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The extracellular role of DJ-1 in the regulation of signaling pathways – implications for Parkinson's Disease

O papel extracelular da DJ-1 na regulação de vias de sinalização – implicações para a Doença de Parkinson

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sandra Anjo, investigadora junior do Centro de Neurociências e Biologia Celular (CNC) da Universidade de Coimbra, do Doutor Bruno Manadas, Investigador Principal do Centro de Neurociências e Biologia Celular (CNC) da Universidade de Coimbra e da Doutora Etelvina Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Este projeto foi realizado no grupo Life Sciences Mass Spectrometry do Centro de Neurociências e Biologia Celular (Universidade de Coimbra, Portugal), orientado pela Doutora Sandra Anjo e Doutor Bruno Manadas.

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Activity Report

During this Master's thesis project, a mini-review article focused on the DJ-1's mechanisms of signaling modulation was initiated and is currently under final preparation. It was also performed the data analysis and the preparation of the manuscript related with redox characterization of cells exposed to oxidative stress. Moreover, the work developed during this thesis was also submitted for presentations at international congresses by Dr. Sandra Anjo.

Publications - manuscripts under preparation:

Margarida Neves, Mário Grãos, Sandra I. Anjo* and Bruno Manadas* "Modulation of signaling pathways by DJ-1: an updated overview" (review article under final preparation)

Margarida Neves, Mário Grãos, Sandra Anjo* and Bruno Manadas* "oxSWATH applied to the study of the alteration of intracellular and extracellular proteome of cells in response to oxidative stress" (research article under preparation)

Communications in peer review meetings:

Margarida Neves, Mário Grãos, Bruno Manadas* and Sandra I. Anjo*, "oxSWATH applied to the study of the alteration of intracellular and extracellular proteome of cells in response to oxidative stress", 20th biennial meeting for the Society for Free Radical Research International (SFRR-I 2021 Virtual Meeting), 15th-18thMarch 2021 (Accepted for Oral Presentation)

S. Anjo, M. Melo, L. Loureiro, L. Sabala, N. Jordão, M. Neves, V.M. Mendes, T. Faria, P. Castanheira, R. Brito, M. Grãos, and B. Manadas, "Deciphering redox-centered regulation of DJ-1 activity and its importance for DJ-1 neuroprotective mechanisms", The 45th FEBS Congress (FEBS2021), 3-8 July 2021 (The congress was postponed from July 2020 to July 2021 due to COVID-19 pandemic)

(*- These authors contributed equally to this work.)

palavras-chave

Doença de Parkinson, DJ-1, neuroproteção, stress oxidativo, vias de sinalização, interactómica, padrões de fosforilação

resumo

A doença de Parkinson (DP) é uma doença neurodegenerativa progressiva sem cura, que é caracterizada pela perda de neurónios dopaminérgicos na *substantia nigra pars compacta* no cérebro. Apesar da ampla investigação, as causas e mecanismos exatos que despoletam o seu desenvolvimento permanecem desconhecidos. Entre as formas hereditárias da DP, mutações no gene *DJ-1* foram associadas a um tipo autossómico recessivo da doença com início precoce. A proteína DJ-1 tem um papel fundamental na resposta defensora ao stress oxidativo, na qual a modulação de vias de sinalização revela ser um mecanismo central. Embora essa função citoprotetora tenha sido mais estudada a nível intracelular, crescentes evidências também comprovam a capacidade da proteína de desencadear um efeito protetor ao nível extracelular quando secretada. Porém, o modo de ação da DJ-1 no ambiente extracelular bem como as implicações patológicas de mutações da proteína nessa função permanecem por clarificar. Neste sentido, o objetivo deste trabalho foi elucidar os mecanismos de ação da DJ-1 extracelular, considerando em particular o seu papel de regulação de vias de sinalização. Para tal, foi realizado um ensaio de interactómica para identificar a rede de interações estabelecidas pela DJ-1 no espaço extracelular, seguido de uma análise semi-direccionada para avaliar o impacto patológico de formas mutantes da proteína na sinalização mediada por cinases.

O ensaio de interatómica realizado em secretomas de células SH-SY5Y após a adição extracelular de DJ-1 na presença ou ausência de stress oxidativo permitiu identificar um grupo de 28 potenciais interatores extracelulares da DJ-1, que poderão estar envolvidos na ação neuroprotetora mediada pela proteína. Além disso, foi também determinada a modulação destas interações em condições de stress oxidativo. Os potenciais interatores encontrados confirmam um importante envolvimento da DJ-1 na modulação de vias de sinalização a partir do espaço extracelular. TSP1, THBR, MIF e CAD11, alguns dos potenciais interatores da DJ-1 identificados neste trabalho, estão envolvidos na regulação de diversas vias de sinalização, tais como: PI3K/Akt, TGF-β, ERK1/2, Wnt e PLD. Por sua vez, da análise “semi-direccionada” de perfis de fosforilação focada na identificação dos eventos de sinalização mediados diferencialmente pela adição exógena de DJ-1 nas formas nativa ou associadas às mutações missense M26I e E163K, foi possível confirmar que ambas as mutações consideradas promoveram alterações no perfil de fosforilação mediada por cinases, em comparação com a adição extracelular da forma nativa da proteína DJ-1 em células SH-SY5Y. De facto, foi observada uma diminuição geral de atividade de cinases, e a isoforma E163K-DJ-1 exibiu um maior impacto levando a um significativo decréscimo de atividade das cinases Akt, ATM/ATR, AMPK, CK2, CDKs e PKC. Porém, os resultados também denotam que as duas formas mutantes da proteína exibem um padrão semelhante de alteração de fosforilação de alguns substratos, o que poderá ser uma indicação de potenciais mecanismos patológicos convergentes.

Assim, o presente trabalho contribuiu para o aumento do conhecimento do papel extracelular da DJ-1 na regulação de vias de sinalização e permitiu verificar o impacto de mutações na atividade da proteína. Deverão ser conduzidos estudos posteriores de forma a validar os resultados obtidos e corroborar as hipóteses apresentadas. Por fim, as evidências adquiridas refletem também informações relevantes para a compreensão dos mecanismos moleculares implicados na DP.

keywords

Parkinson’s disease, DJ-1, neuroprotection; oxidative stress, signaling pathways, interactomics, phosphorylation patterns

abstract

Parkinson’s disease (PD) is a progressive neurodegenerative disorder with no cure, characterized by the loss of dopaminergic neurons in the brain's *substantia nigra pars compacta*. Despite extensive research, the exact causes and mechanisms leading to the development of PD are still unknown. Among the hereditary forms of PD, mutations in the *DJ-1* gene have been associated with an autosomal recessive early-onset disease type. The DJ-1 protein plays an important role in the defensive response to oxidative stress, in which the modulation of signaling pathways is pointed out as a central mechanism. While such cytoprotective function has been most described at the intracellular level, increasing evidence also confirms the ability of the protein to mediate a protective effect from the extracellular space upon its secretion. However, the precise mode of action of the extracellular DJ-1 or the pathological implications of its mutations is still misunderstood. In this sense, the aim of this work was to elucidate the mechanisms of action of extracellular DJ-1 with particular consideration to its role in signaling pathways modulation. To accomplish this, an interatomic study was performed to identify the network of interactions established by DJ-1 in the extracellular space, followed by a phospho-motif profiling analysis to assess the pathological impact of DJ-1 mutants in kinase-related signaling.

From the interactomic analysis of the secretome of SH-S5Y5 cells after the addition of exogenous DJ-1 in the presence or absence of oxidative stress, it was possible to identify a set of 28 potential extracellular DJ-1-interactors possibly involved in the neuroprotective action exerted by DJ-1. Additionally, the modulation of such interactions was also assessed in oxidative stress conditions. The list of potential interactors identified in this work confirms an important involvement of DJ-1 in the modulation of signaling pathways from the extracellular environment. TSP1, THBR, MIF, and CAD11, some of the binding partners of DJ-1 identified in this work, are known to be involved in the regulation of some signaling pathways such as PI3K/Akt, TGF-β, ERK1/2, Wnt, and PLD- related signaling. In turn, from the “semi-targeted” phospho-motif profiling assay aimed to assess the differential modulated signaling events by the exogenous addition of DJ-1 native and missense mutants M26I and E163K, it was possible to confirm that both mutations elicited alterations in the kinase-mediated substrate phosphorylation profile in comparison to the condition of WT-DJ-1 extracellular stimulated SH-S5Y5 cells. In fact, a generally decreased kinase activity was observed, with the E163K-DJ-1 isoform exhibiting a more prominent impact associated with a significant reduction in the activity of Akt, ATM/ATR, AMPK, CK2, CDKs, and PKC. Nevertheless, the results also denote that both mutants of DJ-1 had a similar pattern of alteration in the phosphorylation of some of the substrates analyzed, which could be an indication of convergent pathological mechanisms.

In conclusion, this work contributed with important knowledge into the biological role of extracellular DJ-1 in the modulation of signaling pathways and elucidated the impact of mutations in the activity of the protein. Following studies should be conducted to validate the obtained results and support the hypotheses raised in this work. Ultimately, the present findings also reflect valuable insights into the understanding of the molecular mechanisms implicated in PD.

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List of abbreviations

6-OHDA	6-hydroxydopamine
A	
AADC	L-aromatic amino acid decarboxylase
ACN	Acetonitrile
AEP	Asparagine endopeptidase
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
AP-MS	Affinity purification-mass spectrometry
APOB-100	Apolipoprotein B-100
ASK1	Apoptosis signal-regulating kinase 1
ATM/ATR	Ataxia telangiectasia mutated kinase /Ataxia telangiectasia and Rad3-related kinase
B	
Bax	Bcl-2 associated X
BIM	Bcl-2-like protein 11
C	
C106	Cysteine 106
CAD11	Cadherin-11
CDKs	Cyclin-dependent kinases
CK2	Casein Kinase II
CNS	Central nervous system
CSF	Cerebrospinal fluid
D	
Da	Dalton
DAT	Dopamine transporter
Daxx	Death-associated protein 6
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DLP	Daxx like protein
DUSP1	Dual specificity protein phosphatase 1
E	
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinase 1/2
FBN1	Fibrillin-1
F	
FA	Formic acid
FBS	Fetal bovine serum
FDR	False Discovery Rate
Fis1	Mitochondrial fission 1 protein
FOXO	Forkhead box subgroup O proteins

G

GFP	Green fluorescent protein
GO	Gene ontology

H

His-tag	Histidine tag
HO-1	Heme oxygenase-1
HPLC	High-performance liquid chromatography
Hsp70	Heat shock protein 70

I

IDA	Information dependent acquisition
IDH	Isocitrate dehydrogenase
IS	Internal standard

J

JNK	c-jun N-terminal kinase

K

Keap1	Kelch-like ECH-associated protein1
KEGG	Kyoto Encyclopedia of Genes and Genomes

L

LC-MS/MS	Liquid chromatographic coupled to tandem MS

M

m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
MIF	Macrophage migration inhibitory factor
MKK3	Mitogen-activated protein kinase kinase 3
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass spectrometry

N

NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2–related factor 2
Nurr1	Nuclear receptor-related 1
NQO1	NAD(P)H quinone oxidoreductase-1

P

PANTHER	Protein Analysis Through Evolutionary Relationships
PD	Parkinson’s disease
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K/Akt	Phosphatidylinositol 3-kinase/protein kinase B
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLK	Polo-like kinase
PLMN	Plasminogen
PP2A	Protein phosphatase 2A
PPI	Protein-protein interactions
PTEN	Phosphatase and tensin homolog
PTMs	Post-translational modifications
PVDF	Polyvinylidene fluoride
PXDN	Peroxidasin

R

RNF5	RING-finger protein-5
ROS	Reactive oxygen species
RT	Room temperature

S

SCG1	Secretogranin-1
SG2NA	S/G2 nuclear autoantigen
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SNc	*Substantia nigra pars compacta*
SOD1	Superoxide dismutase-1
SWATH	Sequential window acquisition of all theoretical fragment-ion spectra

T

TGF-β	Transforming growth factor beta
TH	Tyrosine hydroxylase
THRB	Prothrombin
TRAFs	TNF-alpha receptor-associated factors
Trx	Thioredoxin
TSP1	Thrombospondin-1
TUDCA	Tauroursodeoxycholic acid

U

uPA	Urokinase plasminogen activator

V

VMAT2	Vesicular monoamine transporter 2

W

WT	Wild-type

X

XIC	Extracted-ion chromatogram

1. Introduction

1.1. Parkinson's Disease

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disorder, affecting mostly the elderly [1]. This progressive disease occurs in approximately 1% of the population over the age of 60 and growing to 4% at the age of 80 [2]. Until 2040, the cases of PD are predicted to duplicate to 12.9 million patients, which makes it the fastest-growing neurodegenerative disorder, ahead of Alzheimer's disease [3, 4].

PD main pathological features entail the degeneration of dopaminergic neurons occurring in the *substantia nigra pars compacta* (*SNc*) and the accumulation of cytoplasmic protein inclusions (mostly α-synuclein aggregates) in these neurons, known as Lewy bodies (Figure 1.1) [2, 3]. The clinical characteristics consist of a set of motor symptoms, known as *parkinsonism*, including resting tremor, bradykinesia, rigidity, and abnormal gait. Nevertheless, patients can also present non-motor symptoms, such as cognitive dysfunction, psychiatric disturbances, incontinence, and autonomic disorders [1].

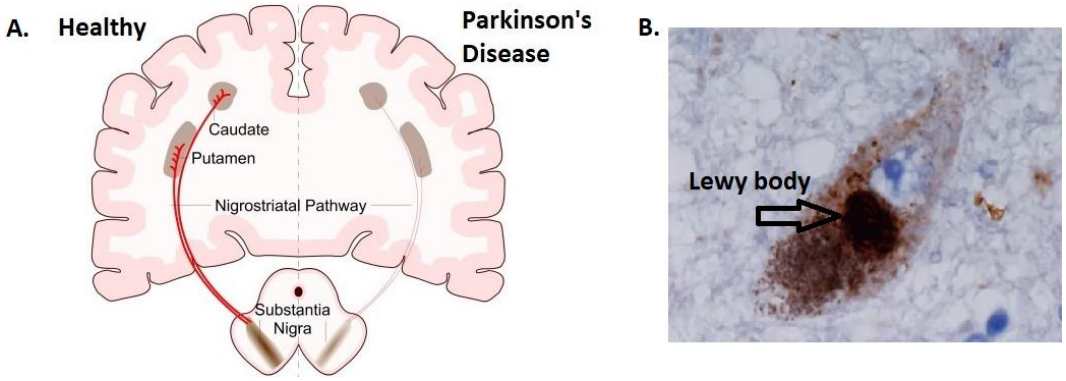


Figure 1.1| Substantia nigra degeneration and Lewy Bodies in Parkinson's disease A) The PD brain is characterized by a selective loss of *substantia nigra* dopaminergic neurons culminating in striatal dopamine depletion due to the degeneration of the dopaminergic nigrostriatal pathway; B) Lewy bodies revealed by α-synuclein immunohistochemistry (Adapted from references [5, 6]).

The diagnosis of PD is usually based on the patient's symptomatology; however, to date, there is no absolute method that can accurately diagnose the pathology. Regarding treatment, current medications are able to control and relieve symptoms, yet they do not prevent progression either provide a cure for the disease. In this sense, scientific investigation in the field is a critical need for the development of new methods of diagnosing and treatments [7].

PD cases can be divided into sporadic and familial forms. Most of the affected individuals present sporadic PD, which is suggested to be mediated through the interaction of aging, environmental,

and genetic susceptibility risk factors. On the other hand, familial forms of the disease account for 5-10% of the total cases and are associated with genetic mutation causes [3, 8].

Over the past decades, genetic studies have provided valuable insights into the pathological mechanisms underlying this disorder, but there is still much information to uncover due to its inherent complexity. Even so, until the date, at least 23 *loci* and 19 disease-causing genes have been associated with PD pathogenesis (Table 1.1). Among monogenic forms of the disease, the genes identified can be autosomal dominant (e.g., *SNCA* and *LRRK2*), autosomal recessive (e.g., *PRKN*, *PINK1*, and *DJ-1*) or X-linked (*RAB39B*), and usually have an earlier age-at-onset than sporadic PD [8-10].

Table 1.1| Gene *locus* and disease-causing genes of the monogenic form of PD (Adapted from [8, 9]).

Locus	Gene	Position	Protein	Inheritance	Onset	Variants
PARK1/4	*SNCA*	4q22.1	Synuclein-alpha	AD	EO, LO*	5 point mutations, genomic multiplications
PARK2	*PRKN*	6q26	Parkin E3 ubiquitin ligase	AR	EO	>250 point mutation, ins/de and exon rearrangements
PARK3	Unknown	2p13	Unknown	AD	LO	Not identified
PARK5	*UCHL1*	4p13	Ubiquitin C-terminal hydrolase L1	AD	EO, LO	1 missense variant in one sibling pair
PARK6	*PINK1*	1p36	PTEN-induced kinase	AR	EO	>100 point mutations, ins/del and exon rearrangements
PARK7	*DJ-1*	1p36.23	DJ-1	AR	EO	>20 point mutations and deletions
PARK8	*LRRK2*	12q12	Leucine-rich repeat kinase 2	AD	LO	7 point mutations
PARK9	*ATP13A2*	1p36.13	ATPase 13A2	AR	EO	>20 point mutations
PARK10	Unknown	1p32	Unknown	Unclear	LO	Not identified
PARK11	*GIGYF2*	2q37.1	GRB10 interacting GYF protein 2	AD	LO	7 missense variants
PARK12	Unknown	Xq21-q25	Unknown	X-linked inheritance	LO	Not identified
PARK13	*HTRA2*	2p13.1	Serine peptidase 2	AD	LO, EO*	1 missense variant
PARK14	*PLA2G6*	22q13.1	Phospholipase A2, group 6	AR	EO	>18 missense variants
PARK15	*FBXO7*	22q12.3	F-box protein 7	AR	EO	4 point mutations
PARK16	Unknown	1q32	Unknown	Unclear	LO	Not identified
PARK17	*VPS35*	16q11.2	Vacuolar protein sorting 35	AD	LO	2 point mutations
PARK18	*EIF4G1*	3q27.1	Eukaryotic translation initiation factor 4 gamma, 1	AD	LO	1 missense variant
PARK19	*DNAJC6*	1p31.3	DNAJ subfamily C member 6	AR	EO	9 missense variants
PARK20	*SYNJ1*	21q22.1	Synaptojanin-1	AR	EO	3 missense variants
PARK21	*DNAJC13*	20p13	DNAJ subfamily C member 13	AD	LO, EO*	1 missense variant
PARK22	*CHCHD2*	7p11.2	Coiled-coil-helix-coiled-coil-helix domain 2	AD	LO, EO*	1 missense variant, 1 truncation
PARK23	*VPS13C*	15q22.2	Vacuolar protein sorting 13C	AR	EO	2 missense variants, 1 truncation

Abbreviations: AD, autosomal dominant; **AR**, autosomal recessive; **EO**, early-onset; **LO**, late-onset; * few cases

Interestingly, genetic and sporadic PD have been shown to exhibit phenotypic similarities, which allows inferring that these forms of the disease have convergent pathological pathways [10]. Along with this fact, familial PD-related proteins have been suggested to be involved in the pathogenesis of the sporadic form of the disease. For this reason, the study of these proteins and their mutations may be an important contribution to the elucidation of the overall molecular mechanisms underlying PD [11, 12].

PD-related mutations have been linked to specific disease pathogenic pathways (Figure 1.2). As in other neurodegenerative disorders, a huge body of evidence suggests that PD arises by multifactorial conditions, specifically by (1) oxidative stress, (2) mitochondrial dysfunction, and (3) protein misfolding and aggregation [13, 14]. Indeed, evidence of oxidative damage has been detected in brain analysis of both sporadic and familial PD patients. Sources of reactive oxygen species (ROS) include dopamine metabolism since tyrosine hydroxylase and other related enzymes are responsible for the production of hydrogen peroxide (H_2O_2) as a by-product of their activity [7, 14]. Moreover, mitochondrial dysfunction is also associated with elevated ROS levels in dopaminergic neurons of the *substantia nigra pars compacta*. Mitochondrial dysfunction in PD is mainly associated with impaired activity of mitochondrial complex I, ultimately leading to cellular oxidative injury and apoptosis. Furthermore, familial forms of PD involving mutations in the genes *DJ-1*, *PINK1*, *PRKN,* and *SNCA* genes, are indicated to be causal of aberrant mitochondrial dynamics, function, and integrity. Finally, PD brain tissues also revealed an abnormal deposition of proteins, in particular of Lewy bodies that consist mainly of oxidatively modified α-synuclein, owing to their misfolding and toxic aggregation. Besides, mutations related to parkin and UCHL1 also suggest the contributing role of the impairment of the ubiquitin-proteasome pathway to PD [7, 14].

Although several pathways and players have already been associated with the development of PD, their relations remain unclear. Consequently, current studies are focusing on the elucidation of the metabolic relationship established between those, along with the study of the PD-linked proteins set, including the mechanisms by which their mutations induce the disease [14]. Ultimately, the understanding of the overall disease etiology will promote the development of potential therapies.

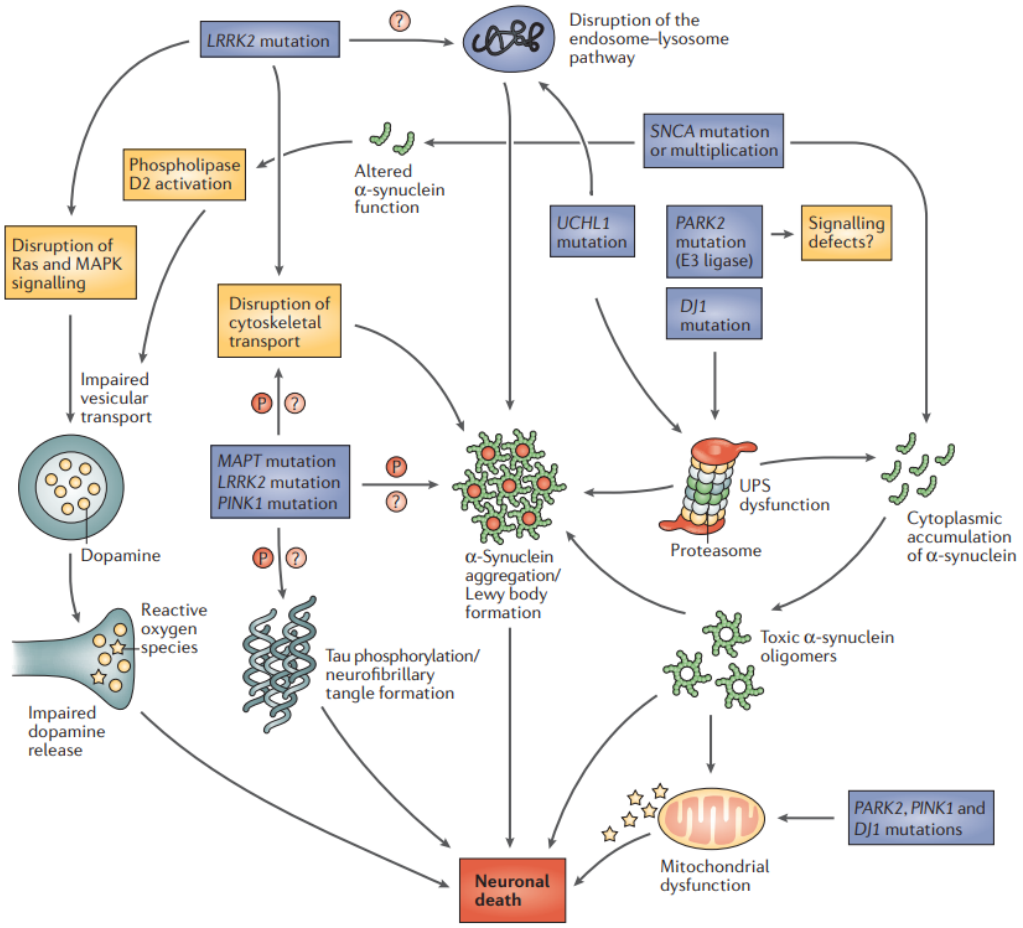


Figure 1.2| Pathways to Parkinson's disease. Missense mutations and genomic multiplications of *SNCA* (the gene that encodes α-synuclein) lead to an increase in the cytoplasmic accumulation of α-synuclein, promoting its toxic oligomerization. The neuron can respond either by rapidly degrading monomeric α synuclein through the ubiquitin-proteasome system (UPS) and/or endosomal–lysosomal pathways or by driving the formation and aggregation of higher molecular weight fibrils. The altered α-synuclein function associated with mutation results in its impaired vesicular binding, which abrogates the inhibition of phospholipase D2, an enzyme that is involved in lipid-mediated signaling cascades and vesicle trafficking. Impaired neurotransmitter release and its accumulation in the cytosol might lead to ROS formation, which triggers neuronal death. Parkin and DJ1 interact and are involved in normal UPS function. Mutations that affect these proteins might, therefore, impair the neuronal response to α-synuclein aggregation. DJ1 also has antioxidative properties, which might provide another link with defective α-synuclein function and its fibrillization. In late-onset Parkinson disease, where there is residual UPS function, α-synuclein aggregates that accumulate within neurites and axons are ultimately sequestered within a central Lewy body in surviving neurons. Ubiquitin carboxyl-terminal esterase L1 (UCHL1) has ubiquitin hydrolase and ligase activities and provides a link between the UPS, endosomal–lysosomal pathways, and Lewy bodies in late-onset Parkinson's disease. It maintains a pool of monoubiquitin for E3 ligase and UPS function while preventing degradation of free ubiquitin in the endosomal–lysosomal pathway. UPS function and aggregate clearance require ATP synthesis by mitochondria, and normal mitochondrial function is notably compromised by the loss of PTEN-induced kinase 1 (PINK1), DJ1, and parkin activities, resulting in early-onset parkinsonism (albeit primarily restricted to dopaminergic neurons). The clearance of neuritic aggregates also requires that normal cytoskeletal architecture, including microtubule stability, is maintained. Tau (encoded by microtubule-associated protein tau (MAPT)) normally stabilizes the microtubule network, promoting transport and intracellular signaling within neurons. Again, its functional ability is compromised by aberrant phosphorylation, leading to the formation of neurofibrillary tangles. Leucine-rich repeat kinase 2 (LRRK2) seems to be central to these events, with an integrative role in cellular trafficking, phosphorylation and intracellular signaling. (Adapted from reference [14])

1.2. DJ-1

Among the familial forms of PD, mutations in the *DJ-1* gene have been shown to lead to a dysfunctional activity of the correspondent DJ-1 protein that contributes to PD development. Therefore, by studying its physiological and pathological functions, new insights may be achieved for the understanding of the disease [15]. The protein DJ-1 is a 189 amino acid length homodimer structure that belongs to the DJ-1/ThiJ/PfpI superfamily [16]. This small conserved protein was initially reported as an oncogene product [17] and only later was associated with an autosomal recessive early-onset type of PD [15]. While DJ-1 is widely distributed in the brain, its highest expression occurs in the brain regions critically implicated in PD pathogenesis, the *substantia nigra* and *striatum* [16].

The structure of DJ-1 contains three cysteine residues at positions 46, 53, and 106 (C46, C53, and C106), that can be targeted for oxidation reactions from the reduced form (-SH) to sulfenated form (-SOH), sulfinated form ($-SO_2H$) and sulfonic form ($-SO_3H$). Among them, C106 is considered the most susceptible to oxidative stress, and it has been demonstrated to play an important role in the function of the protein. Evidences have suggested that changes in the oxidation state in the C106 regulate the neuroprotective action performed by DJ-1, since oxidation to the sulfinated state seems to trigger its neuroprotective function, and the sulfonated state is responsible for the loss of function of the protein [2]. Moreover, several mutations associated with the loss of function of DJ-1 protein have been conclusively demonstrated to being responsible for familial forms of PD (Table 1.2) [18].

DJ-1-related early-onset PD seems to manifest very rarely. To date, studies have reported pathogenic variations of DJ-1 in 18 families and 30 individuals [19]. This low occurrence allows only few insights; thus, little is known about the clinical characteristics, neuropathology, and the correlation between the genotype and phenotype of this type of PD [13]. Moreover, despite the fact that loss of DJ-1 function is related to a rare familial early-onset PD, evidences from post mortem analysis have also reported the presence of accumulated oxidatively modified DJ-1 in sporadic cases by unclear causes [20].

Table 1.2| Overview of DJ-1 associated mutations and polymorphism (Adapted from references [18, 21]).

Mutation	Inheritance	Population	Effect
L166P	Homozygous	Italian	Protein instability
M26I	Homozygous	Ashkenazi Jewish	
L172Q	Homozygous	Portuguese	
L10P	Homozygous	Chinese	
P158del	Homozygous	Dutch	
IVS6-1 G-C	Heterozygous	Hispanic	Altered transcript
Ex5-7del	Heterozygous	Northern Italian	
IVS5 + 2-12del	Heterozygous	Russian	
g.168_185del	Both	Global	Polymorphism
R98Q	Heterozygous	Global	
c.56delC **c.57G→A**	Heterozygous	Hispanic	Frameshift
E163K	Homozygous	Italian	Altered activity
14-kb deletion	Homozygous	Dutch	Loss of protein
D149A	Heterozygous	Afro Caribbean	Unknown
A104T	Heterozygous	Latino	
E64D	Homozygous	Turkish	
g.168_185dup	Homozygous		
A179T	Heterozygous	Dutch	
Ex1-5dup	Heterozygous	Dutch	
A107P	Homozygous	Iranian	
T154K	Homozygous	Italian	
Asp24Metfs*3	Homozygous	Iranian	
Ile31Aspfs*2	Homozygous	Iranian	
Q45X	Homozygous	Turkish	
Ile105Phe	Homozygous	Indian	
Arg98Gln	Homozygous	Indian	

Over the years, research studies have proven that DJ-1 accounts for important functional roles towards neuroprotection. It is well established that DJ-1 functions as an oxidative stress sensor via a variety of mechanisms. In fact, DJ-1 can develop a direct quenching activity by self-oxidation of its cysteine residues, thus operating as a ROS scavenger [22]. Redox-sensitive chaperone activity has also been associated with DJ-1 as a mechanism to prevent the toxic aggregation of α-synuclein, among other proteins [23]. Another role was attributed to the C- terminal truncated form of DJ-1 that is generated upon mild ROS-induced stress, as it presented a cytoprotective protease action [24]. Moreover, DJ-1 is known to be implicated in mitochondrial homeostasis by ensuring complex I activity upon stress-induced translocation to the mitochondria [25]. Lastly, DJ-1 has been reported to modulate gene expression levels and critical signaling pathways to promote cellular survival. For instance, transcription regulation mediated by DJ-1 targets tyrosine hydroxylase (TH), leading to its upregulation and subsequent stimulation of dopamine synthesis [26]. As a response to stress stimulus, the DJ-1 modulation of signaling pathways occurs by regulating specific transcription factors, such as the nuclear factor erythroid 2–related factor 2 (Nrf2), p53, and nuclear factor kappa B (NF-κB), and signaling cascades, namely the extracellular signal-regulated kinase 1/2 (ERK1/2),

phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), apoptosis signal-regulating kinase 1 (ASK1), c-jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase [2].

Ultimately, the study of these cited protective actions of DJ-1 could provide important insights for the understanding of PD pathology and corresponding treatment developments [27]. Despite essential research advances focused on DJ-1, its mechanisms of action are not completely understood yet. Reasonably, an important task relies on the determination of the precise acknowledge mechanisms that confer the neuroprotective role to DJ-1, for which the ability to modulate signaling pathways seems to be of utmost importance.

1.2.1. Modulation of signaling pathways by DJ-1[1]

As stated above, DJ-1 is implicated in the regulation of several signaling pathways responsible for mediating cellular adaptative responses to certain stimuli, such as oxidative stress [11, 27]. In particular, DJ-1 induces cell survival and proliferation by activating ERK1/2 and PI3K/Akt signaling cascades, as well as Nrf2 pathway-mediated antioxidant response. Additionally, inhibition of ASK1 and p53-related apoptotic pathways by DJ-1 leads to attenuated cell death upon critical conditions. Aberrant functioning of these mentioned events is associated with PD, among other diseases [11, 27].

DJ-1 can regulate the referenced pathways in a variety of ways that have been reported over the years. Nevertheless, due to the complex interaction established between these signaling pathways and DJ-1 upon stress conditions, the exact mechanisms are still not fully elucidated. Besides, there may be additional mechanisms and players left to uncover [27]. Further investigations on this matter may, therefore, reveal valuable knowledge.

1.2.1.1. ERK 1/2 pathway

The DJ-1 protein is known to modulate the extracellular signal-regulated kinase (ERK1/2) pathway involved in neuronal protection by regulating cell proliferation, differentiation, and other biological functions (Figure 1.3). The ERK1/2 pathway is one of the main mitogen-activated protein kinase

[1] The information present in this subsection was adapted and further explored to become a review article on the role of DJ-1 in the modulation of signaling pathways. The current draft version of the manuscript is presented in Annex I.

(MAPK) signaling cascades, and its components include Ras, Raf, MEK 1/2, and ERK 1/2 proteins. Its activation occurs upon certain stimuli, such as growth factors and stress conditions. The impairment of this pathway has been associated with the development of PD and other neurodegenerative disorders [28, 29].

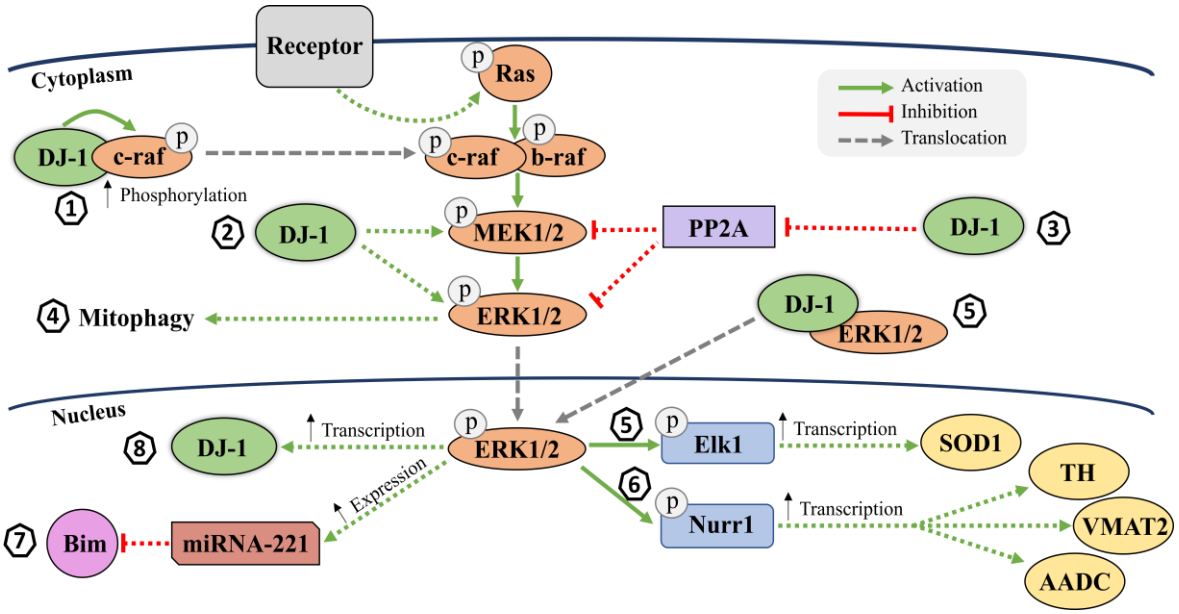


Figure 1.3| Summary of DJ-1's mechanisms involved in the modulation of the ERK1/2 signaling pathway. (1) DJ-1 is able to bind to c-raf, promoting its self-phosphorylation at Ser 338 and activating subsequent pathway components MEK1/2 and ERK1/2. (2) In oxidative conditions, phosphorylation of MEK1/2 and ERK1/2 is also increased by a dual-mechanism that includes the direct action of DJ-1 on these proteins and (3) DJ-1 suppression of PP2A expression, a known inhibitor of MEK1/2 and ERK1/2 family kinases. (4) Upon oxidative stress, DJ-1 can promote pro-survival ERK-dependent mitophagy. (5) DJ-1 interacts directly with ERK1/2, enhancing its nuclear translocation. As a result, phosphorylation of downstream transcription factor Elk1 occurs, and the expression of its target SOD1 is augmented. (6) DJ-1 enhances Nurr1 transcription factor activity through the ERK1/2 pathway activation, triggering the expression of TH, VMAT2, and AADC that are involved in the synthesis and transport of dopamine. (7) DJ-1-mediated activation of ERK1/2 signaling promotes miRNA-22 neuroprotective function by enhancing its expression, in turn downregulating BIM pro-apoptotic protein expression. (8) Finally, ERK1/2 pathway can also be responsible for upregulating DJ-1 upon stress stimulus generating a loop regulatory mechanism. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation. (Adapted from reference [27])

Initial studies revealed that the overexpression of DJ-1 causes the increased phosphorylation of ERK1/2 and its upstream kinase MEK1/2, an event shown to be defensive against oxidative injury in the presence of hydrogen peroxide (H_2O_2) [30]. Contrarily, the overexpression of DJ-1 mutant L166P, causative of PD, presented no effects in the phosphorylation events in the ERK1/2 pathway. In this work, it was also confirmed that DJ-1 could regulate the ERK1/2 pathway, suppressing protein phosphatase 2A (PP2A) expression, a known inhibitor of MEK1/2 and ERK1/2 family kinases [30].

Another DJ-1-mediated mechanism for ERK1/2 signaling activation was pointed out by Takahashi and colleagues [31], entailing a DJ-1-c-Raf association upon epidermal growth factor (EGF) stimulation. EGF is known to bind and activate the epidermal growth factor receptor (EGFR), triggering Ras to transduce the signal to the ERK1/2 pathway components c-Raf, MEK, and ERK1/2 through a phosphorylation cascade. As a result, ERK1/2 is translocated from the cytoplasm to the nucleus, activating gene expression involved in cell survival mechanisms [31]. It was demonstrated that DJ-1 is able to bind to the kinase domain of c-Raf, as both were found to be colocalized in the cytoplasm. These events were shown to be enhanced in cells after EGF treatment. The researchers explained that DJ-1 interacted with and stimulated the kinase activity of c-Raf, by promoting its self-phosphorylation at Ser338, leading to the subsequent ERK1/2 pathway activation. This step was noticed to be decreased in the C106S mutant DJ-1, in comparison to wild-type (WT)-DJ-1, upon the same EGF treatment conditions. However, the oxidation of C106 to the SO_2H and SO_3H forms was not required for this DJ-1-mediate c-Raf activation. These data point out another pro-survival mechanism of DJ-1, in which DJ-1 triggers the ERK1/2 signaling cascade by binding to c-Raf [31].

DJ-1 has been linked to defensive responses implying the enhancement of pro-survival autophagy mechanisms [32], such as an ERK-dependent mitophagy, protective of dopaminergic neurons of rotenone-induced oxidative damage [33]. The action of DJ-1 on the ERK1/2 pathway also results in the stabilization of dopamine levels by the activation of the nuclear receptor-related 1 (Nurr1) protein [34, 35]. The Nurr1 protein is a nuclear transcription factor responsible for regulating the expression of specific components involved in the synthesis and transport of dopamine, namely tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), and L-aromatic amino acid decarboxylase (AADC). In fact, Nurr1 is known to play an important role in neuroprotection associated with PD [36]. Crucial data suggests that overexpression of DJ-1 increases the nuclear translocation of Nurr1 through the ERK1/2 pathway, therefore enhancing its activity and allowing the expression of its target genes [35]. The PD-causative mutant L166P was shown to have no influence on this transcription factor nor on the expression of its target genes. As expected, knockdown of DJ-1 was proved to decrease the action of Nurr1, implying the regulatory effect of DJ-1 via ERK1/2 [35].

Another report points out the DJ-1's capacity to modulate superoxide dismutase-1 (SOD1) expression by the ERK1/2-Elk1 pathway as a neuroprotection mechanism against oxidative insults [37]. In this work, DJ-1 was shown to interact with ERK1/2 and enhance its nuclear translocation under oxidative stress conditions, consequently phosphorylating its substrate, Elk1. The

transcription factor Elk1 regulates the expression of genes associated with antioxidant protection, such as SOD1, accounting for the conversion of superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2). In this sense, it was demonstrated that the DJ-1-mediated activation of Elk1 triggered the expression of SOD1, whereas these events were suppressed in the case of DJ-1 knockdown, leading to cell death. Additionally, salivary samples of PD patients in different stages corroborated a close correlation between the levels of DJ-1 and SOD1 [37].

An additional DJ-1 function was recently described, consisting of the ERK1/2-dependent regulation of cytoprotective microRNA-221 (miRNA-221) in response to oxidative stress [38]. miRNA-221 is responsible for neurite outgrowth and neuronal differentiation, being largely abundant in the human brain [39]. Moreover, miRNA-221 was proved to be neuroprotective upon 1-methyl-4-phenylpyridinium (MPP+)- induced toxicity and also to downregulate the expression of specific pro-apoptotic proteins, such as the three bcl-2-like protein 11 (BIM) isoforms [38]. This research found that the absence of DJ-1, both in cultured cells and mice brains, led to a decrease in miRNA-221 expression. The authors then observed that DJ-1 reintroduction in the knockdown cells caused an increase of miRNA-221 expression, while the PD pathogenic mutant M26I-DJ-1 showed no effect. Lastly, DJ-1 knockdown cells overexpressing ERK1 presented elevated miRNA-221 levels. This fact implies that the DJ-1-mediated activation of the ERK1/2 pathway is actually responsible for stimulating the miRNA-221 cytoprotective effect, preventing cellular apoptosis [38].

On the other hand, ERK1/2 pathway is not exclusive to act downstream of DJ-1. A previous study showed that DJ-1 is upregulated by the activation of the ERK1/2 pathway under oxidative conditions generated by 6-hydroxydopamine (6-OHDA) [40]. This particular work demonstrated that the induced ROS levels led to the upregulation of DJ-1 in mice and cultured cells to counteract the dopamine toxicity through the activation of a MAP kinases pathway, more specifically the ERK1/2 pathway [40].

Overall, evidences indicate that DJ-1 modulates the ERK1/2 pathway by different mechanisms (summarized in Figure 1.3) [30, 31, 33-35, 37, 38], resulting in a combined action to respond to stress conditions, such as oxidative stress, and improving cell survival. Studies also indicate that ERK1/2 activation leads to an increase in DJ-1 levels [40], thus pointing out for a positive feedback loop between DJ-1 and ERK1/2 activation. Therefore, these regulatory mechanisms involving DJ-1 and ERK1/2 signaling can trigger different protective responses, namely by promoting mitophagy [33], enhancing the activity of transcription factors (Nurr1 [34, 35] and Elk1 [37]) and miRNA-221 [38].

1.2.1.2. PI3K/Akt pathway

The PI3K/Akt pathway acts as a defense mechanism against stress insults through a negative control of apoptosis processes. This pathway involves the proteins Phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) and functions via serine and/or threonine phosphorylation of variated downstream substrates. When cells are stimulated, PI3K becomes activated, which triggers the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3). As a result, Akt protein is recruited to the membrane and binds to PIP3. Lastly, Akt activates or suppresses target substrates associated with various cellular functions, namely metabolism, growth, proliferation, and survival [41, 42].

Dysfunctional PI3K/Akt-related signaling plays a part in PD's pathogenesis, contributing to the loss of dopaminergic neurons [43]. Furthermore, research work acknowledges DJ-1 as a key player in the activation of the PI3K/Akt pathway (Figure 1.4), promoting Akt phosphorylation for cellular survival mechanisms upon stress conditions [44-46]. In particular, inhibition of DJ-1 in Drosophila was shown to impair PI3K/Akt pathway, thus leading to a defective oxidative stress response in DJ-1-associated PD phenotype [45]. A second study demonstrated that overexpression of DJ-1 resulted in Akt hyperphosphorylation and cell survival, whereas reduced expression of DJ-1 caused Akt hypophosphorylation and cell death [44]. These latter observations were confirmed by Aleyasin and co-workers that identified a critical role of DJ-1 as an upstream regulator of Akt in cellular and animal models that undergo H_2O_2 treatment [46].

Besides confirming DJ-1's role in Akt phosphorylation, Kim *et al.* also demonstrated that DJ-1 was an antagonist of phosphatase and tensin homolog (PTEN) protein function [44]. PTEN is a lipid phosphatase that acts as a negative regulator of PI3K/Akt signaling cascade by converting PIP3 back into PIP2, hence promoting cell apoptosis [47]. It was demonstrated that DJ-1 prevented PTEN-induced cell death, enhancing the PI3K/Akt pathway and the activity of its downstream survival-related-effectors [44]. Later on, another work confirmed that under oxidative conditions, DJ-1 directly binds to PTEN, inhibiting its phosphatase activity and activating PI3K/Akt pathway [48]. The authors underlined the importance of DJ-1's oxidative status since most bindings of DJ-1 to PTEN occurred in the presence of the DJ-1's C106 reduced form [48].

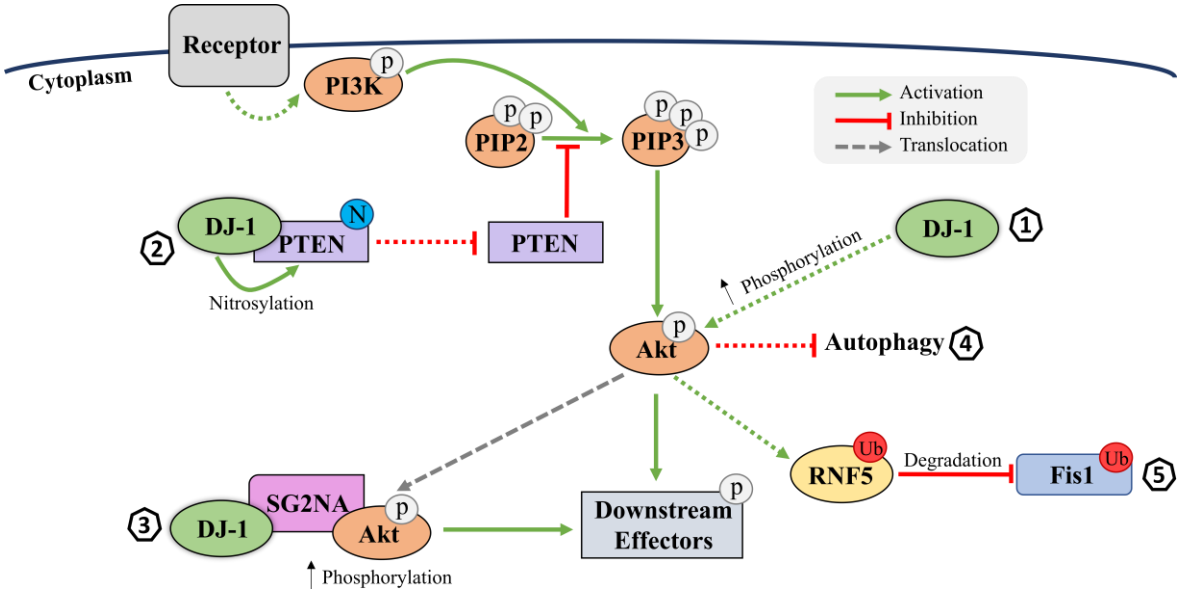


Figure 1.4| Summary of DJ-1's mechanisms involved in the modulation of PI3K/Akt pathway. (1) DJ-1 promotes phosphorylation of Akt, enhancing protective responses executed by the downstream effectors, having an effect, for instance, in mitochondrial well-functioning. (2) On the other hand, DJ-1 can suppress the PI3K/Akt pathway inhibitor's activity, PTEN, by binding to it or by establishing a nitrosylation reaction upon mild nitrosative conditions. (3) The interaction between DJ-1 and Akt may also be promoted by SG2NA, forming a complex by recruiting DJ-1 and Akt mainly to mitochondria and plasma membrane, promoting Akt signaling activity. (4) Defensive responses induced by the DJ-1-dependent activation of PI3K/Akt pathway include the prevention of harmful autophagy processes caused by C2-ceramide insults. (5) Finally, PI3K/Akt pathway activation mediated by DJ-1 is also involved in the proteasomal degradation of Fis1, a protein leading to mitochondrial fragmentation. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

DJ-1 can also interact with PTEN and regulate its activity by a transnitrosylation reaction [transfer of a nitric oxide (NO) group from the cysteine residue of a protein to another], upon nitrosative conditions-inducing of PD-linked neuronal damage [49]. Research developed by Choi and co-workers provided evidence that under mild nitrosative stress, DJ-1 can be S-nitrosylated to form SNO-DJ-1 with subsequent transfer of a NO group to PTEN by transnitrosylation. As a result, PTEN's phosphatase activity is inhibited, leading to PI3K/Akt pathway activation and cell survival. Consistently, levels of transnitrosylated PTEN (SNO-PTEN) were identified in human brains with a sporadic type of PD. In general, DJ-1 was reported to operate also as an NO-sensitive protein to perform its neuroprotective function by enhancing PI3K/Akt pathway [49].

Another way that DJ-1 can promote Akt activity involves a protein from the striatin family, the S/G2 nuclear autoantigen (SG2NA), that recruits DJ-1 and Akt into the mitochondria and to plasma membrane [50]. Subsequent DJ-1-mediated stimulation of Akt activity promoted cell survival upon moderate H_2O_2 conditions. Opposed results were observed in tests involving PD-associated L166P-

and M26I-DJ-1 forms of the protein. These mutants were unable to be recruited to form the SG2NA/DJ-1/Akt complex, therefore increasing cell susceptibility to H_2O_2-induced oxidative stress [50].

One of the defense mechanisms promoted by the DJ-1-mediated PI3K/Akt activation relies on the suppression of harmful autophagy events. This relation was observed in a study conducted on C2-ceramide-generated neurotoxic conditions, known to cause early inhibition of PI3K/Akt signaling, thus enhancing cytotoxicity and ROS production [51]. In this work, CAD cells, a catecholaminergic cell line from mouse, were exposed to the neurotoxic lipid C2-ceramide resulting in cell death, through reduced phosphorylation of PTEN and Akt, while increasing autophagy flux. Interestingly, the overexpression of DJ-1 was able to protect from C2-ceramide insults by phosphorylating PTEN and triggering PI3K/Akt pathway. Along with these events, it was also observed a decreasing occurrence of harmful autophagy processes, therefore prevented by DJ-1-directed PI3K/Akt signaling [51].

Mitochondrial well-functioning can also be assured by a PI3K/Akt signaling-dependent mechanism, activated by DJ-1 [52-54]. Past work has already highlighted DJ-1's ability to interact with and maintain the activity of the mitochondria complex I [25]. Recently, tests conducted on neuron-like cells PC12 and SH-SY5Y showed that loss of DJ-1 causes mitochondrial dysfunction, in part by inhibiting the Akt survival signaling under ROS-induced stress [53]. Additional evidence clarified that DJ-1 maintained mitochondrial function by promoting Akt phosphorylation at its catalytic site (the threonine (Thr) 308) in a neuronal context [54]. Despite the fact that DJ-1-overexpressing cells did not evidence alterations in PI3K and total Akt levels, the phosphorylation of Akt at its catalytic site Thr208 was augmented. As a result, mitochondrial function was enhanced, proved by the increase in mitochondrial mass, activity of mitochondrial complex I, and ATP levels in the cells [54]. Another mitochondrial protective role of DJ-1-mediated activation of PI3K/Akt pathway implicated the proteasomal degradation of the protein mitochondrial fission 1 (Fis1) [52]. Fis1 is a mitochondrial outer membrane protein responsible for mitochondrial fragmentation. In this study, DJ-1 triggered the RING-finger protein-5 (RNF5) ligase activity and subsequent Fis1 degradation through PI3K/Akt's action [52].

Altogether, these research works regarding DJ-1's role in PI3K/Akt signaling allowed the identification of numerous regulation mechanisms, such as those in which DJ-1 may be able to promote Akt phosphorylation and also suppress the activity of the PI3K/Akt inhibitor PTEN by binding to it [44, 46, 48] or by causing its nitrosylation [49]. The interaction between DJ-1 and Akt

may also be enhanced through the formation of a complex mediated by SG2NA, promoting Akt phosphorylation [50]. Among other defensive responses, studies have shown that the DJ-1-mediated activation of PI3K/Akt can be responsible for suppressing harmful autophagy processes [32] and also for maintaining/protecting mitochondrial function [52-54], leading to cellular survival.

1.2.1.3. ASK1 pathway

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPK signaling system that operates in response to cellular insults, namely oxidative stress, endoplasmic reticulum stress, and calcium influx. Under these stress stimuli, ASK1 targets downstream signaling cascades, such as c-Jun N-terminal kinase (JNK) and p38 pathways, which are responsible for apoptosis, differentiation, and inflammation processes [55-57]. ASK1 inactive signalosome complex includes its inhibitors, such as thioredoxin (Trx) and 14-3-3 proteins [58, 59]. In turn, ASK1 and its activators, namely TNF-alpha receptor-associated factors (TRAFs) and death-associated protein 6 (Daxx), form the active signalosome [60, 61]. As a matter of fact, dysfunction of ASK1 has been associated with various neurodegenerative diseases, specifically being involved in neuronal death events occurring in PD [57].

DJ-1 plays an important role as a regulator protein of the ASK1 pathway (Figure 1.5), suppressing its activity and ensuring cellular survival [62-67]. For instance, DJ-1 can control Trx1 interaction with ASK1 upon an oxidative-induced environment [64]. Under basal conditions, Trx1 is found to be bound to ASK1, restraining ASK1's functions. However, when subjected to oxidative stress, the inhibitor Trx1 is separated from ASK1, thus activating its kinase activity and triggering its apoptotic pathway [58]. A study focused on DJ-1's role as a regulator of the Trx1/ASK1 complex found that DJ-1 is able to suppress the ASK1 pathway by avoiding dissociation between Trx1 and ASK1 in response to oxidative stress [64]. This work detailed that contrarily to WT-DJ-1, the PD-causative L166P mutant was ineffective in maintaining the complex Trx1-ASK1 after H_2O_2 treatment, thus leading to Trx1 dissociation and subsequent ASK1 activation and cell death. Once again, central C106 was revealed to be essential for the performance of DJ-1 in regulating the Trx1/ASK1 complex. A final analysis of mice brain homogenates confirmed that Trx1 interaction with ASK1 was more easily disrupted in mice DJ-1 null brains than DJ-1 WT brains, promoting the downstream activation of the JNK pathway [64]. Ultimately, these findings prove DJ-1's critical role in mediating apoptosis by ASK1 signaling cascade.

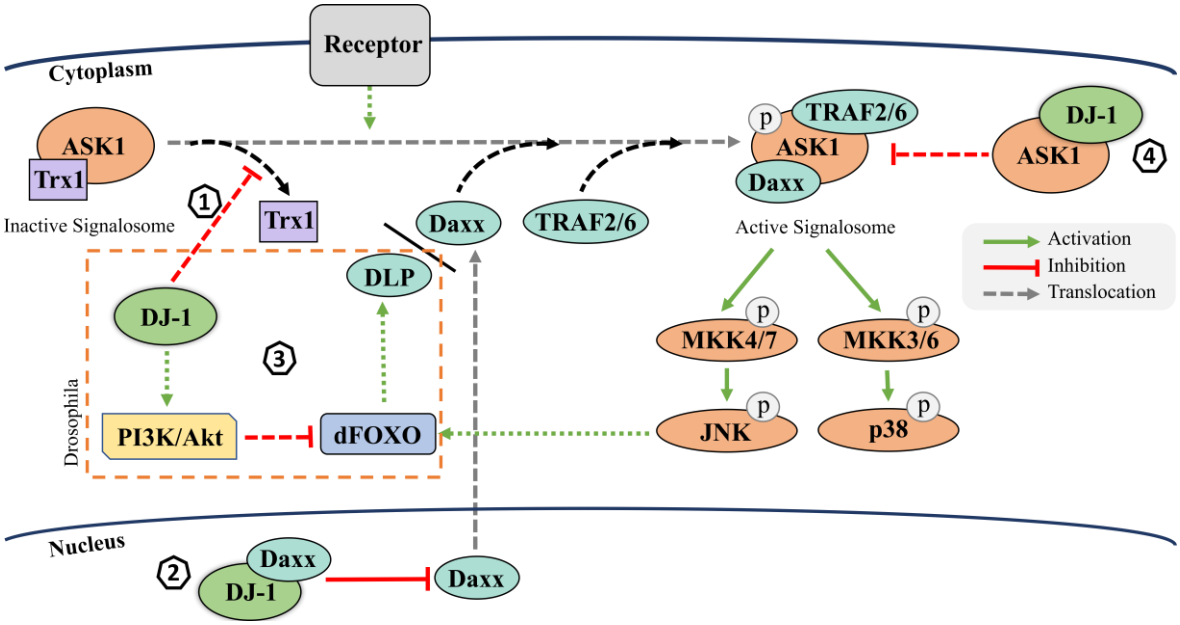


Figure 1.5| Summary of DJ-1's mechanisms involved in the modulation of the ASK1 pathway. (1) DJ-1 prevents the dissociation of the ASK1 inhibitor Trx1 from the inactive signalosome, inhibiting activation of the ASK1 apoptotic pathway. (2) DJ-1 is able to sequester ASK1 activator Daxx in the nucleus under oxidative stress conditions, blocking the formation of the active ASK1 signalosome and ensuring cell survival. (3) A study conducted in drosophila indicated that DJ-1 also suppressed Daxx homolog, DLP, interaction with ASK1, by downregulating the activity of enhancer dFOXO in a PI3K/Akt signaling-dependent manner. (4) Upon oxidative stimulation, DJ-1 may also interact directly with ASK1, disrupting its homo-oligomerization type of activation and avoiding subsequent p38 and JNK-induced cellular apoptosis. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation. (Adapted from reference [27])

An alternative way by which DJ-1 can inhibit the biological functions of ASK1 is by binding and sequestering the ASK1 activator, Daxx [65]. This mechanism further entailed that WT-DJ-1 interacts with Daxx in the nucleus of H_2O_2-treated cultured cells, preventing it from translocating to the cytoplasm and phosphorylating ASK1, ultimately avoiding cell death. In contrast, upon the same oxidative conditions, the PD-causative mutant L166P DJ-1 failed to protect cells from apoptosis since it was unable to associate with Daxx that subsequently led to ASK1-induced cell death [65]. In a similar way, an additional study focused on another PD-causative DJ-1 mutant, the M26I-DJ-1 form, also proved its inability to prevent the activation of the ASK1 pathway favoring cell death since it also failed to suppress Daxx translocation [67].

A different work reported an additional stress-protective manner of DJ-1 to modulate Daxx through the activity of the forkhead box subgroup O (FOXO) transcription factor, impacting the ASK1 cascade [63]. Actually, the *Drosophila* homolog DJ-1b was shown to exert its defensive function against oxidative conditions by controlling not only localization but also the expression of Daxx like

protein (DLP). Interestingly, the researchers stated that DJ-1b negatively regulates *Drosophila* homolog dFOXO, which is in turn, responsible for DLP expression. Moreover, flies with DJ-1b loss of function mutations presented increased levels of DLP under oxidative stress, consequently triggering ASK1 apoptotic events. DLP overexpression was shown to activate apoptosis through the JNK/dFOXO pathway, meaning that the activation of DLP stimulates its further expression by dFOXO [63]. Nevertheless, DJ-1 is known to activate the PI3K/Akt pathway, which is responsible for inhibiting dFOXO [68]. Altogether, these findings imply that DJ-1 is able to downregulate DLP expression via a PI3K/Akt/dFOXO pathway [63].

It has been established that DJ-1 can also interact with ASK1 and mediate its signaling pathway through oxidative-driven direct contact [66, 67]. Waak and co-workers [67] explained that DJ-1 could bind to ASK1 under H_2O_2-induced stress, thus generating mixed disulfide bonds that are, in fact, mediated by the central DJ-1 C106. According to the model proposed by the authors, while the C106 is the main mediator of the interaction, the peripheral C46 and C53 modulate the oxidant-induced activation of DJ-1. In addition to the fact that the PD-associated M26I mutation generated a loss of DJ-1 protein levels, this isoform decreased the cytoprotective effect by failing to inhibit ASK1 apoptotic activity. Although M26I-DJ-1 could actually bind to ASK1, it seemed to occur in a dysfunctional manner and in a different site that ultimately led to a defective action [67].

The direct interaction between DJ-1 and ASK1 was further investigated, confirming that it is also responsible for the DJ-1-mediated downregulation of the p38 pathway [66]. This work provided evidences that H_2O_2-treatment led DJ-1 to bind to ASK1, thus disrupting ASK1 homo-oligomerization type of activation. This event consequently prevented the p38 upstream kinase's phosphorylation, the mitogen-activated protein kinase kinase 3 (MKK3), and therefore p38-related apoptosis. On the other hand, DJ-1 mutant isoform L166P revealed a much weaker connection to ASK1 than WT-DJ-1, failing to suppress ASK1 functions. [66] Altogether, these studies confirm the DJ-1-ASK1 association resulting in ASK1 activity inhibition, in part by suppressing its homo-oligomerization-driven activation [66, 67].

In line with the results reported by Waak and colleagues [67] and other evidences that show that the oxidation status of DJ-1 influences and determines the protein's mode of action in response to ROS stimulus, the importance of C106 in modulating ASK1/DJ-1 complex was once again highlighted [62]. This research focused on a cancer model demonstrated that DJ-1 inhibited ASK1-mediated p38 and JNK pathways upon mild oxidative stress conditions; however, excessive oxidative stimulation led to its loss of function [62].

In general, these studies seem to highlight the DJ-1's relevant role in ensuring cell survival by suppressing the ASK1 apoptotic pathway. DJ-1 can accomplish this by inhibiting ASK1 activator Daxx [63, 65] and by preventing the dissociation of the inactive Trx1-ASK1 signalosome [64]. In addition, evidences also point out a direct interaction established between DJ-1 and ASK1, resulting in the repression of the ASK1 functions [66, 67]. The DJ-1 inhibition of ASK1 signaling pathway blocks, in turn, the activation of ASK1-mediated apoptotic mechanisms, such as JNK and p38 [62, 66].

1.2.1.4. p53 pathway

P53 is a tumor suppressor protein that functions as a transcription factor, hence regulating the expression of a wide variety of genes involved in several biological processes, namely cell cycle control, DNA repair, senescence, and apoptosis. Cells tend to increase the levels and the transcriptional activity of p53 when experiencing a range of stress stimuli, such as oxidative stress, DNA damage, and oncogene activation [69, 70].

Over the years, researchers have been investigating the role of p53 as a pathogenic factor associated with neuronal death in neurodegenerative diseases [70, 71]. In fact, such studies have confirmed the presence of increased levels and activity of p53 in PD patient brains and in both cellular and animal models [72-74]. Moreover, DJ-1 protein has been shown to modulate p53 to control cellular responses to stress insults (Figure 1.6) [75-79]. The majority of research regarding this topic consensually indicates that DJ-1 represses the p53 signaling pathway, with the exception of one work [80] expressing that DJ-1 restores p53 transcription activity inhibited by Topors-mediated sumoylation.

Indeed, the expression of both p53 and its target Bcl-2 associated X (Bax) apoptotic protein was shown to be enhanced in DJ-1 knockdown zebrafish subjected to oxidative stress, resulting in p53-mediated neuronal death [81]. Further studies carried out in DJ-1 overexpressed cultured cells clarified that DJ-1 binds to p53 and decreases its transcriptional activity [76]. As a result, the apoptotic regulator Bax's expression is inhibited, and caspase activation is prevented [76]. These findings allow to conclude that DJ-1 is able to exert its neuroprotective function through the downregulation of the p53-Bax-caspase death pathway.

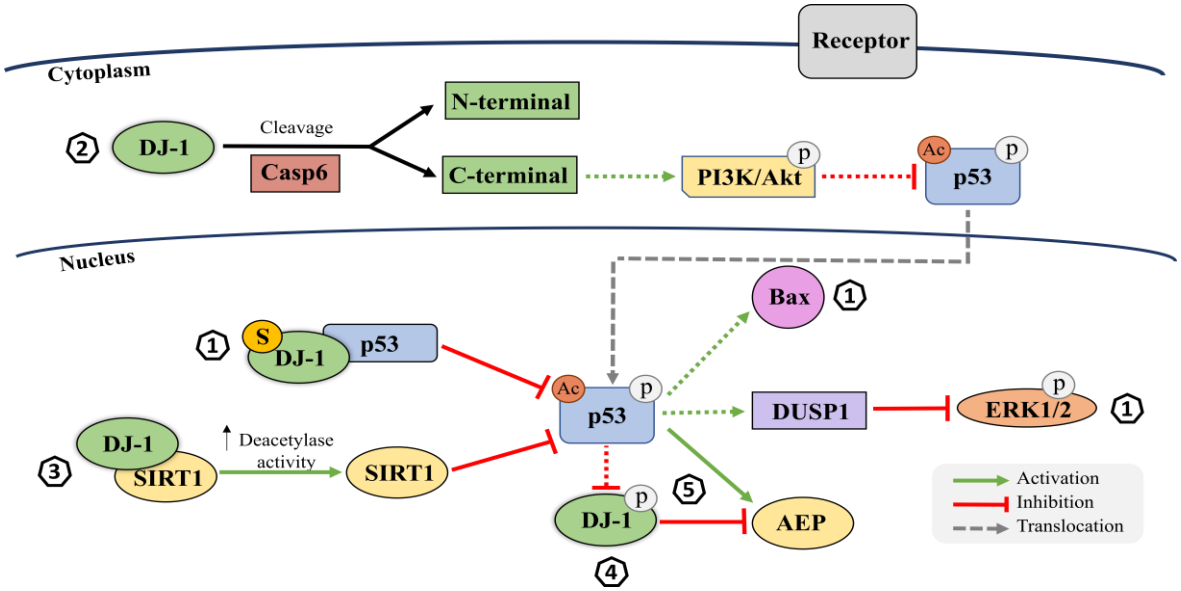


Figure 1.6| Summary of DJ-1's mechanisms involved in p53 pathway regulation. (1) DJ-1 is able to suppress p53 apoptotic signaling by binding to it, which requires proper DJ-1 sumoylation. Consequently, the expression of p53-related targets is blocked, such as Bax, an apoptotic protein, and DUSP1, a phosphatase responsible for suppressing ERK1/2 survival signaling. (2) The DJ-1 C-terminal generated by caspase-6 proteolysis can also suppress p53 activity in a PI3K/Akt-dependent manner. (3) Moreover, the interaction between DJ-1 and SIRT1 enhances the deacetylase activity of SIRT1 towards p53 inactivation. (4) Conversely, p53 has been shown to have a downregulatory effect on DJ-1 expression and mRNA levels, besides targeting the protein for an inhibitory phosphorylation reaction. (5) p53 is responsible for the increase of neurotoxic AEP activity. DJ-1 is able to suppress this p53-mediated activation of AEP by binding to its p53 binding site. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

According to Kato *et al.*, DJ-1 is able to bind to the DNA-binding region of p53 upon oxidative conditions, in a manner dependent on the DJ-1 oxidative state [78]. Oxidation of the central C106 was found to be required to enhance the affinity of DJ-1 to p53 and subsequently to repress p53 transcriptional activity. These facts emphasize once more the part of DJ-1 functioning as a redox-sensitive protein. This work also allowed to verify that the DJ-1 interaction with p53 upon H_2O_2 exposure resulted in the suppression of the expression of Dual Specificity Protein Phosphatase 1 (DUSP1), by detaining p53 to recognize its gene promoter [78]. DUSP1 is a mitogen-activated protein kinase phosphatase that dephosphorylates and inactivates MAPKs, such as the ERK survival pathway, therefore promoting cell death [82]. This way, p53-mediated inhibition of DUSP1 gene expression by DJ-1 also results in the upregulating of the ERK pathway for cell survival [78].

Nevertheless, compelling evidence suggest that the sumoylation of DJ-1 is a fundamental post-translational modification for the protein to suppress p53 transcriptional activity [75, 83]. In fact, DJ-1 has been proposed to undergo a sumoylation reaction in the K130 site to become fully

functional [83]. Another work observed that a sumoylation-deficient mutant of DJ-1 translocated from the nucleus to the cytoplasm upon ultraviolet (UV) stimulus [75]. Consequently, the protein failed to inhibit the nuclear p53 transcriptional function on the promotor of Bax and led to cell death. These findings indicate another defense mechanism of DJ-1 since the protein seems to require a proper sumoylation modification for its localization in the nucleus, subsequently allowing p53 repression [75].

Additional DJ-1-mediated mechanisms aimed at blocking the p53 pathway have been described [77, 79, 84]. Research conducted by Takahashi and co-workers identified Sirtuin 1 (SIRT1), a member of the Sirtuin family of proteins with deacetylase activity, as a binding protein of DJ-1 [79]. In fact, DJ-1 was found to bind to SIRT1 in cultured cells, enhancing its deacetylase activity upon the acetylated p53, therefore suppressing p53 functions and promoting cell growth. Contrarily, DJ-1 knockdown cells were shown to decreased SIRT1 activity, but this effect was reversed upon the introduction of WT-DJ-1, but not C106S DJ-1 mutant. This data implied that the direct interaction between DJ-1 and SIRT1 was dependent on the central C106 [79].

Furthermore, Giaime and colleagues reported that the C-terminal of DJ-1 generated by the caspase-6 cleavage led to p53-associated cell death inhibition in a PI3K/Akt dependent manner [77]. The authors observed that caspase-6 proteolytic activity could be activated under 6-OHDA-induced PD pathology conditions, leading to the cleavage of DJ-1 in a consensus sequence. As a consequence, the newly generated C-terminal DJ-1 fragment leads to the activation of PI3K/Akt survival pathway, which in turn inhibits p53 activity by its sequestration in the cytosol. Oppositely, PD-associated mutants of DJ-1, D149A, and L166P, exhibited impaired caspase-6 proteolysis, suggesting a DJ-1 loss of function impact in PD [77].

A most recent study has pointed out a mechanism of regulation ensured by DJ-1 and p53 regarding the asparagine endopeptidase (AEP) enzyme, also known as legumain [84]. Initially, it was established that p53 upregulates AEP enzyme activity by binding to its gene. In turn, this work demonstrated that DJ-1 was able to suppress AEP by binding to its p53-binding site in AEP's correspondent gene [84]. In fact, increased AEP levels are characteristic of neurodegenerative disorders, and recent work actually indicated AEP to have a pathological influence in PD [85, 86]. AEP was found to cleave α-synuclein in an age-related manner, leading to its aggregation and subsequent neurotoxic effect in dopaminergic cells and motor dysfunction in a mouse model [85, 86]. Altogether, these pieces of evidence may suggest a DJ-1 protective function, regulating AEP

levels by preventing its p53-directed activation. Nonetheless, all these results must be further confirmed.

Apart from the fact that DJ-1 regulates p53 signaling through the mentioned mechanisms, studies have also suggested a p53 role in the modulation of DJ-1 [87, 88]. Regarding this matter, proteomic analysis identified DJ-1 as a p53 phosphorylation target, possibly resulting in a suppressing effect on DJ-1 [88]. Another research stated that p53 is able to inhibit DJ-1 expression and its mRNA levels [87]. It was further explained that parkin, another PD-associated protein, was demonstrated to upregulate DJ-1 in a p53-dependent manner upon ER-stress conditions. Actually, parkin triggers the suppression of p53, which in turn leads to an increase of DJ-1 expression. This mechanism was proposed to be responsible for increasing ER-stress response, which is impaired in PD, probably due to a dysfunctional parkin-mediated control of DJ-1 [87].

All in all, studies indicate that DJ-1 can repress p53 apoptotic activity by different mechanisms, such as by directly binding to it [76, 78], for which proper sumoylation of the protein seems to be required [75, 83]. The C-terminal of DJ-1 generated by the caspase-6 cleavage has also been demonstrated to inhibit p53 in a PI3K/Akt-dependent manner [77]. Another mechanism is based on the DJ-1 binding to SIRT1, enhancing its deacetylase activity, and consequently blocking p53 activation [79]. The p53-dependent activation of the AEP enzyme implicated in PD development can also be inhibited by a mechanism of action of DJ-1 [84]. Therefore, as a result of DJ-1-mediated suppression of p53 activity, DUSP1 inhibitor of ERK1/2 signaling [78] and Bax apoptotic protein [76] can be suppressed, promoting cellular survival. However, p53 may also have an effect on DJ-1, leading to the downregulation of the protein [87, 88].

1.2.1.5. Nrf2 pathway

Nuclear factor erythroid-related 2 (NRF2) is a transcription factor encoded by the *NFE2L2* gene and is involved in the control of antioxidant cellular responses [89]. This protein can be degraded by ubiquitin-protease ligase systems, mediated by the substrate adaptor Kelch-like ECH-associated protein1 (Keap1). In basal conditions, Nrf2 is bound to its inhibitor Keap1 in the cytoplasm, while upon stress stimulus, such as oxidative stress, Nrf2/Keap1 interaction is disrupted. As a result, Nrf2 is activated and translocated to the nucleus, where it associates and controls the expression of target genes that present the antioxidant response element (ARE sequence). Therefore, the Nrf2/ARE signaling cascade is accounted for the expression of detoxifying agents, such as NAD(P)H

quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione-S-transferases (GST), and thioredoxin (Trx). [89-91] Pathogenic events characteristic of PD and other neurodegenerative disorders involve aberrant redox homeostasis instigated by defective Nrf2 signaling [92].

Research work concerning this pathway proved the DJ-1's involvement in its modulation (Figure 1.7), as the protein was described to participate in an antioxidant response mediated by Nrf2 signaling and in promoting the expression of its target genes [90]. The absence of DJ-1 in mouse primary embryonic fibroblasts (MEFs) caused the loss of Nrf2 and, subsequently, the decrease of Nrf2-regulated gene's levels, namely of the NQO1 enzyme. The researchers explained that DJ-1 favors the presence and action of the free Nrf2 form, possibly by blocking the association between the transcription factor and its inhibitor Keap1 [90]. These findings are supported by another study concerning the Nrf2/DJ-1 interaction in cardiomyocyte cell line H9c2 upon hypoxia/re-oxygenation (H/R)-induced oxidative stress [93]. Overexpression of DJ-1 was observed to upregulate the transcription of antioxidant enzymes through Nrf2 activation, attenuating oxidative injury. Besides, Nrf2/Keap1 dissociation and subsequent Nrf2 translocation to the nucleus was also verified [93]. This fact confirms the previous theory proposing that DJ-1 may stabilize Nrf2 by blocking its association with Keap1 [90]. From these data, it is valid to infer that, in a neuronal PD context, the loss of DJ-1 function in oxidative conditions may lead to neuronal death, in part due to the lack of Nrf2-mediated antioxidant protection.

Even though Gan *et al.* [94] argued that the Nrf2 pathway was independent of DJ-1, the majority of research actually consent to this DJ-1/Nrf2 mechanism. Supporting DJ-1 and Nrf2 interaction results kept emerging in recent studies conducted on astrocytes [95, 96] and neuron-like cells [53]. This latter investigation described an impaired mitochondrial function caused by the absence of DJ-1 in PC12 and SH-SY5Y cells, which was proposed to be associated with a deficient Nrf2 nuclear translocation. Consequently, Nrf2 cascade activation was suppressed, as well as the expression of HO-1 and NQO1, essential for the proper mitochondrial function [53]. Furthermore, a PI3K/Akt-dependent regulation of Nrf2 mediated by DJ-1 is reported by the most recent discoveries [97-100].

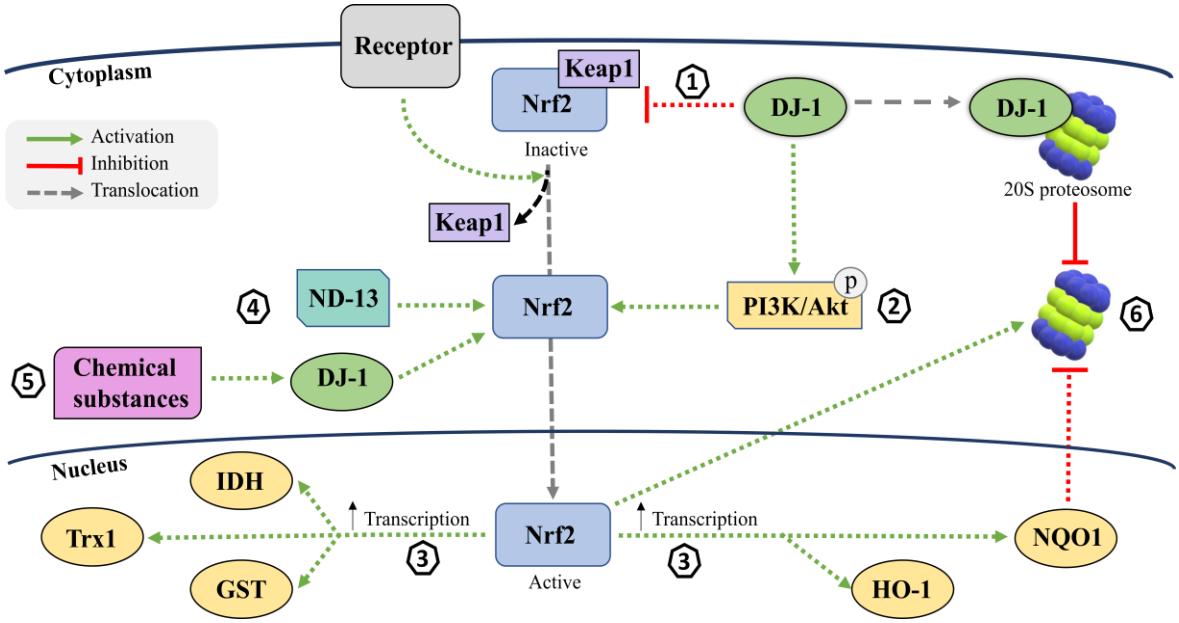


Figure 1.7| Summary of DJ-1's mechanisms involved in Nrf2 pathway regulation. (1) DJ-1 stabilizes Nrf2 by favoring Nrf2 free form, possibly blocking or disrupting the association of inhibitor, Keap1. (2) DJ-1 is also able to modulate Nrf2 signaling, activating it in a PI3K/Akt dependent manner. (3) As a result, Nrf2 triggers the expression of specific enzymes involved in antioxidant protective responses, such as IDH, Trx1, GST, HO-1, and NQO1. (4) ND-13 is a DJ-1 and TAT based peptide with therapeutic potential, promoting DJ-1-dependent activation of Nrf2 antioxidant mechanism. (5) A number of chemical substances have also been described with a promising effect in enhancing DJ-1-mediated Nrf2 signaling activation. (6) Furthermore, DJ-1 is involved in a loop 20S proteasome regulatory mechanism that provides a balance in protein degradation processes. DJ-1 may bind to 20S proteome, inhibiting its action together with NQO1 enzyme. Contrarily, the DJ-1-mediated Nrf2 activation also leads to 20S proteasome enhancement. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

Described cytoprotective mechanisms of action of DJ-1 via the Nrf2 pathway entail the upregulation of the expression of antioxidant isocitrate dehydrogenase (IDH) [101] and of ASK1 apoptotic pathway-related inhibitor, Trx1 [102]. Moreover, Moscovitz and colleagues [103] identified DJ-1 as a 20S proteasome regulator through a C106-dependent interaction, but also by an Nrf2 pathway-mediated modulation. This mechanism relies on a loop regulatory system, in which the protein leads to both upregulation and inhibition of the 20S proteosome activity: DJ-1 promotes 20S activation by enhancing the Nrf2 pathway; DJ-1 may also bind to 20S proteome, inhibiting its action together with NQO1 enzyme, also a 20S proteosome inhibitor. As a result, a balance between the degradation of harmful disrupted proteins and the preservation of intrinsically unstructured ones is provided [103].

A number of studies continued to prove the involvement and importance of DJ-1 in the activation of Nrf2 signaling in a therapeutic context [97-100, 104-108]. One particular work developed a DJ-1 based peptide (ND-13) shown to prevent ROS-induced cellular injury in PD animal and cellular

models by upregulating the Nrf2 pathway [104]. ND-13 is a peptide with clinical potential that comprises a combination of segments of DJ-1 and TAT, a cell-penetrating peptide for favoring the delivery of the peptide to the central nervous system. This novel peptide was able to protect neuroblastoma cells SH-SY5Y from oxidative injury by promoting the Nrf2-dependent expression of detoxifying enzymes, namely HO-1 and NOQ1. The same mechanism was confirmed in mice, in which dopaminergic neuronal loss was decreased following ND-13 treatment [104].

Moreover, additional research has reported the promising effect of chemical substances that target the DJ-1-mediated Nrf2 antioxidant pathway to enhance its activity against oxidative stress [97-100, 105-108]. Cu(II)ATSM is a hypoxia imaging agent validated to have a neuroprotective action in PD animal models by preventing nigral cell loss, enhancing dopamine metabolism, and reducing the motor and cognition symptoms characteristic of PD [109]. Further work provided evidences that Cu(II)ATSM upon oxidative insults was able to trigger the Nrf2 antioxidant pathway in a DJ-1-dependent manner in human coronary artery smooth muscle cells (HCASMC) and cardiac myocytes (HCM) [108]. The authors also explained that Cu(II)ATSM treatment increased DJ-1 levels that were shown to be essential to stabilize and promote the Nrf2 function. In fact, it was explained that DJ-1 was able to bind to Cu(II) in order to modulate Nrf2 activation [108]. Another work identified tauroursodeoxycholic acid (TUDCA) as a protective agent of SH-SY5Y cells exposed to PD pathological-induced conditions [106]. Again, the results showed that this compound led to the upregulation of both Nrf2 and DJ-1 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-affected mice midbrain and striatum, which are indicated as the mediators of the neuroprotective action of TUDCA [106]. These results confirmed the connection between DJ-1 and Nrf2 and the role of DJ-1 in Nrf2 stabilization and antioxidant activity.

More recently, a DJ-1-binding compound, compound B, was shown to enhance the capacity of DJ-1 to promote Nrf2 transcriptional activity in a Keap1-independent manner under oxidative conditions [98]. Indeed, compound B was previously reported to bind to the C106 region of DJ-1 and maintain the protein's active and functional forms by preventing its inactivating superfluous oxidation [110]. In this sense, cells treated with compound B upon oxidative stress proved that the substance facilitates DJ-1-mediated inhibition of PTEN, therefore activating PI3K/Akt pathway that is in turn known to stimulate Nrf2 action. These results suggest that the DJ-1-associated compound B could act as an important neuroprotective drug for PD treatment since it seems to trigger an antioxidant defense Nrf2 mechanism mediated by DJ-1[98].

Based on the described pieces of evidence, DJ-1 seems to be able to modulate the Nrf2 pathway, which may occur through the regulation of the Nrf2-Keap1 complex [90, 93] or in a Keap1-independent manner [98]. Following the DJ-1-dependent activation, Nrf2 exerts its transcriptional activity stimulating the expression of antioxidant enzymes involved in cellular protective responses against oxidative stress [53, 90, 93, 101, 102]. Additionally, triggering of Nrf2 by DJ-1 may also lead to the regulation of the 20S proteasome [103]. A number of studies have been focusing on the antioxidant response promoted by the DJ-1/Nrf2 mechanism for the development of therapeutic strategies. These include the ones involving a DJ-1-based peptide ND-13 [104] and chemical substances focused on enhancing DJ-1 activity, such as Cu(II)ATSM [108], TUDCA [106], compound B [98], and others [97-100, 105, 107].

1.2.1.6. DJ-1 in other potential signaling mechanisms

Previous work from our laboratory focused on studying the mode of action of DJ-1 allowed the detection of other potential mechanisms based on the regulation of signaling pathways, by which the protein may exert its neuroprotective function with possible implications in PD [111]. An interactomics analysis was performed to elucidate the dynamic network of interactions of endogenous DJ-1 under well-defined oxidative stress conditions and, therefore, identify new mechanisms and players involved in its physiological activity [111].

In the interactomics study, SH-SY5Y cells were exposed to a non-lethal H_2O_2 stimulus that would mimic the ROS burst event leading to the activation of protection mechanisms, namely of ERK1/2 and PI3K/Akt signaling pathways [111]. The interactions implicated in the functional activity of DJ-1, associated with the activation of ERK1/2 and PI3K/Akt pathways, were assessed before, during, and after the oxidative stimulus in different time points (0, 5, 15, 20 and 40 minutes). To identify and quantify the interaction established under these conditions, an immunopurification of DJ-1 in each of the 5 time points was used, enabling the isolation of endogenous DJ-1 and its interactors. Following these protein's capture, a mass spectrometry (MS) strategy, short-GeLC-SWATH-MS, was performed, allowing the identification and quantification of 881 proteins as potential DJ-1 interactors. Furthermore, the interaction profiles of these interactors under the time-course experiment were established, and 731 of those interactors were clustered into twelve different groups according to their interaction profiles (Figure 1.8). The results revealed that 365 proteins presented a maximum of interaction that corresponds to the peak of activation of the mentioned pathways (cluster 5 to 8), implying that a large number of DJ-1 interactors may be associated with

the activation of those pathways. Additional gene ontology (GO) enrichment analysis focused on the molecular function of each of the previously defined clusters (Figure 1.9) demonstrates enrichment of proteins associated with a variety of signaling pathways (green arrows) within several of those clusters. The role of DJ-1 in cell signaling regulation was further confirmed by the analysis concerning the biological processes (Figure 1.10), which highlights several different signaling pathways and apoptotic specific processes that may be mediated by DJ-1 [111].

In conclusion, as previously mentioned, a huge body of evidence seems to confirm the role of DJ-1 in the modulation of several signaling pathways through specific mechanisms acting on different levels. However, such acknowledge signaling pathways may be beyond those already described in the literature, for which further studies should be conducted with the intent to elucidate potential new mechanisms associated with this DJ-1 central function.

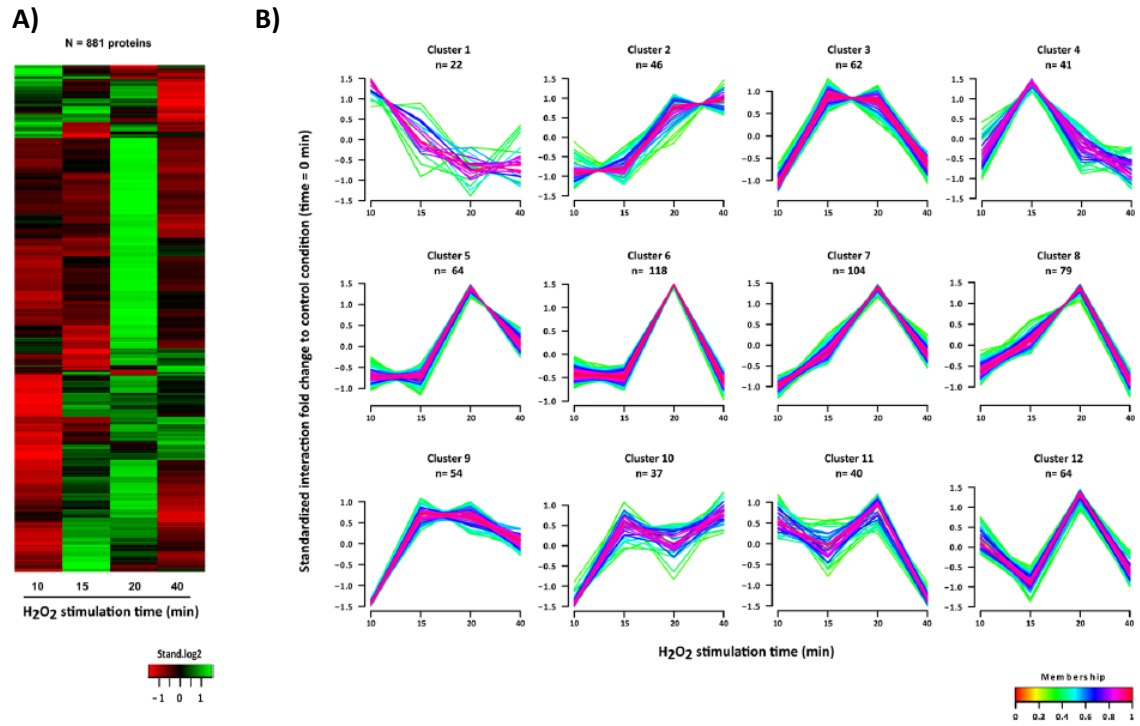


Figure 1.8| Characterization of DJ-1 interacting network under oxidative stress. A) Row-clustered heat map showing the median standardized interaction levels of the 881 proteins involved in the DJ-1 network under oxidative stress identified in this study. The proteins presented have statistically significant differences among the time course considered. Protein intensities were normalized by the amount of immunopurified DJ-1 to determine the interaction levels and further normalized to control conditions (0 min stimulation). B) Dynamic profiles of DJ-1 network o interactions under oxidative stress. Profiles were obtained by an unsupervised clustering analysis performed for the standardized interaction levels, with an upper and lower ratio limit of log2 (2) and log2 (0.5) established for the higher and lower interaction levels of each protein. A membership limit of at least 0.3 was used for protein inclusion into a particular cluster, and the proteins (a total of 150 proteins) that fail the defined criteria were considered “unregulated” during the oxidative stress conditions. ‘‘n” indicates the number of proteins within each cluster. Membership color values represent how well the protein profile fits the average cluster profile [111].

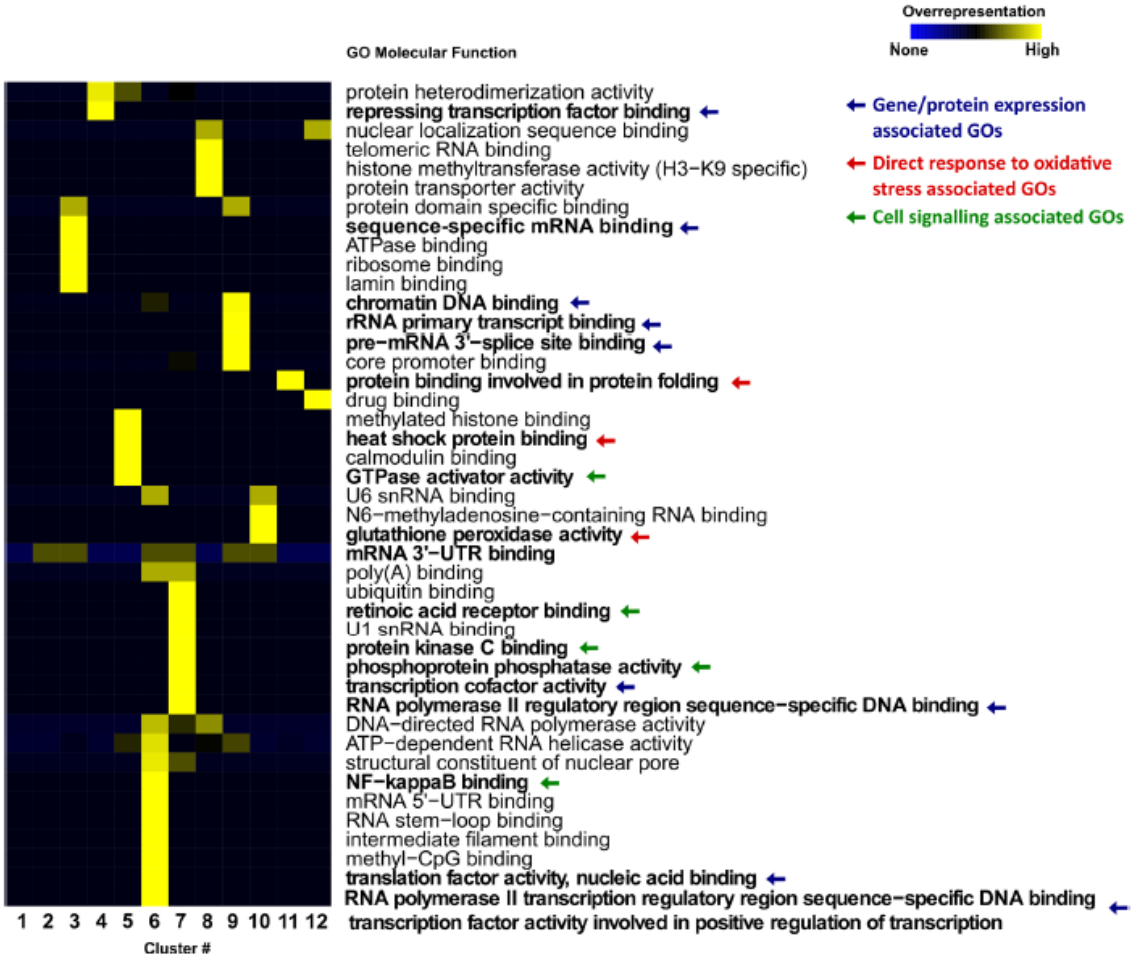


Figure 1.9| Overrepresented gene ontologies for molecular function for each cluster of DJ-1 interactors previously defined. Each cluster from Figure 1.8B was tested for overrepresented GO compared with the unregulated proteins using a Binomial statistical test with Benjamini-Hochberg adjustment and a cut-off of 0.05 p-value and a minimum occurrence of 2. Some of the GOs associated with specific mechanism associated with DJ-1 response to oxidative stress are highlighted (in bold) and group in three important categories: gene/protein expression (indicated by the blue arrow), a direct response to oxidative stress (indicated by the red arrow), and cells signaling (green arrow) associated GOs [111].

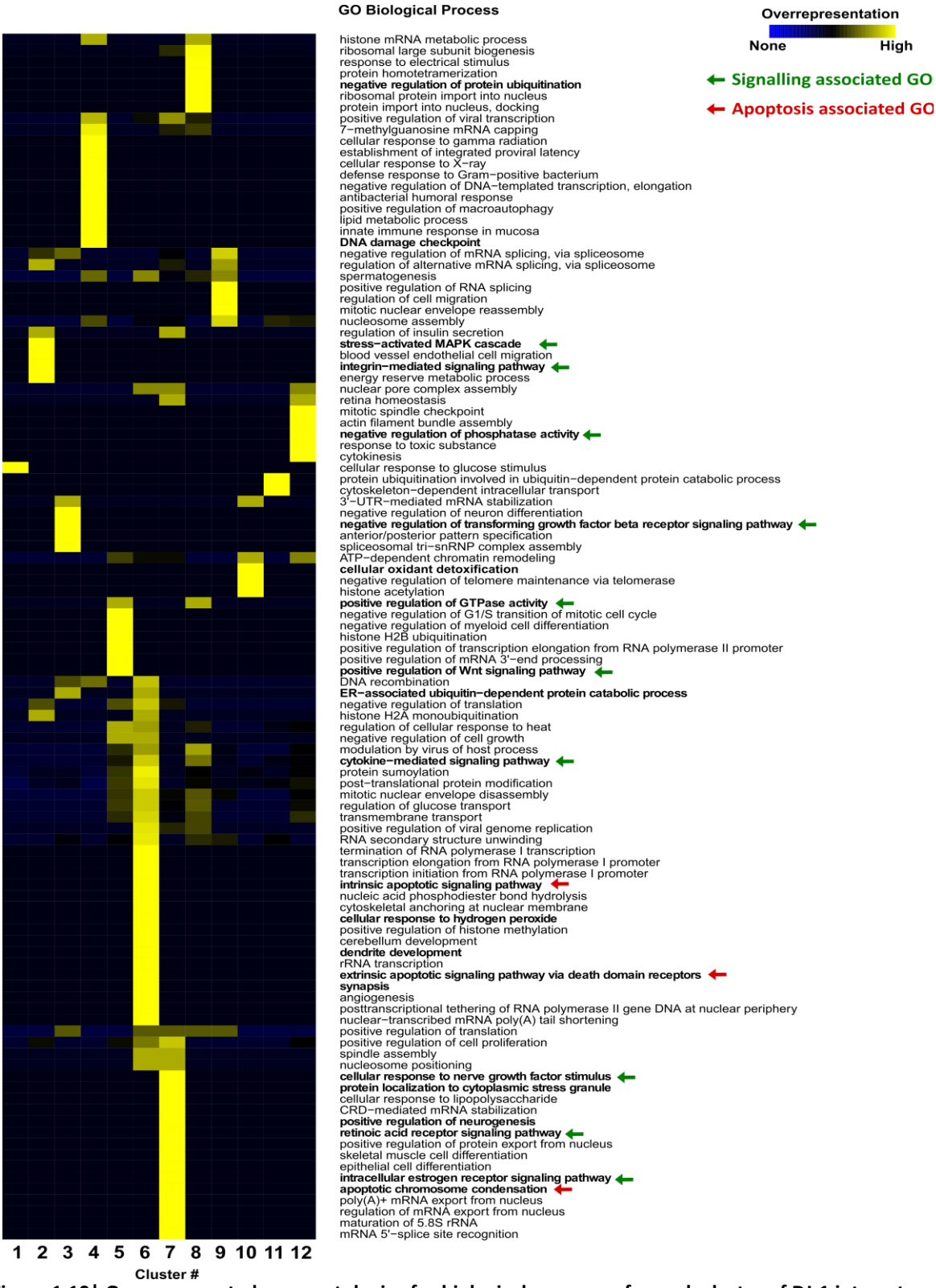


Figure 1.10| Overrepresented gene ontologies for biological processes for each cluster of DJ-1 interactors previously defined. Each cluster from Figure 1.8B was tested for overrepresented GO compared with the unregulated proteins using a Binomial statistical test with Benjamini-Hochberg adjustment and a cut-off of 0.05 p-value and a minimum occurrence of 2. Highlighted GOs (in the bold) corresponds to potential mechanism associated with the neuroprotective role of DJ-1, with a special focus on the mechanisms associated with signaling (red arrows) and apoptosis (green arrows). [111]

1.2.2. Extracellular DJ-1

DJ-1 has been thoroughly investigated as a neuroprotective protein responsible for mediating several survival mechanisms in response to stress conditions; such cytoprotective role has been most evidenced in different compartments at the intracellular level, while its precise functions in the extracellular environment have been poorly comprehended. Multiple studies report that DJ-1 can be extracellularly secreted from cells and found in the cerebrospinal fluid and serum of PD patients [112, 113]. Ultimately, DJ-1 secretion has been proposed to be more than a byproduct of normal secretory processes of cells, as increasing evidence suggests its ability to mediate a protective effect from the extracellular space [114, 115]. As a matter of fact, Yanagida and colleagues demonstrated that DJ-1 secretion from astrocytes was triggered by oxidative stress as part of astrocyte-mediated neuroprotection mechanisms [115].

Studies conducted in the laboratory have also highlighted the potential neuroprotective action of extracellular DJ-1 [116, 117]. These studies intended to evaluate the effects of the exogenous addition of WT and/or mutant DJ-1 isoforms to neuronal cells, under normal and oxidative stress conditions. To do so, different recombinant proteins were produced and structurally characterized, including a WT-DJ-1 and the PD-related mutants M26I- and E163K-DJ-1 variants (Figure 1.11) [116-119]. Such DJ-1 mutations are known to be responsible for the loss of function of the protein leading to neurodegeneration events associated to PD [120, 121].

MGSSHHHHHHDYDIPTTENLYFQGH|MASKRALVILAKGAEEMETVIPVDVMRRAGIKVTVAGLAGKDPVQCS
RDVVICPDASLEDAKKEGPYDVVVLPGGNLGAQNLSESAAVKEILKEQENRKGLIAAICAGPTALLAHEIGFG
SKVTTHPLAKDKMMNGGHYTYSENRVEKDGLILTSRGPGTSFEFALAIVEALNGKEVAAQVKAPLVLKD

Figure 1.11| Sequence of recombinant DJ-1 WT engineered. The vertical black line represents the separation of the hexahistidine tag (6-His-tag, highlighted in grey) and tobacco etch virus (TEV) cleavage sequence N-terminal (left side of the line) from the DJ-1 sequence (right side of the line). Highlighted in yellow are the amino acids that were changed in DJ-1 mutants: M26I [methionine (M) to isoleucine (I)] and E163K [glutamate (E) to lysine (K)] -DJ-1 (Adapted from reference [119]).

In particular, a study conducted by Nuno Jordão aimed to assess the protective role of WT-DJ-1 and the consequences caused by M26I and E163K mutations on a biological level [116]. In this experiment, SH-SY5Y cells were subjected to a 24h extracellular incubation with WT-, M26I- and E163K-DJ-1 forms upon control and oxidative stress conditions induced by 50 µM H_2O_2. A negative control condition (c-) without DJ-1 stimulation was also considered. A luciferase-based ATP quantification was performed to indirectly assess cellular viability, in which the amount of ATP is directly proportional to the number of metabolically active cells (Figure 1.12). The results

demonstrate that while no differences were observed in control conditions, WT-DJ-1 was able to confer higher protection than M26I-and E163K-DJ-1 variants from oxidative insults. Indeed, WT-DJ-1 led to a greater increase of cellular viability, than M26I- and E163K-DJ-1, which, in turn, demonstrated cellular vulnerability to the stress stimulation applied. Moreover, upon oxidative stress, M26I-DJ-1 condition presents significantly lower viability than WT and higher viability than the negative control condition, denoting that despite the biological impairment due to M26I mutation, the protein was still able to lead a protective effect to a certain extent. On the other hand, similar results were observed between the negative control and E163K-DJ-1 stimulation conditions under stress, suggesting that this mutation in the DJ-1 protein caused a loss of oxidative stress protection. Overall, this experiment was able to prove the protective role elicited by the extracellular addition of WT-DJ-1 upon oxidative stress and confirm the impairment of M26I and E163K mutations on its native activity [116].

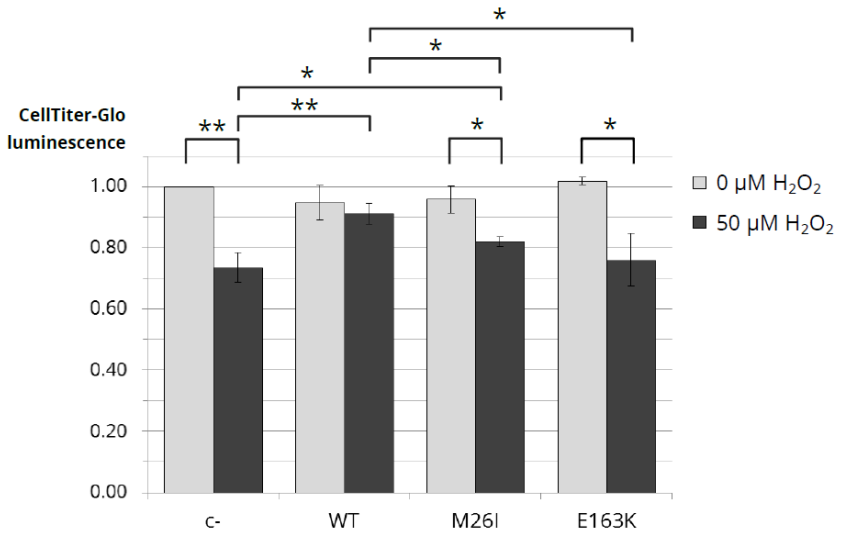


Figure 1.12| SH-SY5Y cells viability assay, under normal (0 µM H_2O_2) or oxidative stress (50 µM H_2O_2) conditions, and in the absence (c-) or presence of different DJ-1 protein forms (WT, M26I, E163K). Values normalized to control condition (c-, 0 µM H_2O_2). Negative and positive error bars of each condition corresponding to the respective standard deviation (n =3). Above the graphic, indication of condition pairs with significant difference after independent samples t-test analysis: * = p-value under 0.05; ** = p-value under 0.001. [116]

Considering these findings, a crucial aspect remains to be clarified with regards to the exact mode of action of DJ-1 in the extracellular space. Interestingly, another previous work in the laboratory carried out by Matilde Melo attempted to understand the mechanisms involved in the neuroprotection of extracellular DJ-1 using recombinant WT-DJ-1 exogenously added to neuronal cells (Figures 1.13 and 1.14) [117]. In a preliminary experiment, SH-SY5Y cells were treated with

H_2O_2 in the absence or presence of the exogenous recombinant DJ-1 protein for 15 minutes. Immunoblot analysis was performed using phospho-motif antibodies to detect potential DJ-1-mediated activation of Akt (Figure 1.13) and ERK1/2 (Figure 1.14) -related signaling. The obtained results show that the extracellular stimulation of cells with a recombinant WT-DJ-1 led to the activation of ERK1/2 and Akt signaling pathways upon oxidative stress conditions. These findings provide important clues into the potential involvement of extracellular DJ-1 in the modulation of intracellular signaling mechanisms [117]. Moreover, a preliminary interatomics study of extracellular DJ-1 allowed to identify a set of proteins involved in cellular processes, such as axon guidance, cell migration and proliferation, carbohydrate metabolism, synaptic process and positive or negative regulation of signaling pathways [117]. In this sense, more work should be carried out to further explore and clarify the DJ-1 function in the extracellular environment, in particular its potential role in signaling modulation carried out at this level.

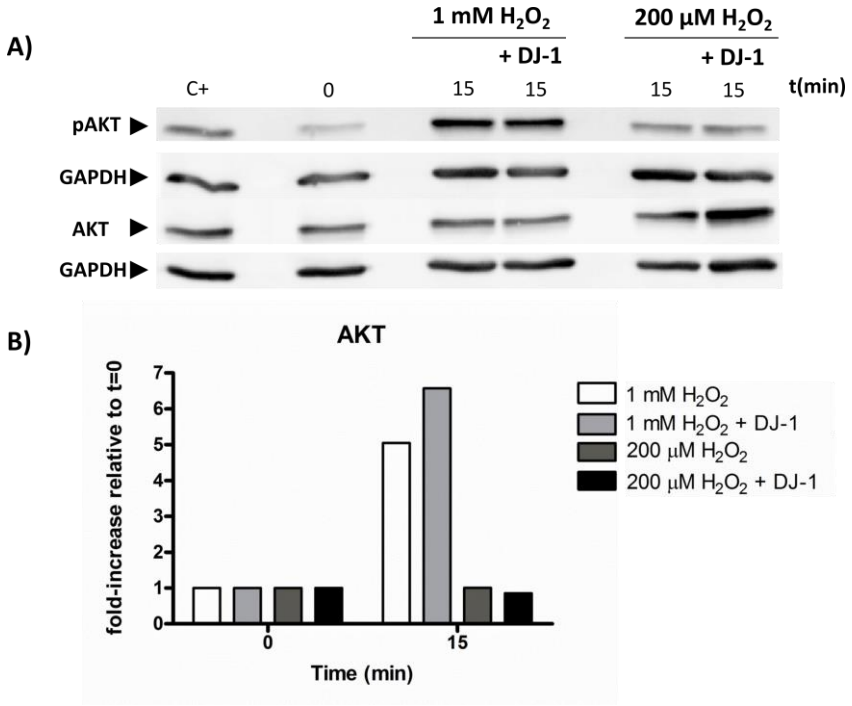


Figure 1.13| H_2O_2-induced Akt activation, in the presence or absence of the recombinant DJ-1, in SH-SY5Y cells. Serum-starved SH-SY5Y cells were treated with 200 µM or 1 mM of H_2O_2, in the presence or absence of 1 µM of 6-His-DJ-1, for 15 minutes. Equal amounts of protein extract were analyzed for Akt (60 kDa) activation by Western blot using phospho-specific Akt (Ser473) antibody or anti-total Akt, and anti-GAPDH antibodies to check protein loading. A) Representative Western blot for the treatment with 200 µM or 1 mM of H_2O_2, in the presence or absence of 1 µM of 6-His-DJ-1; B) Graphic representation of the measured adjusted volume of each band. Data were normalized to the positive control ("C+" - 15% FBS stimulation) for each western-blot. (Adapted and corrected from [117])

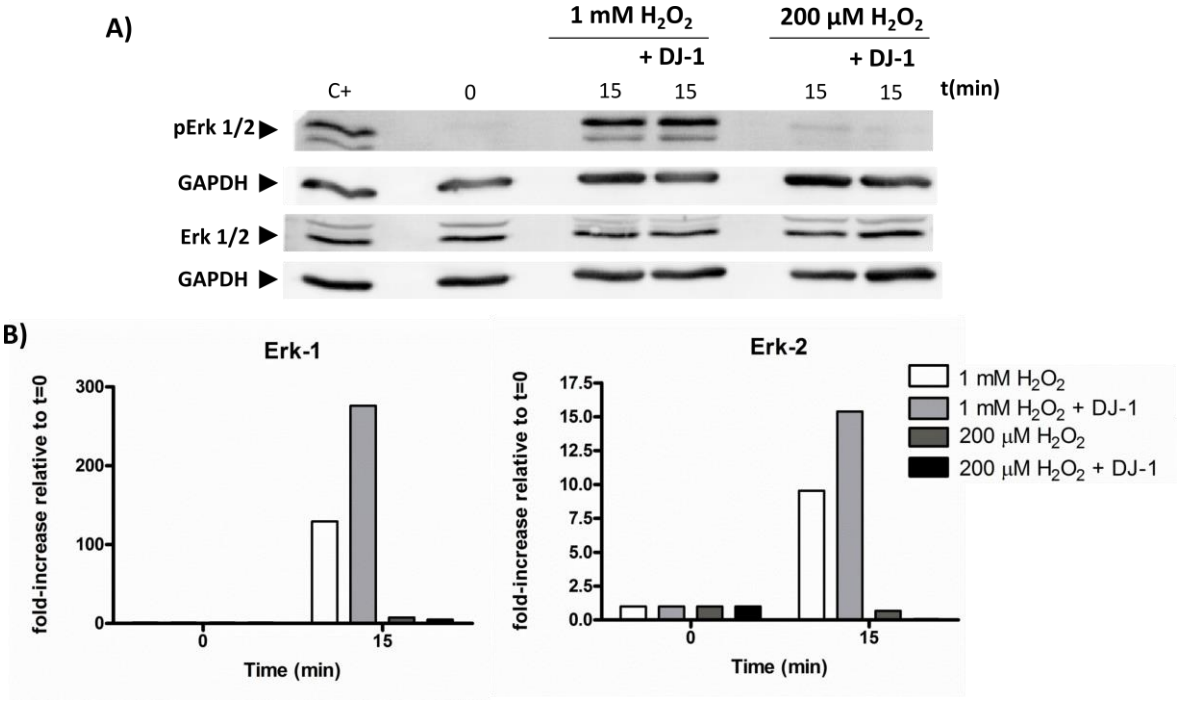


Figure 1.14| H_2O_2-induced ERK1/2 activation, in the presence or absence of the recombinant DJ-1, in SH-SY5Y cells. Serum-starved SH-SY5Y cells were treated with 200 µM or 1 mM of H_2O_2, in the presence or absence of 1 µM of 6-His-DJ-1, for 15 minutes. Equal amounts of protein extract were analyzed for ERK1 (44 kDa) and ERK2 (42 kDa) activation by Western blot using phospho-specific ERK1/2 (Thr202/Tyr204) antibody or anti-total ERK1/2, and anti-GAPDH antibodies to check protein loading. A) Representative Western blot for the treatment with 200 µM or 1 mM of H_2O_2, in the presence or absence of 1 µM of 6-His-DJ-1; B) Graphic representation of the measured adjusted volume of each ERK1 corresponding band; C) Graphic representation of the measured adjusted volume of each ERK2 corresponding band. Data were normalized to the positive control ("C+" - 15% FBS stimulation) for each western-blot. (Adapted and corrected from [117])

1.3. Approaches to identify the mechanisms of action of a protein – its involvement in signaling transduction.

To better understand the neuroprotective role of a protein in a given context, it would be important to identify the exact cellular mechanisms mediated by the protein that confer such effect. Since most proteins act as part of protein complexes, identifying protein's binding partners is an essential primary task to infer and characterize the overall mode of action of the target protein [122]. Moreover, as signaling pathways are crucial to define cellular fate and conventional vias by which protective mechanisms can be achieved, studies conducted to provide further clues into the involvement of a protein in the modulation of signaling pathways can also be important to identify the major neuroprotective roles of the proteins being studied.

As extensively described, signaling pathways are vital biological mechanisms of the cellular system in control of growth, differentiation, metabolism, and apoptosis events, upon health and disease

contexts [123]. Therefore, a clear understanding of the signaling nature and mechanisms would provide vital insights sought after the biological and medical research fields [124]. A signal propagation event is accounted for specific protein changes at three distinct levels: i) regulated protein post-translational modifications (PTMs), namely phosphorylation, acetylation, ubiquitylation, and methylation; ii) protein-protein interactions (PPIs), often owing to PTMs; and iii) signal-induced changes in protein expression [124].

Hence, the priority tasks in studying the mode of action of a protein with potential involvement in the modulation of signaling pathways are the analysis of PPIs and PTMs phenomenons, which can be achieved by interactomics and phosphoproteomics strategies, respectively. The main approaches for recognizing proteins and their modifications include immunoblot detection approaches and mass spectrometry (MS)-based techniques [124].

1.3.1. Interactomics studies

Cells can be envisioned as a series of complex networks of macromolecular interactions, therefore constituting the interactome [125]. Specifically, protein-protein interactions (PPIs) are highly precise and regulated networks responsible for managing the most fundamental molecular and cellular mechanisms [126, 127]. For that reason, determining protein partners has become an urgent matter of investigation towards identifying relevant protein-transmitted biological pathways that contribute to the elucidation of disease pathology. Indeed, expanding the knowledge of signaling pathways often integrates PPIs analysis allowing the reconstruction of the cross-talks occurring in a given signaling event in response to specific cues, from which it is possible to predict novel signaling components or pathways targeted proteins [123, 128].

One challenge in the elucidation of an organism's PPI networks relies on the fact that protein contact ensues by selective molecular docking in a particular condition and time point, making it difficult to be captured and measured. To tackle this issue, steps have been taken for the development of systematic and comprehensive approaches for a precise and unbiased characterization of the interactome within specific cellular contexts [126, 129]. There are a number of described methods for investigating PPIs, of which binary and affinity purification-based strategies are of wide use [125, 127]. Yeast two-hybrid (Y2H) is a genetic high-throughput assay capable of rapidly identifying binary interactions between two proteins *in vivo* [130].

On the other hand, many proteins perform their functional act as a part of protein complexes. Concerning this perspective, mass spectrometry has been pinned at the forefront of technology for the mapping of PPIs, specifically enabling the identification of protein complex components. An MS-based analysis can be coupled to preceding affinity purification (AP) of the proteins of interest resulting in an enrichment of its interactions (Figure 1.15) [125, 127, 131, 132]. This step commonly implies the isolation of the target protein and its bound interactors by using an affinity ligand, such as specific antibodies or epitope tags. In particular, a pull-down assay is an affinity purification method that makes use of a purified tagged protein (called bait) able to bind to interacting proteins (preys), in a series of washing and elution steps [127, 133]. The bait protein is conjugated to a matrix affinity tag, for which some examples are polyhistidine tag, FLAG tag, and glutathione S-transferase (GST) tag. The captured protein complexes are then subjected to a liquid chromatographic-coupled to tandem MS (LC-MS/MS) experiment, from which a list of potential interactors is generated [127]. Actually, a mass spectrometer is able to detect down to the smallest contaminants; therefore, a quantitative MS-based approach is an essential step that allows distinguishing specific from non-specific interactors in the sample. Ultimately, this workflow generates a high-confidence PPI identification [131].

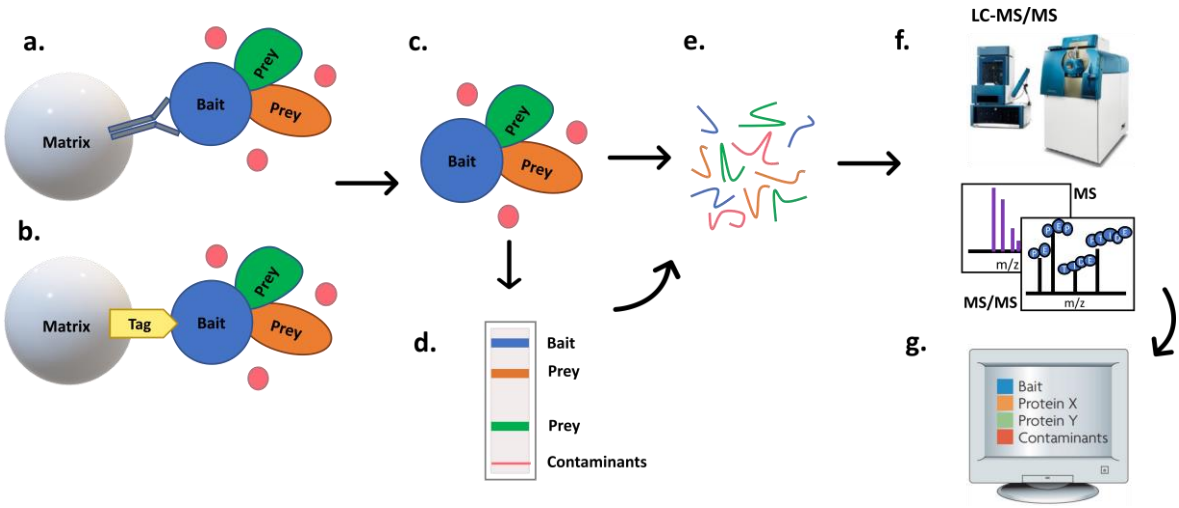


Figure 1.15| Overview of a generic affinity purification-mass spectrometry (AP-MS) approach. The protein of interest (bait-protein; in blue) is immobilized either to a specific antibody (a) or to an epitope tag (b). (c) The generated tagged bait protein is then purified together with its binding partners (prey-proteins; in green and orange), as well as contaminants that may be present in the sample (in pink). (d) An optional extra step implies an SDS-PAGE separation procedure of the multiprotein complex. (e) Proteolytic digestion is accomplished by proteases, usually trypsin, cleaving proteins into peptides. (f) An MS analysis is carried out, in most cases, through an LC-MS/MS experiment, producing a tandem MS (MS/MS) spectrum. (g) Data interpretation and protein identification is performed by database searching, from which a list of the tagged protein, its interactors, and possible contaminants are generated. (Adapted from reference [134])

1.3.2. Study of pathways modulation – focus on determination of protein phosphorylation patterns

Post-translational modifications (PTMs), such as phosphorylation, refer to biochemical processes that alter the properties of proteins in order to modulate cellular signaling pathways as a response to specific cues [135]. These modifications are of extreme importance to determine protein's function by having an effect on its structure, stability, localization, and interactions with other proteins. Over the past decades, efforts have been made to understand how these PTMs, in particular phosphorylation, influence signaling mechanisms in certain contexts [135].

Phosphorylation is the most widespread PTM, and its reaction occurs by a kinase-mediated reversible transfer of a phosphate group from ATP to a certain amino acid residue, mostly serine (S), threonine (T), and tyrosine (Y). [135] Inversely, phosphatases catalyze the opposite reaction, resulting in a regulatory mechanism of phosphorylated protein levels [136]. Dysfunction of this regulated mechanism is likely to occur, usually due to kinases or phosphatases-related mutations, as well as mutations or epigenetic changes in proteins that act at any level of these signaling cascades. Consequent aberrant phosphorylation-related signaling is known to take part in the development of several diseases, including neurodegenerative disorders [135]. For this reason, the study of the complex phosphorylation networks is a relevant matter with the intent to elucidate the molecular mechanisms underlying those diseases [137].

Phosphorylation studies aim to assess the protein phosphorylation status of biological samples upon specific contexts [138]. Traditional biochemical methods include western blotting with phosphospecific stains (e.g., Pro-Q Diamond dye) and phosphospecific antibodies, and *in vitro* kinase reactions encompassing the incorporation of modified adenosine triphosphate (ATP) molecules, for instance, with radio-labeled phosphate [138, 139]. Employed by most laboratories, an immunoblotting-based phosphorylation profiling strategy makes use of phosphorylation-site specific antibodies generated against different phosphorylated amino acids of a target protein [139, 140]. Alternatively, phospho-motif profiling assays employ phosphospecific motif antibodies that can be used as a powerful tool for the study of kinase activity. This type of assay relies on the detection of kinase phosphorylated substrates, reflecting the activation of a specific pathway cascade. This phenomenon relies on the principle that a known kinase is associated with a particular phosphorylation site through a target consensus sequence, thus conferring substrate specificity [141]. Besides, pronounced protein expression alterations can also be inferred by assessing the degree of staining on the western blot experiment [124]. The design and further developments of

phosphospecific antibodies have transformed signaling research, contributing to the understanding of the phosphoproteome's complexity and its changes upon different cues. Still, these studies enable only a semi-quantitative analysis, by which the relative change in protein phosphorylation is determined. Also, a significant limitation relies on antibody availability, which allows a directed analysis and detection of a given protein and phosphorylation alteration per antibody [140, 141].

Technological advances concerning the mass spectrometry (MS) field of proteomics gave rise to MS-based phosphoproteomics strategies that enable the identification and quantification of complex phosphorylation events on a proteome-wide scale. Indeed, this high-throughput technique is able to provide a large amount of information in an unbiased and reproducible manner [136, 142]. An untargeted MS-phosphoproteomics study typically includes a phospho-enrichment step that can be carried out by chromatographic methods, such as immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC). As a matter of fact, phosphospecific or phosphor-motif antibodies can also be applied for the immunoprecipitation-based phospho-enrichment process. Following the isolation of the phosphopeptides, the sample's characterization is concluded by a liquid chromatographic-coupled tandem MS (LC-MS/MS) experiment [142, 143].

1.3.3. Mass spectrometry-based proteomics

Mass Spectrometry (MS)-based strategies have revolutionized the study of biomolecules, notably proteins, their interactions, and posttranslational modifications (PTM) [126]. Mass spectrometry is a valuable analytical technique that measures the mass-to-charge ratio (*m/z*) of ions [144]. A standard mass spectrometer instrument comprises an ion source, a mass analyzer and a detector. Firstly, the samples are ionized into gas phased-ions by ion sources, in particular matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) types. The MALDI type consists of laser pulses responsible for sublimating and ionizing samples through a crystalline matrix, being adequate for simpler peptide mixtures. On the other hand, ESI is suitable for complex samples and operates by ionization of the analytes in a solution, allowing it to be coupled with chromatographic separation techniques, such as High-performance liquid chromatography (HPLC). After ionization, the ionized analytes go through the mass analyzer to measure their *m/z* ratios and are registered by the detector that is connected to computer systems for data analysis of the mass spectra (MS) and fragmentation spectra (MS/MS) produced [144]. Thus, in a generic MS-based proteomics

approach (Figure 1.16), the peptides resulting from sample preparation procedures are subjected to liquid chromatography coupled to the mass spectrometer (LC-MS/MS), leading to the generation of MS and MS/MS spectra characteristic of the eluted peptides [144].

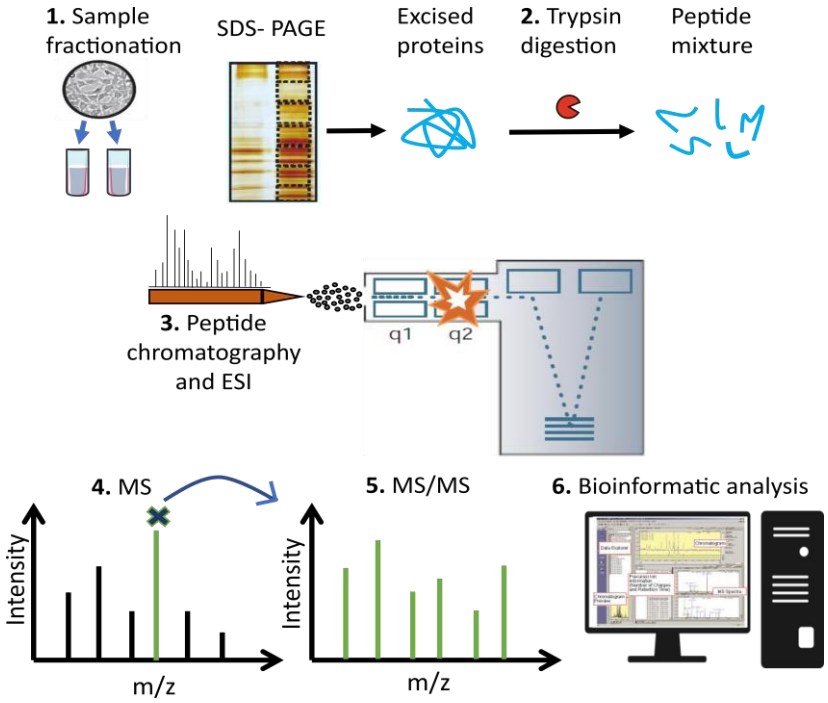


Figure 1.16| Generic mass spectrometry (MS)-based proteomics experiment. (1) The proteins to be analyzed are isolated from cell lysate or tissues by biochemical fractionation or affinity selection. This often includes a final step of one-dimensional gel electrophoresis that defines the 'sub-proteome' to be analyzed. (2) Enzymatic digestion of the proteins into peptides, usually by trypsin. (3) The peptides are separated by one or more steps of HPLC and eluted into an electrospray ion source where they are nebulized in small, highly charged droplets. After evaporation, the ionized peptides enter the mass spectrometer. (4) A mass spectrum of the peptides eluting at this time point is produced. (5) The computer generates a prioritized list of these peptides for fragmentation, and a series of tandem mass spectrometric (MS/MS) experiments ensues. These consist of isolation of a given peptide ion, fragmentation by energetic collision with gas, and recording of the tandem or MS/MS spectrum. (6) The MS and MS/MS spectra are stored for matching against protein sequence databases. The outcome of the experiment is the identification of the peptides that constitute the protein sample (Adapted from reference [144]).

In general, protein identification is accomplished by MS-based strategies that operate in the data-dependent acquisition mode (DDA, also known as information-dependent acquisition – IDA) [145]. In this type of experiment, it is performed a first scan of the intact ionized peptides, followed by the selection of only a number of precursor ions, normally the most intense ones, for further fragmentation, usually via a collision-induced dissociation (CID) process. The identification of the peptides, and consequently the proteins, is determined based on the match of the obtained MS/MS spectra that contain the sequence information, with the theoretical ones generated *in silico* from

information available in databases [144, 145]. Even though this method provides an efficient qualitative study for protein identification, there are some limitations making it less reliable for protein quantification. One of the biggest limitations relies on the fact that the DDA method becomes biased due to the exclusive analyses of the most intense precursor ions, causing it to be influenced by the sample's complexity and/or dynamic range [145].

In this sense, quantitative proteomics MS-based strategies were developed aiming at quantifying protein levels present in a sample in the most reproducible way. To this end, mainly labeled approaches were employed in the past for untargeted quantitative screenings. These methods involve the sample's stable isotope labeling (enzymatic, metabolic, and chemical) before proceeding with an MS experiment. However, in the last few years, with the development of different acquisition modes and the improvement of the MS instruments and data processing algorithms, the label-free approaches have become more relevant for quantitative proteomics [146]. The increase in label-free screenings was mainly associated with the development of data-independent acquisition methods (DIA), consisting of the analysis of the fragmentation spectra of all the precursor ions that are present in a sample. In fact, this acquisition mode does not imply any type of precursor selection, overcoming the DDA disadvantage of biased analysis. Nevertheless, the loss of the link between the precursor ion and its fragments makes it challenging to identify proteins; therefore, these approaches have to rely on highly efficient algorithms [145, 146].

There are 2 types of DIA methods entailing either the simultaneous acquisition of fragmentation spectra from the total mass range or the scan of the *m/z* range through established widths of sequential windows [145, 146]. This latter process provides a beneficial reduction in sample complexity since it allows fewer ions to undergo fragmentation analysis at the same time [146]. This is the case of the sequential window acquisition of all theoretical fragment-ion spectra (SWATH-MS) strategy that presents an innovative data extraction process based on the combination of both DDA and DIA methods (Figure 1.17) [145]. Briefly, SWATH-MS comprises a targeted data analysis, extracting from the DIA data the quantitative information of the peptides identified on an optimized DDA approach. The data acquired from the DDA method is presented as a list (designated "library") of all the identified peptides, displaying parameters such as retention time (tR), precursor *m/z,* and MS/MS spectra for every single positive identification. In a parallel acquisition of the samples in the SWATH mode, the fragmentation spectra are obtained for the total precursor ions generated (called "digital maps"), by scanning sequentially defined precursor windows. Finally, the quantification comes from the DIA file analysis, from which are extracted the

chromatographic elution trace of sets of fragment ions characteristic of a given high confidence peptide, whose information is available at the library created from the DDA analysis [145]. The quantification information is extracted for all the positive identifications (all the peptides/proteins present in the library) for all the samples being analyzed.

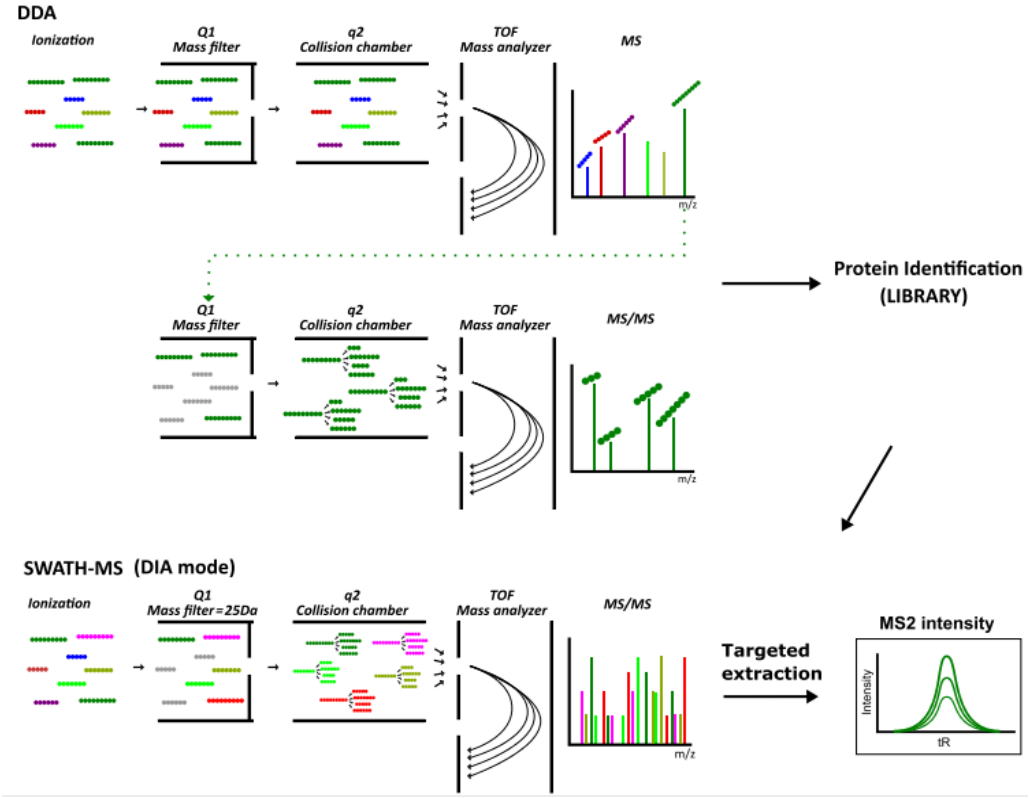


Figure 1.17| Overview of the SWATH-MS approach. The usual SWATH-MS method combines a parallel analysis of samples with an optimized DDA method for peptide identification, followed by a DIA acquisition to be used to extract the quantitative information of the previously identified peptides. In a DDA experiment, the fragmentation spectra are only acquired for a group of selected precursor ions based on their intensities. In SWATH mode, the fragmentation spectra are acquired for all the precursor ions independently of their intensities. Moreover, the fragmentation spectra are acquired by covering the entire mass range in sequential smaller windows of defined size. In the SWATH-MS approach, the quantification of the confident peptides/proteins is determined based on the fragment ions intensities (MS2 intensity), designated as peak groups, of each previously identified peptide (Adapted from reference [145]).

1.4. Objectives

Despite extensive research, the exact molecular mechanisms underlying Parkinson's Disease development remain largely unknown to this day. Mutations in the protein DJ-1 that led to the impairment of its correct function have been linked to an autosomal recessive early-onset form of the disease. DJ-1 has a pleiotropic role in neuroprotection, particularly against oxidative stress, and mainly relying on the modulation of signaling pathways. Even though most of the DJ-1 uncovered functions take place at the intracellular level, an important cytoprotective role led by the protein from the extracellular space has also been addressed. However, there is still limited information concerning the exact mechanisms performed by extracellular DJ-1 or how its PD-related mutations impair such action.

In this sense, this study's main goal was to identify new mechanisms of action of extracellular DJ-1 with a particular focus to assess its mediated regulation of signaling pathways. For this purpose, this work focused on an MS-based interactomics study in view of uncovering DJ-1 protein partners that would allow reconstructing the cross-talks taking place at the extracellular level upon control and oxidative stress conditions. Furthermore, an immunoblot-based phospho-motif profiling analysis was considered to assess the potential impairment of kinases-mediated intracellular signaling induced by exogenous stimulation with M26I- and E163K-DJ-1 pathological isoforms. Therefore, this study was expected to expand the knowledge on DJ-1's extracellular functions of signaling modulation and how PD-associated mutations impact its native activity. Ultimately, new important insights may be provided into the DJ-1-associated PD pathogenesis.

2. Methods

The present work may be generically divided into two parts. The first part entailed the analysis of results acquired from the interactomics study of extracellular WT-DJ-1 previously conducted under the project PTDC/NEU-NMC/0205/2012[2], that had not been analyzed until now (section 2.1). In the second part, a phosphorylation profiling study of kinase substrates was performed considering the WT-, M26I- and E163K- DJ-1 proteins (section 2.2).The recombinant proteins used in this part of the work, including WT-DJ-1 and PD-related mutant isoforms, M26I-DJ-1 and E163K-DJ-1, were produced and characterized in prior master projects developed in the laboratory [116-119].

2.1. Extracellular DJ-1 interatomic study

2.1.1. Cell sample preparation

SH-SY5Y cells were seeded at 9.375×10^4 cells/cm^2 in 148 cm^2 plates in MEM-F12 with 10 % fetal bovine serum (FBS) and maintained in culture for 24h at 37 °C, 95 % air and 5 % CO_2 in a humidified incubator (Sheldon Manufacturing, Inc.). Then, the medium was changed for MEM-F12 medium with 0.1% FBS and cells were either left in culture for 24h (negative control conditions) or subjected to a set of stimuli conditions: i) 1 µM DJ-1; ii) 40 µM H_2O_2 + 1 µM DJ-1; iii) 1 µM His-tag; and iv) 40 µM H_2O_2 + 1 µM His-tag. After 24h, all the conditioned media were collected and centrifuged at 290×*g*, for 5 min at 4 °C to remove cell debris. The conditioned media were further concentrated using 5 kDa cut-off concentrators (Sartorius) upon a 3220×*g* centrifugation at 4 °C, and sample volume was adjusted to 1 mL. Each condition was performed in a total of four replicates.

2.1.2. Pull-down Assay

His-Mag bead (His Mag Sepharose Ni, GE Healthcare) preparation was carried out according to the manufacturer's instructions. Briefly, the bead slurry was thoroughly mixed by vortexing and 200 µl of the homogeneous slurry were dispensed for each condition. The tubes were placed in the magnetic rack and the storage solution was removed, followed by resuspension with 500 µl MEM-F12 medium. Immediately after liquid removal, the concentrated conditioned media were incubated with the His-Mag resin for 4h at 4 °C at 1050 rpm in the thermomixer (Eppendorf). Then, the supernatant was collected using the magnet for the removal of unbound proteins. The beads were washed twice with 1mL of MEM-F12 with 0.1% FBS and transferred to a clean tube. The

[2] Project title: "DJ-1 neuronal rescue under oxidative stress: implications for Parkinson's Disease", PI: Bruno Manadas

collected His-Mag resin was then denaturated with 70 µL sample buffer (2×) (Bio-Rad) at 95 °C, for 5 minutes. All fractions were stored at -20 °C until further use.

2.1.3. Mass spectrometry analysis by SWATH mode

Protein digestion

Protein digestion was performed using the Short-GeLC approach as previously described [147]. First, 10 µL of each replicate were combined to create representative pools of each experimental condition to be used for protein identification. Then, each purified sample along with the prepared pool samples were spiked with 2 µg of the internal standard (IS) mixture (MBP, maltose-binding protein and GFP, green fluorescent protein), as previously described [148, 149], and boiled at 95°C for 5 min to denature the proteins. All the samples were allowed to cool to room temperature and 4 µL and 2.7 µL of acrylamide/bis-acrylamide solution were added to all the condition replicates and pool samples, respectively, to alkylate cysteines. The entire set of samples was loaded in a "4–20% TGX Stain-Free Gel" (Bio-Rad), and a partially electrophoretic separation was performed during 15 min at 110 V, to allow the samples to enter into the gel.

After SDS-PAGE, proteins were visualized with Colloidal Coomassie Blue. Briefly, after electrophoresis, the gel was rinsed in distilled water followed by immersion in the staining solution [10 % (v/v) of 85 % solution of phosphoric acid, 10 % (w/v) ammonium sulfate, 20 % (v/v) methanol, and 0.2 % Coomassie Brilliant Blue G]. The Coomassie powder was added to the solution with a strainer to prevent clotting of the dye and allow the development of colloidal particles. The gel was allowed to stain overnight under agitation, and then the gel was washed with abundant water and maintained in water until further analysis.

The entire lanes were sliced into bands of equal size, and each band was sliced in small pieces and processed individually. Gel pieces were destained, dehydrated and rehydrated in 100 µL of trypsin (0.01 µg/µL solution in 10 mM ammonium bicarbonate) for 15 min, on ice. After this period, 10 µL of 50 mM ammonium bicarbonate were added, and in-gel digestion was performed overnight at RT in the dark. After digestion, the excess solution from gel pieces was collected to a low binding microcentrifuge tube, and peptides were extracted from the gel pieces by sequential addition of three solutions of acetonitrile (ACN) in 1% formic acid (FA) (30%, 50%, and 98% of ACN, respectively). After the addition of each solution, the tubes were shaken in the thermomixer (Eppendorf) at 1050 rpm for 15 min, and the solution was collected to the tube containing the previous fraction. At this stage, the peptides extract from the different bands were pooled together

into 3 peptides mixtures per sample for posterior analysis by LC-MS/MS. The peptide mixtures were dried (preferentially not completely) by rotary evaporation under vacuum.

Before performing the MS/MS analysis, the peptide mixtures from all approaches were cleaned/desalted using OMIX tips with C18 stationary phase (Agilent Technologies) according to the manufacturer's instructions. Each peptide fraction was resuspended in 30 µL of a solution of 2 % ACN and 0.1 % FA (formic acid) (LC-MS/MS mobile phase).

SWATH acquisition

Samples were analyzed on an AB Sciex 5600 TripleTOF® in two modes: information-dependent acquisition (IDA) for protein identification and library generation and SWATH acquisition for quantitative analysis. Peptides separation was performed using liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC ChromXP™ C18AR reverse phase column (300 µm × 15 cm, 3 µm, 120 Å, Eksigent®) at 5 µL/min with a multistep gradient: 0-2 min linear gradient from 5 to 10 %, 2-45 min linear gradient from 10 % to 30 %, and 45-46 min to 35 % of acetonitrile in 0.1 % FA. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, AB Sciex) with a 65 µm internal diameter (ID) stainless steel emitter (AB Sciex).

IDA experiments were performed for all the 3 peptide mixtures of each pooled sample (one per experimental condition). In the IDA experiments, the mass spectrometer was set for IDA scanning full spectra (350-1250 m/z) for 250 ms followed by up to 100 MS/MS scans (100–1500 m/z) using dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 3.3 s. Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 15 seconds. Mass spectrometer operated by Analyst® TF 1.7 (AB Sciex). The rolling collision energy was used with a collision energy spread of 5.

Each biological replicate was set for quantitative analysis by acquisition in SWATH mode. The SWATH setup was essentially as in Anjo *et al*. [147], with the same chromatographic conditions used as in the IDA run described above, and the mass spectrometer operated in a looped product ion mode. The SWATH-MS setup was designed specifically for the samples to be analyzed. A set of 60 windows of variable width (containing 1 m/z for the window overlap; Table 2.1) was constructed covering the precursor mass range of 350- 1250 m/z. A 100 ms survey scan (350-1500 m/z) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were

collected from 100–1500 m/z for 50 ms resulting in a cycle time of 3.15 s from the precursors ranging from 350 to 1250 m/z, which is compatible with the acquisition of at least 8 points per chromatographic peak. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with a collision energy spread of 15.

Table 2.1 | SWATH-MS variable windows (60) used in the acquisition of the samples. For each window is indicated the m/z range and the window width in Dalton (Da).

	m/z range	Width (Da)
Window 1	349.5-368.1	18.6
Window 2	367.1-382.4	15.3
Window 3	381.4-395	13.6
Window 4	394-406.3	12.3
Window 5	405.3-417.1	11.8
Window 6	416.1-427.9	11.8
Window 7	426.9-438.7	11.8
Window 8	437.7-449	11.3
Window 9	448-459.8	11.8
Window 10	458.8-471.1	12.3
Window 11	470.1-482.8	12.7
Window 12	481.8-493.6	11.8
Window 13	492.6-504.4	11.8
Window 14	503.4-514.7	11.3
Window 15	513.7-524.2	10.5
Window 16	523.2-532.7	9.5
Window 17	531.7-540.8	9.1
Window 18	539.8-548.5	8.7
Window 19	547.5-555.7	8.2
Window 20	554.7-562.9	8.2
Window 21	561.9-570.1	8.2
Window 22	569.1-577.3	8.2
Window 23	576.3-584.5	8.2
Window 24	583.5-592.2	8.7
Window 25	591.2-600.3	9.1
Window 26	599.3-609.3	10
Window 27	608.3-617.8	9.5
Window 28	616.8-627.7	10.9
Window 29	626.7-637.6	10.9
Window 30	636.6-648	11.4
Window 31	647-657.9	10.9
Window 32	656.9-668.2	11.3
Window 33	667.2-679.9	12.7
Window 34	678.9-691.6	12.7
Window 35	690.6-704.2	13.6

Window 36	703.2-715.9	12.7
Window 37	714.9-728.1	13.2
Window 38	727.1-738.9	11.8
Window 39	737.9-748.8	10.9
Window 40	747.8-758.7	10.9
Window 41	757.7-768.6	10.9
Window 42	767.6-778.9	11.3
Window 43	777.9-789.3	11.4
Window 44	788.3-800.5	12.2
Window 45	799.5-812.2	12.7
Window 46	811.2-823	11.8
Window 47	822-833.8	11.8
Window 48	832.8-844.2	11.4
Window 49	843.2-854.5	11.3
Window 50	853.5-865.3	11.8
Window 51	864.3-877	12.7
Window 52	876-891.9	15.9
Window 53	890.9-913	22.1
Window 54	912-942.3	30.3
Window 55	941.3-974.7	33.4
Window 56	973.7-1009.8	36.1
Window 57	1008.8-1056.1	47.3
Window 58	1055.1-1106.5	51.4
Window 59	1105.5-1161.4	55.9
Window 60	1160.4-1249.6	89.2

Protein Identification and Library Generation

Protein identification was obtained using ProteinPilot™ software (v5.0, AB Sciex) with the following search parameters: search against the Homo sapiens database from SwissProt (released in August 2020), IS (MBP and GFP), and recombinant DJ-1 sequences; using trypsin as digestion enzyme and acrylamide as an alkylating agent, and a special focus option for gel-based approaches. An independent False Discovery Rate (FDR) analysis, using the target-decoy approach provided by ProteinPilot™, was used to assess the quality of identifications. Positive identifications were considered when identified proteins and peptides reached a 5 % local FDR [150, 151].

A specific library of precursor masses and fragment ions of all the proteins identified in the samples was created by combining all the IDA files from all the pool samples (except the negative control) and used for subsequent SWATH processing.

SWATH data file processing

Prior to data processing, peptides were selected automatically from the library using the following criteria: (i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and (ii) Peptides that contained biological modifications and/or were shared between different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH quantitation was attempted for all proteins in library files that were identified below 5% global FDR from ProteinPilot™ search. In SWATH™ Acquisition data, peptides are confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide.

The target fragment ions, up to 5, were automatically selected following the criteria described in Lambert *et al.* [152]. Peak groups were also scored according to Lambert *et al.* [152], and the peak group with the best score was taken for posterior analysis. The peak group confidence threshold was determined based on an FDR analysis using a target-decoy approach. Peptide features that met the 1% FDR threshold in at least 3 biological replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the samples using a 4 min extracted-ion chromatogram (XIC) window adjusted in order to accommodate the entire chromatographic peaks with 100 ppm XIC error. Additionally, the retention time was adjusted to each sample by the use of the IS peptides.

Protein levels were estimated by summing all the transitions from all the peptides for a given protein (an adaptation of [153]).

2.1.4. Data analysis

Selection of DJ-1 putative interactors

From the acquired data, the selection of potential DJ-1 binding partners was performed based on a combination of the results from two types of analysis, taking into consideration the protein levels and the DJ-1-interacting levels (adapted from [148]).

DJ-1 putative interactors were identified by finding proteins with a dynamic modulation induced by the considered experimental conditions. First, the protein levels were subjected to a step of normalization to the levels of the internal standard (MBP-GFP protein) in each sample (protein/IS ratios), to accommodate for losses during sample processing. A Kruskal-Wallis and Dunn's tests were then performed to compare the protein levels among the control (1 µM DJ-1), stress (40 µM H_2O_2 + 1 µM DJ-1), and negative control conditions (no stimulation), and a statistical significance was considered for p-value bellow 0.1. The results presented in the text correspond to the median of the protein level's fold change, determined for each combination assessed: control vs. negative control; stress vs. negative control; control vs. stress. Proteins were considered as DJ-1 putative interactors if they met one of the following criteria: (i) a p-value under 0.1 and increased (log_{10} fold-change > 0) in DJ-1 conditions when compared to the negative condition; or (ii) a p-value under 0.1 in the control vs. stress condition. An identical analysis was performed considering the control (1 µM DJ-1) and stress (40 µM H_2O_2 + 1 µM DJ-1) conditions, and the respective negative controls in the presence of the His-tag peptide, control (1 µM His-tag) and stress (40 µM H_2O_2 + 1 µM His-tag) conditions. Statistical analysis was performed in R software (version 4.0.2). Venn diagrams were used to identify the putative DJ-1 interactors, combining the proteins selected in each combination assessed in the statistical test. Area proportional Venn diagrams were obtained with BioVenn tool (available at https://www.biovenn.nl/).

On the other hand, the interacting levels parameter was determined for all the proteins under control (1 µM DJ-1) and stress (40 µM H_2O_2 + 1 µM DJ-1) conditions, by normalizing the potential interactor's values by the levels of the recombinant DJ-1 protein in each biological replicate. Then, the ratios of the interacting levels between the control and stress conditions were calculated, and a One-sample student's t-test was performed against a theoretical value of one (i.e., a ratio of 1 indicates no variation). The results presented in the text correspond to the mean of the interaction levels ratio between control and stress conditions, and statistical significance was considered for p-values below 0.1. One-sample Student's t-test was performed in IBM® SPSS® Statistics version 22.

Functional analysis

Subcellular location assessment was performed for the complete set of DJ-1 putative interactors and the proteins with an extracellular space annotation were selected using a Venn diagram combining the protein sets with membrane, intracellular and extracellular spaces annotations. Area proportional Venn diagrams were obtained with BioVenn tool (available at https://www.biovenn.nl/).

A gene ontology (GO) analysis considering biological processes and molecular function was further performed for the extracellular DJ-1 putative interactors. Both analyses were performed using UniProt gene ontology tool and database (available at http://www.UniProt.org/). Functional characterization of the extracellular binding partners of DJ-1 also included a pathway analysis using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system software (version 16.0, available at http://pantherdb.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper tool (available at https://www.genome.jp/kegg/tool/map_pathway2.html).

2.2. Kinase substrate phospho-motif profiling upon extracellular stimulation with DJ-1 WT and PD-related mutants

2.2.1. Cell sample preparation

SH-SY5Y cells were seeded in 55 cm^2 plates at the same density and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS until reaching confluency. Serum starvation was induced by changing the culture medium to DMEM medium without FBS, and cells were maintained in the serum reduced medium for 24 h. Serum-starved cells were then stimulated with 1 µM of either exogenous WT-, M26I- or E163K-DJ-1 for a 24h period of time. Stimulation was stopped by aspiration of the medium followed by a washing step with pre-warmed PBS and addition of ice-cold lysis buffer [RIPA buffer (50 mM Tris-HCl, pH 7.4; 1 % (v/v) Igepal; 0.25 % (v/v) sodium-deoxycholate; 150 mM NaCl; 1 mM DTT; 1 mM EDTA, cOmplete™ Mini protease inhibitor mixture and cOmplete™ Mini phosphatase inhibitor mixture)]. After the addition of RIPA buffer, cell culture plates were placed on ice, and the lysates were obtained by scraping the plate with a rubber cell scraper. The lysates were collected to microcentrifuge tubes, and protein extraction was promoted by sonication (30 seconds (s) with 40% amplitude of cycles of 1 s using an Ultrasonic processor with 750 W), followed by removal of insoluble fraction via centrifugation at 20,000×*g* for 15 min at 4 °C. After centrifugation, the supernatant (protein extract) was collected into new tubes. A sample of each protein extract was used to calculate the protein concentration using Pierce™ 660nm Protein Assay

(Thermo Scientific) according to the manufacturer's instructions, and the remaining extracts were stored at -20°C until posterior use. Each condition was performed in a total of four replicates.

2.2.2. Immunoblots using KinomeView Profiling Kit

The correspondent volume of the 6× SDS Sample Buffer [(0.35 M solution of Tris-HCl with 0.4 % SDS (v/v), pH 6.8, 30 % glycerol (v/v), 10 % SDS (w/v), 9.3 % DTT (w/v) and 0.01 % bromophenol blue (w/v)] was added to the protein extracts to a 1× final concentration. The samples were then denatured by boiling at 95°C for 5 min. Fifteen micrograms of protein per lane were electrophoretically separated on 12.5 % SDS-polyacrylamide gels using a mini-PROTEAN® Tetra Electrophoresis System (Bio-Rad). Proteins in the gels were transferred to low fluorescence polyvinylidene fluoride (PVDF) membranes (TBT RTA TRANSFER KIT, Bio-Rad) using a Trans-Blot Turbo Transfer System (BioRad) for 45 min at a constant voltage of 25 V (with the current limited to 1 A). After the transfer, the membranes were blocked for 1 hour at room temperature (RT) with 5% (w/v) skimmed milk powder in PBS-Tween 20 (PBS-T) [0.1% (v/v)]. Blots were then incubated overnight at 4 °C, followed by 2 h at RT with a set of phospho-motif antibodies provided in the KinomeView kit (Cell Signaling Technology #9812) prepared in blocking solution (Table 2.2). Those antibodies allow the detection of serine, threonine, and tyrosine phosphorylation mediated by known kinase families throughout the kinome (Figure 2.1). Primary antibodies were removed, and membranes were extensively washed with PBS-T (3 times, 15 min under agitation each time). Blots were then incubated for 2 h at RT with the secondary antibodies (Table 2.3) in 5 % (w/v) skimmed milk powder dissolved in PBS-T followed by extensive washes as above.

Protein-immunoreactive bands were developed using the "Enhanced Chemifluorescence (ECF) detection system" (GE Healthcare) and visualized in a Molecular Imager FX System (Bio-Rad).

For determination of the loaded sample's total intensity, the membranes were further stained using the Serva Purple reagent (ref.: 43386, SERVA Electrophoresis GmbH), according to the manufacture's instruction for staining of PVDF membranes. Briefly, the membranes were re-wetted in methanol and rinsed in water prior to a 10 min basification step using a solution of 0.42% (w/v) $NaHCO_3$ and 0.385% (w/v) NaOH. Then, the membranes were incubated in the staining solution composed of 250 µL of Serva Purple reagent in 50 mL of high purity water for 30 min at RT. After incubation, the membrane was transferred to the acidification solution [1% (w/v) citric acid, 15% (v/v) ethanol] for 5 min at RT and finally, rinsed three times in 100 % ethanol for 2-3 min at RT. After

staining, the membrane was dried, and the signal was visualized in a Molecular Imager FX System (Bio-Rad) using the Sypro Orange method.

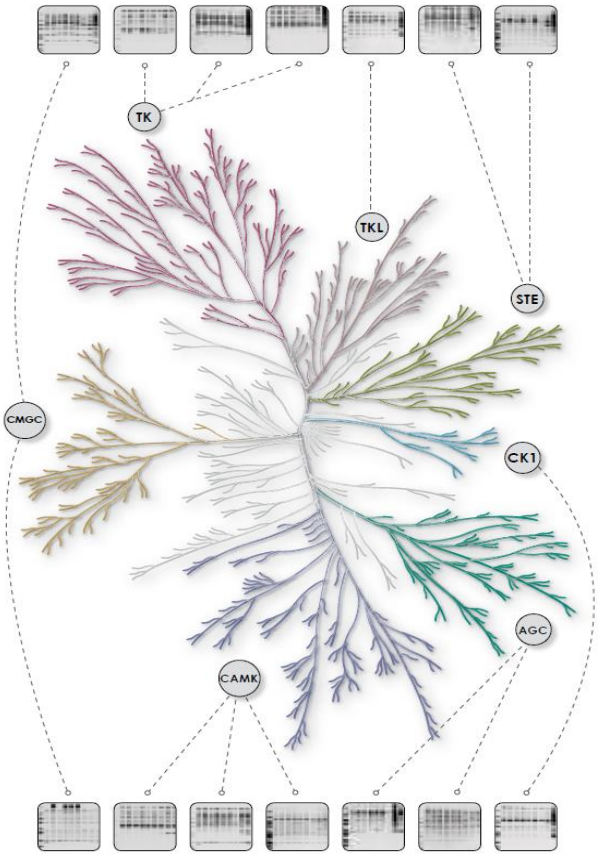
Table 2.2| List of primary antibodies used in kinome-wide detection of kinase-substrate phosphorylation.

Description	Motif	Species	Dilution	Source (ref.)
Phospho-Akt Substrate	RXX(S*/T*)	Rabbit	1:1,000	KinomeView Profiling Kit #9812 Cell Signaling Technology, Inc.
Phospho-Akt Substrate	RXRXX(S*/T*)	Rabbit		
Phospho-(Ser/Thr) AMPK Substrate	(L/M)XRXX(S*/T*), RXX(S*/T*)	Rabbit		
Phospho-(Ser) ATM/ATR Substrate	S*Q	Rabbit		
Phospho-(Ser/Thr) ATM/ATR Substrate	(S*/T*)QG, (S*/T*)Q	Rabbit		
Phospho-(Ser) CDKs Substrate	(K/H)S*P	Rabbit		
Phospho-(Ser/Thr) CK II Substrate	(S*/T*)DXE	Rabbit		
Phospho-MAPK/CDK Substrates	PXS*P, S*PX(K/R)	Rabbit		
Phospho-(Ser/Thr) PDK1 Docking Motif	(F/K)XX(F/Y)(S*/T*)(F/Y)	Mouse		
Phospho-PKA Substrate	(K/R)(K/R)X(S*/T*)	Rabbit		
Phospho-(Ser) PKC Substrate	(K/R)XS*X(K/R)	Rabbit		
Phospho-(Thr) PLK Binding Motif	ST*P	Rabbit		

Table 2.3| List of secondary antibodies conjugated with alkaline phosphatase used in Western blot analysis.

	Dilution	Source	Ref.
anti-rabbit	1:5,000	Jackson ImmunoResearch Laboratories, Inc.	115-055-003
anti-mouse	1:10,000		111-055-003

A)



B)

Kinase groups	Description
AGC	Containing Protein Kinases A, G and C (PKA, PKG and PKC) families
CAMK	Calcium/calmodulin-dependent protein kinases
CK1	Casein kinase 1
CMGC	Containing Cyclin-dependent kinases (CDK), Mitogen-activated protein kinases (MAPK), Glycogen synthase kinase 3 (GSK3) and Dual specificity protein kinase CLK1 families
STE	Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases
TK	Tyrosine kinase
TKL	Tyrosine kinase-like

Figure 2.1| The Human kinome. A) Representation of the kinome coverage provided by the phospho-motif antibodies included in the KinomeView kit (Kinome tree illustration included in the kit's datasheet by Cell Signaling Technology) B) Overview of the seven major kinase groups AGC, CAMK, CK1, CMGC, STE, TK, and TKL.

2.2.3. Data analysis

Analysis of the blots was performed using "Lane and bands" tool, accessible in ImageLab software (version 6.0.1, Bio-Rad). Lanes were manually fit, and most relevant protein bands and sections were selected and quantified considering uniform background signal subtraction. The adjusted phosphorylation values were automatically normalized to total lane protein detected by SERVA purple assay, minimizing any discrepancies in protein amount. The obtained measurements were used for a band/section-focused analysis. A total substrate phosphorylation -focused analysis was

also performed by considering the sum of the bands/sections. The results were presented as the ratio of the M26I- and E163K-DJ-1 conditions to the WT-DJ-1 stimulus condition.

Statistical analysis was performed using IBM® SPSS® Statistics Version 22. Statistical significance was considered relevant for p-values below a significance level of 10% using a Wilcoxon signed-rank test against a theoretical value of one (i.e., a ratio of 1 indicates no variation between conditions). Data is presented as Heatmaps acquired in Permut Matrix software (version 1.9.4, ATGC) [154], showing $\log_{10}$ median fold-change and indicating statistically significant results.

3. Results

Since the establishment of a link between DJ-1-related mutations and the development of an autosomal recessive type of Parkinson's disease, ample attention has been brought towards understanding the protein's native function and the correlation between its loss-of-function variants and the disease pathology [15]. Numerous cytoprotective roles have been attributed to DJ-1 [2, 22-26], unveiling a notable significance of the protein's well-functioning upon stress conditions, particularly of oxidative stress, the leading cause of neurodegeneration events in PD. Although most of the DJ-1 uncovered functions are pinned at the intracellular level, its presence in the extracellular space has been previously reported, hence indicating its secretion [112, 113, 155]. Further studies actually demonstrated the ability of DJ-1 to lead a protective response to stress from the extracellular milieu [114, 115], but there is still scarce information on the exact mechanisms performed by the protein for such action. With this in mind, this project aimed to study the biological role of extracellular DJ-1, primarily by understanding the interactome of exogenous DJ-1 under physiological and pathological conditions and also by characterizing the kinome-directed signaling pathways that can be activated by exogenous DJ-1 WT and PD related isoforms, M26I- and E163K-DJ-1.

3.1. Extracellular Interactome of DJ-1

With the intent to unravel potential DJ-1 extracellular interactors that could be implicated in a neuroprotective effect mediated by the protein at the extracellular level, an interatomic study was performed. To that, SH-SY5Y neuroblastoma cells-derived secretomes were used and biological conditions tested include: (1) cells treated with exogenous recombinant DJ-1 (a His-tag recombinant protein; control condition); (2) cells treated with exogenous DJ-1 and stimulated with 40 µM H_2O_2 (the stress condition); and (3) negative control condition given by non-stimulated cells; and additional negative control conditions: (4) cells in the presence of the His-tag peptide; (5) cells stimulated with 40 µM H_2O_2 and in the presence of the His-tag peptide. The conditioned medium of cells was subjected to a pull-down assay using a His-trap resin, followed by an MS-based proteomic identification and quantification of the purified material. The employed SWATH-MS strategy provides an accurate quantitative evaluation of the proteins, allowing for a high confidence distinction of DJ-1 specific binding partners from background collected proteins. This experimental setup also enabled the quantification of changes in DJ-1 interactor's protein-levels and interacting-levels with DJ-1 in the considered conditions.

A total of 805 proteins were confidently identified in all conditions established, from which 527 (corresponding to 65%) were quantified by the SWATH approach (Figure 3.1A). To eliminate the non-specific interactors, a first analysis considers the protein levels among the relevant experimental conditions (1 to 3): control, stress and negative-control (resin) conditions. The adjusted protein levels were calculated, implying the normalization of protein values to the levels of the internal standard protein used (MBP-GFP) [149]. From this data, a non-parametric Kruskal Wallis statistical analysis coupled to a Dunn's test was applied allowing to compare the 527 quantified proteins between the 3 mentioned conditions (Supplementary Table 7.1). A group of 224 proteins were selected in the Kruskal-Wallis analysis with significant changes (p-value under 0.1) in at least one of those combinations and were considered for further evaluation. Volcano plots in Figure 3.1B display the statistical analysis results of each combination assessed in the following Dunn's test. Proteins were considered as putative DJ-1 interactors if they met one of the following criteria: (i) have a p-value under 0.1 and are increased (log_{10} fold-change > 0) in DJ-1 conditions when compared to the negative control (resin) condition; or (ii) have a p-value under 0.1 in the control versus stress condition. This procedure is expected to discard the abundant proteins in the negative control condition, which may have established a bound to the resin and not specifically to the recombinant DJ-1, and to identify a dynamic modulation of the protein levels upon the control and oxidative conditions. According to the overall criteria, a selection of 21 proteins, highlighted in green, was considered as part of the exogenous DJ-1 interacting network (Figures 3.1B-C and Supplementary Table 7.1). Indeed, a considerable number of the 224 proteins were increased in the negative control sample (log_{10} fold change<0) in comparison to the recombinant DJ-1 conditions. This fact may point out for the resin's high reactivity towards the set of sample's proteins, instead of an exclusive specificity for the His-tagged DJ-1 recombinant protein. This issue of the resin was later confirmed in studies conducted in the laboratory (Supplementary Figure 7.1).

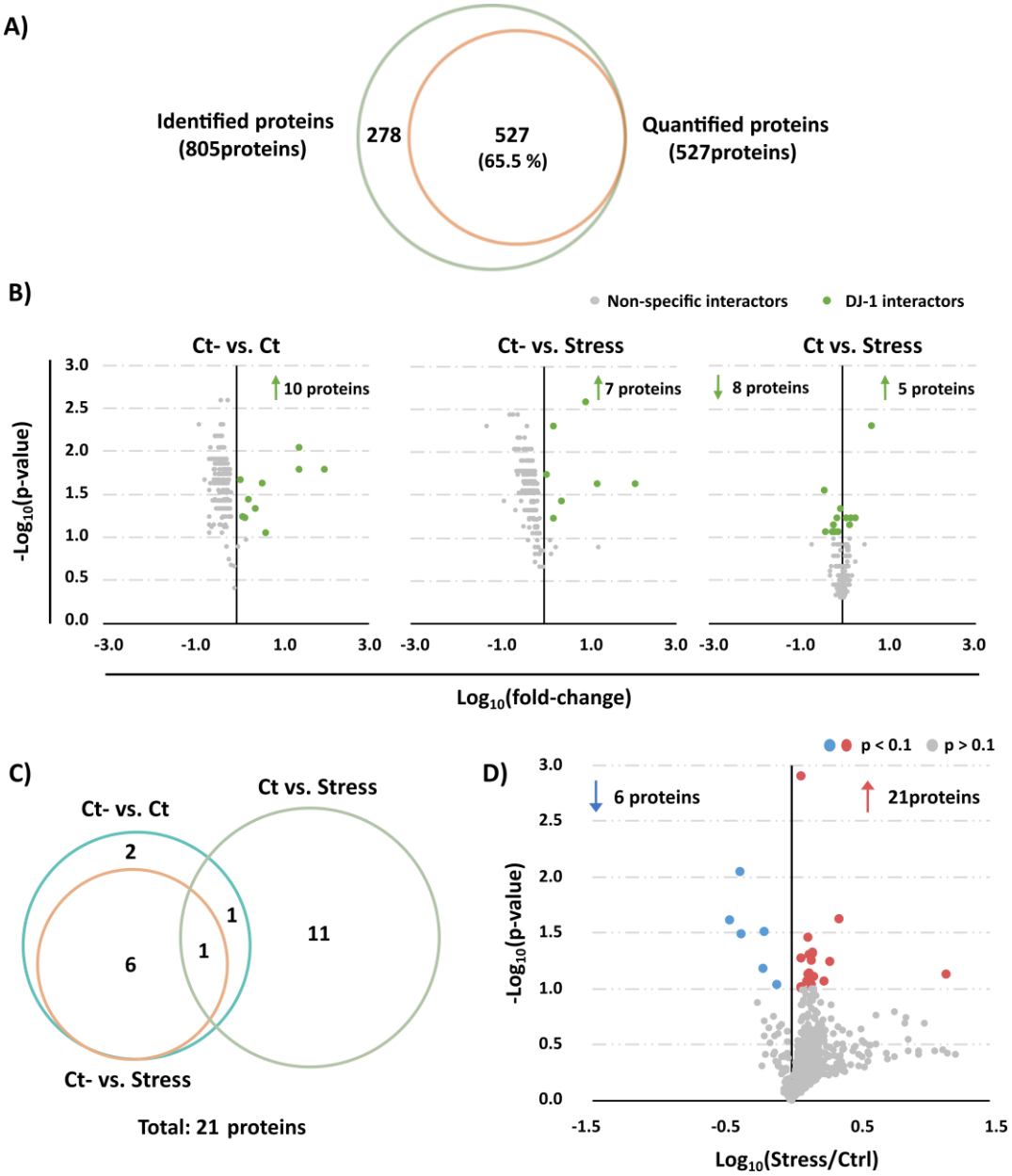


Figure 3.1| Analysis of the results acquired in the SWATH-MS interactomics study of extracellular DJ-1. A) Venn diagram showing the combination of the proteins identified in the IDA screening and in the SWATH analysis. A total of 527 proteins identified and quantified by the SWATH-MS analysis were considered for further analysis. B) Protein levels-based analysis: Volcano plots showing the comparison of 224 proteins (determined in the Kruskal-Wallis analysis with a p-value bellow 0.1) among the experimental conditions considered in this assay [negative control (resin, Ct-), control (Ct) and stress conditions]. Data points in green represent the proteins that met one of the criteria: (i) $p<0.1$ and an increased level (log_{10} fold-change>0) when compared to negative control; (ii) $p<0.1$ between the two conditions with recombinant DJ-1 (Ct vs Stress). Statistical significance was considered for $p<0.1$ using a Kruskal-Wallis statistical analysis coupled to a Dunn's post hoc test. C) Venn diagram combining the proteins selected in each combination assessed in the statistical analysis. A total of 21 highlighted proteins were considered as putative DJ-1 interactors. D) DJ-1 interacting levels-centered analysis: Volcano plot showing the results of a one-sample t-test statistical analysis for the comparison of all the 527 quantified proteins in the control and stress conditions. Statistical significance was considered for p-<0.1 and allowed to highlight 27 potential interacting partners of DJ-1. A total of 21 proteins established a significantly increased interaction with DJ-1 (red dots) upon the stress condition, while 6 proteins decreased their interaction with DJ-1 (blue dots).

Keeping in mind the concerns regarding the non-specificity aspect of the resin employed in the present study, an additional analysis was conducted to evaluate the possibility to avoid these issues with the use of a His-tag peptide as a "blocking agent" of the resin (Supplementary Figure 7.2). Similar statistical analysis was performed for the comparison between control and stress conditions in the presence of recombinant DJ-1, and the correspondent negative controls in the presence of the His-tag peptide (Supplementary Table 7.2). Overall, significant differences were observed for 168 proteins, from which only a total of 9 proteins met the criteria to be classified a putative interactor. This result reveals that the resin's high reactivity was not solved with the addition of the His-tag peptide; thus, the analysis process based on the previous approach (comparison solely against the resin) (Supplementary Figure 7.2 and Supplementary Table 7.3).

To further complement the previous results, a parallel analysis was performed focused on the protein's interacting levels with the recombinant DJ-1, aiming to identify proteins whose interactions with DJ-1 can be significantly modulated upon oxidative stress. From this approach, it could be inferred possible players and mechanisms of action by which DJ-1 is able to promote cell survival under such conditions. For that purpose, the interaction levels were determined by the normalization of the values of the 527 proteins quantified in the SWATH-MS to the levels of DJ-1 in each sample (adapted from [148]). This adjustment reflects a more accurate measurement of the interaction established between each protein and the exogenous recombinant DJ-1. The ratios between the paired datasets of stress and control conditions for each protein were calculated and a one-sample t-test was used against a theoretical value of one to verify for significant differences ($p < 0.1$) (Supplementary Table 7.4). The volcano plot of Figure 3.1D indicates that the statistical method employed allowed to highlight 27 potential interactor proteins of the exogenous DJ-1, which present a significant modulation of their interaction with DJ-1 upon the H_2O_2-induced stress condition. In general, the oxidative stress stimulation seemed to be accountable for the increased interaction of 21 proteins with DJ-1, whereas 6 proteins presented decreased DJ-1-interaction levels upon oxidative stress.

Altogether, the combination of the two analyses revealed a total of 43 potential DJ-1 exogenous binding partners, from which 16 were solely highlighted in the protein levels-focused analysis, 21 in the interacting levels-centred analysis, and 5 were commonly designated in both groups (Figure 3.2A). Since the present study is focused on the analysis of the secretome fraction of cells, the subcellular location of these 43 proteins was verified at the UniProt available database, sorting out a set of 28 potential DJ-1 interactors that have been associated with an extracellular space

localization (Figure 3.2B). The present experimental setup also led to the identification of 15 intracellular and/or membrane -only associated DJ-1 potential interactors, including: transcription regulation proteins (Transcription factor BTF3, Endothelial differentiation-related factor 1 and Host cell factor 1), ribosome-related proteins (40S ribosomal protein S7, Heterogeneous nuclear ribonucleoprotein A0, 60S ribosomal protein L18 and 40S ribosomal protein S24), spliceosome constituents (Serine/arginine-rich splicing factor 1, Splicing factor U2AF 65 kDa subunit, Splicing factor 3A subunit 2 and U6 snRNA-associated Sm-like protein LSm4), a proteosome activator (Proteasome activator complex subunit 3), among others (Disks large-associated protein 3, NAD-dependent malic enzyme, mitochondrial and Reticulocalbin-3). The presence of these proteins in the extracellular space and their interaction with the exogenous DJ-1 may be justified by cell damage/death events normally occurring in *in vitro* conditions that led to their release. For this reason and for the purpose of this work, these were not considered relevant and the remaining analysis is focused on the extracellular set of proteins, given by 28 of the 43 proteins (65% of the potential interactors identified). Protein's ID and its correspondent subcellular location are shown in Table 3.1, along with the determined levels of interaction with DJ-1. Among the set of proposed DJ-1 binding partners, heat shock protein 70 is a well-established interactor of DJ-1 [156], supporting a confident inclusion of the remaining proteins as potential DJ-1 interacting partners.

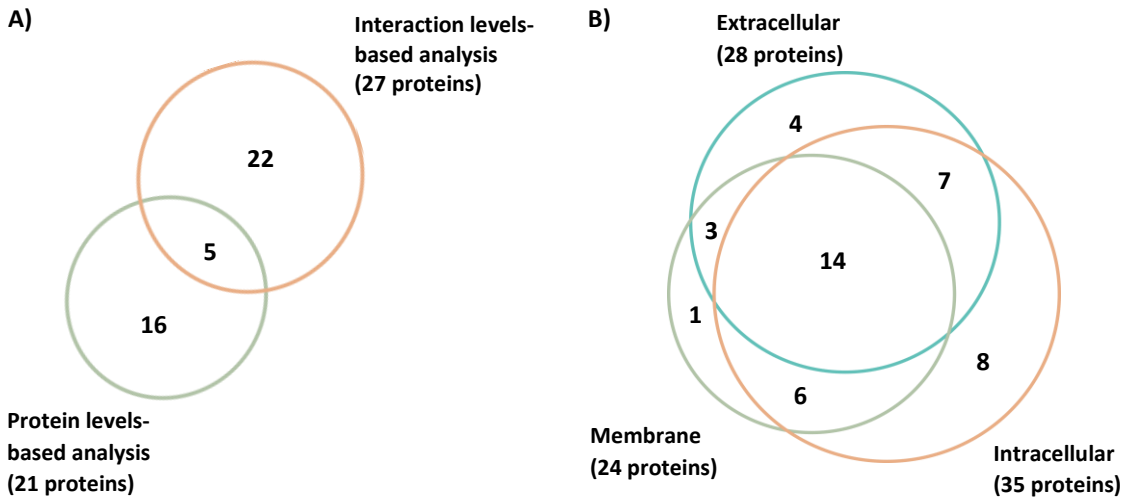


Figure 3.2| Extracellular DJ-1 putative interactors selected from both analyses applied. A) Venn diagram displaying the combination of the DJ-1 putative interactors identified in the two-analysis performed (i) considering the individual protein levels (21 proteins), and (ii) considering the interaction-levels (27 proteins) parameter. A total of 43 proteins were selected as putative DJ-1 interactors in this study; B) Venn diagram displaying the combination of the subcellular locations of the 43 identified proteins, sorted through extracellular (28 proteins), intracellular (35 proteins) and membrane (24 proteins) spaces.

Table 3.1| Proposed interactors of DJ-1 in the extracellular space identified in the SWATH-MS interatomic study, correspondent subcellular location annotation in UniProt database and determined interacting levels.

	Protein ID		Subcellular location			Interacting levels (FC)
	UniProt Accessions (number/name)	Protein name	Extra.	Memb.	Intra.	
Protein levels-based analysis	P07477|TRY1_HUMAN	Trypsin-1	x			
	P11142|HSP7C_HUMAN	Heat shock cognate 71 kDa protein	x	x	x	
	P04114|APOB_HUMAN	Apolipoprotein B-100	x	x	x	
	P14174|MIF_HUMAN	Macrophage migration inhibitory factor	x	x	x	
	Q9UK55|ZPI_HUMAN	Protein Z-dependent protease inhibitor	x		x	
	P62857|RS28_HUMAN	40S ribosomal protein S28	x		x	
	P62081|RS7_HUMAN	40S ribosomal protein S7		x	x	
	Q9Y4Z0|LSM4_HUMAN	U6 snRNA-associated Sm-like protein LSm4		x	x	
	O95886|DLGP3_HUMAN	Disks large-associated protein 3		x		
	P23368|MAOM_HUMAN	NAD-dependent malic enzyme, mitochondrial			x	
	Q15428|SF3A2_HUMAN	Splicing factor 3A subunit 2			x	
	Q96D15|RCN3_HUMAN	Reticulocalbin-3			x	
	P20290|BTF3_HUMAN	Transcription factor BTF3			x	
	P26368|U2AF2_HUMAN	Splicing factor U2AF 65 kDa subunit			x	
	Q13151|ROA0_HUMAN	Heterogeneous nuclear ribonucleoprotein A0			x	
	O60869|EDF1_HUMAN	Endothelial differentiation-related factor 1			x	
Interaction levels-based analysis	O15335|CHAD_HUMAN	Chondroadherin	x			1.20
	P04003|C4BPA_HUMAN	C4b-binding protein alpha chain	x	x		1.34
	P55287|CAD11_HUMAN	Cadherin-11	x	x		0.42
	P19338|NUCL_HUMAN	Nucleolin	x	x	x	1.38
	P00734|THRB_HUMAN	Prothrombin	x	x	x	1.33
	P00747|PLMN_HUMAN	Plasminogen	x	x	x	1.41
	P07996|TSP1_HUMAN	Thrombospondin-1	x	x	x	1.44
	Q9NTK5|OLA1_HUMAN	Obg-like ATPase 1	x	x	x	1.47
	P21333|FLNA_HUMAN	Filamin-A	x	x	x	1.20
	P43034|LIS1_HUMAN	Platelet-activating factor acetylhydrolase IB subunit alpha	x	x	x	1.44
	P15531|NDKA_HUMAN	Nucleoside diphosphate kinase A	x	x	x	1.38
	Q9HB71|CYBP_HUMAN	Calcyclin-binding protein	x	x	x	0.62
	Q00796|DHSO_HUMAN	Sorbitol dehydrogenase	x	x	x	1.43
	P05452|TETN_HUMAN	Tetranectin	x		x	1.45
	Q8NBS9|TXND5_HUMAN	Thioredoxin domain-containing protein 5	x		x	1.95
	Q92626|PXDN_HUMAN	Peroxidasin homolog	x		x	1.20
	Q13126|MTAP_HUMAN	S-methyl-5'-thioadenosine phosphorylase	x		x	1.44
	Q93099|HGD_HUMAN	Homogentisate 1,2-dioxygenase	x		x	1.20
	Q07020|RL18_HUMAN	60S ribosomal protein L18		x	x	14.77
	P51610|HCFC1_HUMAN	Host cell factor 1		x	x	1.33
	P62847|RS24_HUMAN	40S ribosomal protein S24		x	x	0.35
	Q07955|SRSF1_HUMAN	Serine/arginine-rich splicing factor 1			x	1.50
Common to both analysis	P35555|FBN1_HUMAN	Fibrillin-1	x			2.33
	Q12805|FBLN3_HUMAN	EGF-containing fibulin-like extracellular matrix protein 1	x			0.63
	P05060|SCG1_HUMAN	Secretogranin-1	x	x		0.79
	Q09328|MGT5A_HUMAN	Alpha-1,6-mannosylglycoprotein 6-beta-N acetylglucosaminyltransferase A	x	x	x	0.41
	P61289|PSME3_HUMAN	Proteasome activator complex subunit 3		x	x	1.80

Abbreviations: Extra., Extracellullar space; **Memb**., Membrane; **Intra.,** Intracellular space

To achieve a better understanding of the functional significance concerning the 28 putative interactors of the exogenous DJ-1, a gene ontology analysis was performed targeting the biological processes (Figure 3.3) and molecular function (Figure 3.4), in combination with a pathway analysis (Figure 3.5-3.11). In general, most of the proteins are associated with biological processes of cellular process, biological regulation, metabolic process, and response to stimulus (Figure 3.3; Supplementary Table 7.5). Signaling and cell adhesion-related processes have also been linked to some DJ-1 interactors identified.

A more comprehensive characterization was carried out by analyzing the subcategories of the most relevant biological processes. For instance, a set of 12 proteins linked to cellular processes was specified to be involved in the organization of extracellular matrix, membrane and cell junctions, as well as in the biogenesis of ribonucleoprotein complex and central nervous system (CNS) projection neuron axonogenesis (Figure 3.3B; Supplementary Table 7.6). In the context of biological regulation, 7 proteins were referenced in numerous signal transduction processes, namely of apoptosis and intracellular signaling, and pathways mediated by transforming growth factor beta (TGF-β) and integrins (Figure 3.3C; Supplementary Table 7.7). The 21 DJ-1 binding partners associated with metabolic mechanisms were considered to operate at the level of primary metabolism, organic substance and nitrogen compound metabolism, oxidation-reduction processes, among others (Figure 3.3D; Supplementary Table 7.8). Interestingly, mechanisms of response to stress stimulus, such as to endoplasmic reticulum stress, unfolded proteins, starvation, oxidative stress and DNA damage, were considered for 10 extracellular DJ-1 interactors (Figure 3.36; Supplementary Table 7.9). Finally, in the signaling category, the transmission of nerve impulse was highlighted along with a specified transmission mechanism across a chemical synapsis, among the 10 selected proteins (Figure 3.3F; Supplementary Table 7.10).

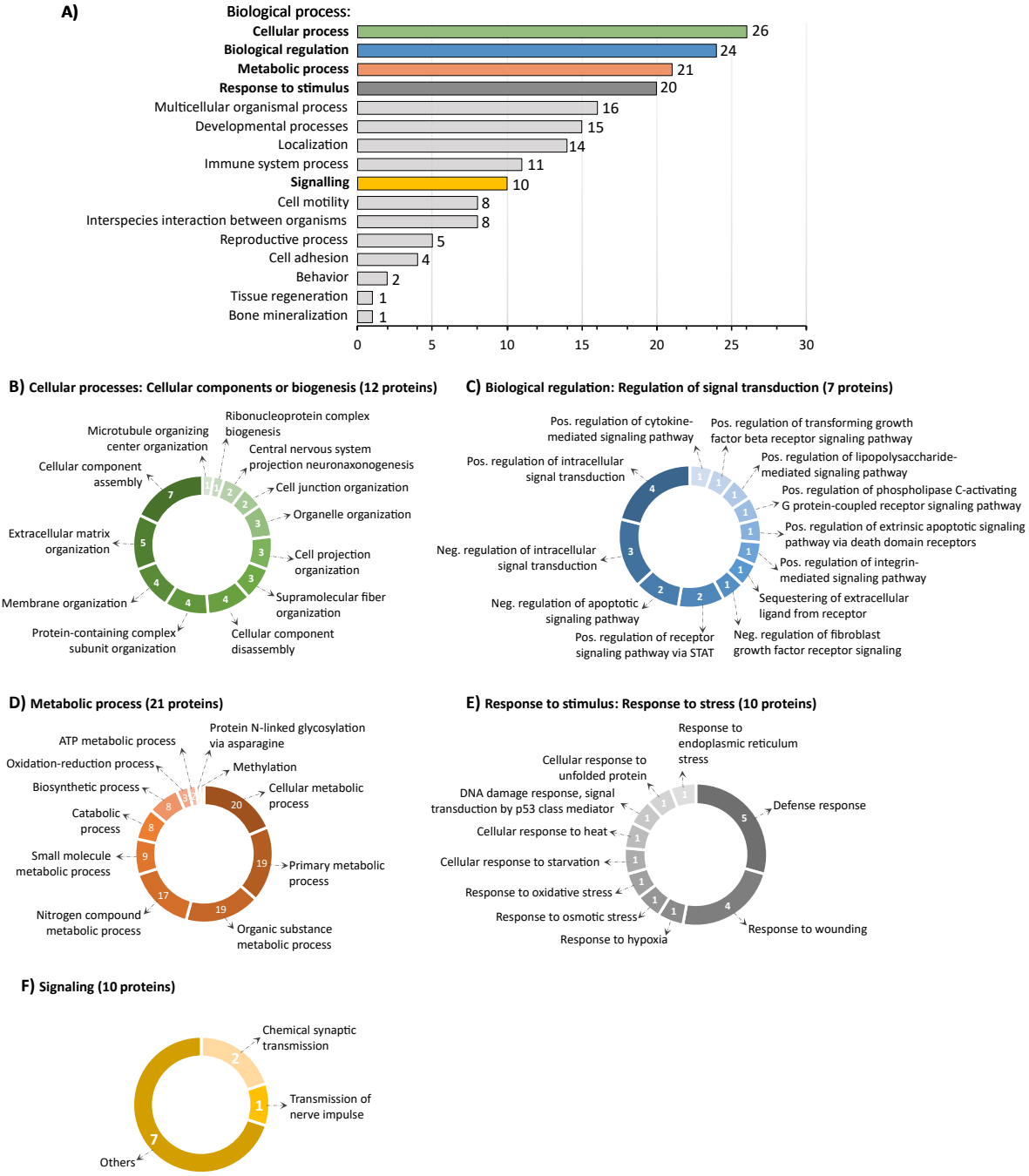


Figure 3.3| Gene ontology analysis for biological processes of the 28 proposed DJ-1 binding partners in the extracellular environment. A) Bar chart displaying the main biological processes attributed to the set of DJ-1 extracellular interactors selected. B) to F) Circular charts detailing the specific biological processes of the main categories, including B) cellular process, C) biological regulation (regulation of signaling transduction), D) metabolic process, E) response to stimulus (response to stress) and F) signaling, respectively. Gene ontology analysis was performed using the UniProt gene ontology tool.

Regarding the main molecular functions associated with the proposed members of the exogenous network of DJ-1, besides the interaction feature attributed to the majority of the proteins, signaling and structural functions were also highlighted (Figure 3.4A; Supplementary Table 7.11). Within the signaling receptor activity group, an association was established with proteins involved in epidermal growth factor and thrombospondin receptors activity, while others present a general receptor ligand activity (Figure 3.4B; Supplementary Table 7.12). In addition, extracellular matrix (ECM) structural components and elasticity conferring constituents were also emphasized within the set of 4 proteins with structural activity (Figure 3.4C; Supplementary Table 7.13). Other significant identified molecular functions among the potential DJ-1 interactors include protein folding chaperone, bromide peroxidase activity, and lipid transporter activity.

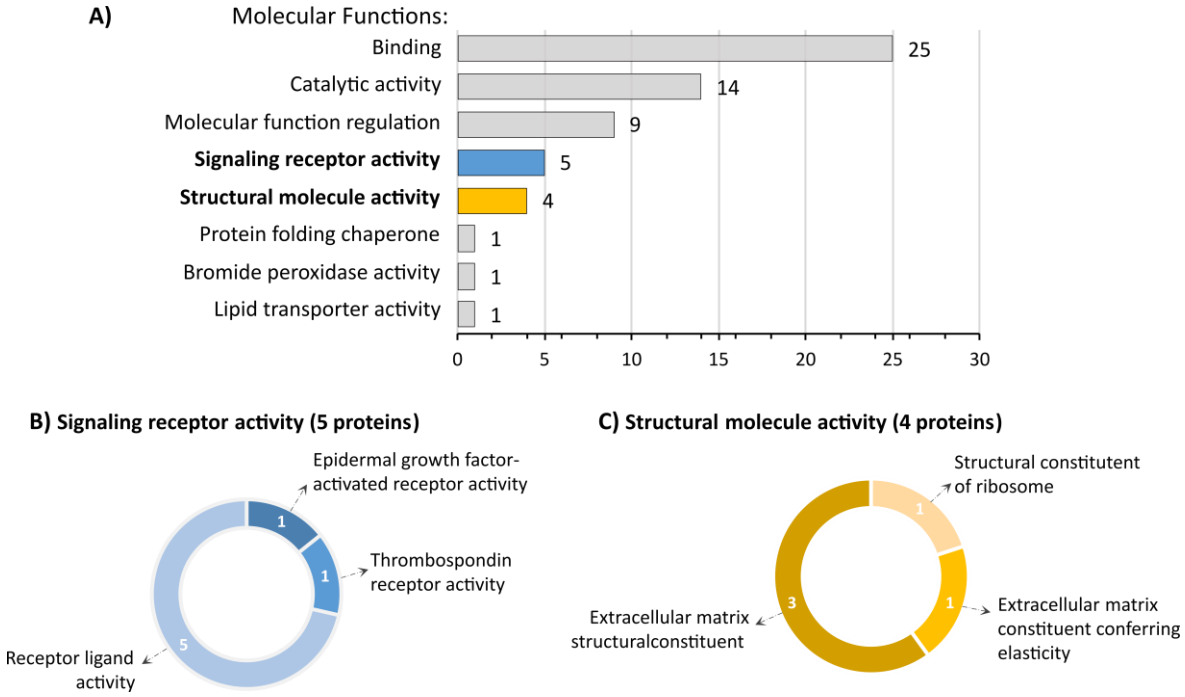


Figure 3.4| Gene ontology analysis for molecular functions of the 28 proposed DJ-1 binding partners in the extracellular environment. A) Bar chart showing the main molecular functions associated with the set of extracellular DJ-1 interactors selected. B) and C) Circular charts detailing the molecular functions of the most relevant categories of signaling receptor activity and structural activity, respectively. Gene ontology analysis was performed using the UniProt gene ontology tool.

Pathway characterization using PANTHER gene ontology tool reaffirms the implication of DJ-1-interactor proteins in the regulation of apoptosis, integrin and p53 -related pathways (Figure 3.5; Supplementary Table 7.14). The results further denote associations to the cadherin, Wnt, plasminogen and dopamine receptor signaling processes, as well as to Parkinson's disease-related pathways.

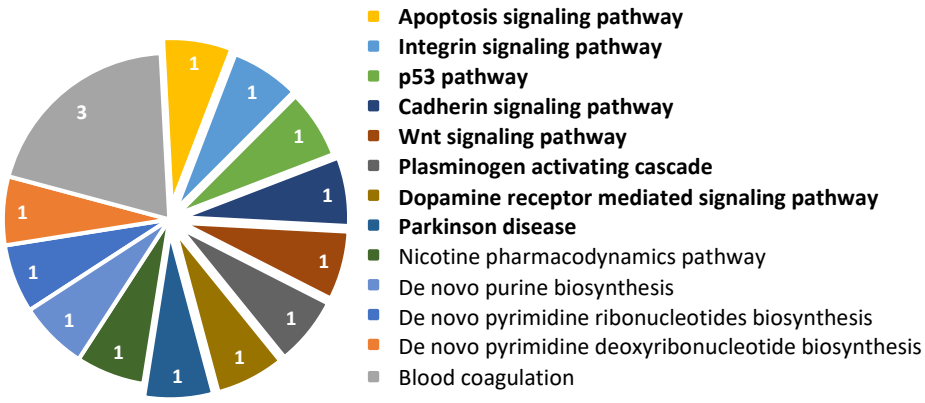


Figure 3.5| PANTHER pathway analysis of the 28 proposed DJ-1 binding partners in the extracellular environment. Highlighted in the chart are the most relevant biological pathways related to the function of the considered DJ-1 extracellular interactors.

An additional KEGG pathway analysis was also performed, and the most relevant annotation schemes demonstrating an extracellular action of the proposed DJ-1 interactors were selected (Figure 3.6-3.11). Thrombospondin-1 (TSP1) and chondroadherin (CHAD) were assigned to the ECM-receptor interaction, focal adhesion and PI3K-Akt signaling pathways (Figures 3.6-3.8). Moreover, TSP1 and fibrillin-1 (FBN1) were associated to the TGF-β pathway displayed in Figure 3.9. Plasminogen (PLMN), trypsin-1 (TRY1) and prothrombin (THBR) were indicated as extracellular components of the neuroactive ligand-receptor pathway (Figure 3.10) and THRB was further mapped to the phospholipase D signaling pathway (Figure 3.11). On the other hand, other interactors identified of the exogenous DJ-1, such as TSP1, Hsp70, filamin-A (FLNA) and calcyclin binding protein (CACYBP), were shown to play a role at the intracellular level in additional pathways, including MAPK, Rasp1, p53 and Wnt pathways, displayed in Supplementary Figures 7.3-7.6.

In summary, despite some problems regarding the pull-down purification technique, this interatomic study allowed to highlight a set of 28 potential DJ-1 binding partners in the extracellular space. In addition, the SWATH-MS-based quantification also allowed to assess the modulation of some of those proposed DJ-1 interactors in response to oxidative stress conditions.

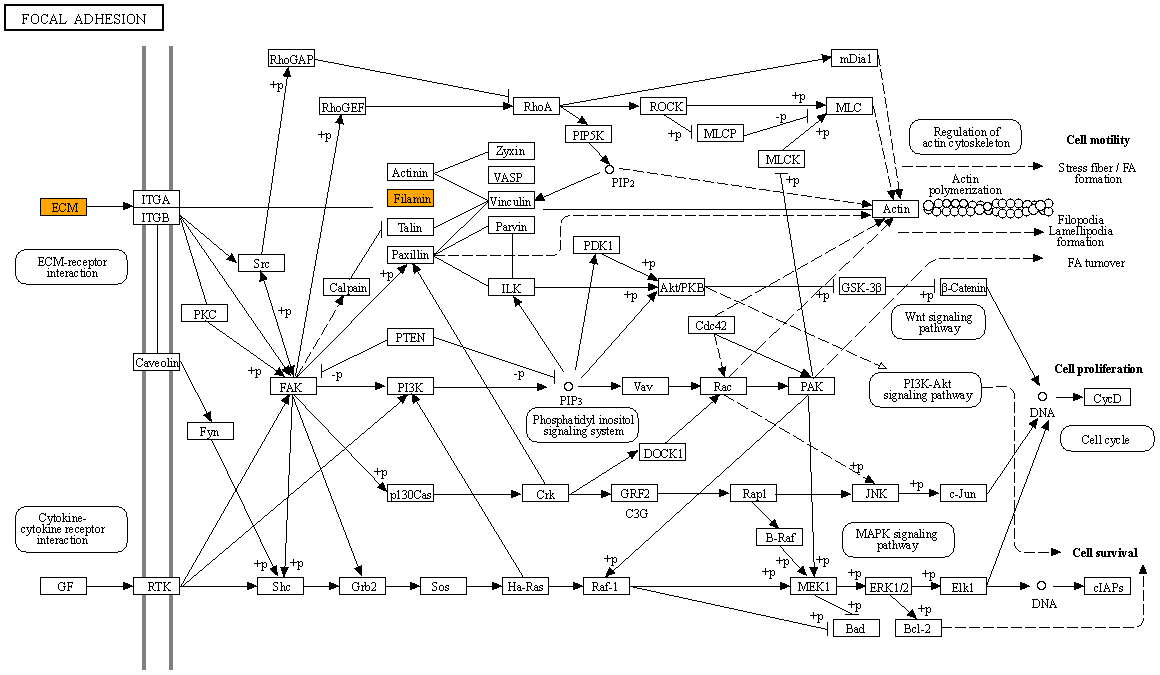


Figure 3.6| KEGG pathway analysis associating chondroadherin (CHAD) and thrombospondin-1 (TSP1/THBS) to ECM-receptor interactors. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

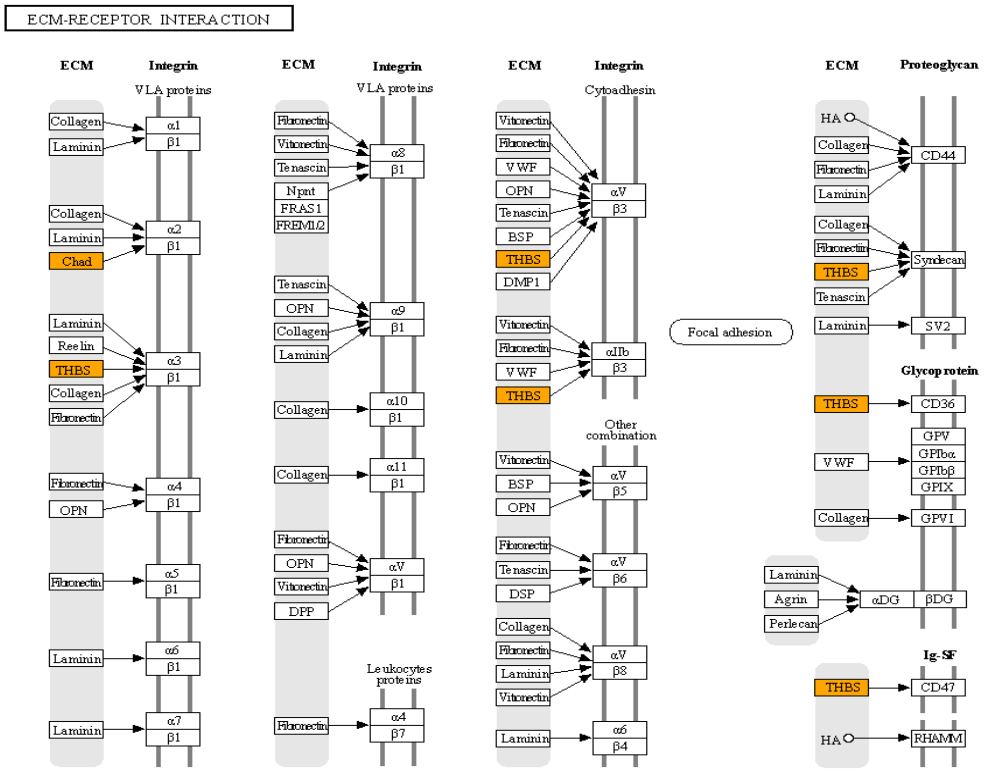


Figure 3.7| KEGG pathway analysis demonstrating the association of ECM components [chondroadherin (CHAD) and thrombospondin-1 (TSP1/THBS1)], and filamin-A (FLNA) to focal adhesion processes. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

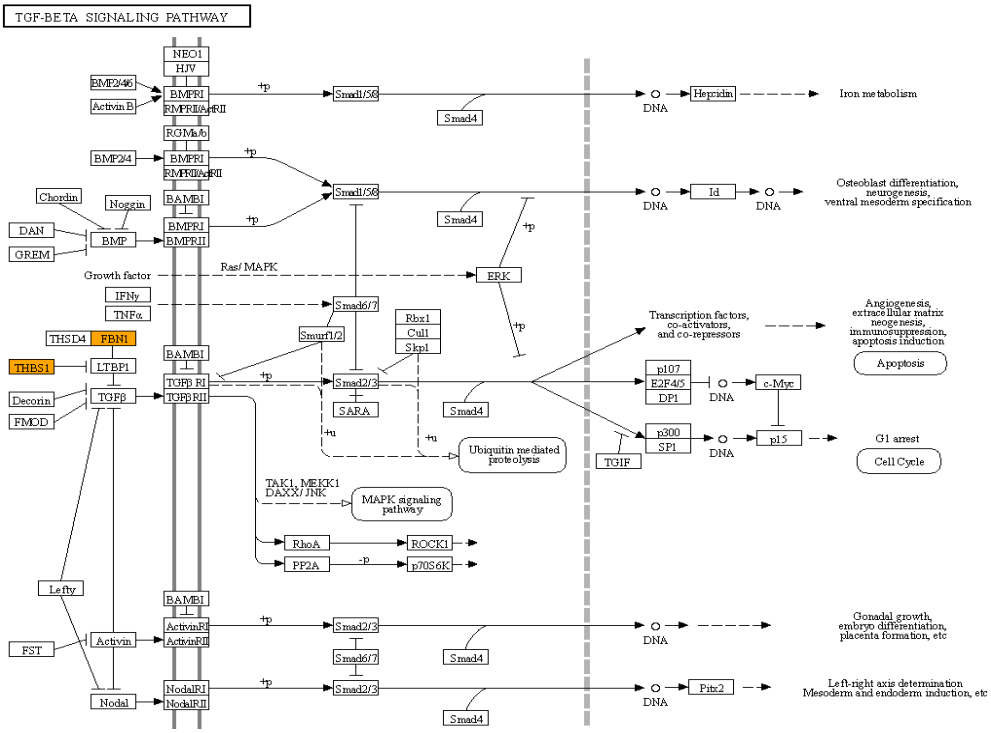


Figure 3.9| KEGG pathway analysis associating thrombospondin-1 (TSP1/THBS1) and fibrillin-1 (FBN1) to the TGF-β pathway. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

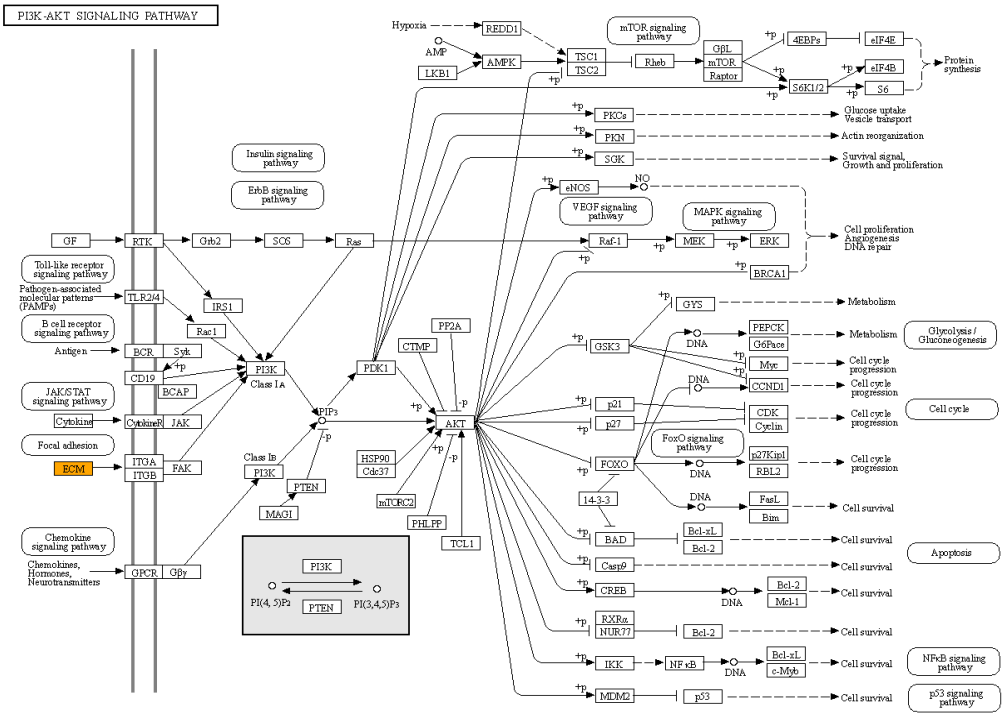


Figure 3.8| KEGG pathway analysis associating ECM components [chondroadherin (CHAD) and thrombospondin-1 (TSP1/THBS1)] to the PI3K/Akt pathway. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

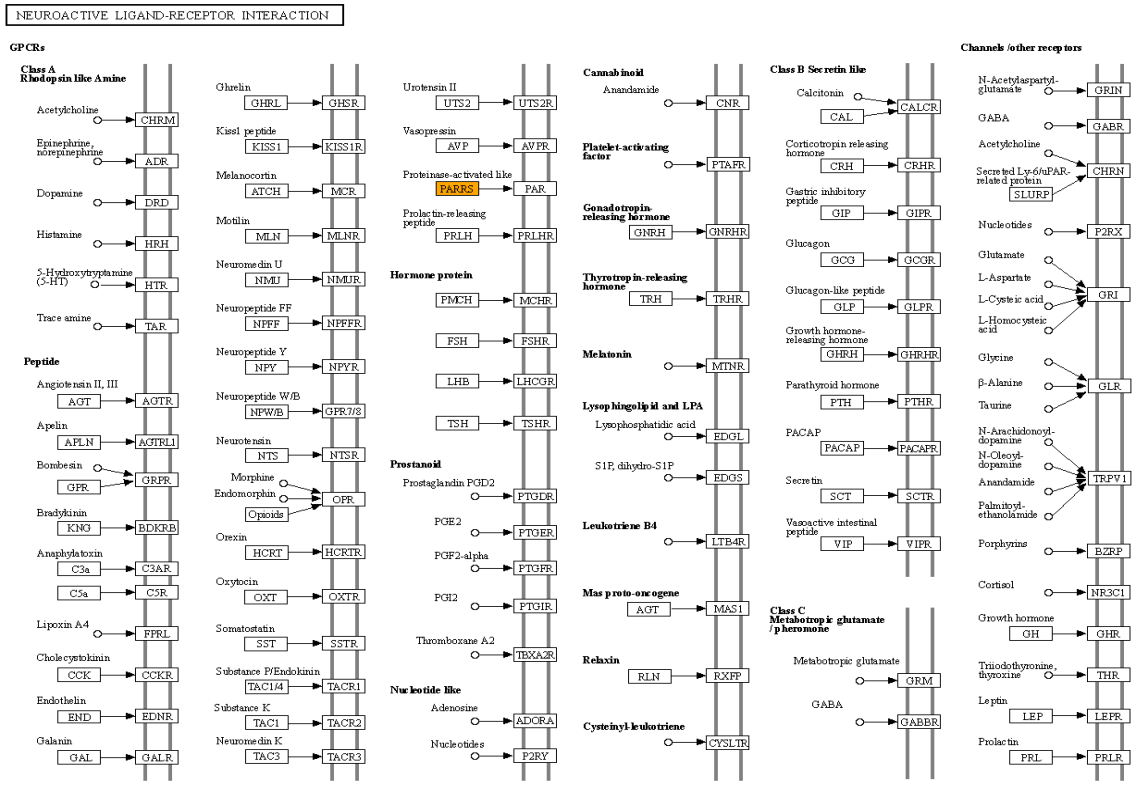


Figure 3.10| KEGG pathway analysis associating proteinase-activated like neuroactive ligands prothrombin (THRB/F2), plasminogen (PLMN) and trypsin-1 (TRY1), also known as serine protease 1, to neuroactive ligand-receptor interactions. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

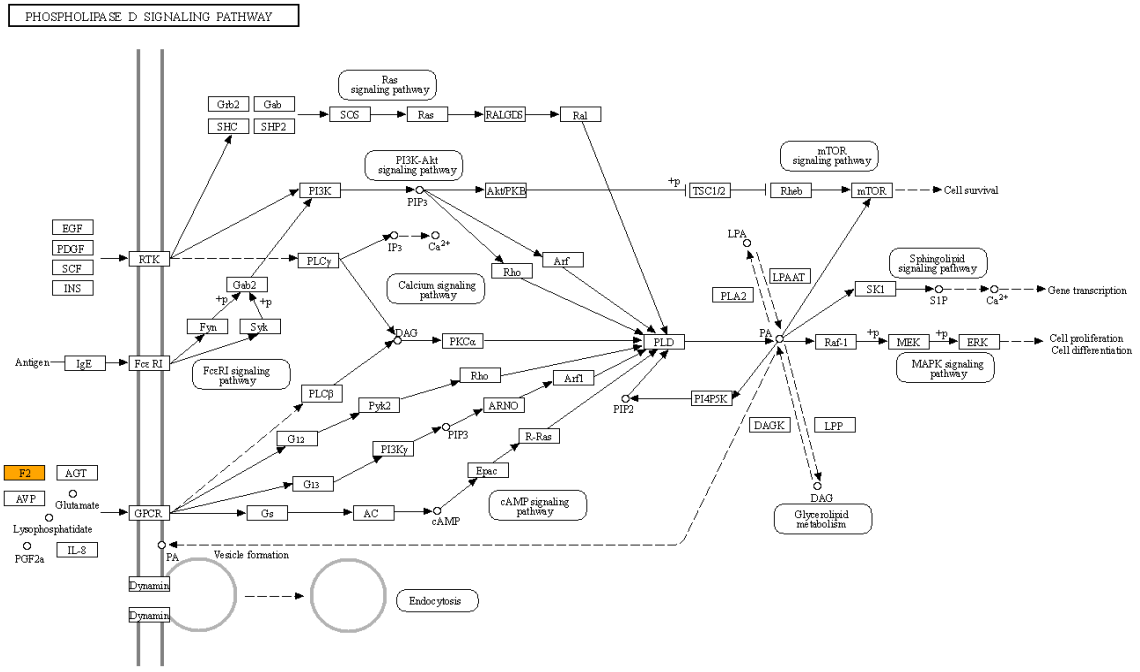


Figure 3.11| KEGG pathway analysis associating prothrombin (THRB), also known as coagulation factor 2 (F2), to the phospholipase D signaling pathway. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

3.2. Differential changes in intracellular kinome signaling induced by extracellular DJ-1 pathological mutants

Further work was conducted to clarify the extracellular role of DJ-1 in a context of PD, focusing on the potential ability of the protein to modulate intracellular signaling from the extracellular space. This study entailed a phospho-motif profiling assay to assess a kinome wide view of cellular phosphorylation, thereby allowing to pinpoint kinase-directed signaling pathways that may be mediated by DJ-1's extracellular activity and altered by PD-related mutations. This experimental setup was intended to verify the impairment of signaling mechanisms led by M26I- and E163K-DJ-1 isoforms.

To that purpose, protein extracts were obtained from SH-SY5Y cells that were stimulated extracellularly with WT-, M26I-, and E163K-DJ-1, and analyzed by immunoblotting using the KinomeView kit of 12 phospho-motif antibodies (Figure 3.12, 3.13, Supplementary Figures 7.7 and 7.8). This procedure allowed to detect the WT and mutant DJ-1-induced phosphoproteins as substrates of a set of kinase families, including: Protein kinase B (PKB/Akt) (Figure 3.13A and Supplementary Figures 7.7A and 7.8A-B), AMP-activated protein kinase (AMPK) (Figure 3.13B and Supplementary Figure 7.8C), Ataxia telangiectasia mutated kinase /Ataxia telangiectasia and Rad3-related kinase (ATM/ATR) (Figure 3.13C and Supplementary Figure 7.7B and 7.8D-E), Cyclin-dependent kinases (CDKs) (Figure 3.13D and Supplementary Figure 7.8F), Casein Kinase II (CK2) (Figure 3.34E and Supplementary Figure 7.8G), Mitogen-activated protein kinases (MAPK)/CDK (Supplementary Figure 7.7C and 7.8H), Phosphoinositide-dependent protein kinase 1 (PDK1) (Supplementary Figure 7.7D and 7.8I), Protein kinase A (PKA) (Supplementary Figure 7.7E and 7.8J), Protein kinase C (PKC) (Figure 3.13F and Supplementary Figure 7.8K) and Polo-like kinase (PLK) (Supplementary Figure 7.7F and 7.8L). The analysis of the results was carried out by quantifying the intensity of immunoreactive bands or sections that correspond to different phosphorylated substrates (Figure 3.13 and Supplementary Figure 7.7). In addition, the combination of those bands/regions per lane was used to determine the overall substrate phosphorylation levels for each kinase (Figure 3.12). From this data, a Wilcoxon Signed-Rank statistical test was applied to allow the comparison of intracellular phosphorylation levels induced by the exogenous addition of DJ-1 PD-related variants, in relation to the WT form results.

In general, from the obtained results, it is observed a decrease in kinase-substrate phosphorylation and, consequently, in kinases' activity, in the presence of mutant DJ-1 isoforms. In detail, the overall phosphoprotein analysis shows that E163K-DJ-1 treatment led to the reduced substrate phosphorylation by Akt, AMPK, ATM/ATR, CK2, CDKs, and PKC in cells, while M26I-DJ-1 condition presented significantly reduced alteration in substrate phosphorylation mediated by PKC.

Notably, however, both mutant DJ-1 variants induced significant alteration of phosphorylation levels in a number of substrates of nearly all of the analysed kinases. Most were noticed to be modulated in the same manner in the two PD-related conditions (Figure 3.13 and Supplementary Figure 7.7). Indeed, the exogenous stimulation of cells with E163K-DJ-1 ultimately caused significant alterations in the phosphorylation of particular substrates or substrate's groups of all the considered kinases in relation to the WT condition. In turn, the M26I-DJ-1 variant induced a significantly altered phosphorylation profile in most analyzed kinase motifs, with the exception of the results related to the PLK and the S*Q phospho-motif ATM/ATR, where no significant alterations were detected in comparison to the WT condition.

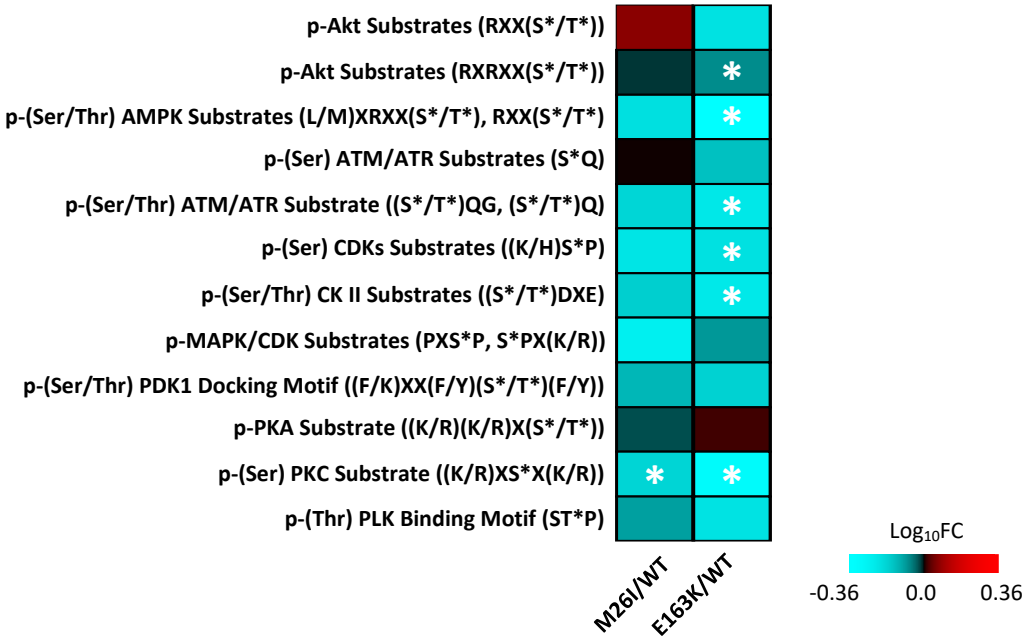


Figure 3.12| Overall kinase substrate's phosphorylation levels induced by extracellular stimulation with M26I and E163K-DJ-1. An immunoblot-based phospho-motif profiling assay, using 12 phospho-motif antibodies of the KinomeView kit, was performed to assess the alteration in the kinase substrate's phosphorylation levels induced by DJ-1 mutant related with the development of PD. The displayed heatmap presents the results of a Wilcoxon signed-rank test applied to compare phosphorylation activity of each kinase between M26I- and E163K-DJ-1 conditions in relation to the WT-DJ-1 condition. Substrates of Akt, AMPK, ATM/ATR, CDKs, CK2, and PKC presented significantly reduced phosphorylation among the DJ-1 mutant conditions compared to the WT-DJ-1 condition. * $p < 0.1$.

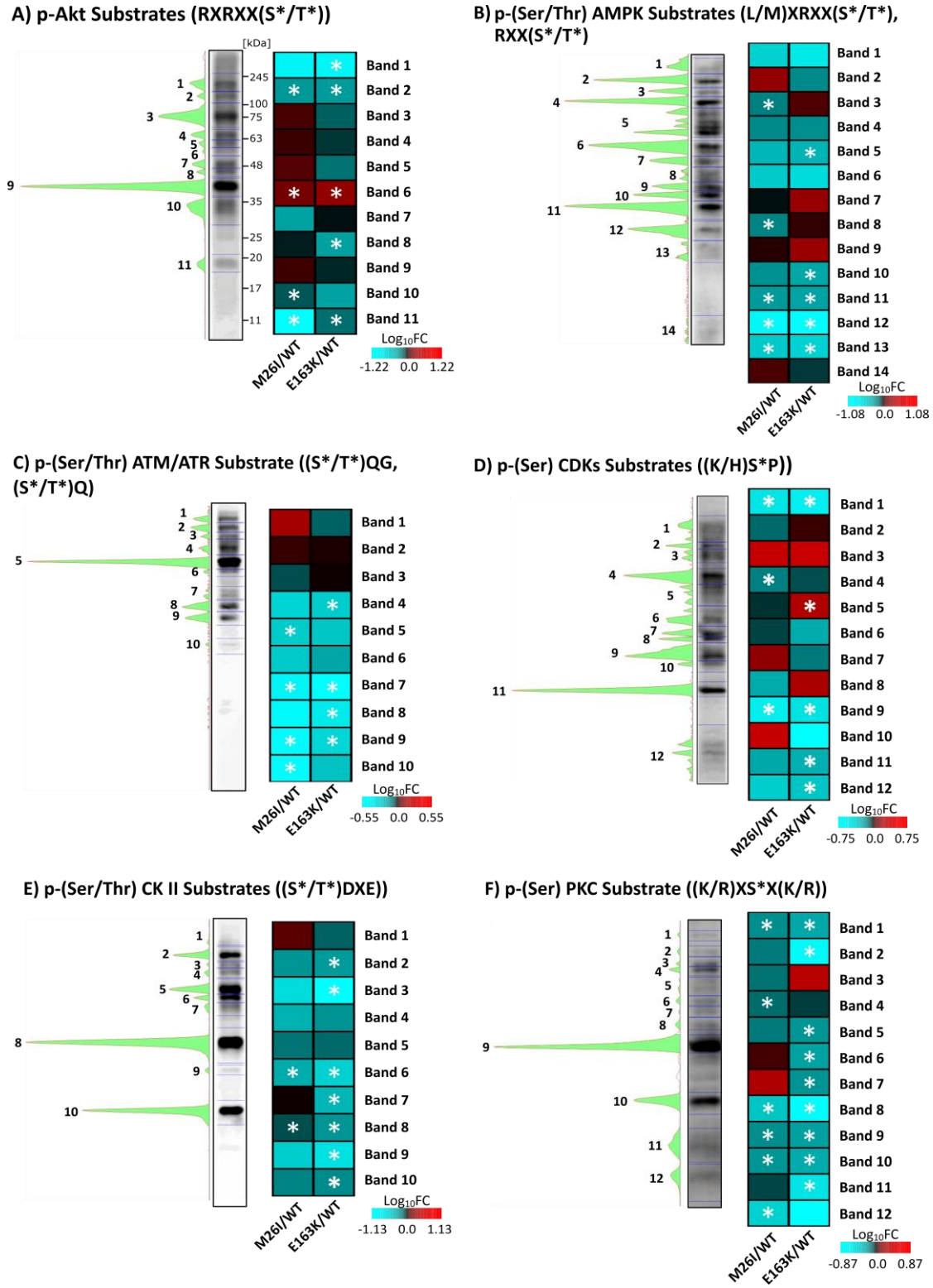


Figure 3.13| Analysis of the phospho-motif profiling assay results of the kinases substrates or substrates groups that displayed an overall significant phosphorylation profile alteration. Heatmaps displaying the extracellular M26I- and E163K-DJ-1 induced changes in the phosphorylation profile of substrates of Akt (A), AMPK (B), ATM/ATR (C), CDKs (D), CK2 (E), and PKC (F) kinase families. Data were analyzed using the ImageLab software and a representative lane with bands' intensities is presented. Statistical analysis was performed by a Wilcoxon signed-rank test to compare the phosphorylation levels of each substrate or substrate group between M26I- and E163K-DJ-1 conditions in relation to the WT-DJ-1 condition. * $p<0.1$.

Ultimately, the observations made based on the present study indicate that the PD-related mutation E163K of DJ-1 caused a more contrasting behaviour in relation to the WT-DJ-1 than M26I-DJ-1, regarding the ability of the protein to modulate signaling mechanisms from the extracellular environment.

4. Discussion

Parkinson's disease is a neurodegenerative movement disorder characterized by the selective loss of dopaminergic neurons at the *substantia nigra pars compacta* and intraneuronal toxic assemblies (mostly of α-synuclein), known as Lewy bodies [2, 3]. Despite extensive research, the exact molecular mechanisms underlying the disease's pathology are still largely unknown; nonetheless, oxidative stress, mitochondrial dysfunction, and proteins misfolding and aggregation have been pointed out as the main pathways that lead up to the neurodegeneration events [13, 14]. Among the monogenic forms of PD, mutations related to DJ-1 have been attributed to the biological impairment of the protein's neuroprotective role [15]. As an oxidative stress sensor, DJ-1 can coordinate protective responses, some of which contribute to mitochondrial homeostasis [25], and mediating crucial signaling pathways and transcription events [2]. The subcellular location of DJ-1 denotes that most of its action takes place at the intracellular level, yet studies have reported the protein's ability to lead a defensive response in the extracellular environment [114, 115]. However, the exact mechanisms by which extracellular DJ-1 provides such protective effect are still unclear.

For this reason, this research project firstly involved an interactomics study of exogenously added DJ-1, which allowed the identification of putative binding partners in the extracellular space. Furthermore, it was also evaluated the modulation of such DJ-1 extracellular interactions upon control and H_2O_2-induced stress conditions, with the intent to unravel potential mechanisms involved in the mitigation of cytotoxic insults. In this way, SH-SY5Y cells-derived secretomes were used for the experimental setup of this study, implying a set of specified treatment conditions. The SH-SY5Y cell line is a well-established *in vitro* model used in PD-related research, a fact owing to its human origin, catecholaminergic neuronal properties, and ease of maintenance [157]. The AP-SWATH-MS methodology applied in this study enabled the quantification of 527 proteins, a considerable fraction given by 65% of the total amount of proteins identified in the precedent IDA scanning (805 proteins) (Figure 3.1A). The selection of putative DJ-1 interactors from the acquired data was based on a combination of the results from 2 types of analysis taking into consideration: (i) the protein levels and (ii) the DJ-1-interacting levels.

Firstly, a protein-level-centered analysis was intended to recognize the truly putative DJ-1 binding partners while excluding potential non-specific ones. From this analysis, no more than 21 proteins were designated as putative DJ-1 interactors (Figure 3.1B-C). Indeed, the presented results point out a high reactivity of the resin with the proteins present in the samples, besides the His-tagged DJ-1, as the great majority of proteins were substantially detected bound to the resin in the negative control sample. This fact suggests that an overestimation of the non-specific DJ-1

interactors might have occurred. Later on, this assumption was confirmed by pull-down methodology tests carried out in the laboratory (Supplementary Figure 7.1). These revealed a great affinity of the His resin with most of the protein content (70%) identified in the test sample, while streptavidin resin showed a much lower background interaction, with less than 8%. With this in mind, the present approach further entailed an evaluation of the use of a condition with the His-tag peptide as a negative control instead of solely the resin, and similar results were achieved (Supplementary Figure 7.2). Altogether, these observations seem to point out a high reactivity issue regarding the matrix used in the pull-down assays, hampering the identification of the complete set of potential DJ-1 binding partners by overestimating non-specific ones.

To study the impact of an oxidative stimuli in DJ-1 interactome, a further analysis was centered on the interacting levels determined between each identified protein and the recombinant DJ-1 upon control and stress conditions. The study of the results allowed to highlight a group of 27 potential interactors of DJ-1 (Figure 3.1D). Such interactions were noticed to be significantly modulated by oxidative stress, hence indicating the triggering of potential mechanisms of action of DJ-1 that may mediate its protective effect. In general, it was observed an enhancement of the interactions with DJ-1 under stress, while only in 6 cases there was a weakening of the interaction.

Overall, the combination of the extracellular DJ-1 interactor candidates achieved from both analyses generated a list of 43 proteins (Figure 3.2A and Table 3.1). The assessment of such proteins' subcellular location disclosed 28 potential DJ-1 binding partners with annotated extracellular localization (Figure 3.2B and Table 3.1). In addition, 15 proteins identified in the secretomes were linked to intracellular and/or membrane-only locations, and their presence in the extracellular environment may be justified by a mediated release by distressed, damaged, or dying cells (Table 3.1). Nevertheless, it should be considered that these proteins might accomplish relevant functional roles through their interaction with DJ-1. This matter should be explored in future work using total protein extracts that may reveal additional important DJ-1-mediated mechanisms carried out by those proteins at other cellular levels.

Henceforth, a functional study was conducted focused on the extracellular 28 proposed DJ-1 interactors. This study revealed a consistent involvement of those proteins in cellular tasks of metabolism, extracellular matrix (ECM)-related and signaling processes, as well as in stress response mechanisms (Figure 3.3-3.11). Among the uncovered DJ-1-interactors with a crucial involvement in stress response mechanisms, heat shock protein 70 (Hsp70) is already a well-established and validated interactor of DJ-1 [156], supporting the reliability of the new DJ-1

interactors identified in this study. In fact, Hsp70 is a molecular chaperone with an anti-apoptotic function that takes part in a protective action in neurodegenerative diseases, including PD [158]. A particular work demonstrated that Hsp70 could chaperone α-synuclein in the extracellular environment and prevent its toxic oligomerization process [159]. Therefore, an association between DJ-1 and Hsp70 in the extracellular space may entail a DJ-1 mechanism to modulate and potentially enhance the activity of Hsp70 against α-synuclein toxicity.

Moreover, the highlighted set of DJ-1 interacting partners involved in ECM organization and functional properties (Figures 3.3B, 3.4C, 3.6 and 3.7, and Supplementary Tables 7.6 and 7.12), includes plasminogen (PLMN), thrombospondin-1 (TSP1), peroxidasin (PXDN), fibrillin-1 (FBN1), chondroadherin (CHAD) and EGF-containing fibulin-like extracellular matrix protein 1 (FBLN3). These ECM-related proteins, along with Hsp70, cadherin-11 (CAD11), thrombin (THRB) and macrophage migration inhibitory factor (MIF), were further implicated in numerous signaling events (Figures 3.3C, 3.3F, 3.4B, 3.5 and 3.8-11, and Supplementary Tables 7.7, 7.10 and 7.13), denoting a clear influence of DJ-1 in such processes in the extracellular environment.

In addition to its structural role, the ECM takes part in most basic cell behaviors, implying cell proliferation, signaling, adhesion, migration, and death mediating processes. Upon specific cues, such as oxidative stress, dynamic interactions between the cells and ECM mediate adaptive ECM remodeling processes assuring tissue function and organization [160]. Even so, the extracellular space elements are understood to be more vulnerable to oxidative stress insults when compared to intracellular compartments due to a less extent of antioxidant defense mechanism [160]. This fact was also evidenced in an experimental work previously conducted in the laboratory and currently under analysis for a research article submission. This work focuses on the study of the alterations of the intracellular and extracellular proteomes induced by an acute stimulation of oxidative stress in cells. From the previously obtained results' analysis we observed a differential adaptation of the intracellular and extracellular proteomes to stress conditions, with a higher number of secreted proteins exhibited both altered protein levels and redox status. Our results confirm that the extracellular space is particularly affected by the stress stimulation, and a clear tendency was denoted for ECM remodeling processes (manuscript under preparation – Annex II).

The process of ECM remodeling is mediated mainly by two proteolytic systems, the plasminogen/plasmin system and the metalloproteins (MMP) system [161]. The present work reported a potential interaction between DJ-1 and plasminogen, which increases upon oxidative stress (Table 3.1). Plasminogen is the zymogen of an extracellular serine protease, plasmin, whose

activation is mediated by tissue-type plasminogen activator (tPA) or urokinase plasminogen activator (uPA) [162]. In the CNS, plasmin is physiologically and pathologically important for neuronal development, synaptic plasticity, and excitotoxicity events [163]. Although a concrete association between the plasmin system in the CNS and PD is yet to be established, studies suggest a dysfunction of the system: plasmin has been shown to be involved in the proteolytic clearance of extracellular α-synuclein, inhibiting its intercellular toxic spreading and glial activation, while suppression of the plasmin system by extracellular α-synuclein was also referenced [164]. To date, DJ-1's involvement with the plasmin system has been reported solely in a cancer model, proposing a positive correlation between DJ-1 and the expression and activity of plasminogen cascade activator, uPA [165]. This study underlined the DJ-1's role of signaling modulation in crucial cellular events, as invasion and metastasis of pancreatic cancer cells were observed to be promoted by a DJ-1-directed activation of the SRC/ERK/uPA pathway [165]. Such DJ-1 role should be further evaluated in neuronal context to elucidate if it provides important insights about the DJ-1-driven modulation of plasminogen cascade (Figure 3.5) and its implication in the pathology of PD. Nonetheless, the present work's observations may be pointing out a different potential manner of DJ-1's-modulation of plasminogen activity by binding to it in the extracellular space and whose interaction was shown to be enhanced upon oxidative stress. In this sense, it might be feasible to hypothesize that DJ-1 could be able to promote plasminogen activation to plasmin, in order to manage defensive ECM remodelling, important signaling processes and the extracellular α-synuclein content in a PD context.

The oxidative stimulus was also accountable for an increased DJ-1 association with peroxidasin (PXDN) (Table 3.1), which may entail another DJ-1 defensive mechanism carried out at the extracellular space by this interactor. Actually, DJ-1-PXDN interaction was already established in a preliminary screening of the DJ-1 extracellular interactome conducted in the laboratory [117]. PXDN is a homotrimeric multidomain heme peroxidase detected in the ECM network, and it is involved in essential tissue biosynthetic processes [166, 167]. PXDN has been reported to catalyze the formation of sulfilimine crosslinks in collagen type IV within the basement membrane, using conventionally toxic hypohalous acid intermediates [166]. Further studies have endorsed the PXDN role as a ROS scavenger preventing cellular apoptosis events [168, 169]. Interestingly, one particular work proposed PXDN as a product protein of the redox-sensitive Nrf2 transcription factor and as a part of its well-characterized antioxidant mechanism [169], which is known to be regulated by DJ-1 [90, 93, 98]. Indeed, a correlation was established between enhanced Nrf2 nuclear translocation and increased PXDN expression in HEK293 and HeLa cells subjected to an H_2O_2 stimulus [169].

Altogether, these findings lead to the assumption that, while DJ-1 may be able to trigger PXDN expression by activating the Nrf2 cascade at the intracellular level, it may also be responsible for modulating PXDN activity in the extracellular space, potentially enhancing its antioxidant effect in response to an oxidative stimulus, as observed in the present work. Although PXDN is ubiquitously expressed in the human body, including the brain [170], to date, no relation has been found between PXDN and a potential neurodegeneration impact. Future work should, therefore, evaluate these propositions in a neuronal setting.

Thrombospondin-1 (TSP1) is another identified ECM-related component that established a stress-modulated interaction with DJ-1 (Table 3.1). TSP1 is an adhesive glycoprotein previously implied in events of synaptogenesis and axonal sprouting during CNS development [171, 172]. Upon specific stimulation, TSP1 functions are modulated by binding to specific ligands, including other ECM components (e.g., plasminogen), cell receptors (e.g., integrins), growth factors (e.g., TGF-beta), and proteases (thrombin) [173]. Moreover, TSP1's role as an ECM component of focal adhesion assemblies (Figure 3.6 and 3.7) is further related to activation of the PI3K/Akt survival pathway (Figure 3.8) [174, 175]. With this knowledge in mind, along with the fact that DJ-1 has a well-established role in the regulation of PI3K/Akt signaling [44-46], it could be perceived that a TSP1-mediated PI3K/Akt activation could be a new regulation mechanism of the pathway exerted by DJ-1 by binding to TSP1. Furthermore, in the context of the current work, TSP1 was observed to establish an increased interaction with the exogenous DJ-1 under stress (Table 3.1), leading to the assumption that DJ-1 may be exerting its neuroprotective function from the extracellular environment by promoting the activation of intracellular PI3K/Akt survival pathway in a TSP1-dependent manner.

Additionally, the potential involvement of DJ-1 in the TGF-β signaling pathway was also identified in the KEGG analysis via DJ-1 interaction with TSP1 along with FBN1, another ECM-related DJ-1 candidate interactor (Figure 3.9). Similarly to TSP1, FBN1 also presented increasing interaction levels with DJ-1 in the secretomes of cells subjected to oxidative stress (Table 3.1). FBN1 is an ECM glycoprotein known to take part in tissue homeostasis by establishing interactions with growth factors, cell surface integrins, and other ECM components [176]. Along the TGF-β-associated cascade, FBN1 is responsible for sequestering TGF-β in the ECM, blocking its association with cell surface receptors and leading to the dysregulation of the pathway [176, 177]. An opposite effect is accomplished by TSP1, as the protein can interact and activate TGF-β in the extracellular environment [178]. Despite the fact that a connection between DJ-1 and both TSP1 and FBN1, or

even TGF-β signaling is yet to be made, these results suggest that DJ-1 could be implicated in a tight regulation mechanism of the pathway, establishing an increased interaction under stress with both an inhibitor and suppressor of TGF-β signaling. Studies performed in a neuronal context advanced compelling evidence for a critical effect of TGF-β pathway in dopaminergic neuron survival upon PD-related conditions [179]. In this sense, studies focused on a potential implication of DJ-1 in the TGF-β pathway, which could be mediated by DJ-1 interaction with TSP1 and FBN1 in the ECM, could shed light on a new mechanism of action of DJ-1 and elucidate its relevance in the PD context.

Results from PANTHER pathway analysis of the uncovered DJ-1 interactors also denote the involvement of DJ-1 in the Wnt signaling pathway (Figure 3.5). An important impact of the Wnt pathway in PD development has been previously established as contributing to dopaminergic neuron survival [180]. In the present work, a DJ-1 connection with Wnt signaling is proposed by a stress-mediated interaction of the protein with cadherin-11 (CAD11), a member of the cell-cell cadherins family adhesion transmembrane proteins [181]. CAD11 plays a distinctive part in the formation of neural networks through the regulation of axon elongation and fasciculation, as well as in synapse formation and plasticity [182, 183]. Cadherin assemblies with cytoplasmic catenins are important complexes involved in the regulation of the Wnt/β-catenin pathway and other major signaling events [184]. However, it is still unclear whether CAD11 positively or negatively impacts β-catenin's action, hence upon the Wnt pathway, as divergent evidence indicates. In a particular work, CAD11 was pointed out as an antagonist of Wnt/β-catenin cascade by facilitating the extracellular sequestration of β-catenin, hampering its nuclear translocation and transcription of target genes upon the Wnt ligand activation at the plasma membrane [185]. An opposite observation was made in a more recent work, stating CAD11 was a positive modulator of β-catenin expression that led to activation of the β-catenin-mediated canonical Wnt pathway [186]. Therefore, additional efforts should be made to clarify the CAD11 role along the Wnt pathway cascade, as well as the potential involvement of DJ-1 in the modulation of this pathway from the extracellular space. In fact, a DJ-1 mediated activation of the Wnt cascade was reported concerning cancer-related research to promote proliferation and invasion of tumor cells [187, 188]. Yet, the described mechanisms accomplished by DJ-1 were taking place at the intracellular level, and no connection has been made between the protein and CAD11. Nonetheless, it could be hypothesized a different modulator role of DJ-1 in Wnt signaling pathway, mediated by its interaction with CAD11, shown to be decreased upon oxidative insults.

Moreover, a DJ-1 role in the phospholipase D lipid signaling pathway was disclosed by the KEGG pathway analysis and assigned by Prothrombin (THRB) (Figure 3.11). Upon oxidative stimulation, increased interacting levels were achieved between DJ-1 and prothrombin, indicating a stress-mediated interaction regulation. Prothrombin and its active form, thrombin protease, have been shown to lead a complex function in the CNS with a strong implication in PD development [189]. Notably, thrombin's deleterious effect on dopaminergic neurons was described in the literature to recapitulate the critical pathological events associated with PD course: oxidative stress and neuroinflammation [190]. However, a protective thrombin function in PD models has also been evidenced, but in a dosage-dependent manner [191, 192]. Furthermore, thrombin is known to be involved in specific signaling events, one of which phospholipase D pathway that is associated with membrane remodeling or biogenesis and cell cycle progression [193, 194]. Most interestingly, a number of reports detail a close involvement of phospholipase D isoforms, PLD1 and PLD2, in PD pathological events, but there is some divergence in these findings [195, 196]. While a neuronal damage effect has been attributed to a PLD2-mediated action [196], PLD1 activity was shown to have an important role in α-synuclein clearance [195]. In this way, in the present context, it may be plausible that DJ-1 may mediate a selective effect of thrombin in response to oxidative insults that could possibly imply the modulation of phospholipase D pathway and α-synuclein toxicity.

This study also identified an association between DJ-1 and a cytokine, Macrophage migration inhibitory factor (MIF). Over the years, this cytokine's role in neurodegenerative diseases has received increasing attention and particular evidence has suggested that MIF exerts complex functions with potential beneficial effects in PD. Studies confirmed a protective anti-inflammatory action in a mouse model of PD by suppressing apoptosis events and inducing protective autophagy mechanisms [197]. Indeed, MIF has been shown to be responsible for inhibiting p53-mediated apoptosis [198, 199], also highlighted in the present study (Figure 3.4F and Supplementary Table 7.9), and to drive the activation of the ERK survival pathway [200]. Given the established involvement of DJ-1 in the regulation of p53 [75-79] and ERK [30, 31, 33] pathways, in the present experimental context, it can be hypothesized that DJ-1's interaction with MIF may regulate its activity and present additional DJ-1 mechanisms behind ERK pathway activation and p53 apoptotic cascade inhibition mediated from the extracellular space. A study conducted in a cancer model has pointed out that DJ-1 and MIF are both implicated in cell invasion and proliferation in cancer, which could indicate a combined action for the regulation of such critical events [201]. With this in mind, further studies should focus on a potential DJ-1 and MIF correlation within PD pathological context.

Other relevant DJ-1 connections identified in this work to be further considered are the ones established with apolipoprotein B-100 (APOB-100) and secretogranin-1 (SCG1). In fact, secretogranin-1 was previously identified in some preliminary studies conducted in the laboratory focused on the extracellular DJ-1 interactome [117], revealing a consistency of this finding. A number of studies have suggested apolipoproteins and secretogranins family groups as candidate biomarkers that may reflect the course of neurodegenerative pathologies [202, 203]. Secretogranins, also called chromogranins, are widely distributed in the CNS and have been suggested to take part in crucial neurosecretory pathways responsible for the controlled delivery of peptides and neurotransmitters, such as dopamine, known to be reduced in PD [204]. Furthermore, the proteolytic processing of secretogranins generates specific extracellular bioactive peptides, which then act as modulators of homeostatic processes [205]. Dysfunction or downregulation of secretogranin-1 in cerebrospinal fluid (CSF) has been reported in PD patients [202, 206] and suggested to be an indicator of reduced dopamine transmission, possibly due to neuronal degeneration [206]. Of note, DJ-1 has been shown to exhibit protease properties [24], and therefore, it may be hypothesized a contribution of the protein in the proteolytic processing of secretogranin-1 in the extracellular environment, yielding important bioactive peptides. Interestingly, plasmin and thrombin have also been suggested as candidate proteases involved in the processing of secretogranins-derived bioactive peptides, though up until now only of chromogranin-A [207, 208]. Further studies should, therefore, evaluate a potential DJ-1-directed modulation of the precursors of plasmin and thrombin identified in this study, plasminogen and prothrombin respectively, in the processing of secretogranin-1. Besides, DJ-1 may also be involved in the transport of secretogranin-1 and derived peptides. However, in the present work it was noticed a weakened DJ-1 interaction with secretogranin-1 upon oxidative stress, which could be instigated by cell damage occurring to a certain extent, that would lead to a decreased release of the secretogranin-1-containing secretory vesicles. Nevertheless, it could also be reasoned that the reduced DJ-1-secretogranin-1 interacting levels may be a result of increased secretogranin-1 processing to bioactive peptides mediated by DJ-1, that may have been triggered upon oxidative stress conditions. For this reason, future work should also consider analyzing some secretogranin-1-derived peptides and verify its protein levels upon control and stress conditions.

Overall, though some issues were faced concerning the pull-down assay methodology that may have hindered the assortment of the complete extent of DJ-1 interactors, this study allowed to reach to a set of 28 proteins that putatively associate with DJ-1 in the extracellular space, upon the considered conditions. Besides the identification of direct DJ-1 binding partners, this strategy is also

likely to detect indirect interactors that may have bound to the DJ-1 direct interactors (for example, in protein complexes), rather than to the target recombinant DJ-1. For instance, it could be considered a DJ-1-plasmin-thrombin-secretogranin-1 protein complex, in which DJ-1 could be enabling the proteolytic activity of plasmin and thrombin for the processing of secretogranin-1. In this sense, further work could be carried out in view of establishing an optimized experimental set-up and validation techniques that would ultimately allow the identification a specific set of DJ-1 interactors and verify their functional relevance in the considered conditions. For instance, one would be using an AP-SWATH strategy employing a different pull-down method, such as the streptavidin resin-based assay capturing of a biotinylated protein. More replicates should also be considered to mitigate the inherent high variability of this type of approaches and thus reach more statistical power and more confident results. Future experiments should also integrate complementary tests, namely of viability assays to confirm the DJ-1-mediated effect for cellular survival. To validate the set of identified interactors, a crosslinking protein interaction analysis could be carried out. Moreover, immunodepletion experiments would allow to assess the contribution of the DJ-1 interactors for the observed DJ-1-mediated neuroprotective effect upon oxidative insults and confirm the proposed assumptions in this work.

The performed interatomic study underscored the extracellular role of DJ-1 as a potential key modulator of important signaling pathways by establishing interaction with specific proteins. A follow-up study was conducted to better understand the extent of such DJ-1 action focused on kinase-mediated signaling cascades and screen for effects of two DJ-1 mutant isoforms on the kinome in control conditions. M26I and E163K are two DJ-1 missense mutations that have been shown to generate structural perturbations of DJ-1 protein, leading to a decreased stability or altered activity, respectively [120, 121]. To date, the exact impact of M26I and E163K pathological DJ-1 mutations in signaling pathways modulation remains to be clarified, for which studies concerning this matter are important and may provide new insights in view of a better understanding of the DJ-1-loss of function effects.

A phospho-motif profiling immunoblot-based experiment was carried out and allowed to assess kinases' substrate phosphorylation, reflecting activation of Akt, AMPK, ATM/ATR, CDKs, CK2, MAPK/CDK, PDK1, PKA, PKC, and PLK -directed signaling pathways (Figures 3.12-3.13 and Supplementary Figure 7.7). Comparison of kinase signaling activity was assessed between cells treated with the PD-related mutants M26I- or E163K-DJ-1 and WT-DJ-1, thus providing a complementary vision for the role of extracellular DJ-1 and its potential importance to PD

development. Overall, the results clearly show that the extracellular action of M26I- and E163K-DJ-1 led to significant alterations in the phosphorylation activity of the considered kinases at the intracellular level, in comparison to the WT-DJ-1 stimulation condition (Figure 3.12).

The comparison between WT-DJ-1 stimulated cells and cells exposed to the PD-related mutants M26I- and E163K-DJ-1 reveals a significant alteration in the overall activity of kinases Akt, AMPK, ATM/ATR, CK2, CDKs, and PKC, indirectly measured by assessing the phosphorylation levels of their substrates (Figure 3.12 and 3-13). E163K-DJ-1 seems to cause a more substantial dysregulation of the regular activation associated with the presence of WT-DJ-1, demonstrating an overall reduced activation of all the kinases previously referred. On the other hand, the addition of M26I-DJ-1 to the extracellular space negatively impacted only PKC-related signaling in SH-SY5Y cells.

Among the considered signaling kinases, DJ-1 has been mainly described to be implicated in Akt cascade through a number of mechanisms culminating in cellular survival. Besides being able to promote Akt phosphorylation, DJ-1 is also known to suppress the activity of PTEN, an Akt pathway inhibitor [44, 46, 48]. As a result, defensive response mechanisms in an oxidative stress context are mediated, including the prevention of harmful autophagy processes [32] and maintenance/protection of mitochondrial function [52-54]. Indeed, dysfunctional PI3K/Akt-related signaling plays a part in the PD's pathogenesis, contributing to the loss of dopaminergic neurons [43]. Since the present results demonstrate an overall decrease in Akt-induced phosphorylation of substrates in the RXRXX(S*/T*) site caused by the E163K mutation, it is possible that a deficient activation of this survival pathway may be occurring (Figures 3.12 and 3.13A).

The activity of AMPK, a major metabolic energy sensor, was also significantly reduced by the E163K-DJ-1 extracellular addition (Figures 3.12 and 3.13B). AMPK is responsible for maintaining cellular energy balance, downregulating energy-consuming pathways, and upregulating energy producing ones [209]. The activation of AMPK has been reported in work conducted on PD animal models upon stimulation with 6-OHDA or MPTP neurotoxic agents [210, 211]. Yet, so far, the role of active AMPK in PD remains controversial, and a potential double role has been suggested: while studies consider the contribution of AMPK in neurodegenerative events, exacerbating neuronal atrophy and cell death; other studies state rather a neuroprotective effect accomplished by the protein against cytotoxic agents, for instance promoting cell metabolism changes, enhancing mitochondrial quality control, and inducing autophagy processes [209]. In particular, AMPK-dependent autophagy activation has been reported to regulate the degradation of α-synuclein, and thus reducing the toxicity related to α-synuclein aggregation [212]. A connection between DJ-1 and AMPK has been

previously established, as specific works reported that DJ-1 is, in fact, able to promote AMPK phosphorylation [213, 214]. Nevertheless, the impact of such action remains to be evaluated in a PD context, as well as the effect of mutant DJ-1 variants. Interestingly, a particular study demonstrated that DJ-1 secretion was increased in SH-SY5Y cells under 6-OHDA conditions via the unconventional secretory pathway accomplished by AMPK-mediated autophagy [155]. This fact could point out a positive feedback loop exerted through DJ-1 extracellular stimulation, which leads to AMPK activation, resulting in DJ-1 increased presence and action in the extracellular space. Taking all these evidences and propositions into consideration along with the phospho-motif profiling results, it may be possible that the E163K DJ-1 isoform may hinder such effects when comparing to the WT-DJ-1 stimulation.

The performed phospho-motif profiling further revealed reduced E163K-dependent substrate phosphorylation on (S*/T*)DXE protein motif by CK2 (Figures 3.12 and 3.13E). CK2 (formerly Casein Kinase 2) is a heterotetrameric kinase comprising two catalytic subunits (α) and two regulatory subunits (β). This kinase is responsible for phosphorylating more than 300 substrate proteins involved in cell division, proliferation, apoptosis, and DNA repair [215]. For instance, CK2 participates in signaling pathways crucial for cell survival, namely PI3K/Akt, Wnt, and NF-kB [216, 217]. Studies carried out on brains of PD patients revealed the colocalization of CK2 β subunits, but not of CK2 α, with α-synuclein in Lewy bodies in *substantia nigra* [218]. Further work explained that CK2 was able to phosphorylate α-synuclein and that such effect was boosted upon cellular toxicity, such as of proteasomal dysfunction [219]. In addition, by phosphorylation of synphilin, another Lewy body component, CK2 was reported to be able to modulate the interaction of α-synuclein and synphilin [220]. However, studies showing opposite results indicate that the physiological relevance of such effects is still being debated and, for that reason, much work is still required to understand the exact correlation between CK2 and the pathological aggregation of α-synuclein in PD [221]. Moreover, the biological outcomes possibly induced by a DJ-1-mediated enhancement of CK2 activity should also be explored, as well as the implications generated by E163K mutation of DJ-1.

Additionally, ATM/ATR and CDKs phosphorylation activity was also considered in this study, and reduced levels were detected upon exogenous addition of E163K-DJ-1 in comparison to the WT-DJ-1 condition (Figures 3.12 and 3.13B-C). ATM/ATR and CDKs are part of cell stress response mechanisms with implications in the control of cell-cycle checkpoints for the maintenance of DNA integrity [222, 223]. Thus, the present results may indicate a possible dysregulation of such essential ATM/ATR and CDKs -associated functions caused by E163K-DJ-1-related mutation.

Lastly, reduced activation of PKC signaling was observed after treatment of cells with both M26I- and E163K-DJ-1 (Figures 3.12 and 3.13F). PKC family is a group of isozymes able to regulate important cellular functions upon activation of membrane-bound phospholipids. The malfunction of downstream signaling pathways of PKC is implied in several disease pathogeneses, particularly neurodegeneration. The PKC family consists of at least 12 isoforms with diverse roles in the pathway, owing to its phosphorylation abilities [224]. Amongst, PKC δ, α, and ε are expressed in SH-SY5Y neuroblastoma cells [225]. PKC α and PKC ε stimulation were shown to be protective against the 6-OHDA-induced cell injury model of PD, and both kinases' action was further clarified to enhance Bcl-2 anti-apoptotic activity [225, 226]. Conversely, PKC δ isoform activation is led by caspase activity and takes part in oxidative stress-induced dopaminergic cell death in PD models, promoting mitochondrial dysfunction [227]. Considering previous experiments conducted in the laboratory demonstrating that extracellular stimulation of M26I- and E163K-DJ-1 did not lead to significant cell death of SH-SY5Y cells in control conditions; it is possible to infer that upon the same conditions in the present work, there may be no apoptosis-associated caspase activity, hence no PKC δ activation. For this reason, results evidencing a decreased level of PKC family activity led by M26I- and E163K-DJ-1 mutations may be correspondent to the activity of PKC α and PKC ε. Furthermore, this evidence reflects a potential impact of such DJ-1 mutations by hindering protective signaling mechanisms that may reduce the cellular capacity of stress response associated with PD events.

While an alteration in the overall phosphorylation activity was only determined for the 6 mentioned kinases (Figures 3.12 and 3.13), M26I- and E163K-DJ-1 induced phosphorylation changes in particular substrates among most of the analyzed kinases (Figures 3.13 and Supplementary Figure 7.7). In fact, the performed phosphorylation profiling assay points out for numerous common targets that were observed to be modulated in the same manner by the considered DJ-1 isoforms. This evidence may lead to the assumption that M26I and E163K mutants of DJ-1 may have convergent pathological mechanisms that culminate in PD development. For this reason, it becomes relevant to identify and study such commonly modulated phosphoproteins as potential players in the molecular events of DJ-1 -associated PD.

In summary, the extracellular stimulation of cells with E163K-DJ-1 has demonstrated to lead to a greater impact on signaling events, suggesting a reduced capacity to lead a protective response, for instance, when challenged with an oxidative stress stimulus. This proposition seems to be in agreement with previous work conducted in the laboratory demonstrating that, while not

impacting cell viability in control conditions, the exogenous addition of E163K-DJ-1 failed to provide protection against oxidative stress-induced cell death and to a greater extent than M26I-DJ-1 (Figure 1.12) [116]. Indeed, despite the fact that M26I-DJ-1 has also been shown to impact oxidative stress response [116], this work suggests a lower impact of this mutation on the native extracellular DJ-1-mediated signaling pathways in control conditions. Of note, in the present work the effect caused by the addition of DJ-1 mutant forms to the extracellular milieu of cells could have been buffered to some extent by the endogenous DJ-1 (WT form of the protein). In this way, follow-up work could consider using neuronal cells expressing the DJ-1 mutations for a more accurate evaluation of the effect led by extracellular stimulation of WT-DJ-1 and a better understanding of the pathological implications of the mutants' extracellular action in a DJ-1-associated PD context.

Overall, future studies should be considered in order to provide further insights into the kinase-mediated signaling modulation by native DJ-1 and its mutant isoforms in response to PD-associated pathological conditions. A particular focus could be considered for the Akt, AMPK, PKC, CK2, ATM/ATR, and CDKs-directed signaling events. Besides phospho-motif profiling using phospho-motif antibodies, an additional immunoblot-based screening could also include a kinase phosphorylation assay. Perhaps a different detection step could be considered, such as immunofluorescence, to decrease the blots' low visualization quality, an issue verified in this study. A phosphoproteomics MS-based untargeted study could also be conducted to identify and quantify the kinase-mediated phosphoproteins upon the established conditions.

5. Conclusions

In the present work, the main goal was to study the biological role of extracellular DJ-1 to provide new insights on the mechanisms by which the protein may exert its neuroprotective effect in the extracellular space. The focus was given to the DJ-1's ability to modulate signaling pathways, a crucial function of the protein that promotes adaptive cellular responses to stress stimulus, such as oxidative stress. Moreover, this study also intended to assess the impairments in such a DJ-1 signaling function led by the protein's pathological mutations. To reach the proposed goals, two complementary strategies were followed: on one hand, an interactomics approach was performed to uncover the network of interactions established by DJ-1 in the extracellular space; on the other hand, a phospho-motif profiling study was performed to identify key signaling pathways that may be affected by PD-related M26I- and E163K-DJ-1 variants.

The performed interactomics study entailed a SWATH-MS based strategy for the identification of DJ-1 binding partners in the extracellular space of SH-SY5Y cells as potential players in the protein's neuroprotective action. This study's quantitative approach further allowed to assess their modulation upon control and oxidative stress conditions. Data analysis was performed considering two parameters, relative protein levels and interaction levels, and successfully allowed to obtain 28 proposed DJ-1-interactors at the extracellular space. Functional analysis revealed a significant involvement of most of those proteins in important signaling pathways, potentially triggering intracellular survival mechanisms. For instance, DJ-1's established interaction with TSP1, THRB, MIF, and CAD11 could potentially mediate signaling events, including those accomplished by PI3K/Akt, TGF-β, ERK1/2, Wnt, and PLD. Nevertheless, many of these hypotheses remain to be confirmed in PD contexts, and for this reason, more studies should be conducted on this topic.

A phospho-motif profiling assay was also carried out to assess intracellular phosphorylation induced by a group of kinases in SH-SY5Y stimulated cells with WT-DJ-1 and the PD-related mutants M26I- and E163K-DJ-1. The results from an immunoblot-based experiment with the KinomeView kit suggests that both mutations of DJ-1 led to alterations in the phosphorylation profile of the kinase substrates analyzed, revealing a differential modulation of the kinase's activity by the different forms of the protein. In fact, an overall decreased kinase activity was observed upon both PD-associated conditions. Interestingly, the cellular stimulation with exogenous E163K-DJ-1 was observed to have a greater impact on the kinase-mediated signaling events, showing a significant overall reduction in the activation of Akt, ATM/ATR, AMPK, CK2, CDKs, and PKC, for which future studies should be performed. Results also show that M26I- and E163K-DJ-1 affected individual substrates of nearly all the considered kinases, and an identical modulation mechanism was further

denoted. These findings may indicate that M26I- and E163K-DJ-1 may have convergent pathological mechanisms, and further studies should aim to explore such common modulated targets that could be implicated in the development of PD.

Overall, the results obtained from these two approaches did provide promising data regarding the mode of action of DJ-1 in the extracellular environment. Most importantly, such mode of action is strongly suggested to be exerted through the modulation of signaling pathways that may be impaired by pathological mutations of the protein. Still, much more work remains to be performed for the validation and further investigation of each discussed mechanism in the context of PD. Ultimately, the study of DJ-1 extracellular mechanisms through the modulation of signaling pathways may contribute to the understanding of the overall disease etiology of PD.

6. References

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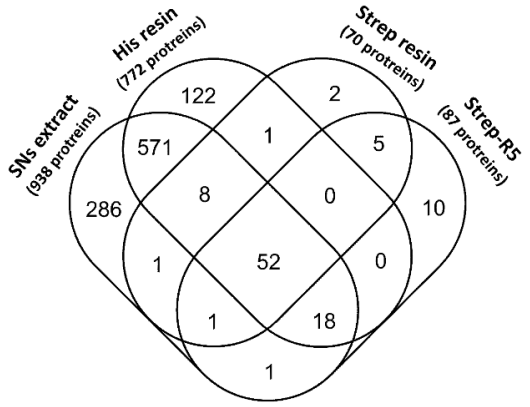
7. Supplementary data

7.1. Extracellular interactome of DJ-1

7.1.1. Protein levels-based analysis

Supplementary Table 7.1| Protein-levels-based analysis of the 527 proteins quantified by SWATH-MS in the Interactomics study of extracellular DJ-1. For each protein it is presented the UniProt accession number and name, the IS-normalized protein levels and the results from the Kruskal-Wallis coupled to Dunn's test statistical analyses. A total of 21 highlighted proteins were considered as putative DJ-1 interactors. Due to the extension of the table, it will be presented in digital format to facilitate the consultation of the data at: https://docs.google.com/spreadsheets/d/1IgzvS8d2dLTsuu5BWhu8pKmEXjByeTiwOJVCvuwFgyw/edit?usp=sharing.

To assess the specificity of the His resin employed in this interatomic study, subsequent work in the laboratory was carried out. The experiments consisted of a set of pull-down assays to test and compare histidine and streptavidin resin types on synaptoneurosomes extracts. The results clearly proved the His-tag-directed resin's high reactivity since it presented affinity with approximately 70% of the total amount of proteins identified in the synaptoneurosomes extracts. On the other hand, streptavidin-based resin was shown to establish a background interaction with less than 8% of the sample's protein content, proving to be a more accurate pull-down methodology.



Total: 1078 identified proteins

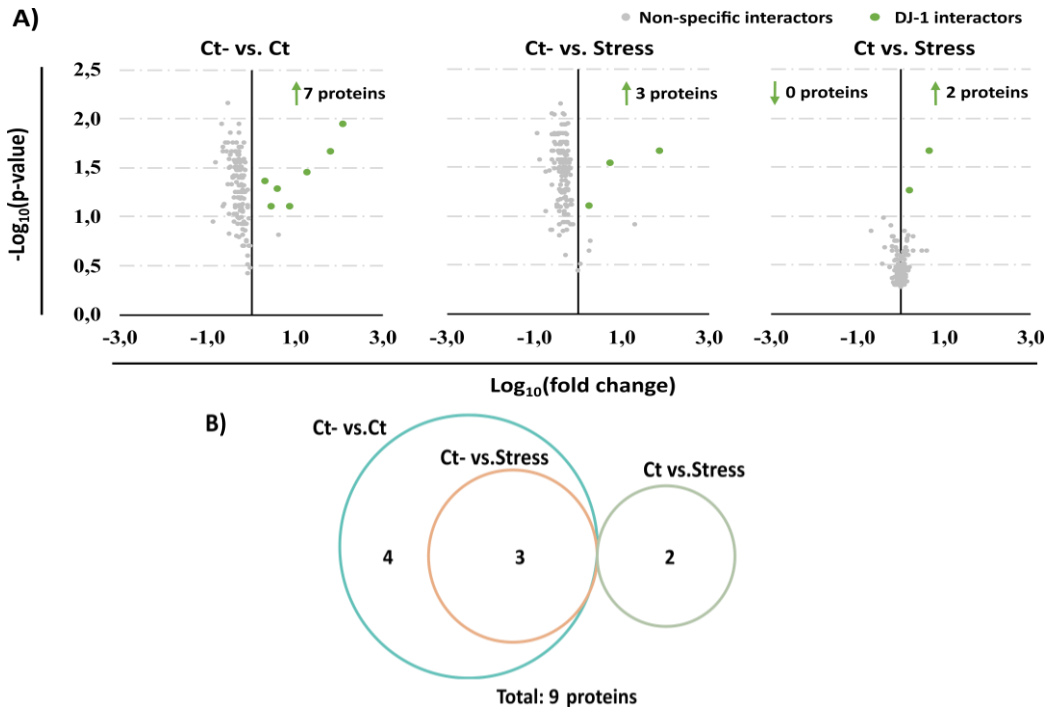
Condition	Total	Common to NPs no. proteins	Common to NPs %
SNs extract	938		
His resin	772	649	**69.2**
Strep resin	70	62	6.6
Strep-R5	87	72	7.7

Abbreviations: SNs, synaptoneurosomes**; R5**, R5/PTG protein

Supplementary Figure 7.1| Evaluation of the specificity of His resin and streptavidin resin: Venn diagram and results table showing the combination between the total set of proteins identified in a synaptoneurosomes sample and the set of proteins identified after pull-down assays of the sample with His resin, streptavidin resin and streptavidin resin bound to the R5/PTG protein.

Subsequent analysis implied the assessment of the specificity of the His-tag peptide as well. In this analysis, it was considered a control and stress conditions with the exogenous DJ-1 and their respective negative controls in the presence of the His-tag peptide. A Kruskal Wallis statistical method coupled to a Dunn's test was applied to the combination of the conditions considered and significant differences between the data of at least one of the combinations were verified for 168 proteins (Supplementary Figure 2). This analysis yielded a total of just 9 putative DJ-1 interactors (Supplementary Table 7.3), and 6 of them were already considered in the previous analysis (Table 1). For this reason, this analysis was chosen not to be considered.

Supplementary Table 7.2| Protein-levels-based analysis of the 527 proteins quantified by SWATH-MS in the Interactomics study of extracellular DJ-1, considering His-tag peptide-based conditions as negative controls. For each protein it is presented the UniProt accession number and name, the IS-normalized protein levels and the results from the Kruskal-Wallis coupled to Dunn's test statistical analysis. A total of 9 highlighted proteins were considered as putative DJ-1 interactors. Due to the extension of the table, it will be presented in digital format to facilitate the consultation of the data at: https://docs.google.com/spreadsheets/d/1IgzvS8d2dLTsuu5BWhu8pKmEXjByeTiwOJVCvuwFgyw/edit?usp=sharing.



Supplementary Figure 7.2| Protein levels-based analysis considering His-tag peptide negative control. A) Volcano plots showing the comparison of 168 proteins (determined in the Kruskal Wallis analysis with a p-value under 0.1) between the experimental conditions considered. Data points in green represent 9 proteins that met the criteria: (i) p-value under 0.1 and an increased level (log_{10} fold-change below 0) when compared to negative control; (ii) p-value under 0.1 between the two conditions with recombinant DJ-1 (Ct vs Stress). B) Venn diagram combining the proteins selected in each combination assessed in the statistical analysis. A total of 9 highlighted proteins were considered as putative DJ-1 interactors.

Supplementary Table 7.3| Proteins selected from the protein levels-based analysis considering the His-tag peptide negative control conditions, and correspondent subcellular location.

Protein ID		Subcellular location		
Accession	**Protein name**	**Extra.**	**Memb.**	**Intra.**
P07477|TRY1_HUMAN	Trypsin-1	x		
Q9UK55|ZPI_HUMAN	Protein Z-dependent protease inhibitor	x		x
P61289|PSME3_HUMAN	Proteasome activator complex subunit 3	x	x	
O95886|DLGP3_HUMAN	Disks large-associated protein 3		x	
P26368|U2AF2_HUMAN	Splicing factor U2AF 65 kDa subunit			x
Q13151|ROA0_HUMAN	Heterogeneous nuclear ribonucleoprotein A0			x
Q13148|TADBP_HUMAN	TAR DNA-binding protein 43			x
O43447|PPIH_HUMAN	Peptidyl-prolyl cis-trans isomerase H			x
Q15366|PCBP2_HUMAN	Poly(rC)-binding protein 2	x	x	x

Abbreviations: Extra., Extracellular space; **Memb.**, Membrane; **Intra.,** Intracellular space

7.1.2. Interacting levels-based analysis

Supplementary Table 7.4| Interacting-levels-based analysis of the 527 proteins quantified by SWATH-MS in the Interactomics study of extracellular DJ-1. For each protein it is presented the UniProt accession number and name, the interacting levels with DJ-1 and the results from the one-sample t-test statistical analysis. A total of 27 highlighted proteins were considered as putative DJ-1 interactors. Due to the extension of the table, it will be presented in digital format to facilitate the consultation of the data at: https://docs.google.com/spreadsheets/d/1IgzvS8d2dLTsuu5BWhu8pKmEXjByeTiwOJVCvuwFgyw/edit?usp=sharing.

7.1.3. Functional analysis

The functional analysis performed on the putative DJ-1 interactors selected in this study entailed an extensive gene ontology analysis (biological processes and molecular function) using the UniProt tool, followed by a PANTHER and KEGG pathway analysis. The results obtained are presented in the Supplementary Tables 7.5-14, specifying the proteins attributed to each biological process, molecular function, and pathway.

Supplementary Table 7.5| Gene ontology analysis for biological processes of the 28 proposed DJ-1 interactors.

Biological Process	N.º proteins	Protein short name
Cellular process	26	TETN, SCG1, HGD, FLNA, FBLN3, PLMN, PXDN, NUCL, APOB, OLA1, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, TXND5, CYBP, CAD11, THRB, ZPI
Biological regulation	24	TETN, SCG1, FLNA, FBLN3, PLMN, PXDN, NUCL, APOB, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, CHAD, TSP1, TXND5, C4BPA, CYBP, CAD11, THRB, ZPI
Metabolic process	21	SCG1, HGD, FLNA, FBLN3, PLMN, PXDN, APOB, OLA1, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, THRB, ZPI
Response to stimulus	20	TETN, SCG1, FLNA, FBLN3, PLMN, PXDN, NUCL, APOB, MTAP, NDKA, MIF, HSP7C, FBN1, DHSO, TSP1, TXND5, C4BPA, CYBP, THRB, ZPI
Multicellular organismal process	16	TETN, FLNA, FBLN3, PLMN, NUCL, APOB, NDKA, LIS1, FBN1, CHAD, DHSO, TRY1, TSP1, CYBP, CAD11, THRB, ZPI
Developmental processes	15	TETN, FLNA, FBLN3, PLMN, NUCL, APOB, NDKA, MIF, LIS1, FBN1, CHAD, TSP1, CYBP, CAD11, THRB
Localization	14	TETN, FLNA, PLMN, APOB, OLA1, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, DHSO, TSP1, TXND5, THRB
Immune system process	11	FLNA, PLMN, PXDN, APOB, MIF, LIS1, HSP7C, TSP1, TXND5, C4BPA, THRB
Signaling	10	SCG1, FLNA, FBLN3, APOB, MTAP, MIF, LIS1, HSP7C, FBN1, THRB
Cell motility	8	FLNA, PLMN, APOB, MIF, LIS1, DHSO, TSP1, THRB
Interspecies interaction between organisms	8	FLNA, PLMN, APOB, MIF, RS28, HSP7C, C4BPA, THRB
Reproductive process	5	FLNA, PLMN, APOB, LIS1, DHSO
Cell adhesion	4	FLNA, FBN1, TSP1, CAD11
Behavior	2	LIS1, TSP1
Tissue regeneration	1	PLMN
Bone mineralization	1	TETN

Supplementary Table 7.6| Cellular biological processes: Cellular component organization or biogenesis.

Cellular processes: Cellular component organization or biogenesis (12 proteins)	N.º proteins	Protein short name
Cellular component assembly	7	FLNA, PXDN, APOB, MIF, RS28, LIS1, CAD11
Extracellular matrix organization	5	PLMN, PXDN, FBN1, TRY1, TSP1
Membrane organization	4	APOB, LIS1, HSP7C, TSP1
Protein-containing complex subunit organization	4	APOB, MIF, RS28, HSP7C
Cellular component disassembly	4	PLMN, LIS1, HSP7C, TRY1
Supramolecular fiber organization	3	FLNA, PXDN, LIS1
Cell projection organization	3	FLNA, LIS1, CAD11
Organelle organization	3	FLNA, RS28, LIS1
Cell junction organization	2	FLNA, CAD11
Central nervous system projection neuron axonogenesis	2	LIS1, CAD11
Ribonucleoprotein complex biogenesis	1	RS28
Microtubule organizing center organization	1	LIS1

Supplementary Table 7.7| Biological regulation processes: Regulation of signal transduction.

Biological regulation: Regulation of biological processes: Regulation of signaling: Regulation of signal transduction (7 proteins)	N.º proteins	Protein short name
Positive regulation of intracellular signal transduction	4	FLNA, MIF, TSP1, THRB
Negative regulation of intracellular signal transduction	3	MIF, LIS1, TSP1
Negative regulation of apoptotic signaling pathway	2	MIF, TSP1
Positive regulation of receptor signaling pathway via STAT	2	MGT5A, THRB
Negative regulation of fibroblast growth factor receptor signaling	1	TSP1
Sequestering of extracellular ligand from receptor	1	FBN1
Positive regulation of integrin-mediated signaling pathway	1	FLNA
Positive regulation of extrinsic apoptotic signaling pathway via death domain receptors	1	TSP1
Positive regulation of phospholipase C-activating G protein-coupled receptor signaling pathway	1	THRB
Positive regulation of lipopolysaccharide-mediated signaling pathway	1	MIF
Positive regulation of transforming growth factor beta receptor signaling pathway	1	TSP1
Positive regulation of cytokine-mediated signaling pathway	1	LIS1

Supplementary Table 7.8| Metabolic biological processes.

Metabolic process (21 proteins)	N.º proteins	Protein short name
Cellular metabolic process	20	SCG1, HGD, FLNA, FBLN3, PLMN, PXDN, APOB, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, THRB, ZPI
Primary metabolic process	19	SCG1, HGD, FLNA, FBLN3, PLMN, APOB, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, THRB, ZPI
Organic substance metabolic process	19	SCG1, HGD, FLNA, FBLN3, PLMN, APOB, MGT5A, MTAP, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, THRB, ZPI
Nitrogen compound metabolic process	17	SCG1, HGD, FLNA, FBLN3, PLMN, APOB, MGT5A, MTAP, NDKA, RS28, LIS1, HSP7C, FBN1, TRY1, TSP1, THRB, ZPI
Small molecule metabolic process	9	HGD, APOB, MTAP, NDKA, MIF, LIS1, FBN1, TRY1, DHSO
Catabolic process	8	HGD, PLMN, PXDN, APOB, RS28, LIS1, HPS7C, DHSO
Biosynthetic process	8	FLNA, APOB, MGT5A, MTAP, NDKA, MIF, RS28, DHSO
Oxidation-reduction process	3	HGD, PXDN, DHSO
ATP metabolic process	2	OLA1, HSP7C
Protein N-linked glycosylation via asparagine	1	MGT5A
Methylation	1	MTAP

Supplementary Table 7.9| Response to stimulus biological processes

Response to stimulus: Response to stress (10 proteins)	N.º proteins	Protein short name
Defense response	5	FLNA, MIF, TSP1, C4BPA, THRB
Response to wounding	4	FLNA, PLMN, THRB, ZPI
Response to hypoxia	1	TSP1
Response to osmotic stress	1	DHSO
Response to oxidative stress	1	PXDN
Cellular response to starvation	1	HSP7C
DNA damage response, signal transduction by p53 class mediator	1	MIF
Cellular response to heat	1	TSP1
Cellular response to unfolded protein	1	HSP7C
Response to endoplasmic reticulum stress	1	TSP1

Supplementary Table 7.10| Signaling biological processes

Signaling (10 proteins)	N.º proteins	Protein short name
Chemical synaptic transmission	2	LIS1, HSP7C
Transmission of nerve impulse	1	LIS1
Others	8	SCG1, FLNA, FBLN3, APOB, MTAP, MIF, FBN1, THRB

Supplementary Table 7.11| Gene ontology analysis for molecular functions of the 28 proposed DJ-1 interactors.

Molecular function	N.º proteins	Protein short name
Binding	25	TETN, SCG1, HGD, FLNA, FBLN3, PLMN, PXDN, NUCL, APOB, OLA1, MGT5A, NDKA, MIF, RS28, LIS1, HSP7C, FBN1, TRY1, DHSO, TSP1, C4BPA, CYBP, CAD11, THRB, ZPI
Catalytic activity	14	HGD, FBLN3, PLMN, PXDN, OLA1, MGT5A, MTAP, NDKA, MIF, HSP7C, TRY1, TXND5, THRB
Molecular function regulation	9	SCG1, FLNA, FBLN3, PXDN, MGT5A, MIF, FBN1, THRB, ZPI
Signaling receptor activity	5	FBLN3, THRB, SCG1, MIF, FBN1
Structural molecule activity	4	PXDN, RS28, FBN1, TSP1
Protein folding chaperon	1	HSP7C
Bromide peroxidase activity	1	PXDN
Lipid transporter activity	1	APOB

Supplementary Table 7.12| Signaling molecular functions

Signaling receptor activity (5 proteins)	N.º proteins	Protein short name
Receptor ligand activity	5	SCG1, FBLN3, MIF, FBN1, THRB
Epidermal growth factor-activated receptor activity	1	FBLN3
Thrombospondin receptor activity	1	THRB

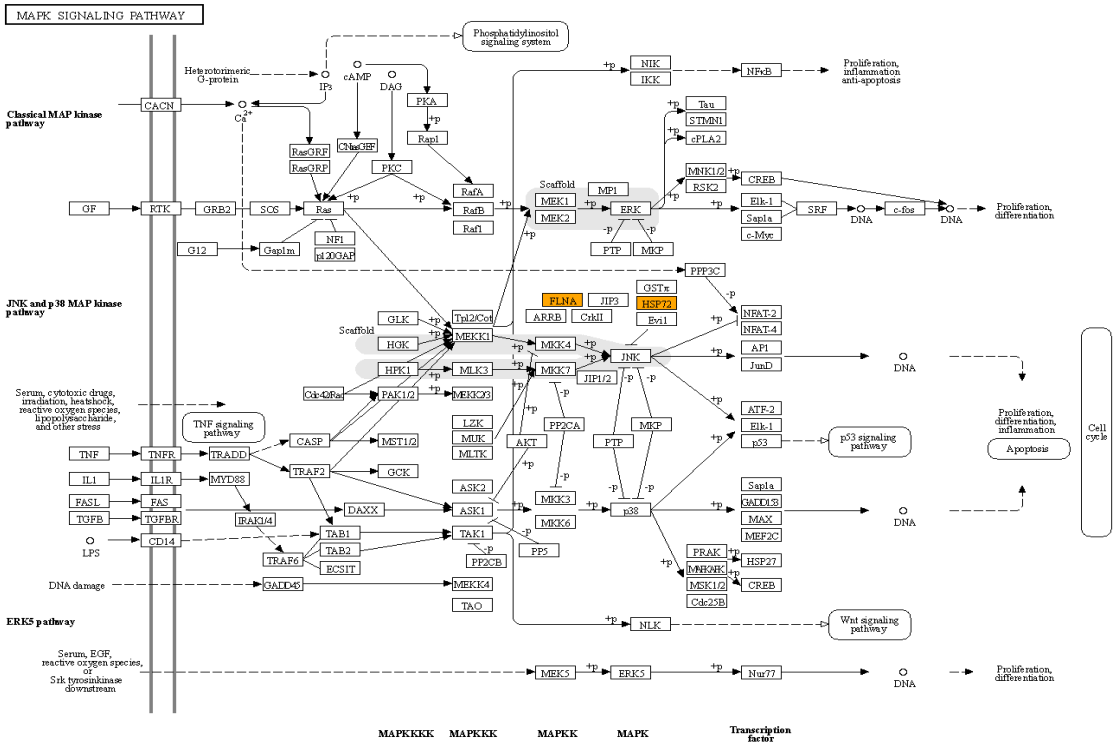
Supplementary Table 7.13| Structural activity molecular functions

Structural molecule activity (4 proteins)	N.º proteins	Protein short name
Extracellular matrix structural constituent	3	PXDN, FBN1, TSP1
Extracellular matrix constituent conferring elasticity	1	FBN1
Structural constituent of ribosome	1	RS28

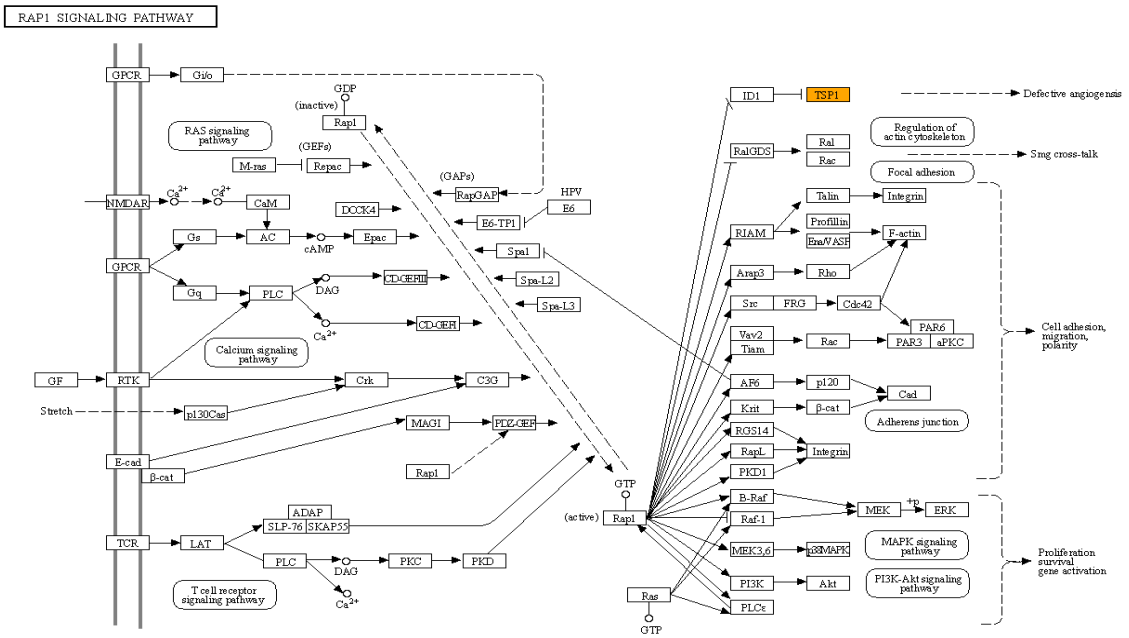
Supplementary Table 7.14| Panther pathway analysis of the 28 proposed DJ-1 interacting partners.

Panther pathways (5 proteins)	N.º proteins	Protein short name
Cadherin signaling pathway	1	CAD11
De novo pyrimidine deoxyribonucleotide biosynthesis	1	NDKA
Blood coagulation	3	ZPI, THRB, PLMN
De novo purine biosynthesis	1	NDKA
De novo pyrimidine ribonucleotides biosythesis	1	NDKA
Apoptosis signaling pathway	1	HSP7C
Integrin signaling pathway	1	FLNA
p53 pathway	1	TSP1
Wnt signaling pathway	1	CAD11
Parkinson disease	1	HSP7C
Dopamine receptor mediated signaling pathway	1	FLNA
Plasminogen activating cascade	1	PLMN
Nicotine pharmacodynamics pathway	1	FLNA

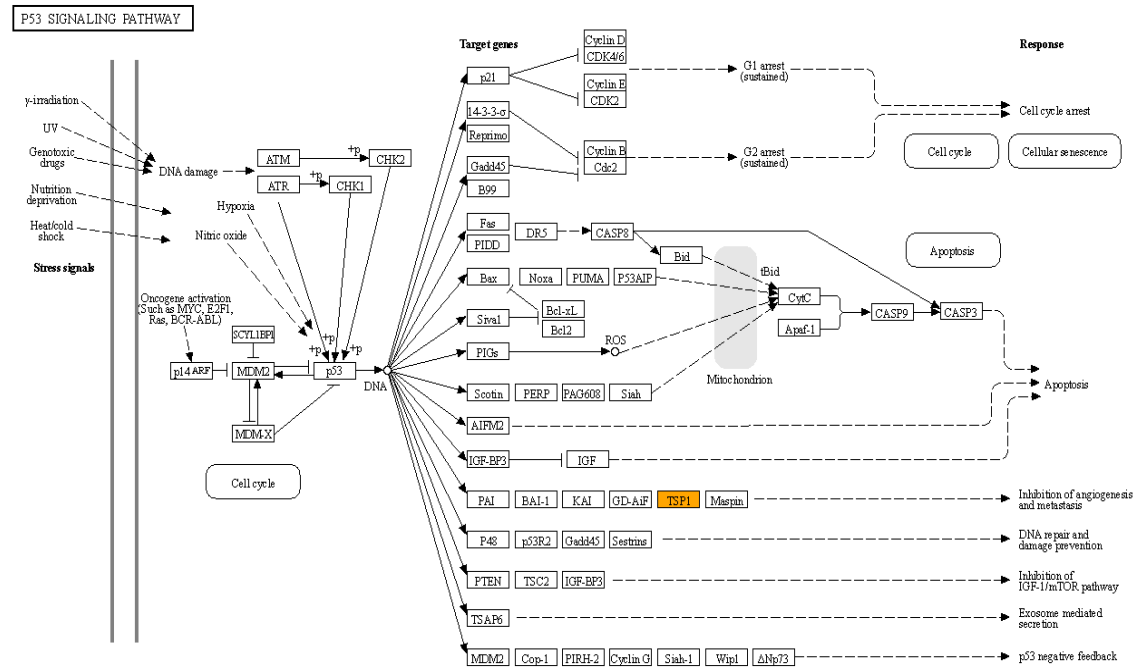
Moreover, MAPK, Rap1, p53, and Wnt KEGG pathways displaying proteins that operate at the intracellular level were not considered for analysis in this work (Supplementary Figures 3-6).



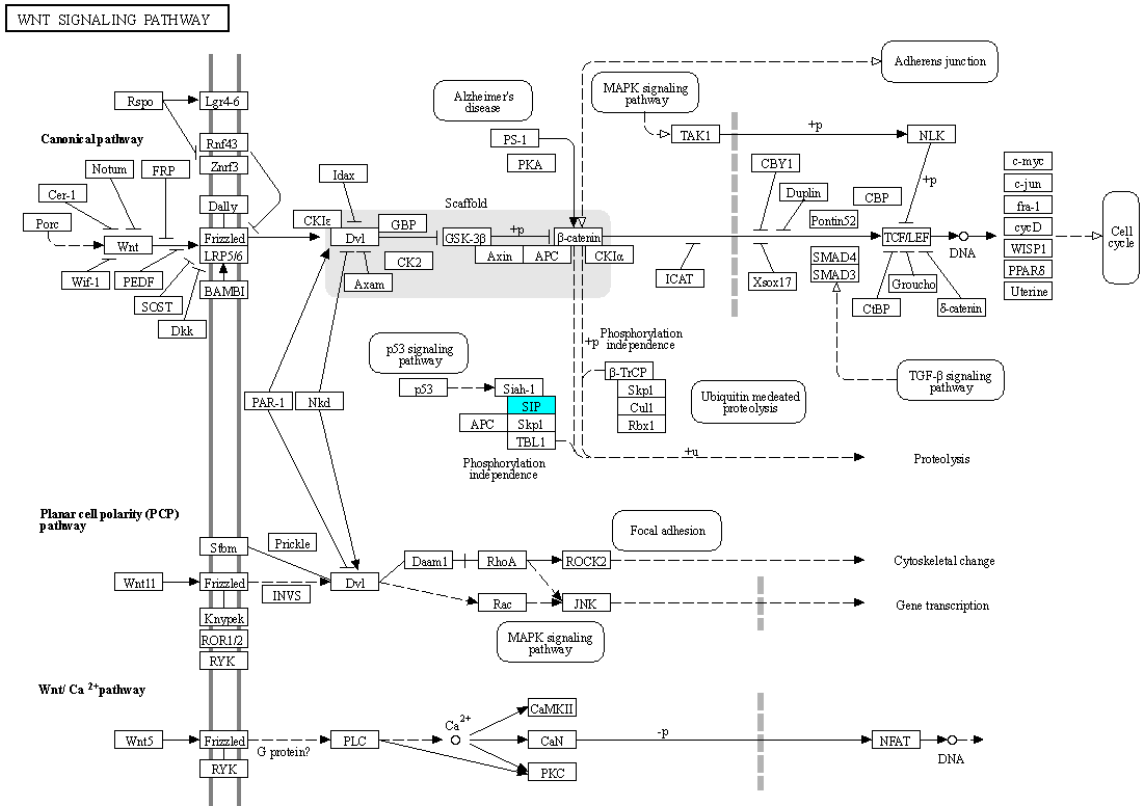
Supplementary Figure 7.3| KEGG pathway analysis associating filamin-A (FLNA) and heat shock protein 70 (Hsp70/HSPA8) to the MAPK signaling pathway in the intracellular space. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.



Supplementary Figure 7.4| KEGG pathway analysis associating thrombospondin-1 (TSP1/THBS1) to the Rap1 signaling pathway in the intracellular space. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

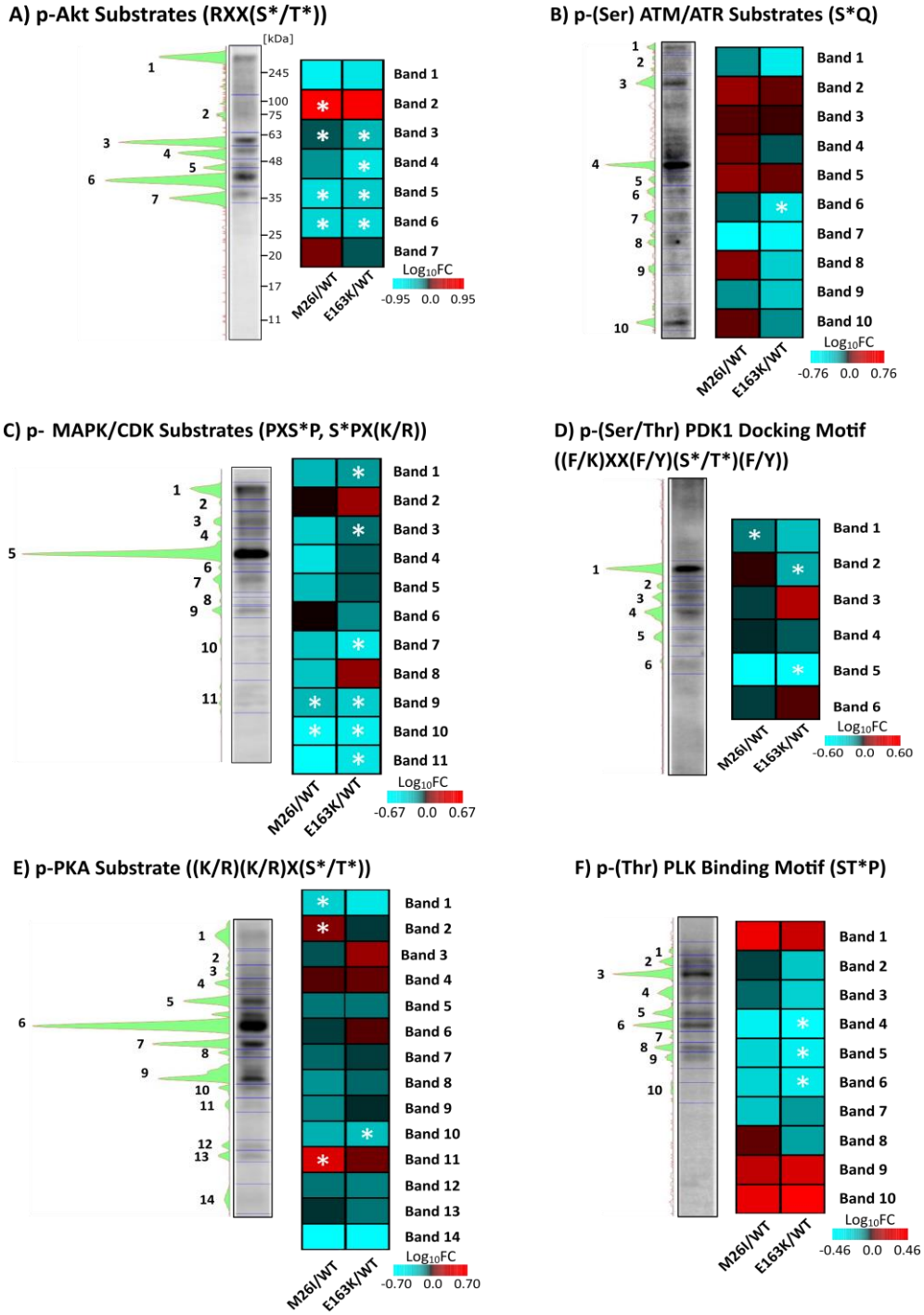


Supplementary Figure 7.5| KEGG pathway analysis associating thrombospondin-1 (TSP1/THBS1) to the p53 signaling pathway in the intracellular space. The represented proteins (in orange) exhibited an increased tendency to interact with DJ-1 upon stress conditions.

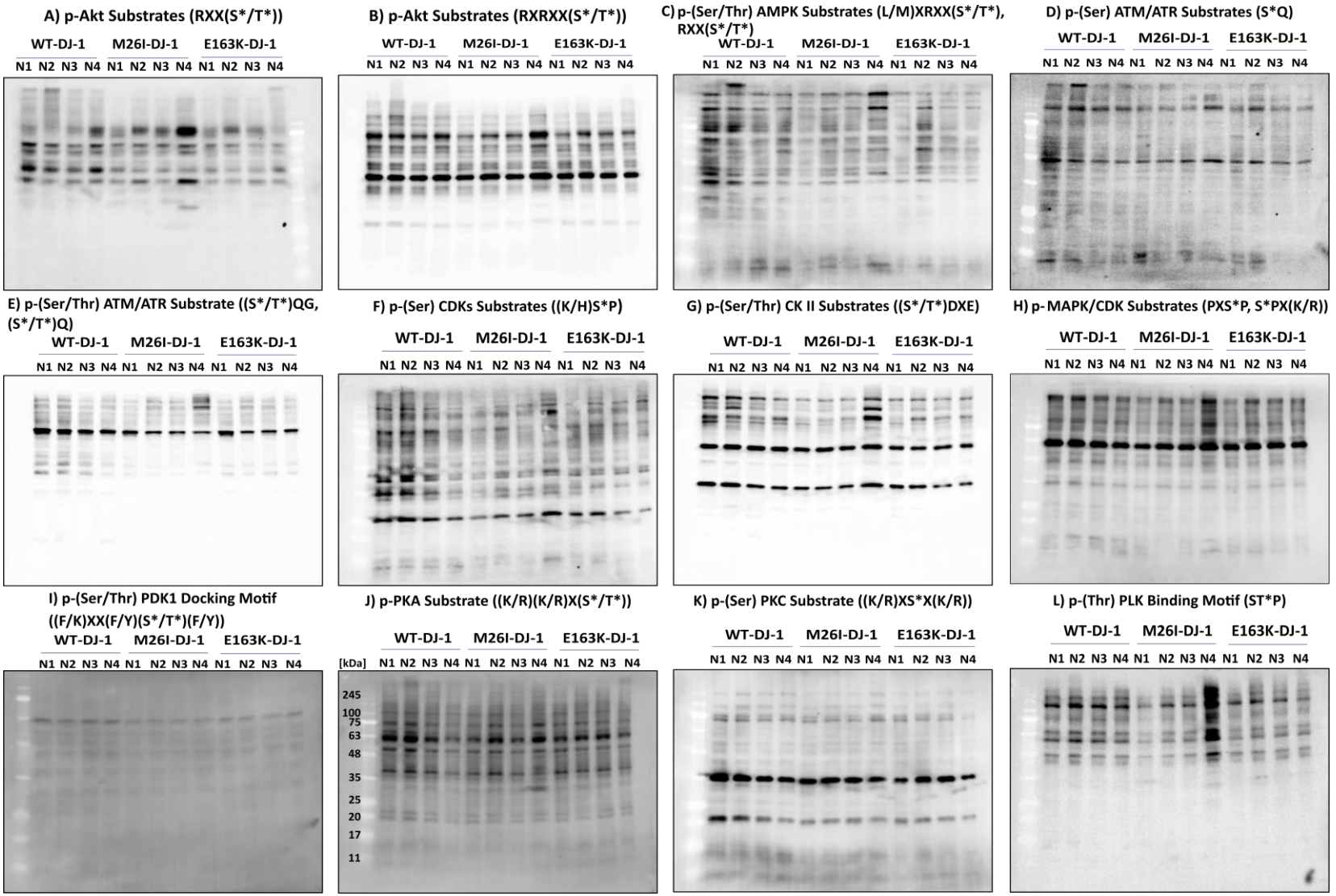


Supplementary Figure 7.6| KEGG pathway analysis associating calcyclin binding protein (CACYBP) to the Wnt signaling pathway in the intracellular space. The represented proteins (in orange) exhibited a decreased tendency to interact with DJ-1 upon stress conditions.

7.2. Differential changes in intracellular kinome signaling induced by extracellular DJ-1 pathological mutants



Supplementary Figure 7.7| Analysis of the phospho-motif profiling assay results of the kinases substrates or substrates groups that did not display an overall significant phosphorylation profile alteration. Heatmaps displaying the extracellular M26I- and E163K-DJ-1 induced changes in the phosphorylation profile of substrates of A) Akt, B) ATM/ATR, C) MAPK/CDK, D) PDK1, E) PKA, F) and PLK kinase families. Data were analyzed using the ImageLab software and a representative lane with bands' intensities is presented. Statistical analysis was performed by a Wilcoxon signed-rank test to compare the phosphorylation levels of each substrate or substrate group between M26I- and E163K-DJ-1 conditions in relation to the WT-DJ-1 condition. * p<0.1.



Supplementary Figure 7.8 | Western blot representations of the phospho-profiling assay using the KinomeView kit's 12 phospho-motif antibodies. This strategy was used to assess phosphorylation levels in kinase substrates upon WT-, M26I-, and E163K-DJ-1 extracellular stimulation.

Annex

Annex I

From the present research project, I started to work on a mini-review article, focusing on the DJ-1's mechanisms of action in the modulation of particular signaling pathways that confer the protein its neuroprotective effect. This work is currently under preparation and a preliminary draft is presented in this section.

Title: Modulation of signaling pathways by DJ-1: an updated overview

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1. Introduction

Over the years, research has been focusing on studying the physiological and pathological role of Parkinson's disease (PD)-associated protein - DJ-1, in an attempt to provide new insights for the understanding of the disease [1]. DJ-1 is a homodimeric structured protein that contains three cysteine residues (Cys46, 53, and 106) sensitive to oxidation reactions, conceding a crucial role to DJ-1 as an oxidative stress sensor able to coordinate adequate protective responses [2]. The oxidation status of Cys106 has been perceived to be particularly fundamental for the performance of the protein [2]. Among its multiple functions, DJ-1 is implicated in the regulation of signal transduction mechanisms, responsible for mediating adaptative cellular actions against stress conditions [3].

In this sense, this mini-review focuses on the crucial neuroprotective mode of action of DJ-1 in the modulation of central signaling pathways. It is provided a detailed overview of the mechanisms by which DJ-1 regulates the signaling pathways mediated by signaling cascades Extracellular signal-regulated kinase 1/2 (Erk1/2), phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and Apoptosis signal-regulating kinase 1 (ASK1); and by specific transcription factors, Nrf2 and p53. In addition, it is also addressed the impact of different DJ-1-associated mutations and the influence of cysteine redox status on the performance of the DJ-1 in signaling modulation.

1.1. DJ-1 modulation of signaling cascades

1.1.1. ERK1/2 pathway

DJ-1 is known to modulate the ERK1/2 pathway (Figure 1, Table 1), involved in neuronal protection, by regulating cell proliferation, differentiation and other biological functions. The activation of this signaling pathway occurs upon specific stimulus, namely growth factors and stress conditions, triggering a phosphorylation cascade throughout its main components Ras, Raf, MEK1/2, and ERK1/2 [4, 5].

Initial studies revealed that the overexpression of DJ-1, unlike its L166P mutant, increases the phosphorylation of ERK1/2 and its upstream kinase MEK1/2, preventing oxidative injury in the presence of hydrogen peroxide (H2O2). DJ-1 can also trigger the ERK1/2 pathway, suppressing the expression of protein phosphatase 2A (PP2A), an inhibitor of MEK1/2 and ERK1/2 family kinases [6]. Another set of evidences demonstrated that DJ-1 was able to bind to c-Raf in the cytoplasm and promote its kinase activity through Ser338 self-phosphorylation upon EGF treatment. Ultimately, c-Raf triggers signal transduction to the following ERK1/2 pathway components, and the nuclear ERK1/2 activates gene expression implicated in survival processes. The oxidation of DJ-1 Cys106 to the SO2H and SO3H forms was found not to be required for this DJ-1-mediate c-Raf activation.[7]

DJ-1 is responsible for different neuroprotective responses mediated by the ERK1/2 pathway [8-12], since the enhancement of pro survival ERK-dependent mitophagy [9], to the enhancement of transcription factors nuclear receptor-related 1 (Nurr1) [10, 11] and ERLK1[8] activities and consequente increase in the proteins' expression involved in dopamine metabolism and response to oxidative stress, respectively.

Overexpression of DJ-1, but not L166P-DJ-1, showed to increase the nuclear translocation of Nurr1 throught the ERK1/2 pathway, allowing the expression of its target genes tyrosine hydroxylase (TH), dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2), resulting in the stabilization of dopamine levels [11]. Another work, states that DJ-1 interacts with ERK1/2 under oxidative conditions, leading to its nuclear translocation and phosphorylation of substrate ELK1[8]. This transcription factor triggers the expression of antioxidant superoxide dismutase-1 (SOD1), accounting for the conversion of superoxide (O2-) radical into hydrogen peroxide (H2O2) [8]. More recently, DJ-1's action has been associated with an ERK1/2-dependent upregulation of cytoprotective microRNA-221 (miRNA-221) in response to oxidative stress and consequent downregulation of the expression of pro-apoptotic proteins. This work further described that the

reintroduction of WT-DJ-1, unlike M26I-DJ-1, in DJ-1 knockout mice brains, caused an increase of miRNA-221 expression [12].

Interestingly, some studies also indicate that DJ-1 can be up-regulated by ERK1/2 signaling activation upon 6-hydroxydopamine (6-OHDA)-induced stress, pointing out a positive feedback loop between DJ-1 and ERK1/2 activation [13].

1.1.2. PI3K/Akt pathway

The PI3K/Akt signaling cascade entails the stress-induced activation of Phosphatidylinositol 3-kinase (PI3K), which triggers the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). Following Akt/protein kinase B (PKB) binding to PIP3, Akt targets downstream substrates associated with metabolism, growth, proliferation, and survival [14, 15]. Dysfunctional PI3K/Akt-related signaling plays a role in the pathogenesis of PD, contributing to the loss of dopaminergic neurons [15]. Importantly, DJ-1 has been indicated as a key player in the activation of the PI3K/Akt pathway (Figure 2, Table 1), promoting Akt phosphorylation for cellular survival mechanisms upon stress conditions [16-18].

Additional work also demonstrated that DJ-1 was able to interact with phosphatase and tensin homolog (PTEN), suppressing its inhibitory action associated with the PIP3 conversion back to PIP2[17, 19]. In this way, DJ-1 proves to prevent PTEN-induced cell death by enhancing Akt activation and subsequent triggering of downstream survival effectors [17, 19]. A specific work demonstrated that the interaction of DJ-1 to PTEN occurred in the presence of the DJ-1's Cys106 reduced form [19], underlining the importance of DJ-1's oxidative status in the inhibition of PTEN. In addition, DJ-1 can also interact with PTEN and suppress its activity by a transnitrosylation reaction upon mild nitrosative conditions associated with neuronal damage [20]. This specific work identified DJ-1 as a NO-sensitive protein that can be S-nitrosylated to form SNO-DJ-1 with subsequent transfer of a NO group to PTEN by transnitrosylation, inhibiting its activity and allowing PI3K/Akt accomplishments for neuroprotection [20]. In fact, levels of transnitrosylated PTEN (SNO-PTEN) were actually identified in human brains with sporadic PD [20]. Another way that DJ-1 can promote Akt activity involves a protein from the striatin family, the S/G2 nuclear autoantigen (SG2NA) that recruits DJ-1, but not L166P and M26I DJ-1 mutants, and Akt into the mitochondria and plasma membrane [21]. The formation of this SG2NA/DJ-1/Akt complex stimulates Akt signaling upon moderate H2O2 stimulation [21].

One of the defensive responses promoted by DJ-1-mediated activation of PI3K/Akt has been reported to be the suppression of harmful autophagy processes under C2-ceramide-generated

neurotoxic conditions [22]. Moreover, DJ-1 is also responsible for improving mitochondrial functional activity in a PI3K/Akt-dependent manner in a neuronal context [23, 24], by promoting Akt phosphorylation at its catalytic site Thr308 [24]. Furthermore, DJ-1 was able to exert its neuroprotective action by targeting RING-finger protein-5 (RNF5) ligase activity and consequent degradation of mitochondrial fission 1 protein (Fis1) via modulation of PI3K/Akt signaling [25].

1.1.3. ASK1 pathway

In response to cellular insults, the activation of ASK1 targets downstream c-Jun N-terminal kinase (JNK) and p38 pathways, leading to apoptosis, differentiation, and inflammation processes [26-28]. The ASK1 inactive signalosome includes its inhibitors, such as thioredoxin (Trx) and 14-3-3 proteins, while its activation is induced by ASK1 activators, namely TNF-alpha receptor-associated factors (TRAFs) and death-associated protein 6 (Daxx). [27, 28]

PD neuronal death can be triggered through the abnormal activation of ASK1[28], while DJ-1 can ensure cell survival by suppressing ASK1 apoptotic signaling (Figure 3, Table 1). DJ-1 can accomplish this inhibition by different mechanisms starting by preventing the dissociation of ASK1 and its inhibitor Trx1 upon oxidative conditions in DJ-1-Cys106 dependent way [29]. DJ-1 can also inhibit ASK1 by binding to and sequestering the activator Daxx in the nucleus, preventing its translocation to the cytoplasm [30, 31]. Studies have reported that, contrary to WT DJ-1, PD-causative L166P and M26I-DJ-1 failed to suppress Daxx activity, leading to ASK1-induced cell death [30, 31]. An additional stress-protective manner of DJ-1 to modulate Daxx occurs via a PI3K/Akt/dFOXO pathway [32]. In fact, the Drosophila homologue DJ-1b was shown to downregulate dFOXO in a PI3K/Akt-dependent way, which in turn blocks Daxx-like protein (DLP) expression and successive ASK1 activation [32].

Additionally, several evidences demonstrate a direct interaction between DJ-1 and ASK1, resulting in the ASK1 repression under oxidative stimulation [31, 33, 34]. Waak and co-workers explained that DJ-1, but not M26I-DJ-1, was able to generate mixed disulfide bonds with ASK1, mediated by DJ-1-Cys106, upon H2O2 conditions [31]. In line with these results, a second work confirmed that oxidative stress induces DJ-1-ASK1 association and subsequent ASK1 inhibition, in part by suppressing its homo-oligomerization-driven activation [34]. This event consequently prevented phosphorylation of the p38 upstream kinase MKK3 (mitogen-activated protein kinase kinase 3) and therefore p38-related apoptosis [34].

1.2. DJ-1 modulation of signaling pathways-mediated by transcription factors

1.2.1. p53 pathway

P53 tumor suppressor protein has an important role as a transcription factor, regulating the expression of genes involved in cell cycle control, DNA repair, senescence, and apoptosis [35]. Cells tend to increase p53 levels and activity when experiencing stress stimulus, such as oxidative stress [36-38].

DJ-1 has been shown to modulate p53 to control cellular responses to stress insults (Figure 4, Table 1) [39-45]. The majority of research consensually indicates that DJ-1 represses p53-associated signaling, with the exception of one work [46] expressing that DJ-1 restores p53 transcription activity inhibited by Topors-mediated sumoylation.

Indeed, DJ-1 is able to exert its neuroprotective function through the downregulation of the p53-Bax-caspase death pathway [40, 47]. DJ-1 can bind to p53 and repress its activity, inhibiting the expression of its target Bcl-2 associated X (Bax) apoptotic protein and preventing caspase activation [40]. According to Kato et al., the oxidation of the DJ-1's central Cys106 is necessary to enhance the affinity of DJ-1 to p53[42]. This work also observed that DJ-1 was able to suppress Dual Specificity Protein Phosphatase 1 (DUSP1), an ERK survival pathway inhibitor, by detaining p53 to recognize its gene promoter [42]. Nevertheless, evidences suggest that proper sumoylation of DJ-1 in the K130 site is required for its interaction with p53[39, 43]. In fact, further research also observed that a sumoylation-deficient mutant of DJ-1 translocated from the nucleus to the cytoplasm upon ultraviolet (UV) stimulus, failing to inhibit p53-induced cell death, implying that sumoylation is fundamental for the localization of DJ-1 in the nucleus and p53 signalling suppression [39].

Other different DJ-1-mediated mechanisms aiming at blocking the p53 pathway have also been described [41, 44, 45]. The deacetylase Sirtuin 1 (SIRT1) was reported to act as a binding protein of DJ-1 in cultured cells, promoting the deactivation of acetylated p53[44]. Moreover, the C-terminal of DJ-1 generated by the caspase-6 cleavage has also been demonstrated to inhibit p53 in a PI3K/Akt dependent manner [41]. Another protective role of the DJ-1-mediated suppression of p53 relies on the inhibition of Asparagine Endopeptidase; Legumain (AEP) [45], an enzyme associated with the PD pathogenesis [48, 49].

On the other hand, p53 may also have a downregulating effect on DJ-1, since a proteomic analysis identified DJ-1 as a p53 phosphorylation target, which resulted in DJ-1's inhibition [50]. A second report also stated that p53 is able to inhibit DJ-1 expression and its mRNA levels [51]. The authors

then proceeded to explain that the parkin protein was able to upregulate DJ-1, by supressing p53's action on DJ-1 [51].

1.2.2. Nrf2 pathway

Nuclear factor erythroid-related 2 (NRF2) is a transcription factor involved in the control of antioxidant responses. In basal conditions, Nrf2 is bound to its inhibitor Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, while upon stress stimulus, Nrf2-Keap1 interaction is disrupted. Nrf2 activation and nuclear translocation allows the expression of target genes, presenting the antioxidant response element (ARE sequence), namely NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione-S-transferases (GST) and thioredoxin (Trx) [52, 53].

Pathogenic events characteristic of PD and other neurodegenerative disorders involve aberrant redox homeostasis instigated by defective Nrf2 signaling [54]. DJ-1 has been shown to promote Nrf2 antioxidant pathway activation by blocking the Nrf2-Keap1 association and favoring the nuclear translocation of Nrf2 and the expression of detoxifying enzymes [52, 55]. These findings were further supported by recent studies conducted on dopaminergic neuron-like cells [23] and astrocytes [56, 57]. Furthermore, emerging evidences also imply a PI3K/Akt-dependent regulation of Nrf2 mediated by DJ-1[58-60].

Other described cytoprotecting mechanisms of action of DJ-1 via Nrf2 pathway activation entail the upregulation of the expression of Isocitrate dehydrogenase (IDH) [61] and the ASK1 inhibitor Trx1 [62] and consequent improvement of cellular survival. Moreover, DJ-1 was identified as a regulator of the 20S proteasome under oxidative stress, acting in a loop regulatory mechanism: in one hand DJ-1 inihibits the 20S proteomose by direct interation; on the other hand, DJ-1 promote its activation by stimulating the Nrf2-dependent expression of NQO1[63]. This critical mechanism provides a balance between the degradation of harmful disrupted proteins and the preservation of intrinsically unstructured ones [63].

In this sense, the majority of research seems to confirm this DJ-1/Nrf2-related mechanism (Figure 5, Table 1), even though Gan et al. [64] argued that the Nrf2 pathway was independent of DJ-1. In fact, a number of studies continued to prove the importance of DJ-1 in the activation of Nrf2 signaling in a therapeutic context [58-60, 65-70], by the use of promising therapeutic compound such as Cu(II)ATSM[70], TUDCA[67], compound B[69], and others[58-60, 66, 68] that acts on DJ-1 to promote the up-regulation of Nrf2. More importantly, a DJ-1 based peptide (ND-13) also

demonstrate to be able to prevent oxidative injury in PD animal and cellular models, by up-regulating Nrf2 pathway [65].

3. Concluding remarks

DJ-1 has been accounted for several functional roles towards neuroprotection, for which the ability to modulate signaling pathways seems to be of utmost importance. In this review we focused on the most relevant mechanisms described in the literature concerning the signaling pathway cascades ERK1/2, PI3K/Akt and ASK1, and Nrf2 and p53 transcription factors-related signaling (Figures 1-5, Table 1). To sum up, studies show that DJ-1 induces cell survival and proliferation by activating ERK1/2 and PI3K/Akt signaling cascades, as well as the Nrf2 pathway-mediated antioxidant response. Alternatively, inhibition of ASK1 and p53-related apoptotic pathways by DJ-1 leads to attenuated cell death upon critical conditions. The aberrant functioning of the mentioned events is known to contribute to the development of multiple diseases, particularly PD. In fact, PD-associated mutations (M26I, L166P and D149A) of DJ-1 have been shown to lead to the loss of the protective function of the protein, implicating the dysregulation of crucial signaling mechanisms (Table 1). Besides, excessive oxidation of the cysteine residues of the protein have also been shown to hinder the native function of DJ-1 on most of the referred pathways (Table 1). Altogether, these facts reveal the importance of the DJ-1 cysteine residue's redox status, mainly of the central Cys106, and the implication of the PD-related mutant form, in the DJ-1 neuroprotective effect mediated by the regulation of signaling pathways. Moreover, it is clear that DJ-1 is able to modulate the addressed signaling pathways in different ways and at various levels.

The role of DJ-1 as a signaling mediator has been widely studied over the years (Figure 6, Table 1), and while the major mechanisms of modulation of DJ-1 in the most common pro-survival and cell death signaling pathways seem to have been gradually established throughout the past 2 decades; an increased interest is denoted in recent years regarding the DJ-1 modulation of the Nrf2-mediated antioxidant pathway. Interestingly, the most recent studies have been focusing on the therapeutic potential of DJ-1, mostly by enhancement of Nrf2 signaling as a cytoprotective mechanism upon PD context. Therefore, it may be expected that future research increases the pursuing of DJ-1-mediated therapeutic strategies for PD treatment based on its neuroprotective function led by signaling modulation. Nonetheless, it remains of great importance to determine the basic mechanisms of action of DJ-1 by which the protein is able to regulate signaling pathways, in order to understand the downstream effects that lead to the protective outcome.

Figure Legends:

Annex I - Figure 1| Summary of DJ-1's mechanisms involved in the modulation of the ERK1/2 signaling pathway. (1) DJ-1 is able to bind to c-raf, promoting its self-phosphorylation at Ser 338 and activating subsequent pathway components MEK1/2 and ERK1/2. (2) In oxidative conditions, phosphorylation of MEK1/2 and ERK1/2 is also increased by a dual-mechanism that includes the direct action of DJ-1 on these proteins and (3) DJ-1 suppression of PP2A expression, a known inhibitor of MEK1/2 and ERK1/2 family kinases. (4) Upon oxidative stress, DJ-1 can promote pro-survival ERK-dependent mitophagy. (5) DJ-1 interacts directly with ERK1/2, enhancing its nuclear translocation. As a result, phosphorylation of downstream transcription factor Elk1 occurs, and the expression of its target SOD1 is augmented. (6) DJ-1 enhances Nurr1 transcription factor activity through the ERK1/2 pathway activation, triggering the expression of TH, VMAT2, and AADC that are involved in the synthesis and transport of dopamine. (7) DJ-1-mediated activation of ERK1/2 signaling promotes miRNA-22 neuroprotective function by enhancing its expression, in turn downregulating BIM pro-apoptotic protein expression. (8) Finally, ERK1/2 pathway can also be responsible for upregulating DJ-1 upon stress stimulus generating a loop regulatory mechanism. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation. (Adapted from reference [71])

Annex I - Figure 2| Summary of DJ-1's mechanisms involved in the modulation of PI3K/Akt pathway. (1) DJ-1 promotes phosphorylation of Akt, enhancing protective responses executed by the downstream effectors, having an effect, for instance, in mitochondrial well-functioning. (2) On the other hand, DJ-1 can suppress the PI3K/Akt pathway inhibitor's activity, PTEN, by binding to it or by establishing a nitrosylation reaction upon mild nitrosative conditions. (3) The interaction between DJ-1 and Akt may also be promoted by SG2NA, forming a complex by recruiting DJ-1 and Akt mainly to mitochondria and plasma membrane, promoting Akt signaling activity. (4) Defensive responses induced by the DJ-1-dependent activation of PI3K/Akt pathway include the prevention of harmful autophagy processes caused by C2-ceramide insults. (5) Finally, PI3K/Akt pathway activation mediated by DJ-1 is also involved in the proteasomal degradation of Fis1, a protein leading to mitochondrial fragmentation. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

Annex I - Figure 3| Summary of DJ-1's mechanisms involved in the modulation of the ASK1 pathway. (1) DJ-1 prevents the dissociation of the ASK1 inhibitor Trx1 from the inactive signalosome, inhibiting activation of the ASK1 apoptotic pathway. (2) DJ-1 is able to sequester ASK1 activator Daxx in the nucleus under oxidative stress conditions, blocking the formation of the active ASK1 signalosome and ensuring cell survival. (3) A study conducted in drosophila indicated that DJ-1 also suppressed Daxx homolog, DLP, interaction with ASK1, by downregulating the activity of enhancer dFOXO in a PI3K/Akt signaling-dependent manner. (4) Upon oxidative stimulation, DJ-1 may also interact directly with ASK1, disrupting its homo-oligomerization type of activation and avoiding subsequent p38 and JNK-induced cellular apoptosis. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation. (Adapted from reference [71])

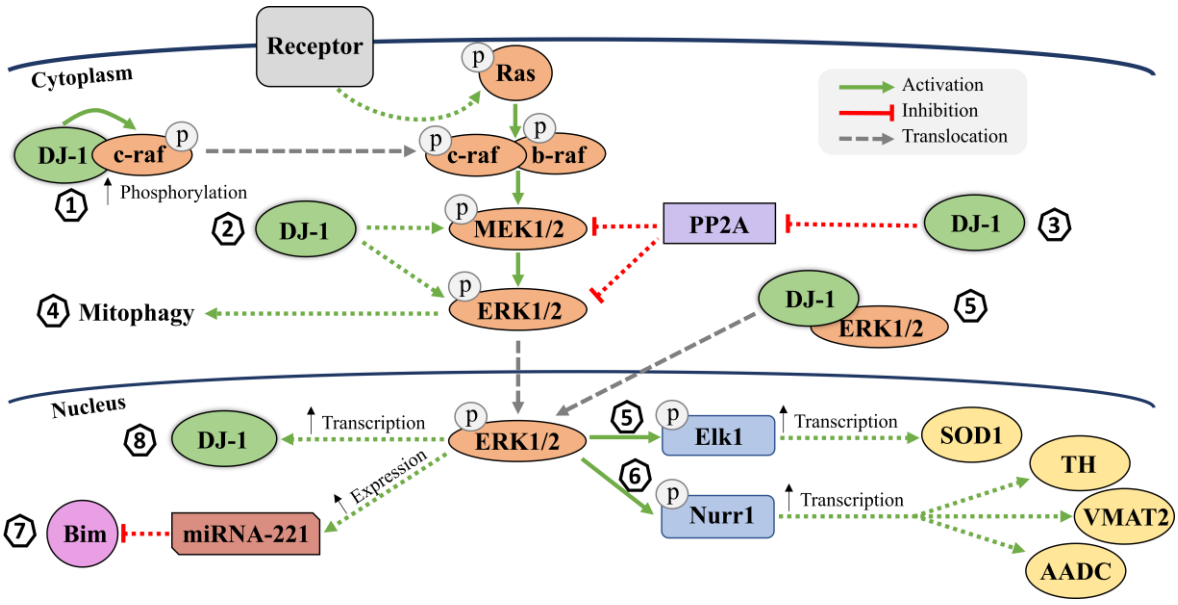
Annex I - Figure 4| Summary of DJ-1's mechanisms involved in p53 pathway regulation. (1) DJ-1 is able to suppress p53 apoptotic signaling by binding to it, which requires proper DJ-1 sumoylation. Consequently, the expression of p53-related targets is blocked, such as Bax, an apoptotic protein, and DUSP1, a phosphatase responsible for suppressing ERK1/2 survival signaling. (2) The DJ-1 C-terminal generated by caspase-6 proteolysis can also suppress p53 activity in a PI3K/Akt-dependent manner. (3) Moreover, the interaction between DJ-1 and SIRT1 enhances the deacetylase activity of SIRT1 towards p53 inactivation. (4) Conversely, p53 has been shown to have a downregulatory effect on DJ-1 expression and mRNA levels, besides targeting the protein for an inhibitory phosphorylation reaction. (5) p53 is responsible for the increase of neurotoxic AEP activity. DJ-1 is able to suppress this p53-mediated activation of AEP by binding to its p53 binding site. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

Annex I - Figure 5| Summary of DJ-1's mechanisms involved in Nrf2 pathway regulation. (1) DJ-1 stabilizes Nrf2 by favoring Nrf2 free form, possibly blocking the association of inhibitor, Keap1. (2) DJ-1 is also able to modulate Nrf2 signaling, activating it in a PI3K/Akt dependent manner. (3) As a result, Nrf2 triggers the expression of specific enzymes involved in antioxidant protective responses, such as IDH, Trx1, GST, HO-1, and NQO1. (4) ND-13 is a DJ-1 and TAT based peptide with therapeutic potential, promoting DJ-1-dependent activation of Nrf2 antioxidant mechanism. (5) A number of chemical substances have also been described with a promising effect in enhancing DJ-1-mediated Nrf2 signaling activation. (6) Furthermore, DJ-1 is involved in a loop 20S proteasome regulatory mechanism that provides a balance in protein degradation processes. DJ-1 may bind to 20S proteome, inhibiting its action together with NQO1 enzyme. Contrarily, the DJ-1-mediated Nrf2 activation also leads to 20S proteasome enhancement. In this schematic representation, solid lines indicate a direct or known regulation, while dotted lines imply an indirect or unknown regulation.

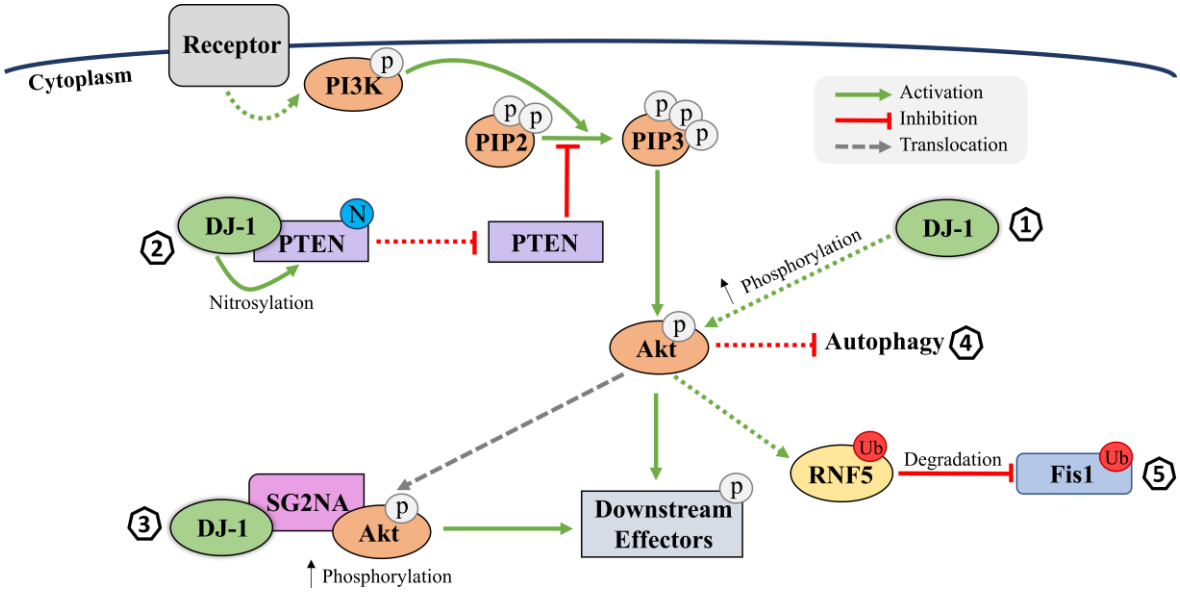
Annex I - Figure 6| Study of signaling pathways modulation by DJ-1 over the years. Graph showing the number of publications of the main DJ-1 mechanisms concerning signaling modulation of ERK1/2, PI3K/Akt, ASK1, p53 and Nrf2 pathways throughout the years.

Annex I - Table 1| Overview of the main described DJ-1 functions in signaling regulation and the influenced of DJ-1 mutations and importance of cysteine residues.

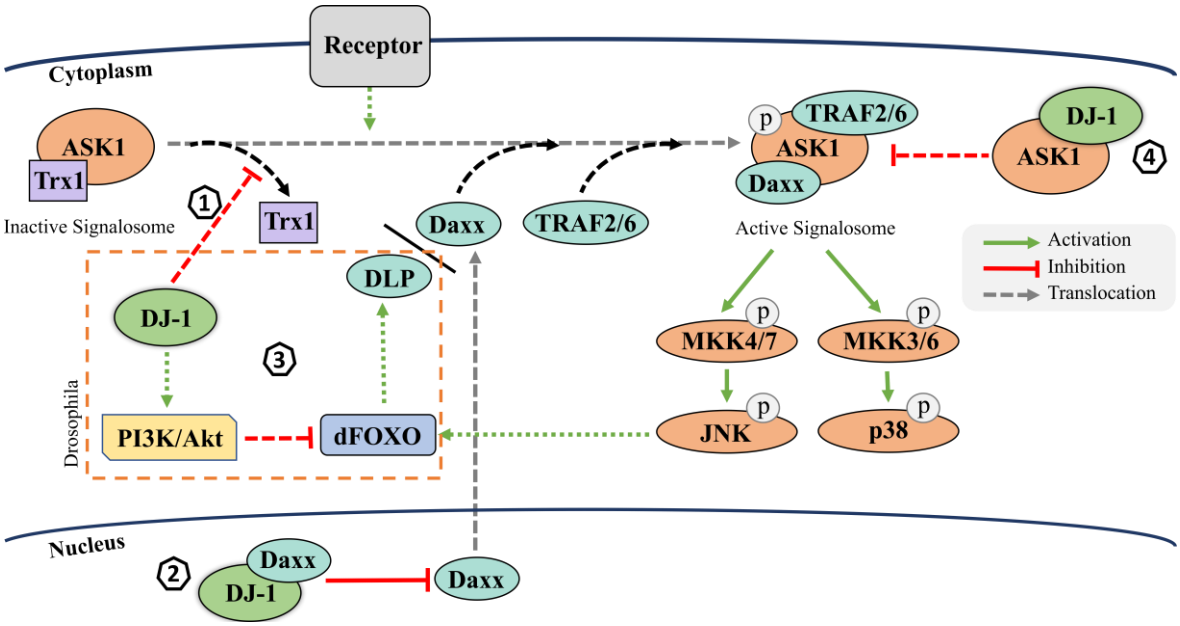
Figures:



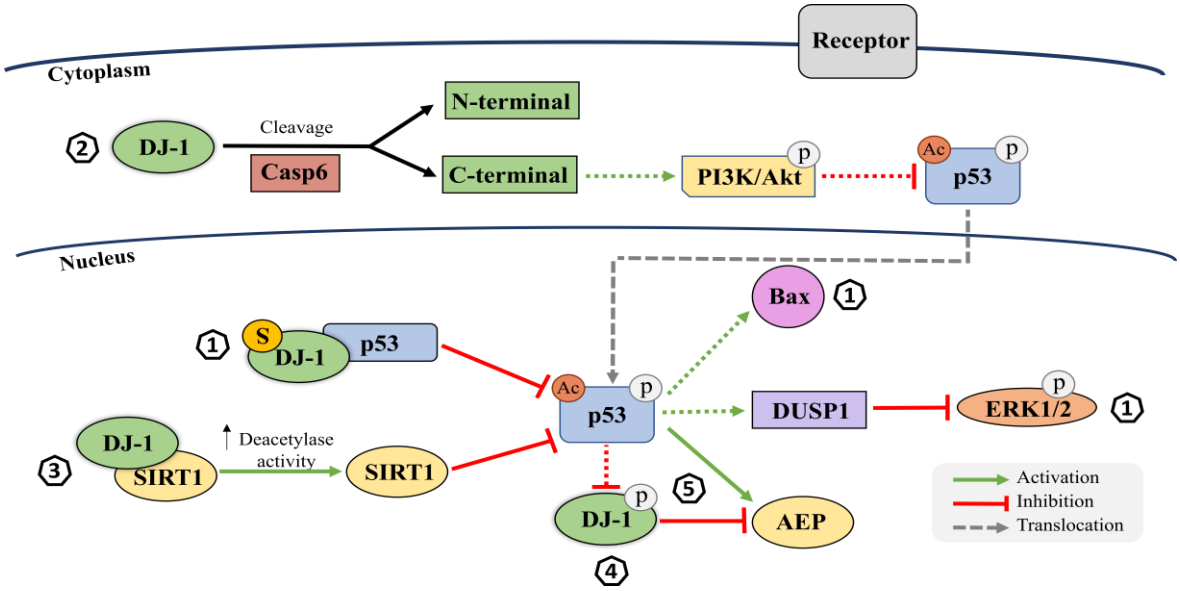
Annex I – Figure 1| Summary of DJ-1's mechanisms involved in the modulation of the ERK1/2 signaling pathway.



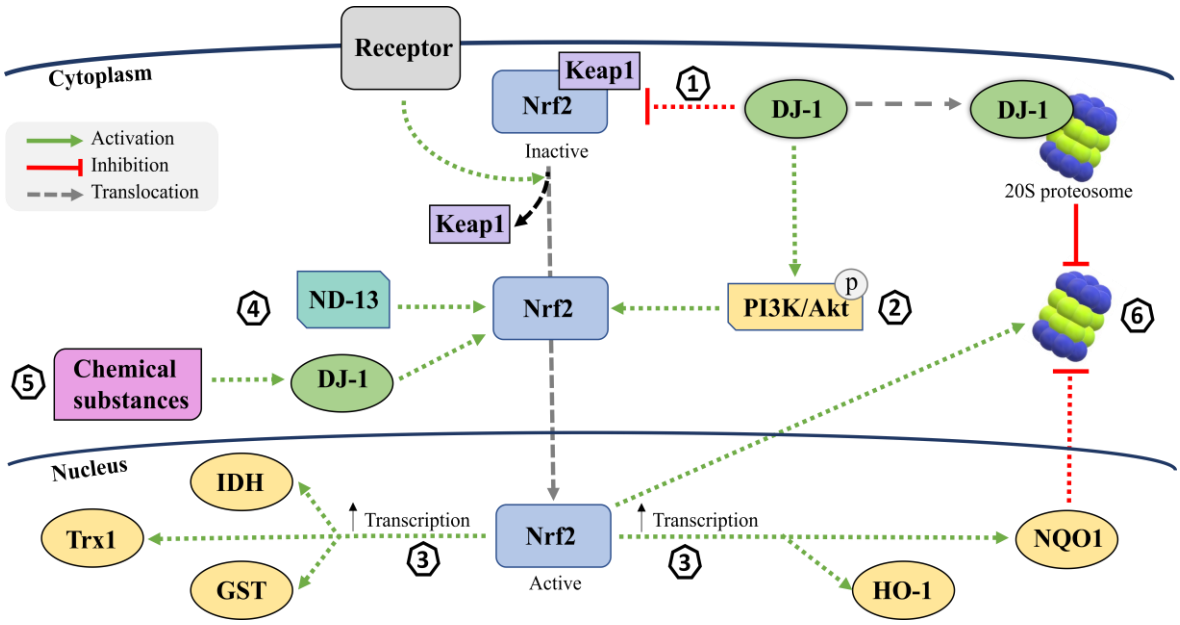
Annex I – Figure 2| Summary of DJ-1's mechanisms involved in the modulation of PI3K/Akt pathway.



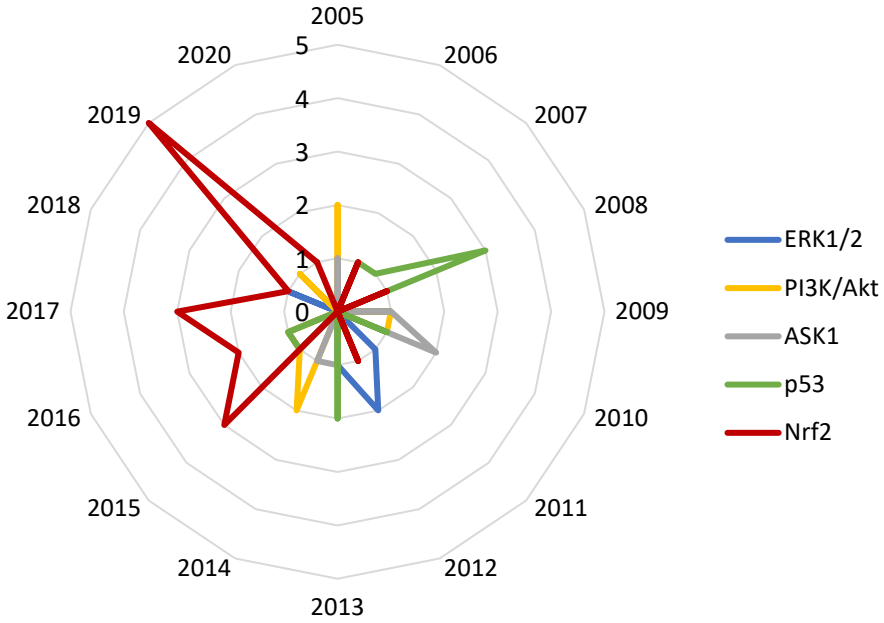
Annex I – Figure 3| Summary of DJ-1's mechanisms involved in the modulation of the ASK1 pathway.



Annex I – Figure 4| Summary of DJ-1's mechanisms involved in p53 pathway regulation.



Annex I – Figure 5| Summary of DJ-1's mechanisms involved in Nrf2 pathway regulation.



Annex I – Figure 6| Study of signaling pathways modulation by DJ-1 over the years.

Annex I - Table 1 Overview of the main described DJ-1 functions in signaling regulation and the influenced of DJ-1 mutations and importance of cysteine residues.

	Function	Mechanism	Influenced by					Year	Ref.:
			PD-related mutantions		Cysteine residues		Other modifications		
			Which	Effect	Which	Details			
ERK 1/2 pathway	ERK pathway activation	Increase of MEK1/2 and ERK1/2 phosphorylation	L166P	Loss of function	-	-	-	2009	[6]
		Decrease of protein expression levels of PP2A	L166P	Loss of function	-	-	-		
		Binding to c-raf and stimulation of its self-oxidation on Ser338	-	-	C106	C106-dependent, but oxidation to SO2H or SO3H was not required	-	2015	[7]
	Enhancement of pro-survival ERK-dependent mitophagy	-	-	-	-	-	-	2012	[9]
	Upregulation of TH, VMAT2, DAT and AADC expression levels for dopamine levels' stabilization	Enhancement of nuclear translocation and activity of transcription factor Nurr1 via the ERK1/2 pathway	L166P	Loss of function	-	-	-	2012, 2016	[10,11]
	Upregulation of SOD1 expression levels, enhancing antioxidant response	Interaction with ERK1/2, enhancing its nuclear translocation and phosphorylation of ELK1 transcription factor	-	-	C106	C106 oxidation not required	-	2011	[8]

	ERK1/2-dependent regulation of cytoprotective miRNA-221	Upregulation of miRNA-221 expression levels and activity, leading to the downregulation of pro-apoptotic proteins	M26I	Loss of function	-	-	-	2018	[12]
PI3K/Akt pathway	Akt pathway activation	Increase of Akt phosphorylation	-	-	-	-	-	2010, 2005	[16-18]]
		Downregulation of PTEN	-	-	-	-	-	2005	[17]
		Binding and downregulation of PTEN	-	-	C106	Requires the presence of the reduced form of C106	-	2009	[19]
		Suppression of PTEN activity via transnitrosylation reaction	-	-	C106	C106-dependent; S-nitrosylation of DJ-1 occuring predominantly at C106	-	2014	[20]
		Formation of DJ-1-SG2NA-Akt complex on the mitochondria and membrane	L166P and M26I	L166P - loss of function); and M26I - decreased function	C106	C106-dependent	-	2014	[21]
	Suppression of harmful autophagy in a PI3K/Akt-dependent manner	Increase of PTEN and Akt phosphorylation	-	-	-	-	-	2015	[22]
	Improvement of mitochondria activity	Enhancement of Akt phosphorylation	-	-	-	-	-	2019, 2016	[23,24]

		Degradation of Fis1 via DJ-1/Akt/RNF5 pathway	-	-	C106	C106 dependent	-	2012	[25]
ASK1 pathway	ASK1 pathway suppression	Prevention of dissociation between ASK1 and inhibitor Trx1	L166P	Loss of function	C106	Dependent of C106 oxidation	-	2010	[29]
		Binding and sequestration of ASK1 activator Daxx in the nucleus	L166P	Loss of function	-	-	-	2005	[30]
			M26I					2009	[31]
		Suppression of Daxx translocation to the cytoplasm and downregulation of its activity via PI3K/Akt pathway	-	-	-	-	-	2013	[32]
		Interaction with ASK1	M26I	Loss of function	C106, C53 and C46	C106 required; C53 and C46 non-essential but modulate C106 activation	-	2009	[31]
		Interaction with ASK1 and disruption of its homo-oligomerization activation	L166P	Loss of function	-	-	-	2010	[34]
	Suppresion of ASK1-driven p38 apoptotic pathway	Binding and suppression of ASK1, and prevention of MKK3 phosphorylation	-	-	-	-	-	2010	[34]
p53 pathway	p53 activity inhibition	Binding and suppression of p53 activity	-	-	C106	C106 oxidation dependent	-	2013	[42]
		Enhancement of SIRT1 deacetylase activity upon the acetylated p53	-	-	C106	C106 dependent	-	2016	[44]
		C-terminal DJ-1-meadiated inhibition of p53 in a PI3K/Akt dependent mechanism	D149A and L166P	Loss of function	-	-	-	2010	[41]

		Sumoylation on the K130 site of DJ-1 allowing its translocation from nucleus to cytoplasm and interaction with p53	L166P	Loss of function	-	-	Sumoylation required	2008, 2006	[39, 43]
	Downregulation of p53-Bax-caspase apoptotic pathway	-	-	-	-	-	-	2007	[47]
		Binding and suppressing p53 transcriptional activity	-	-	-	-	-	2008	[40]
	Suppression of DUSP1, an ERK pathway inhibitor	Binding and suppression of p53 transcriptional activity	-	-	-	-	-	2013	[42]
	Suppression of p53-mediated activation of AEP (legumain)	Binding to the p53 binding site of AEP	-	-	-	-	-	2015	[45]
Nrf2 pathway	Nrf2 activation	Prevention of Nrf2 association with Keap1 inhibitor						2006	[52]
		Disruption of Keap1-Nrf2 complex						2015	[55]
		PI3K/Akt dependent-activation	-	-	-	-	-	2016, 2017, 2020, 2019	[58-60, 69]
		DJ-1 based peptide ND-13 enhancing DJ-1-mediated mechanisms of Nrf2 activation	-	-	-	-	-	2015	[65]
		DJ-1-binding compound B enhances Nrf2 activation through PI3K/Akt pathway by DJ-1-dependent inactivation of PTEN activity	-	-	C106	Compound B binds to the C106 region of DJ-1, preventing inactivating superfluous oxidation	-	2019	[69]
		Other substances (11-Dehydrosinulariolide, bibenzyl compound 20C, Rosmarinic acid,	-	-	-	-	-	2016, 2017, 2020, 2019	[58-60, 66, 68]

		Salidroside, *Morinda citrifolia*'s Active Principle Scopoletin)							
	Upregulation of NQO1 and HO-1	Enhancing Nrf2 activity	-	-	-	-	-	2006, 2015	[52, 53]
	Upregulation of IDH (antioxidant)		-	-	-	-	-	2017	[61]
	Upregulation of Trx1 (ASK1 inhibitor)		L166P and M26I	Loss of function	-	-	-	2012	[62]
	Regulation of 20S proteosome activity	20S proteosome activation by enhancing Nrf2 pathway; 20S proteosome inhibition by binding to 20S proteome together with NQO1 enzyme	-	-	C106	C106 dependent	-	2015	[63]

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Annex II

From an invitation of *Antioxidants* journal (IF: 5.014) to Dr. Sandra Anjo for the submission of a research article to be included in a Special Issue ("Redox and Nitrosative Signaling and Stress"; https://www.mdpi.com/journal/antioxidants/special_issues/redox_nitrosative_signaling_II), I am currently participating in data analysis and preparation of the manuscript to be submitted of the work "oxSWATH applied to the study of the alteration of intracellular and extracellular proteome of cells in response to oxidative stress". This work is summarized in the following abstract based on the analysis of previously obtained results presented in Annex II Figures 1-4. Moreover, this work was submitted and accepted for oral presentation by Dr. Sandra Anjo at the 20th biennial meeting for the Society for Free Radical Research International (SFRR-I 2021 Virtual Meeting), scheduled for 15th-18thMarch 2021.

Title: oxSWATH applied to the study of the alteration of intracellular and extracellular proteome of cells in response to oxidative stress

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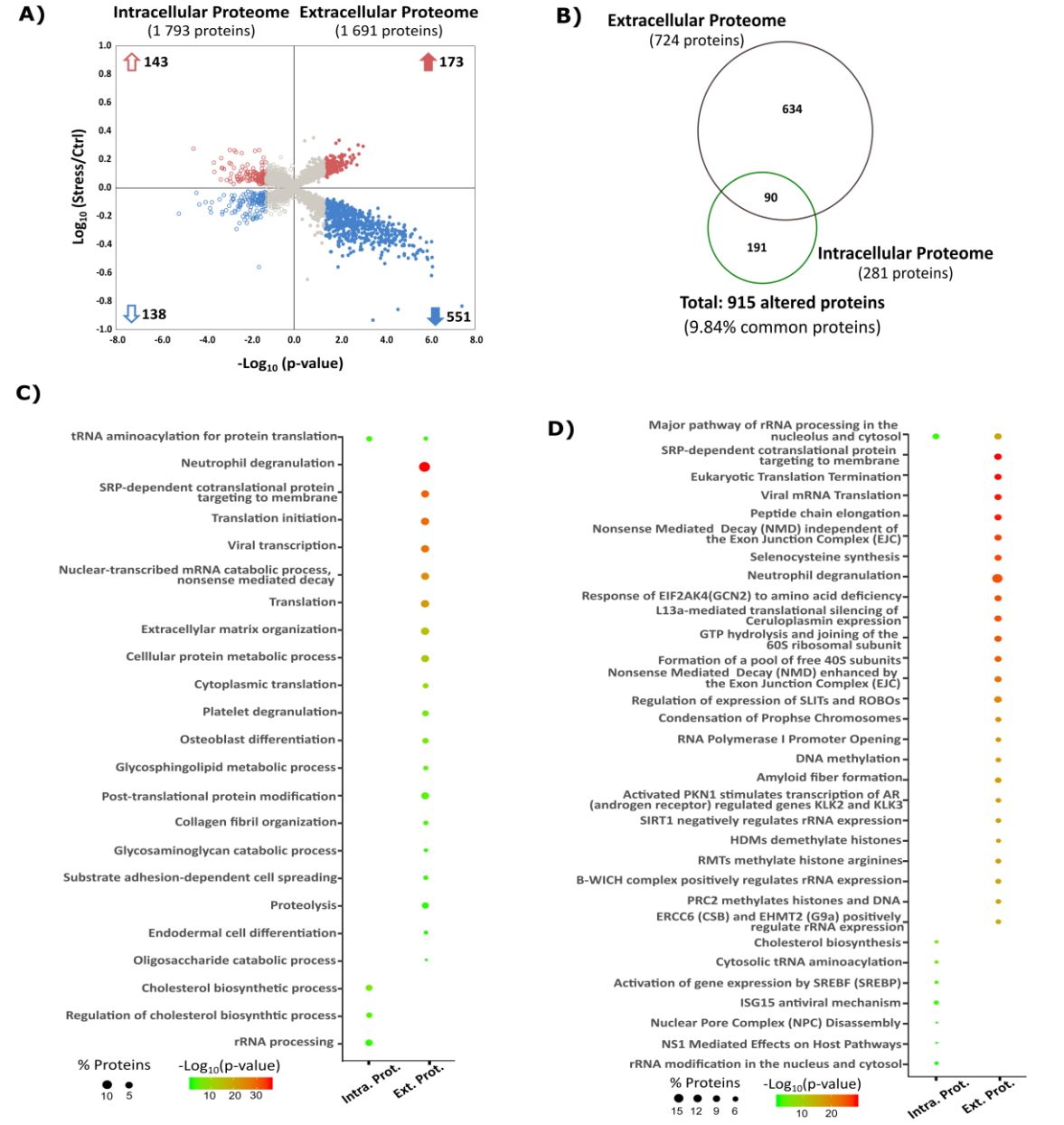
Abstract: In the present work, we apply our novel approach, the oxSWATH[1], to perform an exhaustive characterization of the intracellular and extracellular proteomic alterations of cells exposed to oxidative cells. The oxSWATH method allows integrating the information regarding relative cysteine oxidation with the analysis of the total protein level. Thus, in a single analysis, it was possible to evaluate the alteration considering the redox status of the proteins and performed a generic differential protein expression analysis of the cells exposed to an oxidative stress condition caused by an acute stimulation with hydrogen peroxide. Moreover, to completely characterize the cellular response, both the cells and the secretome were analyzed, covering the intracellular and extracellular responses, respectively. A total of 915 proteins were altered upon oxidative stress, from which 90 were altered in both intra- and extracellular space. Moreover, a clear tendency for a remodeling of the extracellular space was observed, with near 80% of the altered proteins found altered in the secretome. The analysis of the overall redox status of the proteins reveals a tendency to have a reduced environment in the extracellular space, while an

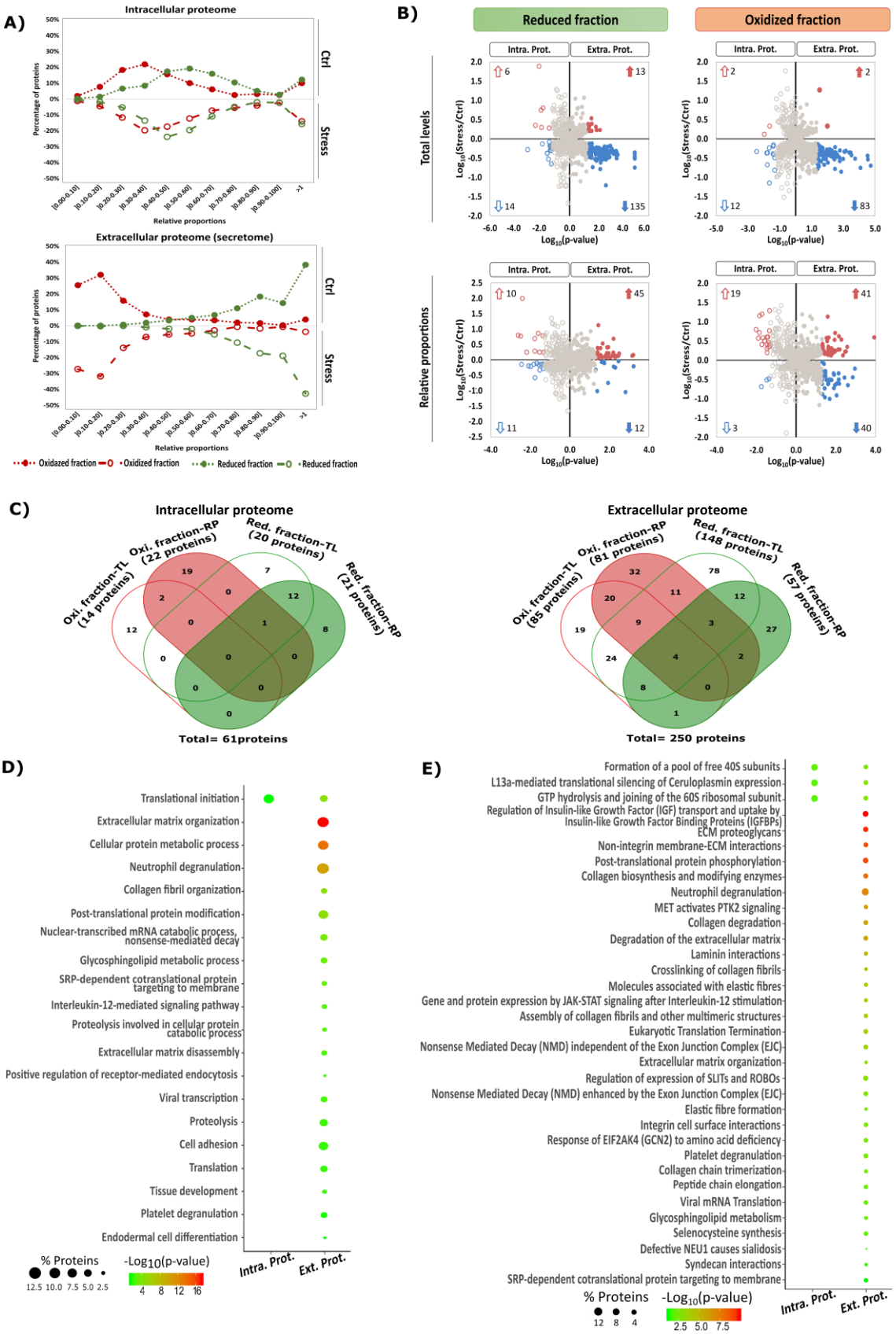
equilibrium between the reduced and oxidized proteins is achieved in the intracellular environment. Again, a higher number of secreted proteins present an alteration of their redox status upon oxidative stress when compared with the intracellular protein (250 and 61 proteins, respectively). From those, only 4 were commonly altered between the two cellular spaces. Overall, these results indicate that there is a differential adaptation of the intracellular and extracellular proteomes, with the extracellular space being particularly affected by oxidative stress. Moreover, the potential of the oxSWATH method was confirmed in this work since a truly comprehensive evaluation of proteomics changes upon the oxidative stimulus was achieved using a single approach.

[1]Anjo, Sandra I et al. "oxSWATH: An integrative method for a comprehensive redox-centered analysis combined with a generic differential proteomics screening." *Redox biology* vol. 22 (2019): 101130. doi:10.1016/j.redox.2019.101130



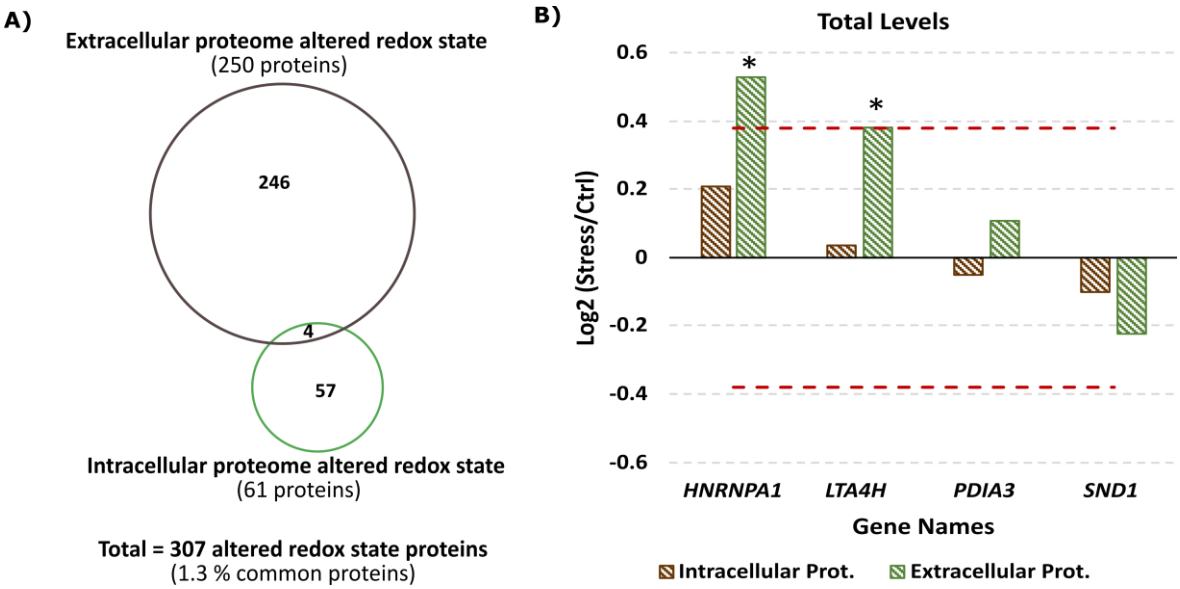
Annex II - Figure 1

Annex II - Figure 1| Differential proteomic analysis of the intracellular and extracellular response to oxidative stress. A) Mirrored volcano plot representing the relative quantification of 1793 intracellular (left side, open dots) and 1691 extracellular (right side, full dots) proteins between the control and stress conditions. Statistical significance was considered for $p<0.05$. A total of 138 and 551 proteins were significantly decreased (blue dots) due to the oxidative stress stimuli in the intracellular and extracellular context, respectively. Conversely, upon the stress condition, 143 intracellular and 173 secreted proteins were shown to be significantly increased (red dots). **B)** Venn diagram comparing the significantly altered proteins in the intracellular and extracellular proteomes. Ninety proteins were altered in both (corresponding to 9.84% of shared proteins), and 915 proteins were considered as altered upon the oxidative stimulus. **C)** and **D)** Biological process and Reactome pathways enrichment analysis, respectively, concerning the total altered proteins: 724 in the intracellular fraction and 281 extracellular proteins. All gene ontology analyses used a minimum p-value of 0.05. **E)** Study of the 90 proteins commonly altered in the intracellular and extracellular components, showing 4 proteins upregulated and 43 proteins downregulated in both. Besides, 35 proteins were shown to be upregulated in the intracellular, but not in the extracellular proteome, whereas 8 proteins were upregulated in the extracellular, but not in the intracellular proteome. **F)** Biological processes, Reactome pathways, and cellular component gene ontology analysis of the groups implying either the same or an opposite relationship established between the intracellular and secreted protein levels. The performed enrichment analysis used a minimum p-value of 0.05.

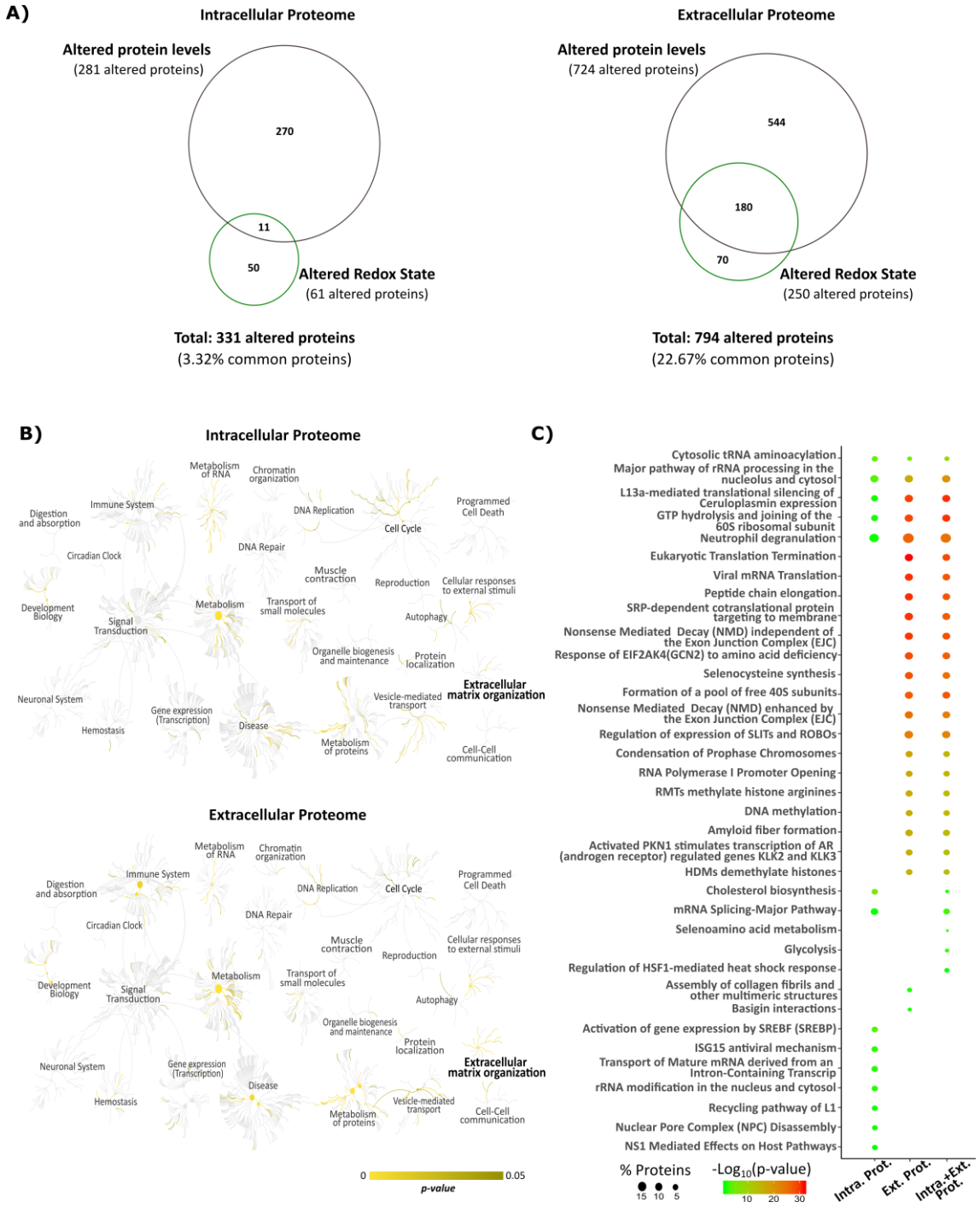


Annex II - Figure 2

Annex II – Figure 2 | Differential redoxomics analysis of the intracellular and extracellular proteomes in response to oxidative stress conditions. A) Graphic representation of the distribution of the reduced (green) and oxidized fractions (red) of the quantified proteins upon control (full dots) and stress (open dots) conditions of each cellular component (intracellular proteome – upper panel -, and extracellular proteome – lower panel). **B)** Mirrored volcano plots representing the differential proteome analysis of the intracellular and extracellular proteomes considering the reduced (volcano plots on the right) and reversible oxidized (volcano plots on the left) fractions of the identified proteins. The results are present considering the total levels (upper panels) and the relative proportions (lower panels). Statistical significance was considered for $p < 0.05$. The number of altered proteins is indicated in the corners of each volcano plot. **C)** Venn diagrams combining all the proteins considered altered in one of the four comparisons accomplished for each cellular component. These results disclose that 61 and 250 proteins in the intracellular (Venn diagrams on the right) and extracellular (Venn diagrams on the left) environments, respectively, present significantly altered expression levels upon oxidative stimulation. **D)** and **E)** Biological process and Reactome pathways enrichment analysis, respectively, in relation to the 61 intracellular and 250 secreted proteins modulated by oxidative stress. The gene ontology analysis performed applied a minimum p-value of 0.05.



Annex II – Figure 3| Analysis of the proteins with an altered redox state in the intracellular and extracellular proteomes in response to oxidative stress. A) Venn diagram combining the proteins significantly altered in the redox-centered analysis of intracellular and extracellular proteomes. Results point out that 4 proteins are commonly modulated by oxidative stress in the intracellular and extracellular space in a total of 307 proteins exhibiting altered redox state upon oxidative stimulation in both environments. **B)** Study of the 4 altered redox state proteins common in the intracellular and extracellular components: Heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1* gene); Leukotriene A-4 hydrolase (*LTA4H* gene); Protein disulfide-isomerase A3 (*PDIA3* gene); Staphylococcal nuclease domain-containing protein 1 (*SND1* gene). The graphic represents the relative quantification of the total protein levels of each of the 4 proteins between control and stress conditions. The results show that the protein levels of hnRNP A1 and LTA-4 hydrolase were significantly increased (above 30% -red line) in the extracellular environment than in the intracellular space upon oxidative stress.



Annex II – Figure 4 | Global characterization of the oxidative stress impact in the intracellular and extracellular proteomes. A) Venn diagrams showing the combination of the proteins altered in the differential proteome analysis with the ones altered in the redox-related analysis, concerning the intracellular (left) and extracellular (right) proteome. As a result, a total of 331 intracellular and 794 secreted proteins were considered altered as a consequence of oxidative-induced stress. **B)** Reactome pathway representation of the intracellular (331 proteins; upper panel) and extracellular (794 proteins; lower panel) proteomes upon the stress condition. **C)** Pathways enriched in the intracellular (331 proteins) and extracellular (794 proteins) proteomes, as well as their combined oxidative stress response (1125 proteins). All gene ontology analyses considered a $p < 0.05$.