). Interestingly, a recent paper has implicated dj-1β in neuronal plasticity under oxidative stress. In fact, elevated ROS concentrations have been observed to stimulate the larval neuromuscular junction, increasing the synaptic boutons number. However, *dj-1β* mutant larvae failed to activate this response under paraquat treatment, suggesting a regulatory role in redox-dependent motoneurons plasticity, function that seems to be mediated through PI3K and PTEN effectors (Oswald et al., 2018). According to these findings, *Drosophila* dj-1β appears to behave similarly to the human counterpart, principally responding to conditions of oxidative stress, via the activation of multiple anti-oxidant pathways.

Differently from the regulatory role in signalling cascades, the mitochondrial aspect of the protein has been less investigated. To date, only one study performed in double-knocked out animals, showed that these flies are defective in mitochondrial respiration and ATP production (Hao et al., 2010), while a report evidenced an altered respiration in *dj-1β* single mutant flies (Stefanatos et al., 2012), accompanied by higher levels of mitochondrial H_2O_2 (Fernandez-Ayala et al., 2009; Sanz et al., 2010a; Stefanatos et al., 2012). Despite these findings, the precise role of dj-1β inside the mitochondrial compartment remains to be elucidated.

Chapter 3

Results

Effects of DJ-1 deficiency in the mitochondrial homeostasis

DJ-1 is a multitasking protein, which participates in a plethora of cellular mechanisms, among which indirect modulation of transcription, regulation of multiple signalling pathways, maintenance of redox homeostasis, and control of glucose levels (Biosa et al., 2017). Among them, the most investigated and supported function is its involvement in the anti-oxidant defence (Raninga et al., 2017). In this regard, the protein has been reported to activate different anti-oxidant pathways and to sustain survival under oxidative stress, repressing cell death mechanisms (Biosa et al., 2017; Raninga et al., 2017). Moreover, DJ-1 has been shown to translocate into mitochondria upon stressful conditions, such as treatment with exogenous pro-oxidants or ischemia-reperfusion injury (Canet-Avilés et al., 2004b; Junn et al., 2009b; Kaneko et al., 2014). Although it is still unclear the exact role of the protein inside this compartment, DJ-1 has been suggested to participate in the mitochondrial functionality and dynamics, contributing to the maintenance of the mitochondrial integrity (Calì et al., 2015; Ding et al., 2018; Hayashi et al., 2009; Irrcher et al., 2010). As many of these findings have been performed *in vitro*, we sought to investigate the mechanisms of action of DJ-1 *in vivo*, using *Drosophila melanogaster* as a model organism.

3.1 dj-1β protein is responsible for the resistance to oxidative insults

As mentioned in the introduction, the *Drosophila* genome shares more than 75% of genes involved in human diseases, among which *DJ-1* (Allocca et al., 2018b). Interestingly, fruit flies have evolved two *DJ-1* homologous genes by duplication, named *dj-1α* and *dj-1β*, both displaying about 70% of similarity to the human protein. Various key amino acids found in human DJ-1 are conserved in both fly homologues, including the redox-sensitive cysteine 106. Nonetheless, the proteins present a different spatial pattern of expression. Indeed, dj-1α is mainly restricted to male testes, while dj-1β is found in all body tissues, reflecting the pattern of expression of the human protein (Meulener et al., 2005). Double knocked-out individuals are viable and fertile but display enhanced sensitivity to high levels of oxidative stimuli. Importantly, this sensitivity was shown to depend on dj-1β protein,

whose absence is sufficient to increase the susceptibility to increased levels of ROS (Meulener et al., 2005).

To confirm this finding, we replicated the experiment, exposing flies to the pro-oxidant molecule paraquat (20 mM) for 4 hours. In accordance with the literature, we found that *dj-1β* single mutant and *dj-1α; dj-1β* double mutant flies present the same sensitivity to the treatment, while *dj-1α* null individuals are more resistant to paraquat, behaving similarly to control flies (Figure 14). This first result confirmed the important role of dj-1β in the protection against oxidative stress.

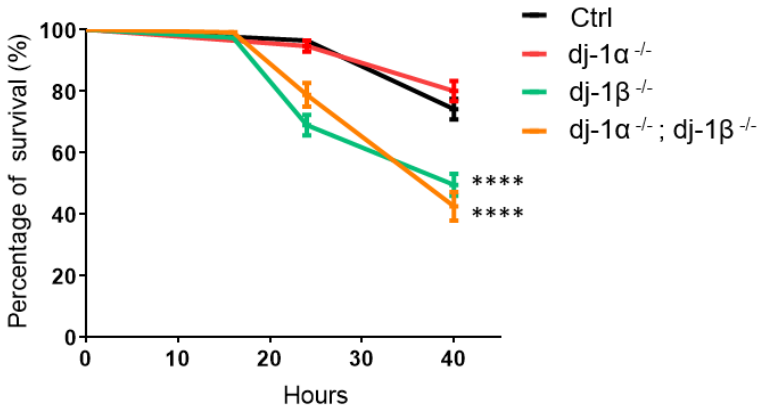


Figure 14. dj-1β protein is responsible for resistance to oxidative insults. *dj-1α; dj-1β* double knock-out, *dj-1α* and *dj-1β* single null flies and control were exposed to 20 mM of paraquat. While *dj-1α* mutants behave like control flies, *dj-1β* mutants and double knock-out animals show a similar sensitivity to paraquat. Data are presented as mean ± S.E.M. For each genotype, 150 flies were used. Mantel-Cox log-rank test, ****$p < 0.0001$.

Therefore, being dj-1β the principal homologue involved in the anti-oxidant defence and showing a shared pattern of expression to the human protein, the following experiments have been performed utilising *dj-1β* null *Drosophila* (*dj-1β*$^{\Delta 93}$). This line carries a 1960bp deletion, generated by P-element insertion, rendering flies null for dj-1β protein (Meulener et al., 2005).

3.2 Phenotypical characterisation of *dj-1β* null *Drosophila*

3.2.1 *dj-1β* deficient flies have a normal lifespan but display increased endogenous ROS levels

As previously mentioned, the fly homologue dj-1β appears to participate in the anti-oxidant defence, similarly to the human protein. To phenotypically characterise the loss of function in flies, in the anti-oxidant context, we compared the behaviour of *dj-1β* null flies to both control individuals (w^{1118}) and mutant flies for Sod1 enzyme (Sod^{n1}). This mutant line carries a point mutation in the Sod1 protein at position 49, where a glycine is replaced by a serine. This substitution interferes with the process of dimerization, rendering the enzyme unstable and, therefore, inducing its rapid degradation (Phillips et al., 1995). Lacking the enzymatic activity of Sod1, these mutants experience premature mortality and are characterised by infertility and hypersensitivity to different oxidative insults (Phillips et al., 1989). Differently, control flies express a functional Sod1 and present a complete anti-oxidant defence. Therefore, these two lines represent extreme and opposite conditions. To analyse how the loss of the anti-oxidant-associated protein dj-1β affects the fruit fly behaviour, we evaluated ROS levels, lifespan, and locomotor abilities, under physiological conditions.

Firstly, through electron paramagnetic resonance (EPR) analysis, we quantified the endogenous ROS levels in flies. To this aim, we evaluated the concentration of the superoxide anion in the whole fly body, by using a specific probe for this ROS species. As expected, due to the lack of the Sod1 enzyme, *Sod1* null flies display the highest $O_2^{\bullet-}$ amount. Interestingly, *dj-1β* deficient individuals present a reduced $O_2^{\bullet-}$ content as compared to *Sod1* mutants, but double than control flies (Figure 15A), showing *in vivo* that the protein plays a role in the maintenance of the ROS homeostasis. Nonetheless, *dj-1β* null flies display a normal lifespan, similarly to controls, while *Sod1* mutants are short-lived, displaying a half-life of approximately 10 days (Figure 15B).

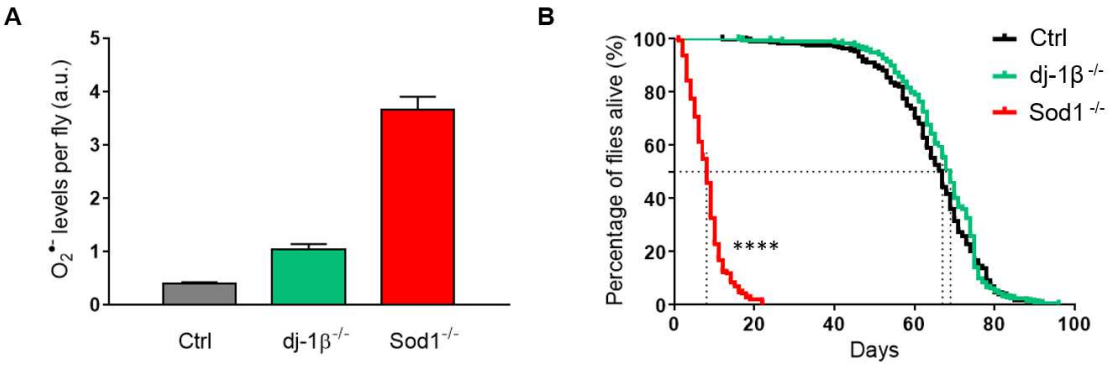


Figure 15. *dj-1β* null flies display higher endogenous levels of superoxide anion than control flies but display a normal lifespan. (A) EPR analysis shows that the lack of Sod1 leads to high endogenous levels of $O_2^{\bullet-}$, while the loss of dj-1β has a milder effect though higher than control flies. Data are presented as mean ± S.D. n=3 for control and *dj-1β* null flies, n=2 for *Sod1* mutants. For each biological replicate, 60 flies were used. (B) Lifespan of control, *dj-1β* null, and *Sod1* deficient flies, which present a half-life of 67, 69, and 8 days, respectively. For each genotype 150 flies were used. Mantel-Cox test, **** $p < 0.0001$.

Considering that the absence of dj-1β leads to a milder increase in ROS levels compared to Sod1, these data suggest that there is a threshold above which ROS levels become incompatible with life and assume mainly toxic properties. On the contrary, a mild augmentation in the ROS content, as observed in *dj-1β* null flies, appears to be compatible with life.

3.2.2 *dj-1β* null flies show an altered locomotor ability

Considering the higher endogenous levels of ROS of *dj-1β* and *Sod1* null mutants, we successively assessed locomotor performance using multiple assays. Flies have been initially evaluated for their climbing ability and total locomotion index. In the former, the negative geotaxis capability is measured by gently tapping flies to the bottom of a vial and counting the number of climbing individuals that reach a defined height. In the latter, referred to as *Drosophila* activity monitoring (DAM) system, single flies are located in small tubes, placed horizontally, and their movements

recorded thanks to an infrared light beam positioned at the centre of the tube. Each time the fly crosses the centre of the tube, the light beam is interrupted, and the signal is converted into a locomotor event.

Our results indicate that the loss of dj-1β does not affect locomotion, with the deficient flies behaving similarly to controls (Figure 16). In contrast, *Sod1* mutants are characterised by a low climbing index and show an increased locomotor activity, suggesting that *Sod1* null flies cannot climb properly but tend to move more frequently in the horizontal plane.

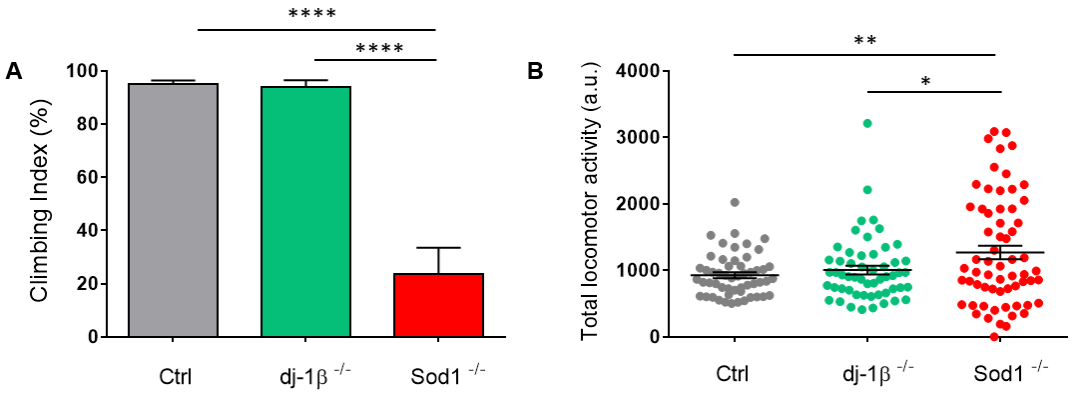
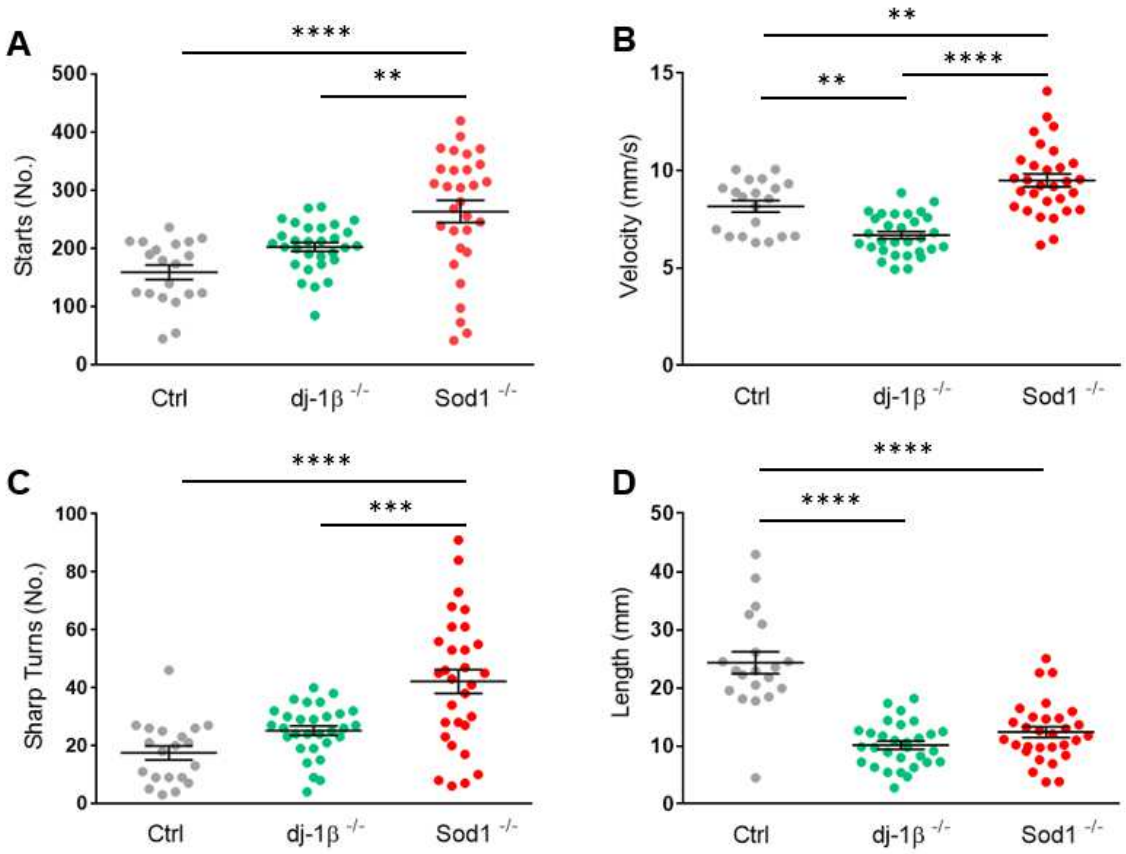


Figure 16. *dj-1β* null flies do not show gross locomotor dysfunctions when assessed via negative geotaxis and DAM system. Locomotor abilities were evaluated by (A) climbing and by (B) total locomotor activity. While Sod1 mutant show an altered locomotor behaviour in both assays, *dj-1β* deficient flies behave as the control. Results are presented as mean ± S.E.M. For each genotype at least 60 flies were used for the climbing assay, and at least 50 were utilised for the total locomotor activity. One-way Anova, Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

To obtain more information on subtle locomotor phenotypes, we performed a different apparatus in which groups of 10 flies were placed in an open field arena, where they could move freely. With this set-up, we were able to evaluate finer locomotor parameters, including the path length walked, velocity, the number of times that flies start moving and changes in direction. As seen in Figure 17A-D,

control flies present a continuous path with moderate velocity, while *Sod1* mutants exhibit a discontinuous movement, frequently stopping and changing direction, with higher velocity. Interestingly, *dj-1β* null individuals have a coordinated path but move less and more slowly than the other genotypes. To obtain a comprehensive view of the locomotor performance of the three genotypes all together, we carried out the principal component analysis (PCA), in which it is highlighted how control, *dj-1β*, and *Sod1* null flies are differently clustered accordingly to their locomotor behaviour (Figure 17E).



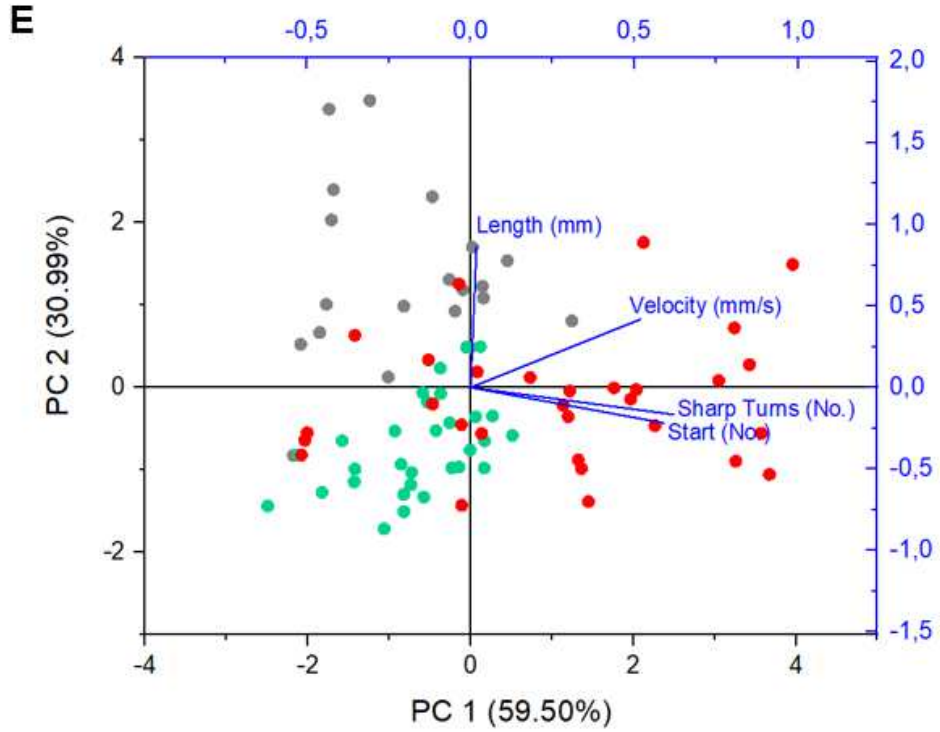


Figure 17. *dj-1β* null flies show altered locomotion in open flied arena. (A, B, C, D) Graphs showing the behaviour of flies according to each single locomotor parameter. The parameters evaluated are: starts (No. of start movements, (A), velocity (B), sharp turns (changes of direction, C), length (length of the path walked, D). Results are shown as mean ± S.E.M. 30 flies were used for *dj-1β* and *Sod1* null flies and 20 for controls. One-way Anova, Tukey's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (E) Principal component analysis (PCA) showing the different distribution of control, *dj-1β* deficient flies and *Sod1* mutant individuals. The locomotor parameters are represented as blue vectors in the graph.

Therefore, although not conferring evident locomotor alterations, the loss of dj-1β confers minor defects. Multiple reasons could cause these deficits, including bioenergetic impairments due to alterations in the mitochondrial functionality, alterations in the muscle strength or in the nervous system. As DJ-1 has been frequently implicated in the mitochondrial homeostasis, we decided to focus our attention on this aspect.

3.2.3 The loss of dj-1β affects the mitochondrial cristae morphology

To assess the involvement of dj-1β in the mitochondrial homeostasis, we first decided to investigate, by electron microscopy, the consequences induced by the loss of protein on the mitochondrial morphology. To this end, we utilised the fly thorax muscle, in which mitochondria are regularly distributed along the muscle fibres, rendering easier the morphological evaluation. Moreover, with this analysis, we also assessed the integrity of the muscle fibres to evaluate whether *dj-1β* null flies present some muscular alterations. As reported in Figure 18, none of the genotypes presents evident muscular abnormalities and the differences observed are mainly related to the orientation of the sample and to the sectioning process. Therefore, the absence of dj-1β does not seem to affect the muscular integrity in the fly thorax. From the mitochondrial point of view, control individuals present mitochondria that are tightly packed between the muscular fibres and with cristae properly organised (Figure 18A), while *Sod1* null flies display swollen mitochondria with disrupted cristae (Figure 18C). Interestingly, *dj-1β* deficient flies show some variability in the mitochondrial morphology, having both larger and smaller organelles, and are frequently characterised by a peculiar cristae organisation (Figure 18B). Indeed, with respect to controls, null flies tend to display a different ultrastructural rearrangement, in which cristae are not completely elongated along the mitochondrion but appear organised in small circles, showing a “fenestrated” pattern.

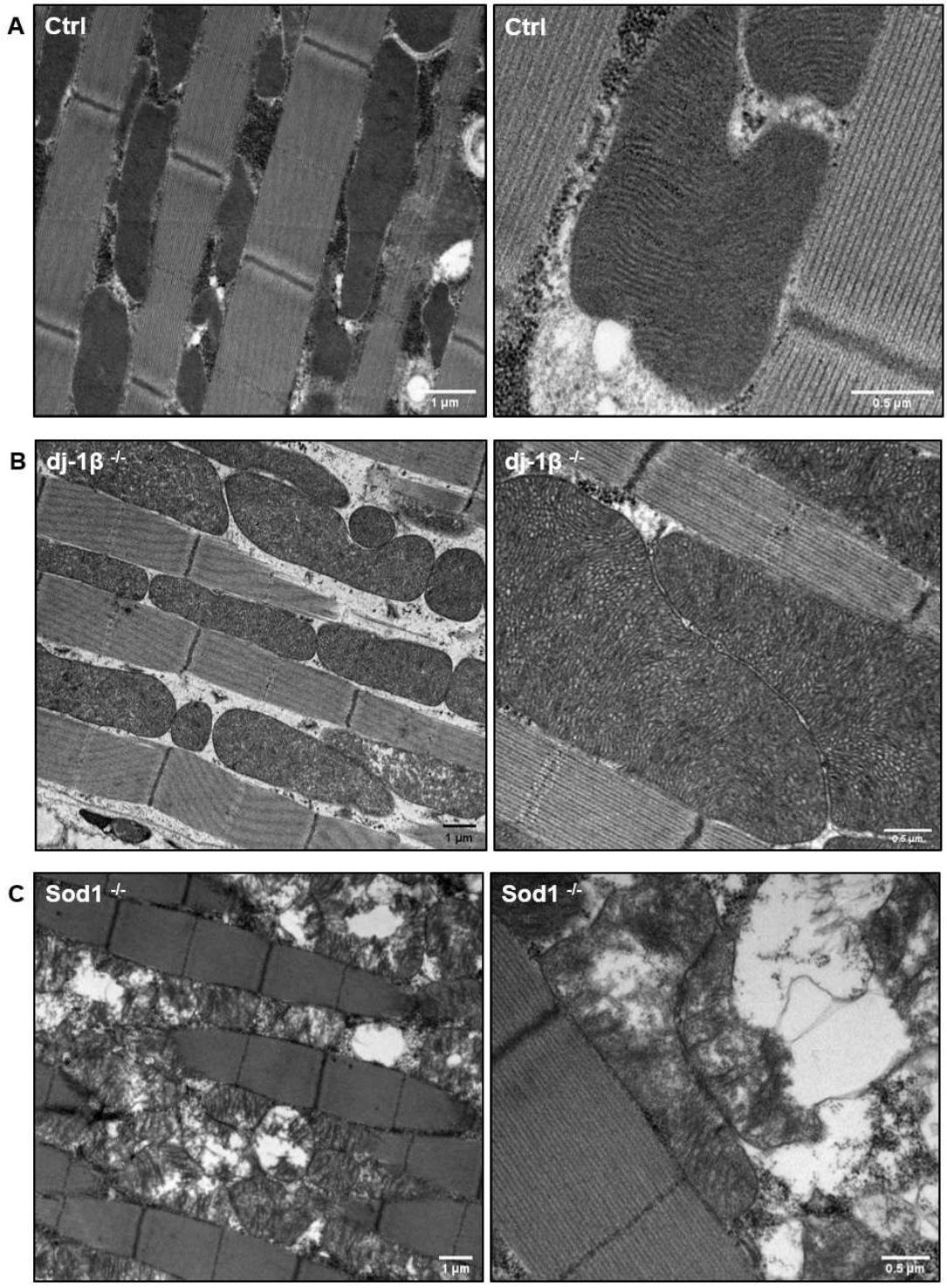


Figure 18. *dj-1β* null mitochondria have an altered cristae organisation. Electron microscopy analysis of the morphology of the thoracic muscle mitochondria of (A) control, (B) *dj-1β* null (C) *Sod1* null flies. Images on the right were acquired at higher magnification to show the cristae organisation in each genotype. For each genotype 3 individuals were analysed.

3.3 Effects of dj-1β deficiency on the mitochondrial homeostasis

Cristae remodelling is a phenomenon through which mitochondria adapt to the cellular needs. Indeed, these morphological changes are known to impact on the organisation of the respiratory complexes, and are capable of influencing the rate of mitochondrial respiration (Cogliati et al., 2016). In this regard, considering the described relationship between DJ-1 and mitochondria and our morphological results, we hypothesised that *dj-1β* deficient flies may display some respiratory alterations. To assess the mitochondrial effects of dj-1β deficiency, we measured different mitochondrial parameters, including respiration and metabolomic responses. Moreover, we also investigated the relevance of the protein in the presence of oxidative insults, by using two approaches: the pro-oxidant molecule paraquat, as exogenous ROS source, and the exposure to anoxia-reoxygenation, as pathophysiological mechanism of ROS production. Being Sod1 mutants extremely sensitive and short-lived, we carried out the following package of experiments specifically on *dj-1β* null individuals and control flies.

3.3.1 Effects of dj-1β loss on the mitochondrial functionality

A first set of experiments were performed by rearing young adult flies either in standard food or in the presence of a chronic sublethal concentration of paraquat (0.1 mM). After 7 days of treatment, flies were sacrificed to assess the mitochondrial functionality. For the respiration assay and ATP quantification we used the whole-body organism, while the abundance of mitochondrial complexes and complex I activity were assessed on purified mitochondria.

3.3.1.1 dj-1β deficiency leads to impairments in the mitochondrial respiration

Real-time mitochondrial respiration was measured using the Oroboros O2k oxygraph. Specifically, we measured complex I- and complex II-dependent oxygen consumption. For the former, we used glycine, proline, malate, and ADP as substrates, while, for the latter, we utilised succinate, as substrate, and rotenone,

as complex I inhibitor. The addition of proline as a substrate for complex I was used since it has been shown to enhance respiration in insects (Teulier et al., 2016).

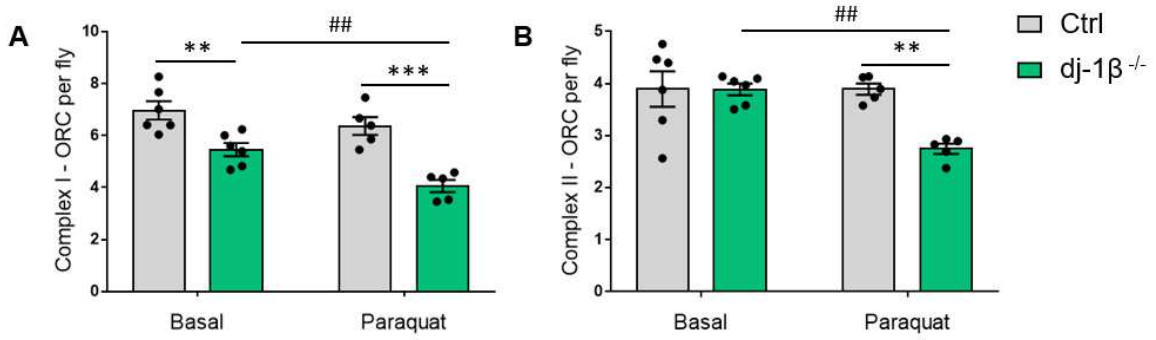


Figure 19. *dj-1β* deficiency leads to an impaired respiration. Oxygen consumption rate (OCR) of (A) complex I and (B) complex II in control and *dj-1β* null flies under basal conditions and after treatment with paraquat (0.1 mM - 7 days). Results are presented as mean ± S.E.M. For each genotype at least 5 replicates were performed. Two-way Anova, Bonferroni's multiple comparisons test. [*] comparison between genotypes, ** $p < 0.01$, *** $p < 0.001$; [#] comparisons between conditions, ## $p < 0.01$, ### $p < 0.001$.

Our data indicate that under basal conditions, complex I-associated oxygen consumption is reduced in *dj-1β* null flies compared to controls and that this deficit is further worsened in the presence of an oxidative insult (Figure 19A). Differently, in the absence of dj-1β, complex II displays a normal rate of basal oxygen consumption but becomes impaired after the paraquat treatment, supporting a role of the protein in mitochondrial respiration, particularly under oxidative conditions (Figure 19B).

3.3.1.2 dj-1β deficiency does not alter the abundance of the respiratory chain complexes

As alterations in the mitochondrial respiration could be due to variations in the respiratory chain components, we successively performed a BN-PAGE to separate the mitochondrial complexes and evaluate their relative abundance in control and mutant flies. According to this analysis, we did not evidence any obvious alteration

in the abundance of the ETC complexes, neither under basal condition nor after paraquat exposure (Figure 20A). In particular, we quantified complex I protein level (relative to complex V) and we did not noticed variations in any condition (Figure 20B), suggesting that dj-1β does not influence complex I abundance. Therefore, considering the respiration data, we next sought to investigate whether dj-1β deficiency causes impairments in the complex I activity.

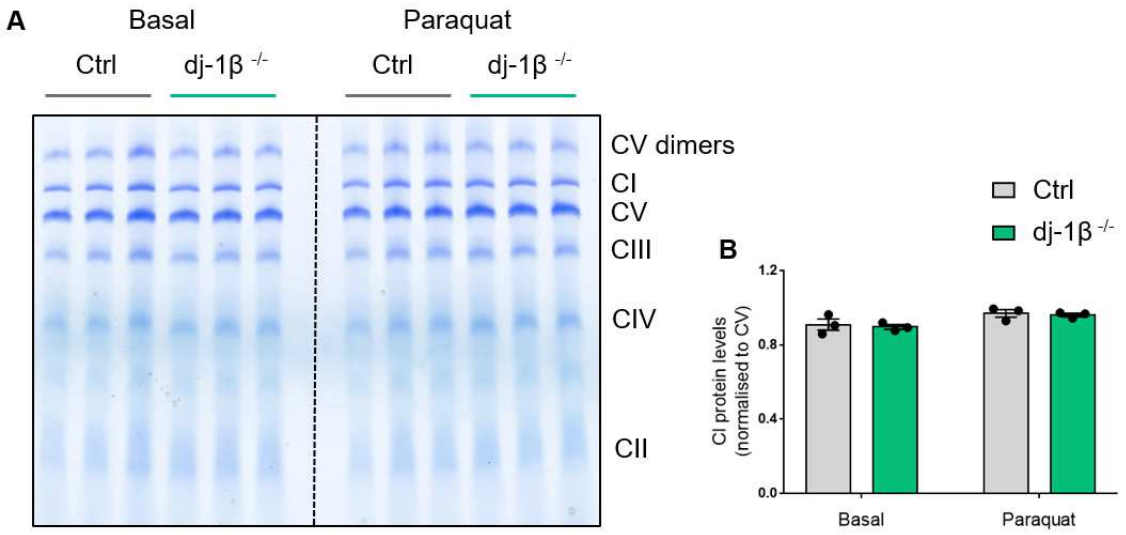


Figure 20. *dj-1β* deficiency does not affect the protein levels of the respiratory complexes. (A) BN-PAGE showing the different mitochondrial complexes performed on control and *dj-1β* null flies, under basal conditions and after treatment with paraquat (0.1 mM - 7 days). (B) Quantification of Complex I (CI) protein levels. Results are presented as mean ± S.E.M. For each genotype 3 replicates were carried out. Two-way Anova, Bonferroni's multiple comparisons test.

3.3.1.3 Loss of dj-1β protein reduces complex I activity

To evaluate complex I activity, we used two different approaches: the in gel activity (IGA) assay and the kinetic measurements. In the former, we firstly separated mitochondrial complexes via BN-PAGE and then we incubated the gel with the complex I substrate NADH and nitro-blue tetrazolium salt (NBT) as electron acceptor. The dehydrogenase activity of complex I is revealed by the colour change of NBT, which forms, upon reduction, a blue-purple precipitate at the level of

complex I band. With this assay, we did not observe significant variations in the complex I activity among the different genotypes or conditions tested, suggesting that the NADH dehydrogenase function is not affected (Figure 21A). In parallel, we also performed kinetic measurements, which represent a more sensitive technique. In this case, isolated mitochondria were incubated in the presence of NADH, as substrate, and CoQ as electron acceptor. Measurements were carried out also in the presence of rotenone to extrapolate the complex I-associated dehydrogenase activity with respect to other cellular dehydrogenases present in the mitochondrial lysate. Differently, from the IGA, with this approach, we observed that complex I activity is impaired in *dj-1β* null mitochondria under paraquat treatment (Figure 21B).

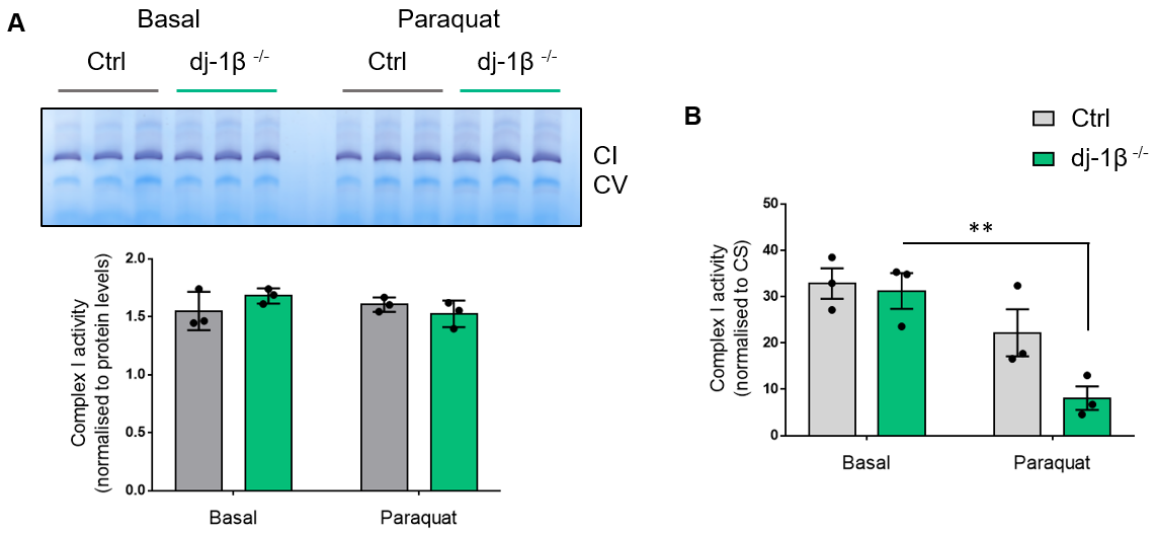


Figure 21. dj-1β deficiency affects complex I activity. (A) In gel activity (IGA) showing complex I activity of control and *dj-1β* null flies under basal conditions and after treatment with paraquat (0.1 mM - 7 days), with relative quantification below. (B) Complex I activity measured by kinetic measurements. Results are presented as mean ± S.E.M. For each genotype 3 replicates were carried out. Two-way Anova, Bonferroni's multiple comparisons test, ** $p < 0.01$.

The apparently contrasting results obtained could be attributed most likely to the different methods used. In fact, in order to sustain complex I-dehydrogenase activity, CoQ needs to bind to complex I and receive electrons, while this is not required in the IGA. Therefore, since the NADH dehydrogenase activity is not altered, according

to the IGA data, the loss of dj-1β seems to influence the binding of CoQ to complex I, thus, probably, affecting the electron flux.

3.3.1.4 dj-1β loss does not affect total ATP levels but enhances sensitivity to starvation

To evaluate whether the observed alterations in mitochondrial respiration are associated with defects in the oxidative phosphorylation process, we then quantified the total ATP levels in the whole fly body. Quantifications showed that dj-1β deficiency does not impact on ATP amount neither under basal conditions nor under oxidative insults (Figure 22A). To observe if dj-1β plays any role in energetic metabolism, we exposed flies to water starvation. Interestingly, we observed that the loss of dj-1β induces an earlier lethality with respect to control flies (Figure 22B), suggesting that, despite the unaltered total ATP levels, these flies display an impaired mobilisation of the energetic reservoirs.

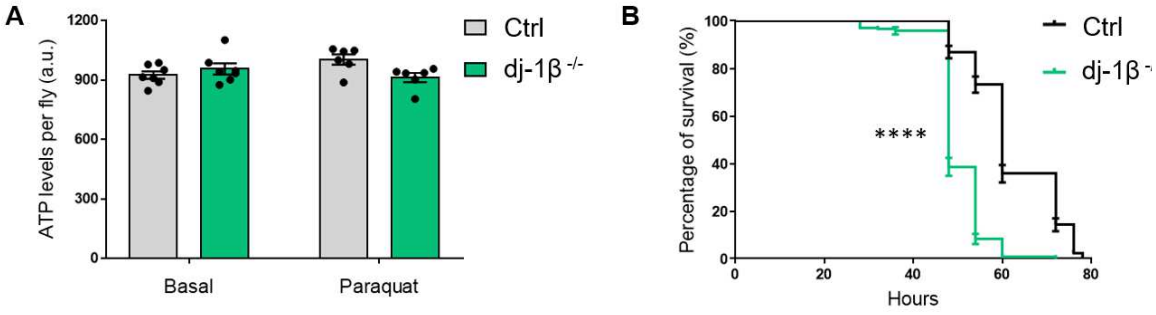


Figure 22. dj-1β loss does not affect total ATP levels but enhances sensitivity to starvation. (A) Total ATP levels of control and *dj-1β* null flies under basal conditions and after treatment with paraquat (0.1 mM - 7 days). Results are presented as mean ± S.E.M, expressed as arbitrary units (a.u.). For each genotype at least 6 replicates were carried out. Two-way Anova, Bonferroni's multiple comparisons test. (B) Starvation assay performed with filter paper embedded with water. For each genotype, 160 flies were used. Mantel-Cox log-rank test, **** $p < 0.0001$.

3.3.2 Effects of dj-1β loss in the response to anoxia-reoxygenation

Considering that DJ-1 has been described to protect against ischemia-reperfusion injury (Dongworth et al., 2014; Peng et al., 2019; Shimizu et al., 2016; Yanagisawa et al., 2008) and that ischemic events could be associated to PD pathogenesis (Shafqat et al., 2017; Shill and Stacy, 2002), in a second set of experiments, we used anoxia-reoxygenation injury as pathophysiological form of oxidative insult. Differently from mammals, fruit flies can resist to low oxygen concentrations, so that they represent a suitable model to study the responses to anoxia-reoxygenation and the associated metabolic adaptations. To evaluate the mitochondrial response to anoxia, we first treated young adult flies with a short exposure to anoxia and, then, reintroduced them into the normoxic atmosphere. A series of preliminary experiments were carried out to optimise the duration of the anoxic treatment in order to allow the complete recovery of our control individuals in terms of viability.

3.3.2.1 dj-1β deficiency impairs the post-anoxic recovery

To firstly assess the survival rate of *dj-1β* null individuals to the anoxic treatment, we exposed control and null flies to 1 hour of anoxia and then recovered them in normoxia. The day after, we started recording death events to evaluate possible variations in life expectancy. While most of control flies present a lifespan similar to untreated animals (Figure 23A), several *dj-1β* deficient animals display a premature death after the anoxic recovery (Figure 23B).

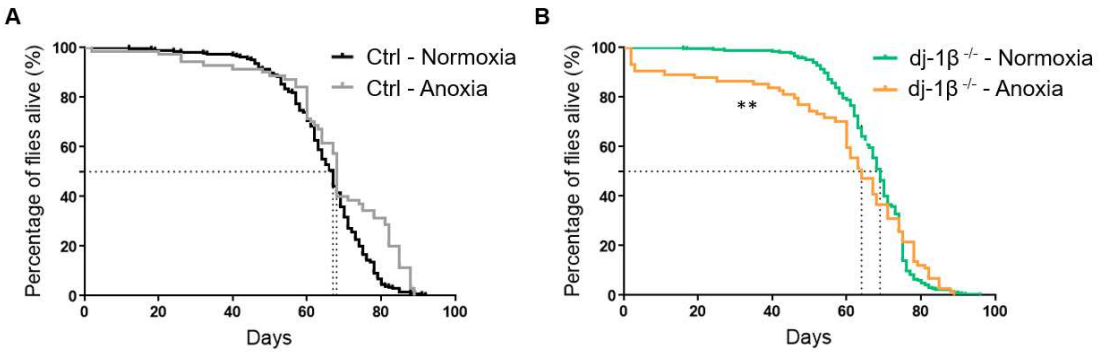


Figure 23. dj-1β loss impairs the post-anoxic recovery. Lifespan analysis after anoxic treatment. (A) Control and (B) *dj-1β* null flies were exposed to 1 hour of anoxia and recovered in normoxia; after that longevity was evaluated. Under this condition, control flies

have a half-life of 68 days (67 under normoxia), while the half-life *dj-1β* null flies is of 64 days (69 under normoxia), showing higher mortality in the first days after the anoxic recovery. For each genotype, at least 70 flies were used. Mantel-Cox log-rank test, ** $p < 0.01$.

This result suggests that dj-1β could play an important role in the first phases of the post-anoxic recovery, which are known to be highly damaging due to the increased levels of ROS produced.

3.3.2.2 dj-1β absence alters the cristae morphology under anoxia-reoxygenation

Working with oxygen, mitochondria are highly sensitive to variations in oxygen concentrations. Being *dj-1β* null flies more sensitive to the anoxic exposure, we investigated the effect of the absence of dj-1β on the morphology of the thoracic muscle mitochondria after anoxia-reoxygenation. To discriminate the contribution of both anoxia and reoxygenation, flies were exposed to 1 hour of anoxia and immediately fixed or re-introduced to normoxia for 5 minutes to promote reoxygenation-derived ROS production and, subsequently, processed. As reported in Figure 24, under anoxia, control and *dj-1β* null flies display a similar mitochondrial morphology: organelles principally present disrupted cristae and, sometimes, they appear swollen (Figure 24A). Nonetheless, the loss of dj-1β confers a peculiarity in the cristae distribution. In fact, we observed that *dj-1β* null mitochondria show fragments of cristae more tightly packed than that of control mitochondria. After 5 minutes of reoxygenation, we did not notice evident changes in the mitochondrial shape, but *dj-1β* null mitochondria still present fragments of cristae localised more closely than the control organelles (Figure 24B). As discussed before, a variation in the cristae arrangement may be associated with a different mitochondrial functionality. Since the anoxic set up did not allow the investigation of the respiratory chain functionality, we decided to evaluate the mitochondrial metabolic response under anoxia, and to this aim, we first set up the conditions to carry out the analysis.

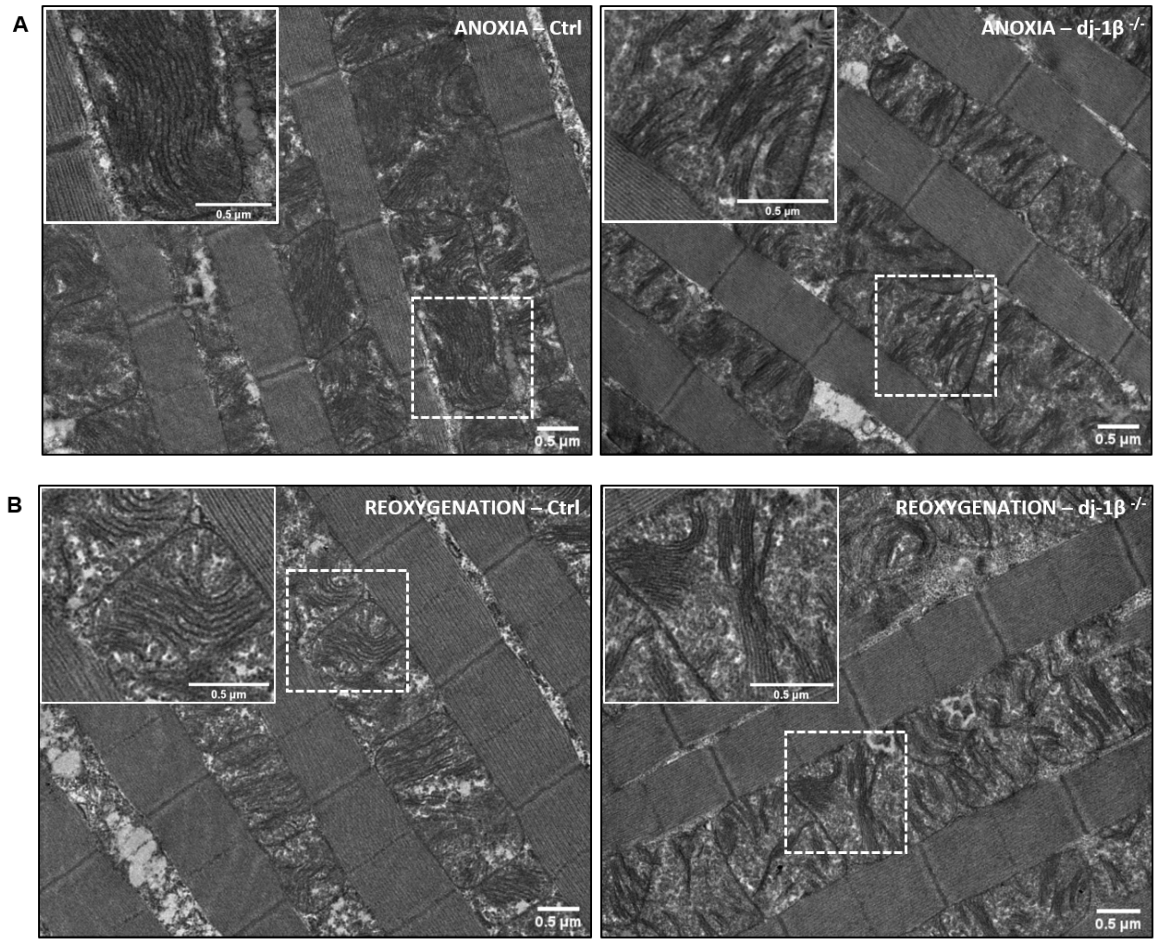


Figure 24. dj-1β loss influences the mitochondrial cristae distribution. Electron microscopy analysis on thoracic muscle sections showing mitochondria of control and *dj-1β* null flies, under (A) anoxia and (B) after 5 minutes of reoxygenation. Enlarged views of the inserts (dotted boxes) are reported on the left upper side of each image to show the cristae pattern. For each genotype, 3 individuals were analysed.

3.3.2.3 Setting up the conditions for assessing the involvement of dj-1β in the anoxic metabolism

During anoxia, mitochondrial respiration is inhibited due to the lack of oxygen, with a consequent drop in the ATP production. An anoxic-associated metabolic response is the build-up of succinate, due to the reverse activity of complex II, which uses fumarate as electron acceptor (Chouchani et al., 2014). Albeit the anoxic-derived accumulation of succinate is a recognised event in mammals, few studies have addressed this issue in fruit flies and most of them did not report succinate as the primary metabolite accumulated under anoxia (Campbell et al., 2019; Feala et al., 2009, 2007). To explore how the *Drosophila* metabolism responds to anoxia and whether dj-1β plays a role in its regulation, we exposed flies to 1 hour of anoxia and evaluated both succinate and ATP content. For this explorative trial, flies were frozen in liquid nitrogen, immediately after the anoxic treatment, and, successively, lysed on ice. As reported in Figure 25A, we did not observe significant accumulation of succinate upon anoxia, although *dj-1β* null flies appear to have a higher amount of succinate, as well as lower of fumarate, than controls. Also, the ATP and ADP content did not seem to be affected by the loss of the protein and, in general, we observed just a mild ATP depletion under anoxia (Figure 25B). In consideration of these results, we tried a longer anoxic exposure (3 hours). Moreover, in order to exclude a possible underestimation of these parameters, we compared two different experimental approaches. In the former, flies were frozen immediately after the anoxic treatment, and lysed as previously described, while, in the latter, flies were lysed inside the anoxic hood, and the obtained homogenate was frozen directly in liquid nitrogen. Interestingly, with this second method, we found increased succinate and fumarate levels, while ATP and ADP contents seem diminished (Figure 25C-D). However, we did not confirm the trend found before, in which the mutant flies generally displayed higher levels of succinate as compared to controls. As these data derive from just one sample, further replicates are required to confirm the validity of this approach and to evaluate whether dj-1β plays a role in this mechanism.

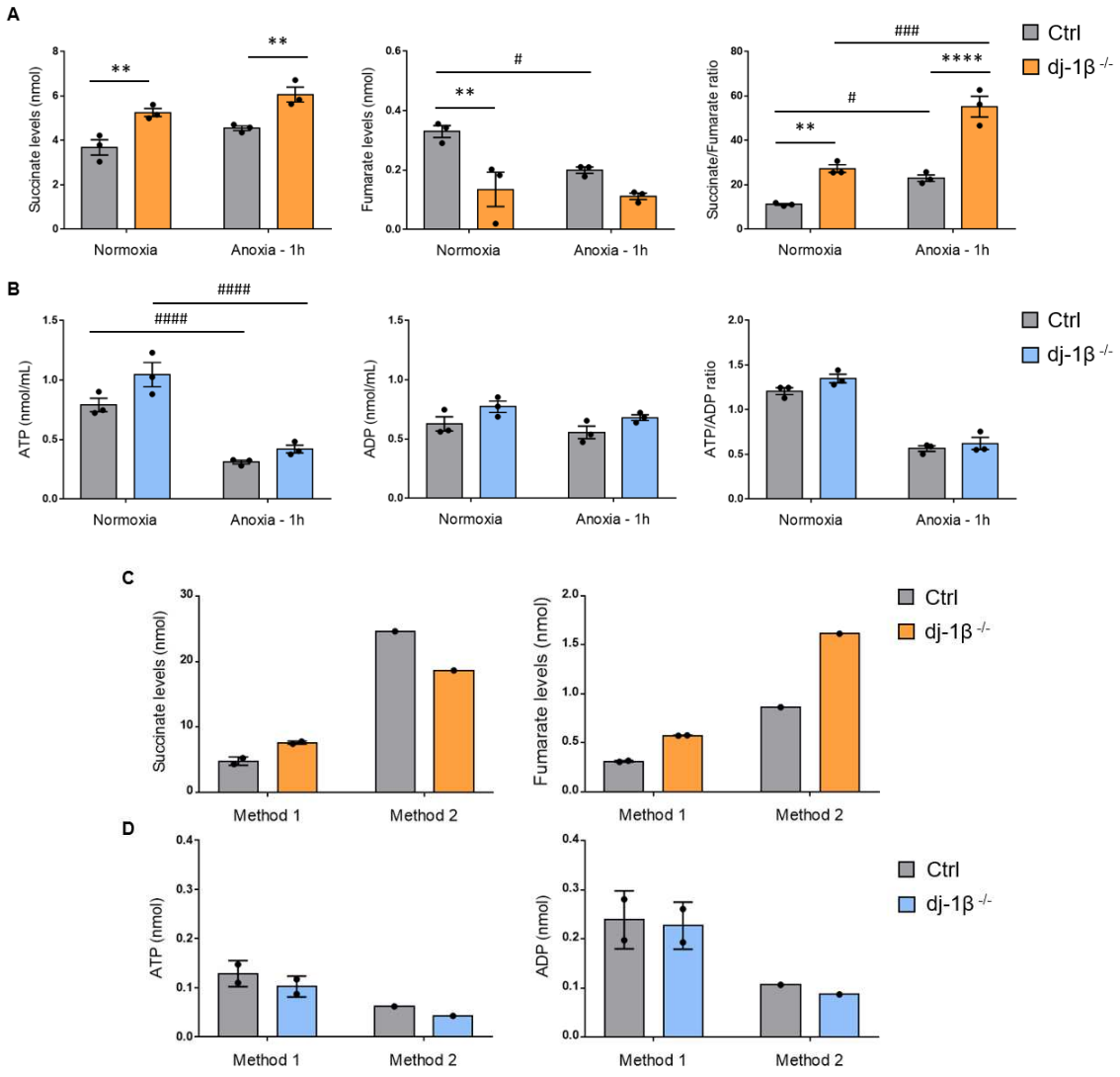


Figure 25. Setting up the conditions for assessing the involvement of dj-1β in the anoxic metabolism. (A) Quantification of succinate and fumarate, with relative ratio, in controls and *dj-1β* null flies, under normoxia and after 1 hour of anoxia. (B) Quantification of ATP and ADP levels, with relative ratio in control and *dj-1β* null flies under normoxia and after 1 hour of anoxia. Results are presented as mean ± S.E.M. For each genotype 3 replicates were carried out. Two-way Anova, Bonferroni's multiple comparisons test, [*] control between genotypes, ** $p < 0.01$, **** $p < 0.0001$; [#] comparison between normoxia and anoxia, ## $p < 0.01$, ### $p < 0.0001$. (C and D) Effects of lysing flies outside the anoxic hood after freezing (Method 1), or inside the anoxic hood (Method 2) on (C) succinate-fumarate and (D) ATP-ADP levels. Results are presented as mean ± S.D. For Method 1, 2 replicates were performed, while 1 replicate was carried out for Method 2.

3.4 Discussion

In this chapter, we have evaluated the effect of the loss of the DJ-1 homologue in fruit flies, focusing in particular on the consequences of DJ-1 deficiency on the mitochondrial homeostasis. *Drosophila* possesses two DJ-1 homologous proteins, named dj-1α and dj-1β. As reported in literature, we observed that dj-1β is primarily involved in the protection against oxidative stimuli, probably due to its ubiquitous pattern of expression, similarly to the human protein. Hence, we proceeded to specifically characterise how alterations in redox homeostasis due to the loss of dj-1β affects the lifespan of fruit flies and their locomotor behaviour. To this end, we used control and *Sod1* mutant flies, as positive and negative control, respectively. Indeed, while the former display a normal anti-oxidant defence, the latter are devoid of Sod1 protein and are unable to counteract ROS accumulation. Therefore, we analysed the behaviour of *dj-1β* null flies with respect to these lines and evaluated whether the absence of dj-1β leads to a phenotype more similar to *Sod1* mutants or to control flies. Interestingly, we found that, although displaying a normal lifespan, ROS levels in *dj-1β* null individuals are twice as high in comparison to control flies. To explore if these alterations in the redox homeostasis have an impact on locomotion, we then assessed locomotor abilities by exploiting different sets up. Albeit not showing any evident locomotor abnormality when using the climbing and DAM devices, where flies are forced to follow an obligated path, *dj-1β* null flies present several alterations when they are free to move without constraints. In fact, in the experiments performed with open flied arena, we found that *dj-1β* null flies present a continuous path, but they walk for shorter distances and with reduced velocity with respect to the other genotypes.

These alterations may be related to different causes, such as an impaired bioenergetic, reduced muscle strength or defective neuronal signalling. Considering the described role of DJ-1 in mitochondrial homeostasis, we decided to evaluate how the loss of the protein affects mitochondria morphology. Control flies have mitochondria with elongated cristae, while *Sod1* mutants principally present disrupted mitochondria, with few disassembled cristae. Our results indicate that, as control flies, *dj-1β* null individuals display more intact mitochondria than *Sod1* mutants, but with a cristae organization that differs from controls. In fact, in *dj-1β*

deficient flies mitochondria present a “fenestrated” cristae pattern. Interestingly, a similar phenotype has already been observed in other studies and it has been considered a response of the mitochondrion to oxidative stimuli. Indeed, fruit flies adapted to hyperoxia as well as the *ATP6* fly mutants, which lacks the subunit 6 of the ATP synthase, display a “honey-comb-like” pattern of cristae (Celotto, 2006; Perkins et al., 2012). Similarly, a mouse model of the Barth syndrome, a cardiomyopathy caused by a dysregulated lipid metabolism, presents the same pattern (Acehan et al., 2011). Considering that the absence of dj-1β confers higher levels of endogenous superoxide anion, the alteration of the redox homeostasis may explain the mitochondrial pattern observed in *dj-1β* null individuals. According to this morphological characterisation, *dj-1β* deficient flies appear to have an intermediate phenotype between control flies and *Sod1* mutants, displaying a milder phenotype as compared to Sod1 null individuals but being more sensitive to oxidative insults than control flies. It could appear, therefore, quite surprising that the absence of dj-1β does not affect the longevity of flies. A plausible explanation might be due to *mitohormesis*, according to which mild mitochondrial stress could promote longevity, rather than being harmful, through the stimulation of the metabolism and of the immune system (Bárcena et al., 2018). The altered organisation of mitochondrial cristae that we observed in *dj-1β* null flies may reflect an impairment in mitochondrial functionality. Indeed, variations in the cristae pattern are known to mirror changes in the assembly of the mitochondrial complexes and, therefore, in the respiration rate (Cogliati et al., 2016). In light of these considerations, we decided to investigate the mitochondrial homeostasis in *dj-1β* deficient flies under basal conditions and in the presence of oxidative stimuli. To this end, we used two different methodological approaches: the exposure to the pro-oxidant molecule paraquat, which we used to investigate the mitochondrial functionality, and the anoxia-reoxygenation treatment, that we utilised to evaluate the mitochondria morphology and metabolism. Being Sod1 mutants short-lived and extremely sensitive to oxidative insults, this genotype was excluded from the analysis.

The analysis of the mitochondrial respiratory capacity revealed that the loss of dj-1β impairs the respiration process at the level of both complex I and complex II under paraquat treatment, although not affecting the abundance of the respiratory

complexes. Interestingly, we observed that *dj-1β* null flies show a reduced complex I-dependent oxygen consumption also under basal conditions. In accordance with our results, several studies have reported that human DJ-1 can bind to and sustain complex I activity, *in vitro* (Ding et al., 2018; Hayashi et al., 2009; Zhang et al., 2018). Therefore, we investigated the consequences of the absence of dj-1β on complex I activity, by IGA and by kinetic measurements. Surprisingly, we noticed that the dehydrogenase activity of complex I is preserved, as IGA data did not show differences between control and null flies. Nonetheless, the kinetic measurements highlighted a remarkable reduction in complex I activity of *dj-1β* deficient flies, under oxidative conditions. These contradictory results can be rationalised considering the different technical approaches used. Indeed, IGA assay allows the measurement of the NADH dehydrogenase activity, using NBT as electron acceptor. In contrast, in the kinetic analysis, we used the more physiological CoQ as electron acceptor, which needs to bind to complex I to exert its function, differently from NBT. It follows that, if CoQ does not properly bind complex I, its activity is impaired. Therefore, with these two techniques, we have obtained complementary results, which suggest that the loss of dj-1β affects the electron flux at the level of complex I. According to some literature reports, dj-1β may exert this function through a direct interaction with complex I (Hayashi et al., 2009; Heo et al., 2012). Alternatively, this result could represent an indirect consequence of higher ROS levels, which accumulate due to the loss of dj-1β. Indeed, under oxidative insults, cardiolipin, a mitochondria lipid involved in the assembly and functionality of the respiratory complexes, becomes oxidised losing its protective activity and leading to respiratory impairments (Paradies et al., 2019). As both explanations are plausible, and they are not mutually exclusive, further investigations are required to elucidate if only one of them or both are related to our results.

Notwithstanding the mitochondrial impairment observed, the loss of dj-1β does not seem to affect the total amount of ATP. Although mitochondria account for most of the ATP production, we cannot rule out that other anaerobic pathways may be involved. In agreement with this consideration, in the *ATP6* mutant flies previously mentioned the total ATP levels remain unaltered during the asymptomatic phase, despite the mutation in one subunit of the ATP synthase enzyme. Indeed, the

reduction in the mitochondrial ATP synthesis appears to be compensated by enhanced glycolysis and ketogenesis (Celotto et al., 2011). For this reason, we cannot exclude the participation of the aerobic metabolism in the ATP production in *dj-1β* null flies. While performing our metabolic analysis, we have also observed that the loss of dj-1β enhances the sensitivity to starvation, supporting the hypothesis of an altered mobilisation of the energetic reservoirs that might be due to a reduced sugar availability in mutant flies. Interestingly, a similar phenotype has been previously observed in a *Drosophila* mutant for the uncoupler protein 5 (*UCP5*), which presents unaltered ATP levels, but diminished sugar content (Sánchez-Blanco et al., 2006). Considering that few studies have proposed a role of DJ-1 in the regulation of metabolism, further analysis could provide interesting insights about this emerging function of the protein (Shi et al., 2015; Wu et al., 2017; Xu et al., 2018).

In parallel with this analysis, we also characterised the response of *dj-1β* null flies to anoxia-reoxygenation treatment, used as a different approach to generate an oxidative insult. Being fruit flies resistant to hypoxia, and even to short anoxic treatments, we exposed flies to 1 hour of anoxia and evaluated variations in mitochondrial morphology and metabolic response. Initially, we assessed how the loss of dj-1β affects longevity, following anoxia/reoxygenation injury. We observed that many *dj-1β* null flies display a premature death with respect to the controls, even though the surviving animals managed to live as long as the untreated individuals. Hence, our data suggest that dj-1β could be important in the first phases after reoxygenation, when most of ROS-derived damages occur. Being mitochondria strictly dependent on oxygen for their functionality, we then investigated how mitochondrial morphology responds to the anoxic treatment. The TEM analysis revealed that the mitochondrial architecture is similarly affected under anoxia, in both controls and mutant flies. Moreover, the exposure to 5 minutes of reoxygenation did not further change the mitochondrial ultrastructure. Although during this period of time most of ROS are produced, most probably the ROS-induced damages manifest successively, over a period of hours or even days. Nonetheless, we noticed that the loss of dj-1β influences the cristae organisation. In fact, both under anoxia and following reoxygenation, cristae appear mostly

fragmented in both genotypes but in dj-1β null flies mitochondria have cristae more tightly packed with respect to controls. This phenotype is different from what observed in *dj-1β* null mitochondria under basal condition (Figure 18B), but it further supports the possible involvement of the protein in the cristae organisation. As previously discussed, an altered cristae organisation often reflects a change in the mitochondrial functionality. Since with this set up we could not assess mitochondrial respiration or complexes activity, we decided to investigate the metabolic response because during anoxia metabolism is adapted to the temporary absence of oxygen. Two key events observed in mammals when exposed to ischemia are the depletion of ATP with the parallel accumulation of succinate (Chouchani et al., 2014). Albeit succinate is a recognised key metabolite of the ischemic response in mammals, its relevance in the fruit fly metabolism is less understood. In fact, investigations conducted so far did not report succinate as a primary metabolite accumulated in *Drosophila* under anoxia (Campbell et al., 2019; Feala et al., 2009, 2007). Considering the possible involvement of dj-1β in the metabolic regulation, as suggested by our previous data, we, therefore, investigated whether the protein plays a role in the regulation of succinate under anoxia. In a first trial, we observed that *dj-1β* null flies tend to display a higher succinate content and lower fumarate levels than controls, while ATP and ADP levels were just mildly affected, without significant differences between control and mutant individuals. Nonetheless, we noticed that the metabolic response is not strongly altered by the anoxic treatment. Since a possible caveat of this experiment arises from the rapid oxidation of succinate upon oxygen reintroduction, we repeated the experiment on flies exposed to a longer anoxic treatment (3 hours), and then we compared the effect of lysing flies outside (method 1) or inside (method 2) the anoxic chamber. Interestingly, we noticed that succinate and fumarate levels are increased when flies are lysed directly under anoxia in comparison to the previous procedure, while, there seems to be a reduction in the ATP and ADP levels. Nonetheless, *dj-1β* null flies appear to behave differently from the first trial. Considering that this second method has been tried just once, further replicates are needed to confirm its validity and to assess whether dj-1β influences the metabolic response to anoxia. However, this preliminary result may suggest that succinate/fumarate levels are particularly

sensitive to oxygen and that this feature should be taken into consideration for future assays to obtain a *bona fide* estimation of these metabolites in *Drosophila*.

In conclusion, our data support a role of dj-1β, *in vivo*, in the mitochondrial homeostasis. Interestingly, this function appears to become particularly relevant under oxidative insults, whereas, under basal conditions, the protein only slightly influences the mitochondrial functionality and alters the mitochondria ultrastructure. In view of these results, we can speculate that the loss of dj-1β leads to a mild increase of ROS levels, which may be more beneficial than detrimental, as proposed by the *mitohormesis* theory (Bárcena et al., 2018). In fact, other studies have reported that the absence of dj-1β in *Drosophila* promotes a higher production of mitochondrial H_2O_2, which does not negatively affect lifespan (Fernandez-Ayala et al., 2009; Sanz et al., 2010a). However, when RONS levels are increased beyond the physiological threshold, as observed in PD, the role of dj-1β could become relevant to confer protection.

Chapter 4

Results

Investigation of the involvement of DJ-1 in SOD1 maturation

SOD1 is the first line of defence against ROS, being involved in the detoxification of superoxide anion, the “primary” ROS, from which most harmful “secondary” ROS derive (De Lazzari et al., 2018). After translation, the nascent SOD1 peptide needs to be post-translationally modified to become stable and active. In the process of maturation, SOD1 acquires zinc, copper and a disulphide bridge (Culotta et al., 2006). While zinc is inserted through an unknown mechanism, the acquisition of copper and the formation of the disulphide bond are mediated by a dedicated protein called copper chaperone for SOD1 (CCS) (Banci et al., 2012; Sala et al., 2019). Albeit CCS is the main factor responsible for SOD1 maturation, a residual activity in the murine, *Drosophila* and *C.elegans* SOD1 homologues has been observed in the absence of CCS, suggesting the existence of an alternative pathway of maturation (Leitch et al., 2009a). In particular, in *CCS* null mice, SOD1 has been estimated to retain approximately 15% of the total activity (Wong et al., 2000). Although the players involved in the alternative pathway have not been clearly identified yet, our group has demonstrated the capability of DJ-1 to bind and transfer copper to SOD1, *in vitro,* rendering the enzyme active and suggesting a possible involvement of DJ-1 in this process (Girotto et al., 2014). In support of this hypothesis, another group, who worked with both the human proteins and the *A. thaliana* DJ-1 and SOD1 homologues, reported similar results (Xu et al., 2010). Furthermore, more recently, DJ-1 and SOD1 interaction has been shown to be increased upon exposure to the copper-binding compound $Cu^{(II)}$ATSM (Srivastava et al., 2016). Considering these promising results, we sought to investigate this mechanism *in vivo*.

4.1 Characterisation of the *Ccs* null fruit flies

In order to explore the role of DJ-1 in the CCS-independent maturation process of SOD1 in *Drosophila*, we firstly characterised the behaviour of *Ccs* deficient flies.

4.1.1 Loss of Ccs affects life expectancy, sensitivity to oxidative stimuli, and mitochondrial morphology

As previously shown, *Sod1* null flies display higher levels of endogenous ROS than controls and are extremely short-lived. When we evaluated the lifespan of *Ccs* null individuals, we observed that they show a premature mortality with respect to controls, although living significantly longer than *Sod1* mutants (Figure 26A).

Furthermore, the absence of Ccs induces sensitivity to oxidative stimuli (1 mM paraquat) but milder than the loss of Sod1 protein itself (Figure 26B). Given that *Sod1* null mutants normally present highly damaged mitochondria, we then evaluated, by electron microscopy, the consequences of the loss of Ccs on the mitochondrial morphology. As previously observed in *Sod1* null mutants, the absence of Ccs leads to swollen mitochondria with a disrupted cristae organisation (Figure 26C). In spite of the mitochondrial morphological alterations observed, the fact that *Ccs* deficient flies are long-lived as compared to Sod1 mutants, suggests that they still present a protective mechanism against oxidative stress, which is able to prolong lifespan. Our results are in agreement with previously published data showing that *Ccs* null flies present a residual Sod1 activity (Kirby et al., 2008) and support the hypothesis that SOD1 maturation can be achieved through an alternative CCS-independent pathway.

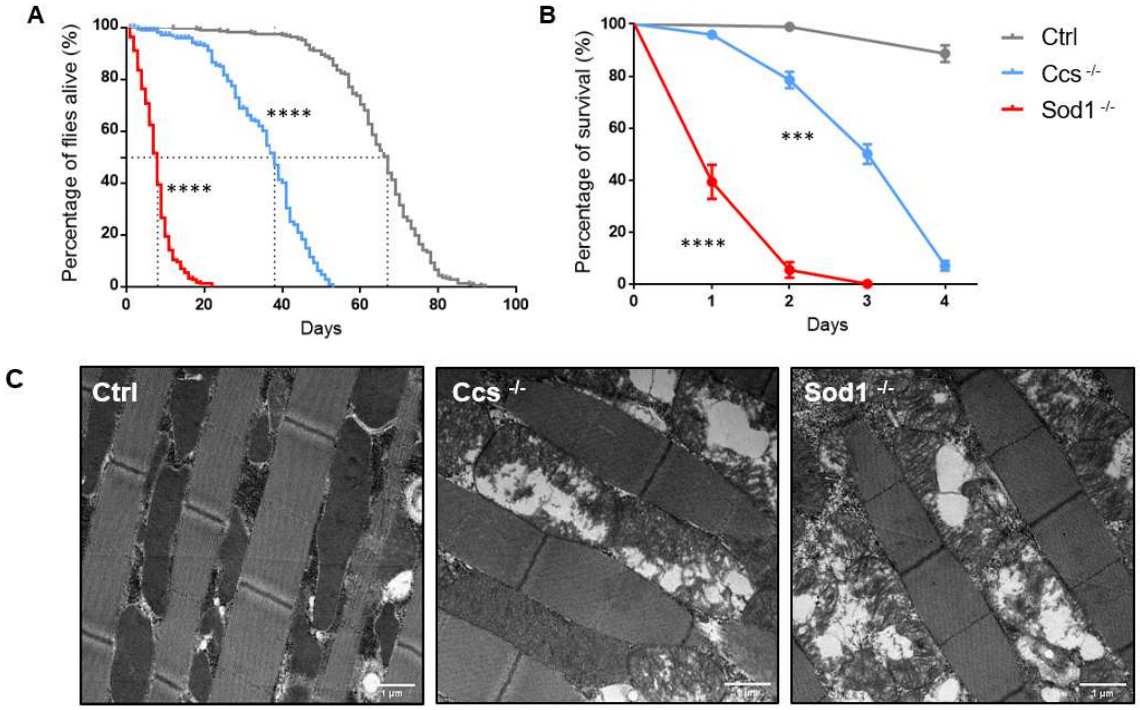


Figure 26. *Ccs* null flies present a milder phenotype than *Sod1* mutants but show a similar mitochondrial damage. (A) Lifespan, (B) survival under mild oxidative stress (1 mM paraquat), and (C) electron microscopy analysis of the mitochondrial morphology of control, *Ccs* and *Sod1* null flies. For lifespan 150 flies were used per genotype, while for survival at least 50 flies were assessed per genotype. Mantel-Cox log-rank test, *** $p<0.001$, **** $p < 0.0001$. For the mitochondrial analysis, at least 3 individuals were analysed per genotype.

4.1.2 Loss of Ccs renders Sod1 unstable and rapidly degraded

As previously described, upon Ccs depletion, *Drosophila* Sod1 protein becomes unstable so that its levels are dramatically reduced (Kirby et al., 2008). To confirm whether Ccs deficiency alters Sod1 protein levels also in our strain, we 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 27), indicating that, in the absence of its copper chaperone, the enzyme becomes unstable. Nevertheless, the residual amount of Sod1 seems to be able to prolong lifespan and to increase resistance to mild oxidative stimuli, as described in the previous paragraph.

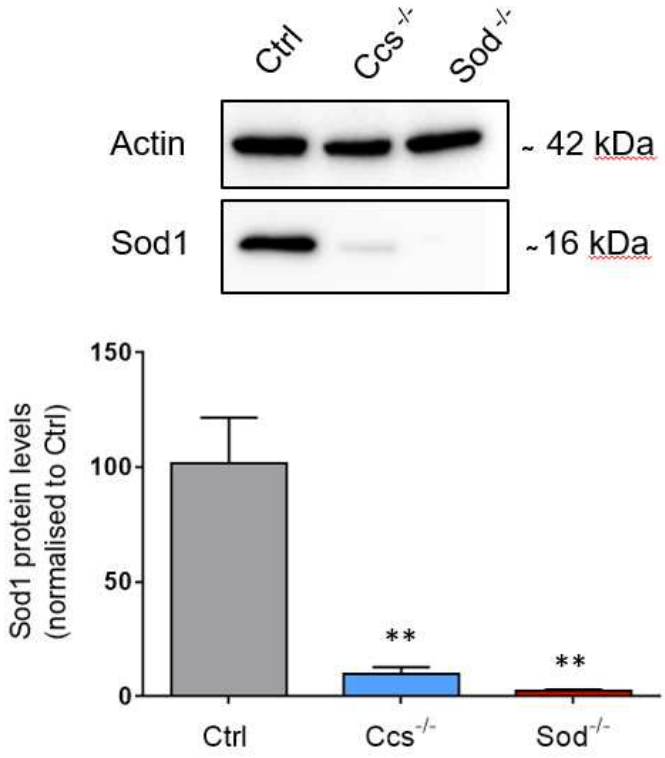


Figure 27. Sod1 protein is unstable under the absence of its copper chaperone Ccs. Immunoblot analysis and relative quantification of Sod1 levels in control, *Ccs* deficient and *Sod1* mutant flies. *Ccs* null individuals present a reduced amount of Sod1 protein, suggesting that the apo-form of the enzyme is unstable in *Drosophila*. Sod1 protein levels are reported as Sod1/actin ratio normalised to control (mean ± S.E.M.). n=3, One-way Anova, Tukey's multiple comparisons test, ** $p < 0.01$.

4.2 Role of dj-1β in the Sod1 maturation pathway

4.2.1 dj-1β is essential under the absence of Ccs copper chaperone

Considering that different *in vitro* studies have shown the capability of DJ-1 to activate SOD1, we next evaluated the role of the protein in the CCS-independent SOD1 maturation pathway, *in vivo*. First, we generated double knock-out flies for both Ccs and dj-1β proteins, referred to as *Ccs* $^{-/-}$*; dj-1β* $^{-/-}$. Interestingly, we observed that the loss of dj-1β in the *Ccs* null background confers lethality (Figure 28), indicating that dj-1β is essential under the absence of the dedicated copper chaperone for Sod1.

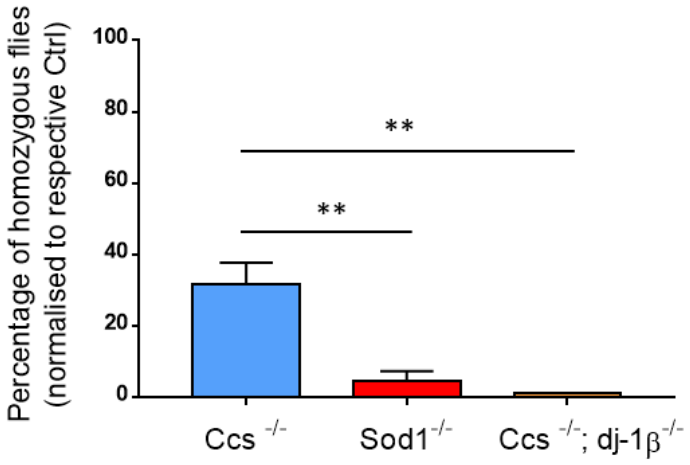


Figure 28. dj-1β is essential under the absence of the copper chaperone Ccs. Percentage of homozygous *Ccs*$^{-/-}$, *Sod1*$^{-/-}$, and *Ccs*$^{-/-}$*; dj-1β*$^{-/-}$ null flies, each one normalised to the respective control. The control used varies according to the mutation considered, for further information see Materials and Methods (Chapter 6, paragraph 6.4.7). The percentages shown derive from the ratio of the number of homozygous flies over the total number of flies counted, normalised to the relative control and multiplied by 100. Values are presented as mean ± S.E.M. For each mutant and control genotype at least 350 flies were counted. One-way Anova, Bonferroni's multiple comparisons test, ** $p < 0.01$.

4.2.2 The overexpression of dj-1β in *Ccs* null background recovers Sod1 protein levels

In the light of the aforementioned results, we decided to explore the effect of overexpressing dj-1β in the absence of Ccs, to assess if the protein can rescue the phenotypes observed in *Ccs* null flies. To this aim, we generated a new fly line, referred to as *Ccs*$^{-/-}$*; da > dj-1β*, which ubiquitously overexpresses dj-1β (*daGal4* driver) in a *Ccs* null background. Interestingly, preliminary data indicate that the overexpression of dj-1β is able to restore Sod1 protein levels (Figure 29). Considering that *Drosophila* Sod1 is unstable when not properly loaded with copper, the observed rescue of the protein levels suggests that dj-1β may incorporate such a mental into the enzyme. Hence, although we need to add the appropriate controls (Ccs$^{-/-}$; daGAL4/+ and Ccs$^{-/-}$; UAS-dj-1β/+, as separated lines) to exclude any

construct-related effect, to this data seem to support a role of dj-1β in stabilising Sod1 in the absence of the dedicated chaperone Ccs.

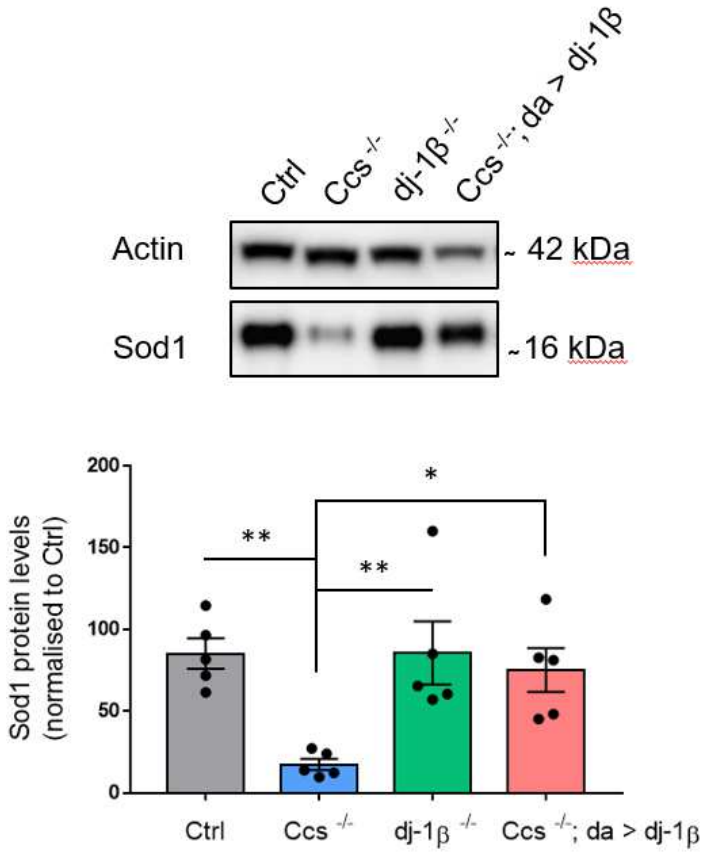


Figure 29. dj-1β overexpression seems to restore Sod1 protein levels in *Ccs* null background. Immunoblot analysis and relative quantification of Sod1 protein levels in control, *Ccs*, *dj-1β* single knock-out flies, and *Ccs* null individuals overexpressing *dj-1β* ubiquitously. Sod1 protein levels are reported as Sod1/actin ratio normalised to control (mean± S.E.M.). n=5. One-way Anova, Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

4.3 Discussion

Our group previously reported the ability of human DJ-1 to transfer copper to SOD1, *in vitro,* rendering the enzyme active (Girotto et al., 2014). Moreover, this function has been reported by another study (Xu et al., 2010). Considering these findings, within this thesis, we sought to explore this function *in vivo*. Beyond DJ-1 (dj-1β), the *Drosophila* genome encodes homologue proteins for CCS (Ccs) and SOD1 (Sod1). Furthermore, fly Sod1 shares a similar mechanism of maturation with mammals, being subjected to both Ccs-dependent and independent activation (Leitch et al., 2009b). For all these reasons, it represents a suitable model to study the involvement of dj-1β in the activation pathway of Sod1.
Initially, we characterised *Ccs* null flies with respect to control and *Sod1* flies. While Sod1 null flies display a premature mortality and are extremely sensitive to oxidative insults, in comparison to our controls, *Ccs* deficient flies show milder effects. Their lifespan is reduced with respect to controls but they live significantly longer than *Sod1* null flies. In addition, they are more sensitive to oxidative treatments than controls but less than *Sod1* mutants. As concerns the mitochondrial morphology, *Ccs* null individuals display damaged mitochondria. These findings suggest that *Ccs* null flies preserve a residual Sod1 activity, which enables a higher survivorship.

We successively assessed Sod1 protein stability in Ccs deficient flies. In the absence of Ccs, we observed that Sod1 levels are markedly reduced, indicating that the protein is highly unstable and degraded. Interestingly, the instability of fly Sod1, which was already reported in the literature, seems to be a unique feature of the fly enzyme (Kirby et al., 2008). Considering that *Ccs* flies display a milder phenotype than *Sod1* null individuals, it is conceivable that the residual Sod1 present in these flies can be activated through an alternative pathway. As DJ-1 has been reported to activate SOD1 *in vitro*, we decided to explore its involvement in this process, *in vivo*. To investigate this function, we generated double knock-out flies for Ccs and dj-1β (*Ccs* $^{-/-}$*; dj-1β* $^{-/-}$) and we observed that this genotype is lethal, signifying that dj-1β becomes essential under the loss of Ccs protein. Different reasons could explain this result. On the one hand, it is possible that the absence of dj-1β completely abolishes the residual activation of Sod1 observed in *Ccs* null background,

supporting a role of dj-1β in the maturation process of the enzyme. On the other hand, a similar phenotype could be the result of the loss of the protective activity of dj-1β in organisms characterised by a compromised anti-oxidant defence, suggesting that dj-1β confers anti-oxidant protection through other mechanisms, independent from Sod1. To investigate whether dj-1β plays a role in Sod1 activation, we generated a fly line that ubiquitously overexpresses dj-1β in *Ccs* null background, via the GAL4/UAS system (*Ccs* $^{-/-}$*; da > dj-1β*). Although further confirmation is needed, adding also the adequate controls, our preliminary data indicate that the overexpression of dj-1β leads to higher Sod1 levels with respect to *Ccs* null flies, suggesting that dj-1β might participate in the stabilisation of Sod1. Albeit the human DJ-1 has been reported to induce the expression of SOD1 (Z. Wang et al., 2011), we do not think that the observed rescue is the result of a higher expression rate, given the high instability of the *Drosophila* protein under the loss of Ccs. Indeed, being dj-1β overexpressed in a *Ccs* null background, we should expect a similar instability of the enzyme. Moreover, we have indication in our lab that neither the ubiquitous overexpression of dj-1β (through the GAL4/UAS system under a wild-type background) nor its deficiency affects the expression levels of Sod1. To confirm the role of dj-1β in Sod1 activation a key experiment will be the measure of the Sod1 enzymatic activity in the *Ccs* $^{-/-}$*; da > dj-1β* individuals. Furthermore, future experiments should be addressed to evaluate whether dj-1β overexpression rescues *Ccs* null flies-related phenotypes, such as the premature mortality and the altered mitochondrial morphology.

Overall, with this package of experiments, we shed some light on a possible additional mechanism of protection associated with DJ-1. Although further experiments are needed to verify this function, the possible participation of DJ-1 in the maturation of SOD1 could be considered as a specific mechanism of protection. Indeed, being SOD1 considered the first line of defence against RONS, the maintenance of its activity, although at low levels, could be mandatory for cell survival. This observation has relevance if we consider that the CCS-dependent SOD1 maturation pathway relies on oxygen, while the CCS-independent one does not (Leitch et al., 2009a). Thus, when oxygen availability is reduced, such as under hypoxia/anoxia, a partial SOD1 activity can be guaranteed through the presence of

DJ-1. Under a similar condition, in which RONS production is reduced, SOD1 could become relevant in the RONS-mediated signalling. As tissues experience a wide range of oxygen concentrations, from normoxia to marked hypoxia, a partial activation of the enzyme could ensure the maintenance of redox signalling also under this condition. As DJ-1 has been implied in the hypoxic response, its possible involvement in the SOD1 maturation pathway might represent a mechanism of protection activated specifically under limited oxygen availability.

Chapter 5

Conclusions

5.1 Conclusions

A tight regulation of the redox homeostasis represents a pivotal process to preserve the cellular integrity. RONS can be considered as a double-edged sword, playing beneficial roles in redox signalling, at the physiological level, while assuming detrimental functions, at higher concentrations (Weidinger and Kozlov, 2015). Among the cellular RONS sources, mitochondria are considered the major contributors, due to their respiratory activity. Accordingly, mitochondrial dysfunctions are frequently associated with several pathological states, including neurodegenerative diseases (Liu et al., 2017). Under this scenario, an appropriate anti-oxidant defence becomes critical to avoid the propagation of the oxidative state and its related damages. A first line of cellular defence relies on SOD enzymes, given their ability to detoxify the superoxide anion, from which more dangerous reactive species can derive. The sophisticated anti-oxidant machinery includes numerous protective players, among which, the PD-associated protein DJ-1 is gaining more and more attention. Indeed, consistent studies have highlighted the participation of the protein in the anti-oxidant defence (Raninga et al., 2017). The experimental findings are in line with a multifaceted nature of DJ-1, which appears to orchestrate several anti-oxidant responses, under multiple stressful conditions. In fact, given its ability to shuttle among different subcellular compartments and being at the crossroad of numerous signalling pathways, the protein seems to exert a broad spectrum of actions. Despite the great steps made in the elucidation of the cellular role of DJ-1, the precise mechanism of action of the protein remains still partially elusive. In the attempt to better elucidated the physiological functions of the protein, *in vivo*, within this work we exploited *Drosophila melanogaster* as a model organism. To this end, we utilised fruit flies lacking the expression of the DJ-1 homologue dj-1β. Our study supports the central role played by the protein in the redox homeostasis at multiple levels. First, we reported that dj-1β is involved in the maintenance of the mitochondrial morphology and functionality, especially under oxidative insults. In addition, we observed that the protein plays a role in the preservation of complex I activity, under oxidative stimuli, probably by influencing the electron flux. Moreover, our investigation has highlighted the possible

participation of the protein in the energetic metabolism, a role that is currently scarcely explored in literature, which may be worthy of further analyses.

Besides the role of dj-1β at the mitochondrial level, the protective function of the protein might rely on the activation of the enzyme Sod1. Our results indicated that dj-1β is essential under the absence of the copper chaperone for Sod1, Ccs. Moreover, we observed that the ubiquitous overexpression of dj-1β in *Ccs* null background seems to rescue Sod1 protein levels. These data may support a role of the protein in the stabilisation of the enzyme, although more validations are needed.

Overall, from our research, the picture that emerges suggests that DJ-1 may preserve the redox homeostasis through multiple mechanisms, which ranges from the maintenance of the mitochondrial homeostasis to the possible involvement in the maturation of the essential enzyme SOD1. As we are still lacking a comprehensive characterisation of DJ-1, with this project, we contributed to shed some light on the physiological functions of such a puzzling protein.

Chapter 6

Materials and Methods

6.1 Fly stocks, husbandry, and genetics

6.1.1 Fly stocks

Flies were raised on agar, cornmeal and yeast food, at 23°C, under 70% relative humidity 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
$dj\text{-}1\alpha^{\Delta 72}$	Deletion in *dj-1α* gene	33600	BDSC
$dj\text{-}1\beta^{\Delta 93}$	Deletion in *dj-1β* gene	33601	BDSC
Ccs^{n29E}	Deletion in *Ccs* gene	24755	BDSC
$Sod1^{n1}$	Point mutation in *Sod1* gene (null allele)	24492	BDSC
da-Gal4	Ubiquitous driver *(daughterless)*	8641	BDSC
UAS-dj-1β	UAS line to express *dj-1β*	33604	BDSC
2^{nd} *balancer*	*Cyo/Sco*	2555	BDSC
3^{rd} *balancer*	*MKRS/TM6B*	7304	BDSC
$2^{nd};3^{rd}$ *balancer*	*Cyo/Sco; MKRS/TM6B*	3703	BDSC
Ccs^{n29E}*; dj-1*$\beta^{\Delta 93}$	Double knock-out	Generated in this study	
Ccs^{n29E}*; daG4 > UAS- dj1β*	Ubiquitous *dj-1β* expression under *Ccs* deficiency	Generated in this study	

For all experiments, male flies were utilised since they normally display a higher sensitivity to oxidative insults than females (Chaudhuri et al., 2007; Niveditha et al., 2017).

6.1.2 Genetic crosses for combining transgenes or mutations together

To assess the effects of the absence of dj-1β on flies deficient for Ccs protein we generated double knock-out flies referred to as *Ccs$^{-/-}$; dj-1β $^{-/-}$*. To this end, the *Ccs^{n29E}* (localised on 2nd chromosome) line was crossed with 2nd balancer flies (*Cyo/Sco*) to generate *Ccs^{n29E}/Cyo* balanced individuals. Successively, *Ccs^{n29E}/Cyo* flies were crossed with 2nd and 3rd balancer flies (*Cyo/Sco;MKRS/TM6B*) to produce *Ccs^{n29E}/Cyo;MKRS/+* and *Ccs^{n29E}/Cyo;TM6B/+* flies, which were following mated together to obtain the *Ccs^{n29E}/Cyo;MKRS/TM6B* line. Similarly, *dj-1β^{Δ93}* (localised on 3rd chromosome) flies were crossed with 3rd balancer flies (*MKRS/TM6B*) to produce *dj-1β^{Δ93}/TM6B*, which were successively mated with *Cyo/Sco;MKRS/TM6B* to obtain *Cyo/Sco;dj-1β^{Δ93}/TM6B* flies. Finally, *Ccs^{n29E}/Cyo;MKRS/TM6B* line was crossed with *CyO/Sco; dj-1β^{Δ93}/TM6B* flies to establish a line carrying both null alleles (*Ccs^{n29E}/CyO; dj-1β^{Δ93}/TM6B*). From this line, only homozygous male flies were used for all the experiments.

To ubiquitously overexpress dj-1β protein under Ccs null background (line referred to as *Ccs$^{-/-}$; da > dj1β*), we exploited the Gal4-UAS system. To this end, daGal4 and UAS-dj-1β (both localised on 3rd chromosome) lines were firstly balanced as described above for *dj-1β^{Δ93}* flies to obtained *Cyo/Sco; daGal4/TM6B* and *Cyo/Sco; UAS-dj-1β/TM6B* flies. Subsequently, these lines were separately mated with *Ccs^{n29E}/CyO;MKRS/TM6B* to generate *Ccs^{n29E}/CyO;daGal4/TM6B* and *Ccs^{n29E}/CyO;UAS-dj-1β/TM6B* lines, respectively. Finally, these two lines were crossed to flies carrying the following genotype: *Ccs^{n29E}/Ccs^{n29E}; daGal4/UAS-dj-1β*. From this cross, only the male progeny was utilised.

6.2 Western blot analysis

6.2.1 Protein extraction

To extract proteins, approximately 10-20 male flies were homogenised manually with a pestle in lysis buffer (see below for the composition) and then centrifuged at maximum speed for 20 minutes at 4°C. Supernatants were collected and re-

centrifuged at maximum speed for 5 minutes at 4°C. The solutions obtained were stored at -80°C to prevent protein denaturation.

Lysis buffer composition	Working concentration
Hepes pH 7.5	20 mM
KCl	100 mM
EDTA pH 8.0	2,5 mM
Glycerol	5 % (v/v)
Triton-X 100	0,5 % (v/v)
DTT	1 mM
protease inhibitors (PMSF)	20 µl/mL

6.2.2 Bicinchoninic acid assay (BCA)

Protein concentration was estimated through Pierce® BCA Protein Assay kit (Thermo Scientific). The assay combines the reduction of Cu^{2+} to Cu^{+} by peptide bonds in an alkaline medium with the chelation of the reduced Cu^{+} by two molecules of bicinchoninic acid (BCA), forming a purple coloured product which has a maximal absorption at a wavelength of 562 nm. The formation of the coloured product is proportional to the amount Cu^{+} formed during the first reaction, which is in turn directly proportional to the amount of peptide bonds, i.e. proteins, present in the lysates. The quantification relies on a standard curve built using known concentrations of bovine serum albumin (BSA). Absorbance was recorded through the plate reader VICTORTM X3 (Perkin-Elmer).

6.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE allows the separation of proteins according to their size in polyacrylamide gels in the presence of SDS and applying an electric field. SDS-PAGE is constituted of a stacking (125 mM Tris-HCl pH 6.8, 0,1% w/v SDS, 5% w/v acrylamide, 0.1% w/v APS, 0.01% v/v TEMED) and a resolving gel (375 mM Tris-

HCl pH 8.8, 0.1% w/v SDS, acrylamide (desired concentration), 0.1% w/v APS, 0.01% v/v TEMED). The former is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometre thin layers when they reach the resolving gel, which allows proteins separation. Then, Tris-Glycine buffer (25 mM Tris, 250 mM glycine, and 0,1% w/v SDS) is poured into the electrophoresis chamber until it just covers the top of the casting tray. This solution slot allows the electric current to flow from the cathode, through the buffer to the anode. Before loading protein samples into the gel, samples were boiled about 10 minutes in the presence of Laemmli loading buffer 1X (4X: 200 mM Tris pH 6,8, 400 mM DTT, 8% w/v SDS, 0.4% w/v bromophenol blue, 40% v/v glycerol. As reference, molecular weights were used (GeneTex, Trident Prestained Protein Ladder, GTX50875). After electrophoresis, separated proteins were transferred from polyacrylamide gels to PVDF membranes.

6.2.4 Immunoblotting

After protein transfer, membranes were soaked in agitation for 1 hour in 5% w/v skimmed milk dissolved in TTBS to saturate non-specific binding sites. After this step, membranes were incubated overnight at 4°C in agitation with primary antibodies dissolved in 5% w/v skimmed milk in TTBS. After incubation, membranes were washed 3 times for 10 minutes with TTBS to remove the excess of antibodies or non-specific bindings. Membranes were then incubated with secondary antibodies conjugated with HorseRadish-Peroxidase (HRP), diluted in 5% w/v skimmed milk in TTBS for 1 hour in agitation at room temperature. Finally, membranes were washed in TTBS 3 times for 10 minutes. Proteins detection was performed by ECL-Plus detection kit (Amersham), and images acquired by VWR® CHEMI Premium analyser. Protein levels were quantified by densitometry using ImageJ software (US National Institutes of Health). The primary antibodies used for western blot are: rabbit α-SOD1 (HPA001401, Sigma Prestige, 1:1000), rabbit α-dj-1β (gently obtained from Yuzuru Imai, Dept. of Juntendo, Japan 1:40000), mouse α-actin (MAB1501, EDM Millipore 1:5000). The secondary antibodies used are: α-Rabbit IgG (A9169, Sigma,1:15000) and α-Mouse IgG (A9044, 1:80000).

6.3 ROS measurement by Electron Paramagnetic Resonance (EPR)

Adult males (1-3 day-old) were collected under brief CO_2 exposure and placed in fresh food vials (50-60 flies/vial). The following day, flies were transferred in 1.5 ml eppendorf and homogenised in the presence of 300 uL Krebs-Hepes buffer (KHB) containing 25 μM deferroxamine methane-sulfonate salt (DF) chelating agent and 5μM sodium diethyldithio-carbamate trihydrate (DETC), pH 7.4, with 1mM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics, Germany), which is a specific probe for superoxide anion. This probe can “trap” and transform the superoxide radical in a more stable radical species, rendering it EPR-detectable. After 30 minutes the homogenized flies were placed in the centre of 1 mL plastic syringe, in according to (Dikalov et al., 2018), and snap frozen and stored at -80°C.Then frozen block was removed by gentle push from the warmed up syringe and analysed in the quartz dewar with liquid N_2. Spectra were recorded at 77 K by using an X-band (9 GHz) EPR spectroscopy (E-scan-Bruker BioSpin GmbH, Billerica, MA, USA). The EPR signal, generated by the reaction of the spin probe (CMH) with whole fly homogenate ROS, was then recorded (acquisition parameters: modulation amplitude: 5 G; centred field: 2.0023 g, sweep time 10 s, field sweep 60 G, microwave power: 1 mW, number of scans: 10; receiver gain: 1*103). Spectra were recorded and analysed by using Win EPR software (2.11 version) standardly supplied by Bruker.

6.4 Behavioural assays

6.4.1 Paraquat treatment

Flies were treated with paraquat for 7 days to induce sublethal oxidative stress. Briefly, a 100 mM water solution of paraquat was added to standard food at a final concentration of 0.1 mM. To preserve paraquat activity, paraquat-added food was changed after 3-4 days.

6.4.2 Lifespan assay

Adult males (1-3 day-old) were collected under brief CO_2 exposure and placed in fresh food vials (20-25 flies/vial, 150-200 flies/genotype). Flies were transferred to fresh food vials every 3-4 days and the number of dead flies counted daily.

6.4.3 Survival under paraquat treatment

For survival experiments, adult flies (1-3 day-old) were starved for 4 hours and then placed in vials with filter paper soaked with 20 mM paraquat in 5% sucrose for 4 days (20-25 flies/vial, 150-200 flies/genotype).

6.4.4 Negative geotaxis (Climbing)

Adult males (1-3 day-old) were collected and transferred to new tubes containing standard food. After 7 days, flies were starved for 4 hours and placed in climbing vials (20 flies/vial, 50 flies/genotype). Negative geotaxis was evaluated by gently tapping flies to the bottom and counting the number of individuals that reached 10 cm up the vial in 10 seconds.

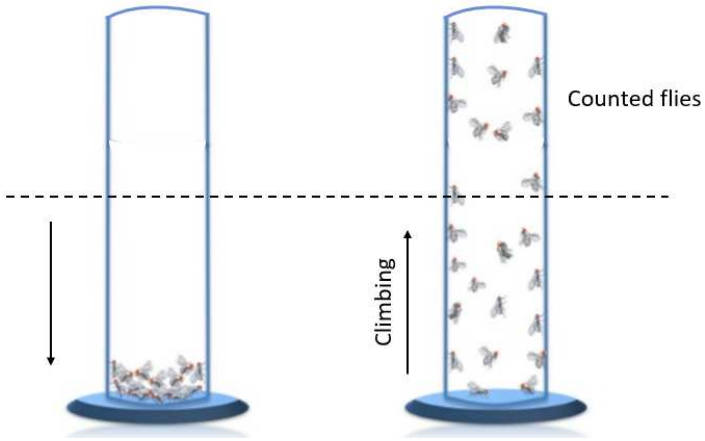


Figure 30. Negative geotaxis (climbing) apparatus. Representative image of the set up utilised for assessing the climbing ability of flies. In this assay, flies are gently tapped to the bottom of the experimental tube and the number of flies that reach 10 cm up the vial in 10 seconds are counted. Figure adapted from Madabattula et al., 2015.

6.4.5 Locomotor assay (DAM system)

Locomotor activity of adult males (1-3 days old) was analysed with *Drosophila* Activity Monitoring System (DAM TM Trikinetics, Waltham, MA). The DAM system is based on monitoring the movements of single flies in a small, closed assay tube, which contains standard food at one end and a sponge foam stopper at the other. Tubes are inserted in a monitor composed of 32 independent channels, each of which crossed by an infrared light beam, which records the number of times a fly passes the midpoint of the tube. Recording was carried out at 23°C, under constant humidity (70%). The locomotor behaviour was evaluated under a diurnal-light/dark cycle of 12 hours and signals recorded every minute for a period of 7 days. Data were acquired using the DAMSystem v3.0 software and subsequently processed by the program DAM Filescan102X (Trikinetics, Inc.).

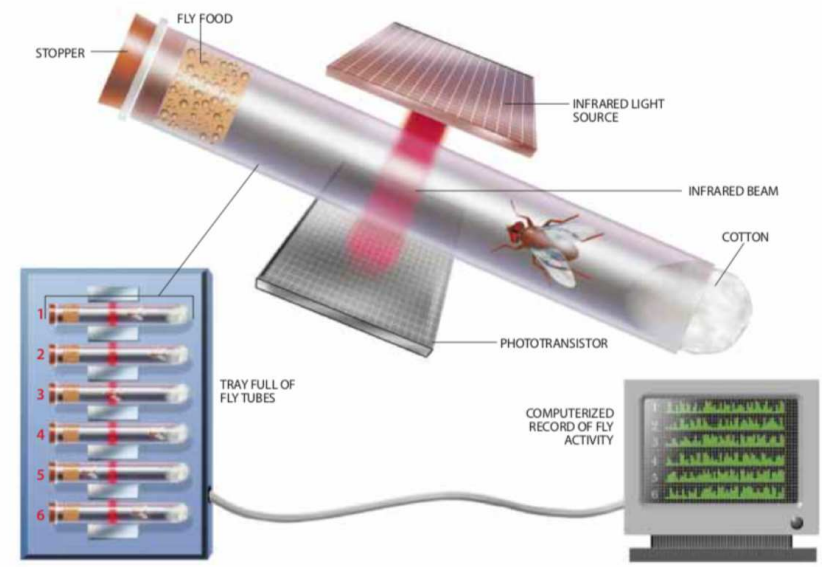


Figure 31. *Drosophila* Activity Monitoring (DAM) System. In this assay, single flies are located in small tubes, placed horizontally, and their movements recorded thanks to an infrared light beam positioned at the centre of the tube. Each time the fly crosses the centre of the tube, the light beam is interrupted, and the signal is converted into a locomotor event. The figure derives from Young, 2000.

6.4.6 Locomotion in open field arena

Open field arena locomotion. 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 assessed for free walking behaviour. Locomotion was investigated in an open field arena of 10 cm diameter custom designed in order to confine flies in 2D space. Groups of 10 flies were loaded into the arena by means of a mouth aspirator, and then left to acclimatize in complete darkness for 5 minutes. Recording started at light on and lasted 10 minutes (15 frames/sec; video resolution: 1296 x 964 pixel). All experiments were conducted between zeitgeber time 2 and 4 at a room controlled-temperature ranging between 22-23°C. The videos obtained were analysed by the CTRAX software (California Institute of Technology Fly Tracker) for fly positions tracking (Branson et al., 2009). Subsequently, eventual errors made by the tracking process were manually corrected with a MATLAB Toolbox (FixErrors), provided by the developers of CTRAX. A series of locomotor parameters were finally computed with another MATLAB script (BehaviouralMicroarray) 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 are listed below:

Velocity: general speed of the fly movement (mm/s).

Bouts: number of times that flies start moving (velocity > 1 mm/s);

Bout Length: distance (mm) walked by flies without interruptions after each walking step (velocity > 1 mm/s);

Sharp Turn: number of times that flies change direction with a velocity > 60 deg/sec.

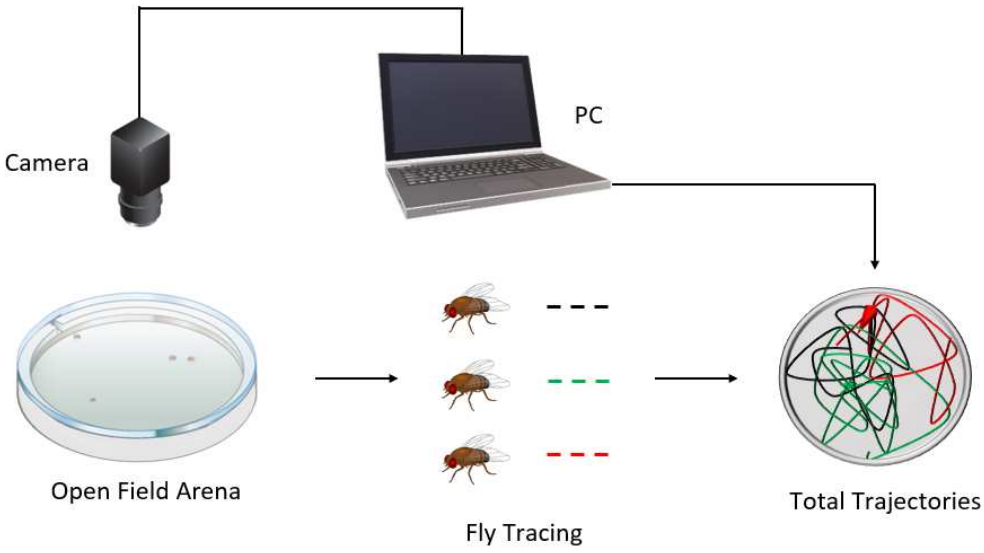


Figure 32. Open field arena set up. In this assay, groups of 10 flies are located in a flat arena, in which the flies are free to move. Movements are monitored thanks to a camera that records short videos, transferring the information to a computer. A different colour is associated with each fly in order to distinguish and compare their paths. The figure representing the open field arena has been adapted from Corthals et al., 2017.

6.4.7 Lethality assay

To estimate the lethality associated with *Ccs; dj-1β* null genotype, we evaluated the effect of removing the second copy of *Ccs* gene from the Ccs^{n29E}/CyO; dj-$1\beta^{\Delta 93}$/dj-$1\beta^{\Delta 93}$ background, which is heterozygous mutant for *Ccs* gene and homozygous mutant for *dj-1β* gene. As *dj-1β* null flies are viable and fertile, while *Ccs* null flies are more sensitive, we specifically focused on the lethality effect associated with *Ccs* mutation. To this end, we calculated the number of homozygous Ccs^{n29E}/Ccs^{n29E} single and Ccs^{n29E}/Ccs^{n29E}; dj-$1\beta^{\Delta 93}$/dj-$1\beta^{\Delta 93}$ double mutant flies obtained from the [Ccs^{n29E}/CyO] and [Ccs^{n29E}/CyO; dj-$1\beta^{\Delta 93}$/dj-$1\beta^{\Delta 93}$] parental lines, respectively. In this way, we assessed the lethality of *Ccs* mutation, as single mutant and when combined to *dj-1β* deficiency. Similarly, as comparison, we estimated the percentage of homozygous $Sod1^{n1}$/$Sod1^{n1}$ mutant flies obtained from $Sod1^{n1}$/TM3 heterozygous parental line. To take into consideration the effect of balancers (Cyo, and TM3), which are chromosomes carrying inversions and breakpoints, we

evaluated the percentage of wild-type flies that were born from these control lines: [+/CyO], [+/CyO; dj-1β$^{\Delta 93}$/ dj-1β$^{\Delta 93}$] and [+/TM3], which are referred to as +/+. As balancers are lethal in homozygosity, the expected percentage of homozygous flies is 33% for non-lethal alleles. Hence, for each mutant line we counted the number of flies carrying the genotype of interest and divided this value by the total number of flies that were born. The obtained value was then normalised to the number of wild-type flies that were born from the relative control crosses and presented as percentage in the graph. For this assay, both males and females were used.

6.5 Mitochondrial analysis

6.5.1 Mitochondrial respiration (Oroboros)

Adult males (1-3 day-old) were collected and transferred to new tubes containing standard food or supplemented with paraquat (0.1 mM) for 7 days. Mitochondrial respiration was monitored at 30°C using an Oxygraph-2k 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 adenosine diphosphate (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.

6.5.2 Quantification of total ATP levels

Adult males (1-3 day-old) were collected and transferred to new tubes containing standard food or supplemented with paraquat (0.1 mM) for 7 days. The ATP assay was performed homogenising five male flies per genotype in 100 µL 6 M guanidine-Tris/EDTA extraction buffer and subjected to rapid freezing in liquid nitrogen. Homogenates were diluted 1/100 with the extraction buffer and mixed with the luminescent solution (CellTiter-Glo Luminescent Cell Viability Assay, Promega).

Luminescence was measured with a SpectraMax Gemini XPS luminometer (Molecular Devices). The average luminescent signal from technical triplicates was expressed relative to protein levels. Data from 3 independent experiments were averaged and the luminescence expressed as a percentage of the control.

6.5.3 Mitochondrial isolation from adult flies

Adult males (1-3 day-old) were collected and transferred to new tubes containing standard food or supplemented with paraquat (0.1 mM) for 7 days. Approximately 200 males were homogenised in 7 ml of BSA-added Mannitol-Sucrose buffer with 15 strokes at 700 rpm. The fly homogenate was centrifuged at 1000 g for 10 minutes at 4°C and supernatant subsequently filtered through a fine cotton mesh and centrifuged for at 6000 g for 10 minutes at 4°C. Pellet was then washed with 10 ml of BSA-added Mannitol-Sucrose buffer and centrifuged at 6000 g for 10 minutes at 4°C. This step was repeated using Mannitol-Sucrose Buffer without BSA addition and centrifuged at 7000 g for 10 minutes 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.

Mannitol-Sucrose buffer pH 7.4	**Working concentration**
D-Mannitol	225 mM
Sucrose	75 mM
Hepes	1 mM
EGTA pH 8.0	5 mM
BSA	1% (w/v)

6.5.4 Blue Native Page 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 solubilisation to extract the mitochondrial complexes. Mitochondrial pellet was resuspended using Solubilisation Buffer to obtain a final concentration of 10 mg of mitochondrial protein/ml. To preserve supercomplexes the solution was properly resuspended with 10 µl of 10% Digitonin and incubated on ice for 5 minutes. The solubilised mitochondria were then centrifuged at 20000 x g at 4°C for 30 minutes. Supernatants were transferred into clean tubes and mixed with 10 µl of Blue-Native Sample Buffer. The equivalent volume of 50 µg was subsequently loaded into NativePAGE™ 3-12% Bis-Tris Protein Gels. Gel was run at 90 V using the Dark Blue Cathode Buffer and the Anode Buffer (50 mM bis-Tris/HCl pH 7.0). When the front was about halfway (after about 40 minutes) the dark blue cathode buffer is substituted with the Light Blue Cathode Buffer and the voltage increased to 150 V till the end of the run. The total procedure required about 4 hours to complete the separation of mitochondrial complexes at 4°C. The gel was subsequently stained with Staining Solution to reveal mitochondrial complexes. After destaining, the gel was scanned for quantification analysis.

The composition of the utilised buffers is indicated below:

Solubilisation buffer	**Working concentration**
Aminocaproic acid	1.5 M
Bis-Tris/HCl pH 7.0	50 mM
Digitonin	2%
Blue-Native Sample buffer	
Aminocaproic acid	0.75 M
Bis-Tris/HCl pH 7.0	50 mM
EDTA	0.5 mM
SERVA Blue G	5%
Dark Blue Cathode Buffer	
Tricine	50 mM
Bis-Tris/HCl pH 7.0	15 mM
SERVA Blue G	0.02%
Light Blue Cathode Buffer	
Tricine	50 mM
Bis-Tris/HCl pH 7.0	15 mM
Serva Blue G	0.002%
Anode Buffer	
Bis-Tris/HCl pH 7.0	15 mM
Staining Solution	
Coomassie Blue G250	0.2%
Methanol	50%
Acetic Acid	7%
Destaining Solution	
Methanol	50%
Acetic Acid	7%

6.5.5 Complex I – In Gel Activity (IGA)

IGA was performed after complexes separation by BN-PAGE as reported in paragraph 6.5.4. Briefly, the gel was rinsed quickly with ultrapure water and incubated with Complex I Reaction Solution for about 10 minutes 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.

Complex I Reaction Solution	Working concentration
Tris–HCl pH 7.4	5mM
NADH	0.14 mg/ml
Nitro Blue Tetrazolium (NBT)	1 mg/ml

6.5.6 Complex I Activity – Kinetics measurements

For this assay mitochondrial pellet was resuspended in Potassium Phosphate buffer to a final concentration of 0.5 µg/µl and subjected to one freeze/thaw cycle in liquid nitrogen. Successively, 10 µg were mixed to Complex I Reaction Buffer and Co-enzyme Q_1 to monitor Complex I activity. To avoid non-specific activity, measurements were performed with and without the Complex I inhibitor rotenone. Kinetic spectrophotometer measurement of Complex I activity was carried out in 96 well plates at 30°C and following the oxidation of NADH as the decrease in the absorbance at 340 nm in 3 minutes of reaction.

Complex I Reaction Solution	Working concentration
Potassium-Phosphate Buffer pH 8.0	20 mM
NADH	0.2 mM
NaN_3	1 mM
BSA (in EDTA 10mM pH 7.4)	1 mg/ml
Co-enzyme Q_1	50 µM
Rotenone	5 µM

6.5.7 Citrate Synthase – Kinetics measurements

For this assay mitochondrial pellet was resuspended in Potassium Phosphate buffer to a final concentration of 0.5 µg/µl and subjected to one freeze/thaw cycle in liquid nitrogen. Successively, 2 µg were mixed to Citrate Synthase (CS) Reaction Buffer and oxaloacetate. Kinetic spectrophotometer measurement of Citrate Synthase activity was carried out in 96 well plates at 30°C and following the appearance of NTB, proportional to the amount of liberated Acetyl CoA, at 412 nm in 3 minutes.

CS Reaction Solution	Working concentration
Tris-HCl Buffer pH 8.0	75 mM
DNTB	0.1 mM
Triton X-100	0.1%
Acetyl-CoA	0.4 mM
Oxalacetate	0.5 mM

6.6 Anoxia-Reoxygenation assays

6.6.1 Exposure to anoxia

Adult males (1-3 day-old) were collected under brief CO_2 exposure and placed in fresh food vials 1 day before the experiment. Vials were covered with gauze to let gas exchange, but to avoid the escape of flies. The day of the experiment flies were incubated in the anoxic hood for 1 hour, and, subsequently, reintroduced into the normoxic atmosphere to monitor lifespan. For metabolic analysis, flies were exposed to either 1 hour or 3 hours of anoxia.

6.6.2 Quantification of total ATP and ADP levels

5 flies were homogenised in 1 ml of ice-cold perchloric acid extractant (3% v/v $HClO_4$, 2 mM Na_2EDTA, 0.5% Triton X-100). This step was carried out either directly inside the anoxic hood or outside, after having frozen the flies in liquid nitrogen, to

compare the yield of two methods. Samples, ATP, and ADP standards were pH neutralized using a potassium hydroxide solution (2 M KOH, 2 mM Na_2EDTA, 50 mM MOPS), vortexed until formation of a white precipitate ($KClO_4$), then centrifuged at 17000 X g for 1 minute at 4°C. For ADP measurements, 250 µl neutralised sample supernatant was mixed with 250 µl ATP sulfurylase assay buffer (20 mM Na_2MoO_4, 5 mM GMP, 0.2 U ATP sulfurylase (New England Biolabs), in Tris-HCl buffer (100 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8.0))), incubated for 30 minutes at 30°C with shaking (500 rpm), heated at 100˚C for 5 minutes and then cooled on ice. Standards (100 µl), samples for ATP measurement (100 µl) or samples for ADP measurement (200 µl) (in duplicate) were added to 400 µl Tris-acetate (TA) buffer (100 mM Tris, 2 mM Na_2EDTA, 50 mM $MgCl_2$, pH 7.75 with glacial acetic acid) in luminometer tubes. 10 µl pyruvate kinase solution (100 mM PEP, 6 U pyruvate kinase suspension (Sigma # P1506)) were added to one set of samples for ADP measurement and incubated for 30 minutes at 25°C in the dark to convert ADP to ATP. The other duplicate tube (without addition of pyruvate kinase solution) served as an ADP 'blank' value. The samples were then all assayed for ATP content in a Berthold AutoLumat Plus luminometer by addition of 100 µl Luciferase/Luciferin Solution (7.5 mM DTT, 0.4 mg/ml BSA, 1.92 µg luciferase/ml (Sigma #L9506), 120 µM D-luciferin (Sigma # L9504), made in TA buffer (25% v/v glycerol)), delivered via auto-injection, protected from light. Bioluminescence of the ATP-dependent luciferase activity was measured for 45 seconds post-injection and the data quantified against standard curves.

6.6.3 Quantification of succinate and fumarate levels

To quantify succinate and fumarate levels, metabolites were analysed by liquid chromatography coupled to mass spectrometry (LC-MS). 5 male flies were lysed in 800 µl of ice-cold extraction solution (50% methanol, 30% acetonitrile and 20% water). This step was carried out either directly inside the anoxic hood or outside, after having frozen the flies in liquid nitrogen, to compare the yield of two methods. Samples were then centrifuged at 16,162 x g for 10 minutes at 4°C. The supernatant was transferred to glass vials (Microsolv Technology Corp., Leland, NC 28451, USA) and stored at -80°C until LC-MS analysis. LC-MS analyses were performed

on a Q Exactive Orbitrap (Thermo Scientific) mass spectrometer coupled to an Ultimate 3000 RSLC system (Dionex). The liquid chromatography system was fitted with either a ZIC-HILIC column (150 mm × 4.6 mm) or a ZIC-pHILIC column (150 mm × 2.1 mm) and respective guard columns (20 mm × 2.1 mm) (all Merck, Germany). The mass spectrometer was operated in full MS and polarity switching mode. Samples were randomised in order to avoid bias due to machine drift and processed blindly. The acquired spectra were analysed using XCalibur Qual and and XCalibur Quan Browser software (Thermo Fisher Scientific) by referencing to an internal library of compounds. Absolute quantification of selected metabolites was performed by interpolation of the corresponding standard curve obtained from serial dilutions of commercially available standards (Sigma Aldrich) running with the same batch of samples. All the extractions and analyses were done under the same conditions and relative to their respective internal standard. The succinate/fumarate ratios are presented as relative changes in the ratio of the ion current for these metabolites, which is proportional to, but not the same as, the true ratio of the metabolite levels.

6.7 Imaging

6.7.1 Dissection of the fly thorax

To assess the mitochondrial morphology under basal conditions, adult males (1-3 day-old) were fixed for 2 hours in 0.1 M sodium cacodylate (pH 7.4), containing 2.5% glutaraldehyde and 2% paraformaldehyde, and then dissected to isolate thoraces. Briefly, 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.

To evaluate the mitochondrial morphology under anoxia-reperfusion, adult males (1-3 day-old) were fixed in 0.05 M sodium cacodylate (pH 7.4), containing 2% glutaraldehyde and 2% formaldehyde, and then dissected as described above. Thoraces were then fixed in the same fixative solution overnight at 4°C. To discriminate the role of anoxia and reoxygenation on mitochondrial morphology, flies exposed to anoxia were fixed directly inside the anoxic hood, while to induce

reoxygenation, anoxia-treated flies were reintroduced in the normoxic atmosphere for 5 minutes, before starting the fixation procedure. For each genotype/condition at least 3 thoraces were prepared.

6.7.2 Transmission electron microscopy (TEM)

Dissected thoraces of flies grown under basal condition 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 hour at room temperature and then post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour 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.

Dissected thoraces of flies exposed to anoxia-reoxygenation were fixed overnight in 0.05M sodium cacodylate pH 7.4, containing 2% glutaraldehyde and 2% formaldehyde. Successively, samples were post-fixed in a solution of 1 % osmium tetroxide and 1.5 % potassium ferrocyanide for 1 hour at room temperature and then stained with 2% uranyl acetate and 0.5 M maleate buffer (pH 5.5) overnight at room temperature. After three water washes, samples were dehydrated in a graded ethanol series and embedded in Quetol 651 resin (Agar scientific Ltd., Stansted, UK). Ultrathin sections (90 nm) were obtained with a Leica Ultracut UCT ultramicrotome, post stained with aqueous 2% uranyl acetate and lead citrate, and viewed using a Tecnai G20 (FEI) transmission electron microscope, operating at 200kV. Images were captured with an AMT XR60B digital camera.

6.8 Statistics analysis

The data were expressed as the mean ± standard error of the mean (S.E.M). for experiments composed of three independent replicates or mean ± standard deviation (S.D). for experiments composed of two independent replicates. Graphs and statistical analysis were performed using GraphPad PRISM 7. Principal Component Analysis (PCA) was carried out with Origin 8.5.

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Abbreviations

CCS Copper Chaperone for SOD1

CI Complex I

CII Complex II

CIII Complex III

CIV Complex IV

CV Complex V

CoQ Co-enzyme Q

$CoQH_2$ Reduced form of Co-enzyme Q

CS Citrate Synthase

CV Complex V

DA Dopaminergic

EPR Electron paramagnetic resonance

FMN Flavin Mononucleotide

GAL4 Galactose-responsive transcription factor

IGA In Gel Activity

PD Parkinson's Disease

RET reverse electron transfer

RNS Reactive Nitrogen Species

RONS Reactive Oxygen and Nitrogen Species

ROS Reactive Oxygen Species

SOD1 Superoxide Dismutase 1

SOD2 Superoxide Dismutase 2

UAS Upstream Activating Sequence

Notes

Drosophila DJ-1 homologues are referred to as dj-1α and dj-1β

Drosophila SOD1 homologue is referred to as Sod1

Drosophila CCS homologue is referred to as Ccs