Parkinson's disease-associated DJ-1 is required for the expression of the Glial cell line-Derived Neurotrophic Factor receptor Ret in human neuroblastoma cells

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CANDIDATE

Rossana Foti

SUPERVISOR

Prof. Stefano Gustincich

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1. INTRODUCTION

1.1 Parkinson's disease

Parkinson's disease (PD) is the second most common human neurodegenerative disorder, after Alzheimer's dementia. This disease is progressive, with a mean age at onset of 60, and its incidence increases markedly with age [1].

Clinically, most patients present with a motor disorder and suffer from slowness of movement, rest tremor, rigidity, and disturbances in balance. A number of patients also suffer from anxiety, depression, autonomic disturbances, and dementia [2]. Additional common symptoms included sleep difficulties, loss of sense of smell, constipation, speech and swallowing problems, unexplained pains, drooling, constipation, and low blood pressure when standing.

Parkinson's symptoms manifest differently in different patients and the pace at what the disease worsens varies on an individual basis.

Typically, symptoms begin on one side of the body and migrate over time to the other side. There is no test (such as a blood test, brain scan or EEG) to make a diagnosis of PD.

Frequently, the doctor will also look for responsiveness to Parkinson's medications as further evidence that Parkinson's is the correct diagnosis.

PD was first described in the Western world in 1817 by James Parkinson, a British physician who published an essay on what he called "the shaking palsy".

The biochemical changes underlying the disease were elucidated in the 1950s: the discovery of dopamine as a neurotransmitter in the brain by Arvid Carlsson, and the subsequent insight provided by Paul Greengard into the cellular signalling mechanisms triggered by dopamine, gained these researchers the Nobel Prize for Medicine in 2000.

The discovery of dopamine was followed very quickly by reports of markedly depleted levels of dopamine in the basal ganglia of individuals with PD.

The main PD pathological hallmarks are the preferential loss of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) and formation of Lewy bodies — intracytoplasmic inclusion bodies that are mainly composed of fibrillar a-synuclein (SNCA).

Clinical symptoms of PD arise by a threshold effect, whereby denervation of the corpus striatum by dopaminergic neuronal loss reduces dopamine levels to below 70% of wild type [3].

1.1.1 Neuropathological features

The pathologic examination of brains from PD patients demonstrates depigmentation of the *substantia nigra* (SN), caused by the selective and progressive loss of dopaminergic neurons projecting in the striatum. Importantly, approximately 60-80% of dopaminergic neurons are lost before the motor signs of PD emerge. Depletion of dopamine within the striatum causes dysregulation of the motor circuits that project throughout the basal ganglia, resulting in the clinical manifestations of PD.

Dysregulation of the motor circuits

The severity of dopamine depletion and the consequent pathophysiologic changes that occur in basal ganglia circuits determine the severity of parkinsonian signs. Restoring the dopamine deficit or the downstream physiologic abnormalities improves Parkinson's Disease (PD) main motor features. Therefore, both the magnitude and duration of the motor response are a function of the degree of motor severity, which is primarily governed by the loss of tonic dopaminergic activity and disruption of basal ganglia homeostatic mechanisms [4].

The basal ganglia motor circuit modulates cortical output necessary for normal movement: signals from the cerebral cortex are processed through the basal ganglia-thalamocortical motor circuit and return to the same area via a feedback pathway. The basal ganglia are composed of four principal nuclei: the *striatum*, the *globus pallidus* (GP) consisting of external and internal segments (GPi and GPe), the *substantia nigra* (SN) consisting of pars compacta and reticulata (SNc and SNr), and the *subthalamic nucleus* (STN). The *striatum* is the major recipient of input to the basal ganglia from the *cerebral cortex*, *thalamus* and brainstem. Its neurons project to the GP and SNr. The output nuclei of the basal ganglia are GPi and SNr. Their inhibitory output is directed to the thalamocortical pathway and suppresses movement.

SN dopaminergic neurons project to ipsilatral *striatum* (*caudate nucleus* and *putamen*). Dopamine release from SN neurons stimulates D1 receptors and inhibits D2 receptors, resulting in the *striatum* sending impulses to the motor cortex (called the basal ganglia-

thalamocortical motor circuit) in a direct excitatory pathway via thalamic nuclei. Concomitant inhibitory impulses to the motor cortex in a polysynaptic indirect pathway are sent via *globus pallidus externa*, *subthalamic nucleus*, and *thalamic nuclei* are also sent. Loss of dopaminergic nigral cells leads to striatal dopamine depletion and overall decreased motor cortex excitation. The loss of excitatory stimulation decreases excitatory activity of the direct pathway to the motor cortex and increases inhibitory activity of the indirect pathway. Not yet completely understood, the increased inhibitory input to the motor cortex causes bradykinesia [5].

Basal ganglia circuitry in normal conditions   **Basal ganglia circuitry in PD**
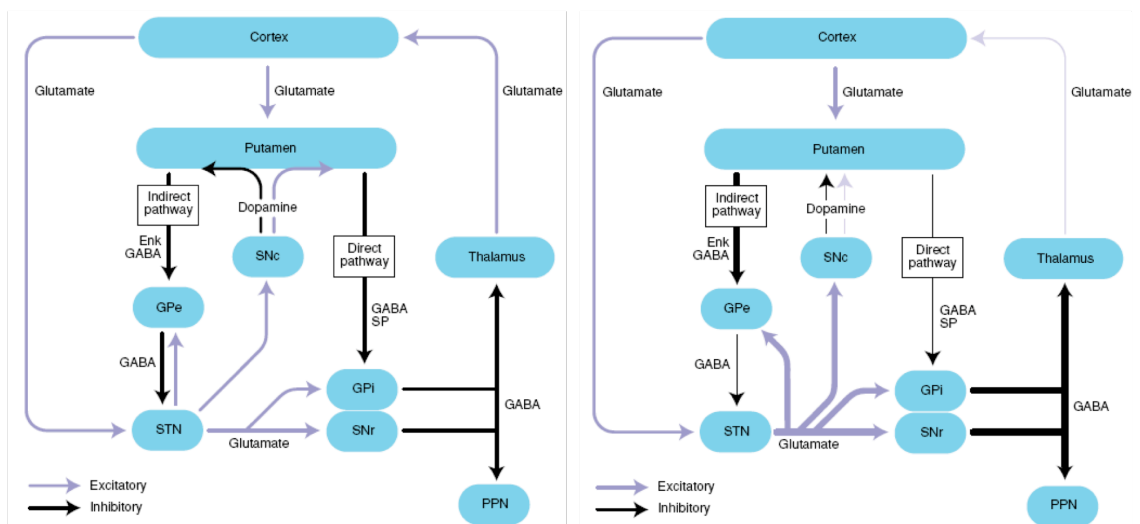
Figure 1. Basal ganglia circuitry in normal conditions (left) and in Parkinson's disease (right). Adapted from: Expert Reviews in Molecular Medicine C 2003 Cambridge University Press.

Lewy Bodies

Brains from PD patients are indeed characterized by the presence of intraneuronal proteinaceous inclusions known as Lewy bodies within the surviving neurons of the SN and other brain regions (dorsal motor nucleus of the vagus, *locus coeruleus*, *raphe* and reticular formation nuclei, *thalamus*, amygdala, olfactory nuclei, pediculopontine nucleus, and cerebral cortex, among others). These inclusions are enriched in filamentous SNCA and other proteins.
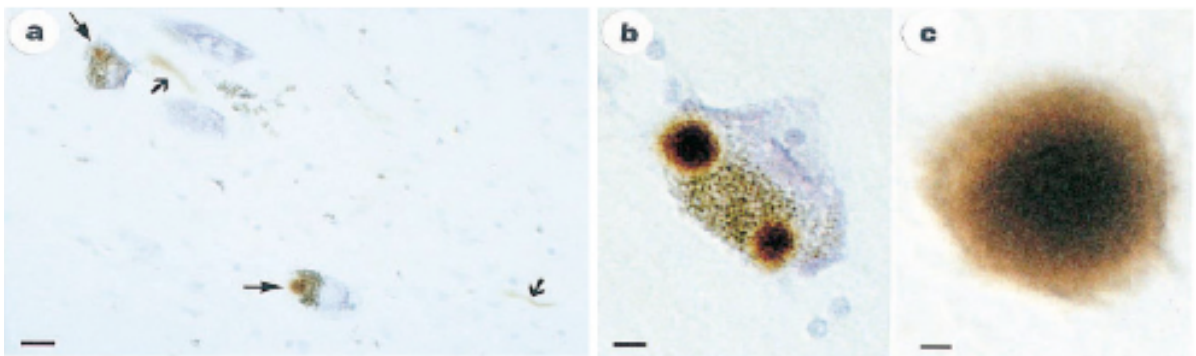
Figure 2. Substantia nigra from patients with Parkinson's disease (from the MRC Cambridge Brain Bank) immunostained for SNCA. **a**, Two pigmented nerve cells, each containing an SNCA positive Lewy body (thin arrows). Lewy neurites (thick arrows) are also immunopositive. Scale bar, 20 mm. **b**, A pigmented nerve cell with two SNCA-positive Lewy bodies. Scale bar, 8 mm. **c**, SNCA positive, extracellular Lewy body. Scale bar, 4 mm.

Lewy body (LB) pathology evolves in six consecutive stages affecting multiple neuronal systems. Lesions initially occur in the *medulla oblongata*, namely the dorsal motor nucleus of the glossopharyngeal and vagal nerves, and in the anterior olfactory nucleus (stage 1). In stage 2, monoaminergic nuclei of the brainstem (*raphe nuclei*, *locus coeruleus*) are involved, whereas the SN becomes affected only at stage 3. In subsequent stages, the pathological alterations take an ascending course with increasing involvement of the cerebral cortex, beginning with the anteromedial temporal mesocortex followed by neocortical areas [6].

The novel neuropathological findings characterizing PD as a multisystemic disorder starting in the *medulla oblongata* and the anterior olfactory nucleus correspond to the recognition that non-motor symptoms are a frequent, if not inevitable, feature of PD. Most remarkably, early symptoms of PD are typically non-motor, implying that parkinsonism is not the first manifestation of PD. The neuropathological alterations outside the SN are well correlated with the non-motor symptoms of PD. The findings of abnormal olfactory

function in early stages of PD correspond to the neuropathological observation that the occurrence of Lewy bodies in the anterior olfactory nucleus is an early feature of the PD .

Braak Parkinson's disease stages 1 & 2
PRECLINICAL

Braak Parkinson's disease stages 3 & 4
CLINICAL PARKINSON'S DISEASE

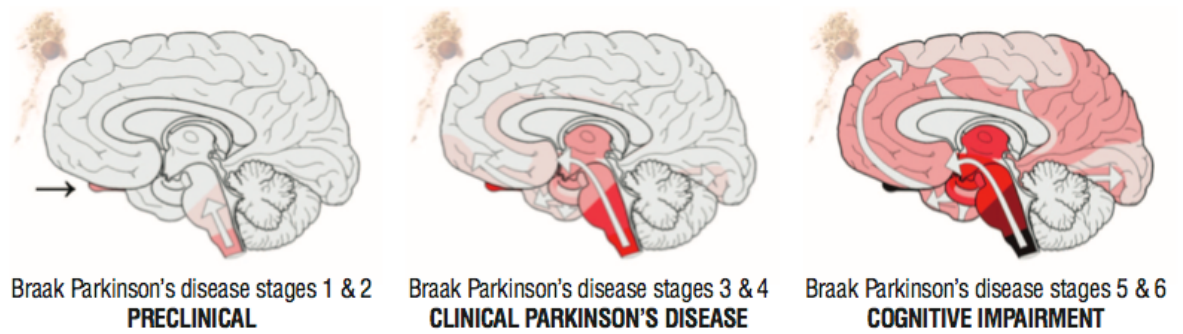Braak Parkinson's disease stages 5 & 6
COGNITIVE IMPAIRMENT

Fig. 3 – Progression of PD-related intraneuronal pathology[7].

1.1.2 Pathogenesis

The exact cause of PD is unknown, although research points to a combination of genetic and environmental factors.

PD is generally a sporadic disorder, but in a significant proportion of cases (10–15% in most studies) it segregates as a Mendelian trait with either autosomal dominant or recessive inheritance. The common form of PD is therefore likely to be a complex trait, determined by several genetic as well as non-genetic factors.

Age of onset tends to be younger in familial PD compared with sporadic PD, particularly so in autosomal recessive PD.

In the last few years family-based linkage analysis and positional cloning have led to the identification of several loci and genes for the rare monogenic forms and more recently of two loci for the classical, non-Mendelian forms (Tab.1).

Table 1. Loci, genes and susceptibility factors involved in parkinsonism

PARK loci	Gene	Map position	Forms of PD	Mutations
PD-associated loci and genes with conclusive evidence				
PARK1/ PARK4	SNCA	4q21	EOPD AD and sporadic	A30P, E46K, A53T, Genomic duplications/triplications
PARK8	LRRK2	12q12	LOPD AD and sporadic	>40 missense variants, >7 of them pathogenic, including the common G2019S
PARK2	Parkin	6q25−q27	Juvenile and EOPD AR and sporadic	>100 mutations (point mutations, exonic rearrangements)
PARK6	PINK1	1p35−p36	ARPD	>40 point mutations, rare large deletions
PARK7	DJ-1	1p36	EOPD AR	>10 point mutations and large deletions
PARK9	ATP13A2	1p36	Juvenile AR Kufor−Rakeb syndrome and EOPD	>5 point mutations
PD-associated loci and genes with unknown relevance				
PARK3	Unknown	2p13	LOPD AD	Not identified
PARK5	UCHL1	4p14	LOPD AD	One mutation in a single PD sibling pair
PARK10	Unknown	1p32	Unclear	Not identified
PARK11?	GIGYF2	2q36−q37	LOPD AD	Seven missense variants
PARK13	Omi/HTRA2	2p13	Unclear	Two missense variants
PARK14?	PLA2G6	22q13.1	Juvenile AR levodopa-responsive dystonia-parkinsonism	Two missense mutations
PARK15?	FBXO7	22q12−q13	EO AR parkinsonian-pyramidal syndrome	Three point mutations
PARK12	Unknown	Xq	Unclear	Not identified
PD-associated genes proposed by candidate gene approach				
Not assigned	SCA2	12q24.1	Unclear, dominant for SCA2	Low-range interrupted CAG expansions in SCA2
Not assigned	GBA	1q21	Unclear, recessive for GD	

Tab. 1 – Loci, genes and susceptibility factors involved in parkinsonism [8].

Although the monogenic forms account for a very small fraction of PD patients, they are promoting the understanding of the molecular pathways in sporadic cases. Even if older age and neurotoxins are established risk factors, several causative genes and susceptibility factors have been identified in rare families with Mendelian inheritance, and suggest that abnormal handling of misfolded proteins by the ubiquitin-proteasome and autophagy-lysosomal systems, mitochondrial dysfunctions and increased oxidative stress, and other pathogenic dysfunctions, contribute to PD [8].

Misfolded proteins

Normal balance between the formation and degradation of cellular proteins is required for cell survival. The pathways by which most cytosolic and misfolded proteins are degraded are carried out by ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP) [9,10]. Impairment of either of these systems may lead to the accumulation and aggregation of proteins. Misfolded accumulated proteins are present in specific populations of neurons and in brain areas physiologically linked to the observed clinical manifestations. Each neurodegenerative disorder has a unique histopathological signature defined by the constituent protein and the distribution and cellular localization of the deposits. The contribution of these aggregated proteins to neuronal death has been debated; for example it has been proposed that the nuclear deposition of huntingtin in Huntington's disease may be neuroprotective, while soluble oligomeric forms of the amyloid β peptide found in senile plaques in Alzheimer's disease are thought to be the primary cytotoxic species in the disease[11].

Increased oxidative stress, associated with depletion of ATP, is considered to contribute to the reduction of proteasome activity and aggregation of abnormal proteins [12,13,14,15].

UPS

The UPS is responsible for a highly selective degradation of short-lived intracellular and plasma membrane proteins under basal metabolic conditions, as well as misfolded or damaged proteins in the cytosol, nucleus or endoplasmic reticulum. The system involves the targeting of susceptible proteins by ubiquitin and only the unfolded ubiquitinated proteins can pass through the narrow pore of the proteasome barrel.

PD and other synucleinopathies (Dementia with Lewy Bodies, Multisystem Atrophy) are characterized by the accumulation of filamentous aggregates of SNCA in Lewy bodies, which co-stain with ubiquitin, pointing to a role of the ubiquitin-proteasome system in the disease. Selective loss of the 20S subunit and of the functional activity of the proteosome is also evident in PD *post mortem* brains. Beside SNCA, other genes involved in the familiar cases are closely linked to the degradation system. The ubiquitin carboxyl-terminal esterase L1 (UCH-L1) gene encodes an ubiquitin hydrolase and ligase, it is mutated in rare familial cases of PD and the protein is found in Lewy bodies of sporadic PD. Parkin, the most common cause of familial cases of PD, appears to function as an ubiquitin E3 ligase. Parkin is a 465 amino acid protein containing two RING fingers separated by an in-between RING (IBR) domain at the carboxyl terminus. The amino terminal bears a ubiquitin-like domain that binds to the RPN10 subunit of the 26S

proteasome. It has been proposed that parkin dysfunction might lead to the toxic accumulation of its substrate. Many substrates for parkin have been discovered, among which the septins CDCREL1 and PAEL-R [16]. Over-expression of CDCREL1 and PAEL-R *in vivo* mediates dopaminergic neurodegeneration [17,18], and both also accumulate in the brains of patients with parkin-related autosomal recessive Parkinson's disease (ARPD) [19,20], but not in *Drosophila* or mammalian parkin knockout models. By contrast, the parkin substrate aminoacyl-tRNA synthetase cofactor p38 is upregulated in the midbrain of parkin null mice as well as in the brains of patients with ARPD and idiopathic PD and its adenovirus-mediated overexpression in the *substantia nigra* of mice induces loss of dopaminergic neurons [21]. Interestingly, it has been observed that parkin can rescue neurons from SNCA-induced proteasomal dysfunction [22]. Moreover, mutations of *ATP13A2* that encodes for a lysosomal ATPase [23,24] have also been shown to result in impaired protein degradation.

ALP

The ALP can be divided into three distinct pathways based on the ways substrates reach the lysosomal lumen: macroautophagy (generally referred to as autophagy), microautophagy and chaperone-mediated autophagy (CMA) [25,26]. Autophagy can be induced within short periods of nutrient deprivation, and CMA can be induced after prolonged nutrient deprivation, while microautophagy is not activated by nutritional deprivation or stress. In contrast to the UPS, the major inducible autophagy is likely to be the primary mechanism involved in the degradation of long-lived, stable proteins and is the only mechanism by which entire organelles such as mitochondria are recycled. Large membrane proteins and protein complexes (including oligomers and aggregates) that fail to pass through the narrow proteasome barrel can be degraded by autophagy [27,25,26,28].

In addition to the UPS, SNCA is also cleared by autophagy [29,25,30,31], which supports the hypothesis that impaired autophagic degradation of SNCA is an important mechanism of neurodegeneration in Parkinson's disease [25]. Indeed, lysosomal malfunction accompanies SNCA aggregation in a progressive mouse model [32], and mutations in *ATP13A2*, a lysosomal ATPase, lead to a failure of autophagy execution and aggregation of SNCA in Parkinson's disease [23],[24] further supporting the hypothesis that ALP dysfunction is an important mechanism of neurodegeneration.

Mitochondrial dysfunctions and oxidative stress

Mitochondrial dysfunction

Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. An important feature of the PD *post mortem* brains is a reduction of mitochondrial complex I activity, discovered in 1983 when the accidental exposure of drug abusers to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) resulted in acute and irreversible parkinsonian syndromes [33]. MPTP inhibits the first enzymatic complex of the mithochondrial electron-transfer chain. After systemic administration, it crosses the blood brain barrier in minutes and, once in the brain, is oxidized to MPDP+ by monoamine oxidase B (MAO-B) in glia and serotoninergic neurons. It is then converted to the active toxic molecule MPP+, likely by spontaneous oxidation. MPP+ is a high affinity substrate for the dopamine transporter DAT and for norepinephrine and serotonin transporters. It then accumulates in dopaminergic neurons, where it confers toxicity and cell death through complex I inhibition and the resulting energy crisis and generation of free radicals. Furthermore, other environmental neurotoxins and inhibitors of complex I such as rotenone and paraquat are also able to induce dopaminergic loss. Rats administered with rotenone develop a PD-like syndrome characterized by neuronal degeneration and the formation of SNCA-rich inclusion bodies. Recently, strong evidences supporting the role of mithochondrial dysfunction in PD came from the discovery of some familial genes: although SNCA and parkin mutations confirm that protein misfolding and UPS dysfunction play a major role in dopaminergic degeneration, these genes seem to be also implicated in mitochondrial dysfunction. The discovery of HtrA serine peptidase 2 (Omi/HtrA2), PINK1 and DJ-1 mutations confirmed that mitochondrial dysfunction is a main upstream pathway to parkinsonism.

SNCA has been found to interact with cytochrome C oxidase (complex IV), the last protein in the mitochondrial electron transport chain, in a yeast two hybrid screening [34]. In transgenic mice, over-expression of SNCA impairs mitochondrial function, increases oxidative stress and enhances nigral pathology induced by MPTP [35]. Moreover, in mice over-expressing A53T SNCA, degenerating mitochondria were found to be immunostained for SNCA, raising the possibility that mutant SNCA might damage mitochondria directly [36]. Whereas over-expression of SNCA increases sensitivity to MPTP, SNCA null mice are resistant to MPTP [37] as well as to other mitochondrial toxins such as malonate and 3-nitropropionic acid [38]. Thus, SNCA seems to mediate some of the toxic effects of MPTP.

Parkin deficiency or mutations have been shown to lead to mitochondrial dysfunction and oxidative stress. Leukocytes from individuals with Parkin mutations present a selective impairment in complex-I activity [39]. Parkin can associate with the outer mitochondrial membrane and prevent mitochondrial swelling, cytochrome C release and caspase activation, and this protective effect is abrogated by proteasome inhibitors and Parkin mutations [40]. Parkin has also been localized to mitochondria in proliferating cells, where it has been shown to associate with mitochondrial transcription factor A and to enhance mitochondrial biogenesis [41]. A *Drosophila* model has revealed a role for Parkin in maintaining mitochondrial function and preventing oxidative stress. Parkin null mutants had severe mitochondrial pathology associated with reduced lifespan, apoptosis, flight muscle degeneration and male sterility [42]. Microarray analysis in these flies revealed up-regulation of genes involved in oxidative stress and electron transport, including a homologue of the mammalian peripheral benzodiazepine receptor, which has been linked to the regulation of mitochondrial integrity and is thought to be involved in the regulation of mitochondrial swelling, respiration, trans-membrane potential and the prevention of oxidative damage to the mitochondria. A genomic screen for modifiers of lifespan in the Parkin null flies found the strongest modifier to be the loss-of-function mutations of glutathione S-transferase (GSTS1) [43]. Re-analysis of the same flies revealed progressive degeneration of a select cluster of dopaminergic neurons and evidence of increased oxidative damage with increased protein carbonyls compared with controls. Furthermore, neurodegeneration was enhanced in GSTS1 null mutants, whereas GSTS1 over-expression significantly rescued the Parkin phenotype [44]. Mammalian models also support a role for Parkin in maintaining mitochondrial function. Deletion of exon 3 of Parkin in mice results in nigrostriatal dysfunction and reduced expression of several proteins involved in mitochondrial function and oxidative stress, including subunits of complexes I and IV. These mice also had decreased mitochondrial respiratory capacity and showed evidence of increased oxidative damage [45]. Intriguingly, Parkin deficiency in these mice did not cause dopaminergic degeneration, which is also observed in exon 2 and exon 3 deletion models [46,47,48]. It will therefore be interesting to determine whether neurodegeneration in these models requires an environmental insult such as an oxidative stressor.

Very recently, several mono-and double-mutant mouse lines expressing high levels of doubly mutated human SNCA and/or a targeted deletion of Parkin have been generated. These mice show genotype-, age- and region-dependent morphological alterations of

mitochondria in neuronal somata, which are restricted to the brain and lacking in skeletal muscle cells. These alterations are not accompanied by alterations of the number or the size of the mitochondria or by leakage of cytochrome C but in the SN coincide with a reduced complex I capacity.

None of the transgenic animals however developed overt motor disabilities or any gross histopathological abnormalities in the analyzed brain regions.

PINK1 encodes a ubiquitously expressed 581 amino acid protein, which consists of an N-terminal mitochondrial targeting motif, a highly conserved serine/threonine kinase domain and a C-terminal autoregulatory domain. Loss of PINK1 function adversely affects mitochondrial function and cell viability under stress. Mitochondrial membrane potential ($\Delta\psi$m) and levels of cell death were measured in a neuroblastoma cell line over-expressing mutated PINK1 (G309D) after exposure to an exogenous source of cellular stress, MG-132, a proteasome inhibitor. Cells over-expressing G309D PINK1 had significantly reduced $\Delta\psi$m compared with the wild type and increased levels of cell death following exposure to stress, but not under basal conditions. Furthermore, cells over-expressing wild-type PINK1 had higher $\Delta\psi$m and lower levels of cell death than cells transfected with vector alone [49]. Consistent with these results, over-expression of wild-type PINK1 was subsequently shown to reduce the release of cytochrome C from mitochondria under basal conditions and staurosporine-induced stress.

Omi/HtrA2 protein has been previously implicated in neurodegeneration, and recently associated with predisposition to PD [50]. It is a PDZ domain-containing serine protease, and also contains an N-terminal mitochondrial targeting motif and a reaper-like motif. Omi/HtrA2 is thought to localize to the mitochondrial intermembrane space, where it is released into the cytosol during apoptosis to relieve the inhibition of caspases by binding to inhibitor of apoptosis proteins (IAPs). Omi/HtrA2 is also able to induce cell death through its proteolytic activity. OMI/HTRA2 knockout mice have been shown to display parkinsonian phenotypes, including rigidity and tremor [51].

Oxidative stress

Inhibition of complex I increases the production of reactive oxygen species (ROS) that can react with proteins, nucleic acids and lipids causing cellular damage.

More in general, impaired mitochondrial function is likely to increase oxidative stress and might render cells more vulnerable to this and other related processes, including excitotoxicity.

The most noticeable feature of the PD *post mortem* brains is the presence of oxidative stress as indicated by the presence of elevated levels of lipid peroxidation markers such as HNE and malondialdehyde, oxidative damage to proteins (such as nitration of tyrosine residues) and nucleic acids, reduced levels of glutathione and oxidized glutathione.

Dopaminergic neurons may be a particularly fertile environment for the generation of ROS, as the metabolism of dopamine (DOP) produces hydrogen peroxide and superoxyde radicals, and auto oxidation of dopamine produces DOP quinone and other species, which can react with proteins and damage them. Mitochondrial dysfunction may disrupt vesicular storage of DOP, causing the increase of free cytosolic concentration of DOP allowing harmful DOP-mediated damage to cellular macromolecules.

Recently, altered ubiquitination and degradation of proteins have been implicated as key to dopaminergic cell death in PD. Oxidative stress can impair these processes directly, and products of oxidative damage, such as HNE, can damage the 26S proteasome. Furthermore, impairment of proteasomal function leads to free radical generation and oxidative stress.

Parkin knockout mice have revealed an essential role for Parkin in oxidative stress [45] and Drosophila Parkin mutants show increased sensitivity to oxidative stress [52].

Implication of *PINK1* in oxidative stress processes has also been strongly suggested: inactivation of Drosophila PINK1 using RNAi suggested that PINK1 maintains neuronal survival by protecting neurons against oxidative stress [53]. In mammalian cell culture, PINK1 protects against oxidative stress-induced cell death by suppressing cytochrome c release from mitochondria, with the protective action of PINK1 depending on its ability to phosphorylate the mitochondrial chaperone TRAP1 [54].

Mutations in the leucine-rich repeat serine–threonine-protein kinase 2 gene (*LRRK2*) represent the most common known cause of familial PD; these mutations also account for cases of sporadic, late-onset PD.

LRRK2 has a conserved serine–threonine kinase mitogen-activated protein kinase kinase kinase (MAPKKK) domain, and is a member of the Roc GTPase family. The commonly occurring Gly2019Ser substitution takes place in the MAPKKK domain, and the mutation augments kinase activity. Although the majority of LRRK2 is present in the cytoplasm, approximately 10% of these proteins are associated with the outer mitochondrial

membrane, raising the question of whether mutant LRRK2 kinase hyperactivity might directly affect mitochondrial function [51].

1.2 DJ-1

DJ-1 gene and protein distribution

DJ-1 gene spans 24 kb and contains eight exons, the first two are non-coding and subject to alternative splicing in mRNA [69]. It encodes a small 189-aminoacid protein that is ubiquitously expressed and is widely distributed in brain and other tissues. It is conserved through different species [70] and determination of crystal structure of human DJ-1 has demonstrated that it exists in homodimeric form, essential to retain its biological function [71]. In human brain, DJ-1 has a marked astrocytic expression [70] while neuronal labelling is very weak. It never localizes at LBs [72]. On the contrary, in the murine brain DJ-1 presents both neuronal and glial expression.

Bader *at al*. found DJ-1 expression in murine neurons of different neurotransmitter phenotypes and in all glial types, such as astrocytes, microglia, and oligodendrocytes. The high DJ-1 expression is not confined to a single anatomical area, considering that positive immunoreactivity was found in cortical areas, *hippocampus*, olfactory bulb, amygdala, *thalamus*, *locus coeruleus*, *caudate*, *putamen*, *globus pallidus* and tinhe deep nuclei of the cerebellum [73,74]. Within the SN, DJ-1 is localized in both neuronal and glial cells. At the cellular level DJ-1 immunoreactivity is found in both cytoplasm and nucleus, and in the mitochondrial matrix and intermembrane space [57]. This staining is increased in oxidative stress conditions suggesting that oxidation promotes the mitochondrial localization on DJ-1 [75].

DJ-1 functions

The exact role of DJ-1 protein in health and disease remains mostly unknown. DJ-1 was first discovered as a novel oncogene in cooperation with activated *ras* [76] and its overexpression has been reported in several cancers including breast, lung and prostate [77].

DJ-1 is involved in various cellular processes including cellular transformation, regulation of RNA stability, transcriptional activation and in oxidative stress response.

Initial computational analysis revealed that DJ-1 belongs to the ThiJ-PfpI Superfamily containing a ThiJ domain. This domain has been first described in the Large Glutamine Amidotransferase superfamily (GAT), enzyme that is involved in the Thiamine synthesis in bacteria. However it is also present in chaperones, catalases and proteases. This heterogeneity of functions of the members of the ThiJ-PfpI superfamily limits the ability to predict the cellular role of the human ortholog. Structural data suggests that DJ-1 conformation seems to be unfavourable for catalytic activity. Furthermore, the catalytic triad Cys-His-Asp/Glu is not conserved across evolution. Olzmann *et al.*, reported that DJ-

1 exhibits protease activity instead of a molecular chaperone function, which is abrogated by the mutation of Cys106Ala [71]. The current consensus is on the lack of catalase or protease activity.

Structural comparisons between DJ-1 and Hsp31, a member of the ThiJ-PfpI family, may point to a chaperone activity [78]. Shendelman *et al.* showed that DJ-1 acts as a redox-regulated chaperone. It is activated in an oxidative cytoplasmic environment and inhibits the formation of SNCA aggregates *in vitro* and *in vivo* [63]. These data suggests that DJ-1 can mitigate the molecular insults downstream ROS burst suppressing the early step of protein aggregation.

DJ-1 chaperone activity prevents SNCA fibrillation, but only when Cys106 is oxidized to sulfinic acid (Cys106-SO_2H), while the native DJ-1 and the highly oxidized form of DJ-1 resulted to be ineffective [79]. These results suggest that the oxidation level can affect its structure thus impairing its normal function.

DJ-1 is linked to oxidative stress response. DJ-1 undergoes a pI shift from 6.2 to 5.8 upon ROS exposure, indicating that it may function as an indicator of oxidative stress [80]. Cys 106 is the most sensitive among all the three cystein residues (i.e. Cys 46, Cys 53 and Cys 106). Oxidation induces a mitochondrial relocalization of DJ-1 and protection against cell death is abrogated in Cys106Ala but not by Cys46Ala or Cys53Ala [58]. Two *post mortem* studies of brain samples from PD brains found that acidic isoforms of DJ-1 are more abundant in PD compared to controls [70,81]. The accumulation of acidic isoforms of DJ-1 monomer is followed by the enrichment of basic isoforms of DJ-1 dimer in PD/AD brains due to protein carbonylation [81].

DJ-1 displays a protective role against ROS-induced cell death. DJ-1 knock-down by short RNA interfering rendered SH-SY5Y susceptible to both hydrogen peroxide (H_2O_2) and 1-methyl-4-phenylpyridinium (MPP^+). While overexpression of wild type DJ-1 protects from oxidative stress, proteasomal inhibition and ER stress [56,80], cells harboring Leu166Pro DJ-1 mutant become susceptible to death induced by hydrogen peroxide and do not show oxidized forms of DJ-1.

Moreover, a number of studies performed on DJ-1 deficient mouse model supports this hypothesis.

Dopaminergic neurons derived from *in vitro* differentiated [82] DJ-1-deficient embryonic stem cells showed a decreased survival and an increased sensitivity to oxidative stress. Comparing the responses to H_2O_2 of primary cortical neurons from brains of DJ-1$^{+/+}$, DJ-

$1^{+/-}$ and DJ-$1^{-/-}$ mice embryos exposed to H_2O_2 [83], DJ-$1^{-/-}$ deficient neurons showed a 20% increase in cell death compared to wild type DJ-$1^{+/+}$ neurons, whereas an intermediate amount of cell death was observed in DJ-$1^{+/-}$ neurons suggesting a gene-dosage effect.

A similar study analyzed the effect of pesticide rotenone on dopaminergic neurons. This pesticide inhibits mitochondrial complex I, increasing ROS. Upon rotenone treatment, the number of surviving dopaminergic neurons in DJ-1-deficient mice was decreased by 30% compared to control DJ-$1^{+/+}$ neurons. In addition, it has been shown that DJ-1 knock-out (KO) mice are more vulnerable to MPTP and restoration of DJ-1 expression in DJ-1 deficient mice via adenoviral vector delivery mitigated cell death [83].

Several DJ-1 KO mice were produced independently but none of them showed degeneration of dopaminergic neurons *per se*. Although they showed an age- and task-dependent motor deficits, and a marked reduction in evoked dopamine overflow in the striatum probably due to an enhancement of DAT function in DJ-1 null mice [84,85], dopaminergic neuronal loss was present only when they were treated with drugs that cause toxicity through mitochondrial complex I inhibition.

Findings from triple KO studies demonstrated that inactivation of Parkin, DJ-1 and PINK1 is still insufficient to cause dopaminergic degeneration in mice during their lifespan. These results further supported the notion that Parkin, DJ-1 and PINK1 are not absolutely required for the survival of nigral neurons. Rather, they likely provide protection to nigral neurons against insults, such as oxidative stress, during the decades-long aging process in human patients [86].

A complementary approach to elucidate DJ-1 role in PD is the study of its homologs in *Drosophila,* DJ-1α and DJ-1β. DJ-1α shares 56% of homology with human DJ-1 while DJ-1β 52%. They are characterized by distinct temporal and spatial expression pattern. Menzies *et al.* found that DJ-1α is expressed predominantly in testis, with high expression level only in the late stages of development, whereas DJ-1β is ubiquitously present, with no relevant changes in expression during the development, closely resembling the human DJ-1 expression pattern [87]. *Drosophila* lacking DJ-1 activity are fertile, have a normal life span and normal number of dopaminergic neurons. Meulener *et al.* indicated that double knock out (DKO) is not deleterious, but it is much more sensitive to agents that induce oxidative stress (i.e Paraquat and Rotenone) [88]. Loss of DJ-1β expression results in an increased survival of dopaminegic neurons due to a compensatory up-regulation of DJ-1α in the brain, which is also associated to a decreased sensitivity to Paraquat [87]. Yang *at al.* analyzed a DJ-1α knock out fly strain: the specific inhibition of DJ-1a in dopaminergic

neurons led to a decrease of TH- positive neuron number, to elevated ROS accumulation and hypersensitivity to oxidative stress. These data clearly indicate a protective role of DJ-1 mostly in oxidative stress response [59].

DJ-1 protective role is also studied through the analysis of its protein-protein interaction network. It has been shown that DJ-1 is indispensable for the stabilization of the transcriptional regulator NF-E2-related factor-2 (Nrf2). In physiological conditions, its expression is maintained at low level by Keap-1 that targets Nrf2 to protein degradation. Upon exposure to oxidative stress, Nfr2 translocates into the nucleus, where it forms hetero-dimers with other transcription factors (TFs) inducing the expression of antioxidant genes whose promoters contain the ARE (Antioxidant Responsive Element) [89].

The anti-apoptotic function of DJ-1 is exerted through its interaction with the death associated protein Daxx. DJ-1 sequesters Daxx in the nucleus, preventing its binding and consequential activation of Apoptosis Signal Regulating Kinase 1 (ASK-1) [62]. DJ-1 is also a transcriptional co-regulator of several transcription factors acting on various promoters, functioning as a co-activator and as a co-repressor. Xu *et al.* demonstrated that DJ-1 interacts with the nuclear proteins p54nrb and pyrimidine tract-binding protein associated splicing factor (PSF), two multifunctional regulators of transcription and RNA metabolism, highly expressed in brain. DJ-1 blocks the transcriptional silencing and apoptosis induced by PSF-binding protein p54nrb antagonizing PSF effects. Interestingly, DJ-1 can activate the transcription at the TH promoter, the rate-limiting enzyme for dopamine biosynthesis [90]. DJ-1 prevents the post translational modification of PSF by the Small Ubiquitin Modifier SUMO-1. Mutations that abolish the sumoylation of PSF relieve the transcriptional repression of the TH promoter by PSF. Interestingly, the same study demonstrated that DJ-1 regulates sumoylation *in vivo.* Lymphoblast from PD patients (both carrying deletion of exon 1-5 and the missense mutation Leu166Pro) have a slight reproducible increase of SUMO-1-modified high molecular weight complexes, compared to patients with wild type DJ-1 or carrying the non-pathogenic mutation Arg98Gln. These data indicate that DJ-1 loss results in an accumulation of SUMO-1-modified PSF, leading to a decreased DA synthesis. It is worth noting that this effect is specific for SUMO-1 conjugated proteins, while it does not affect SUMO2/3 conjugated proteins.

Shinbo *et al.* found that DJ-1 is bound to p53 *in vitro* and *in vivo* and that this binding is

stimulated by UV radiation. Moreover, DJ-1 restores p53 transcriptional activity inhibited by Topors [91], which sumoylates both DJ-1 and p53. DJ-1 is sumoylated by SUMO-1 on the conserved Lys 130 and its post-translational modification is induced by UV radiation in a p53-dependent manner. The mutant DJ-1 Lys130Arg abrogates cell growth-promoting activity with activated *ras* [92].

Furthermore, Kim *et al.* 2005 demonstrated that DJ-1 modulates the phosphatidylinositol 3-kinase survival pathway by negatively regulating the function of the tumor suppressor gene PTEN [77]. Abstrakt, a RNA helicase ubiquitously expressed, has been also found to stimulate the transforming activity of DJ-1 in rat 3Y1 cells transfected with DJ-1 and activated ras [93].

Indeed, it has been show that DJ-1 affects cell viability by regulating the ERK 1/2 signalling pathway. Overexpression of wild type (WT)- DJ-1 but not of the L166P mutant dramatically increases the phosphorylation of Extracellular signal-regulated kinases (ERK 1/2). The ERK1/2 signaling pathway is critical for protecting cells against oxidative stress; therefore, the increase of ERK1/2 phosphorylation caused by DJ-1 might provide protection against subsequent oxidative stress [94].

By interacting with protein inhibitor of activated STAT member x (PIASx) alpha and with DJ-1 binding protein (DJBP,) proteins predominantly expressed in testis, DJ-1 was also able to alter their binding to the Androgen Receptor (AR) [95].

DJBP directly binds to AR in a testosterone-dependent manner and it negatively modulated the AR transcription activity by recruiting the histone-deacetylase complexes. DJ-1 antagonizes this inhibition by the abrogation of the complex and restoring the AR activity.

DJ-1 in Parkinson's Disease

After the identification of the PARK-7 locus by van Dujin *et al.*[96], Bonifati *et al.* have shown mutations in the DJ-1 gene in two PARK-7 linked families. The Dutch family displayed a large homozygous deletions of exons 1-5 of the DJ-1 gene and an Italian kindred harbored a single missense mutation at an highly conserved position, Leu166Pr [97].

Till now different mutations affecting DJ-1 (including missense, truncating and splice-site mutations and large deletions) have been linked to autosomal recessive PD.

PD families may present missense mutations of DJ-1 in homozygous (L166P, M26I and E64D) and heterozygous forms (A104T and D149A) [98]. M26I and L166P are the most studied DJ-1 missense mutations. Although M26I dimer formation and protein levels reported in the literature may vary, differences with WT DJ-1 are small and of unclear

biological significance. On the contrary, L166P is very unstable and its expression level lower than WT [99,100,101,102].

Several studies indicate that the mutant Leu166Pro is unstable and thus degraded through the UPS [71,103,75]. It is worth noting that position 166 is localized in the penultimate C-terminal a-helix near the dimer interface and the mutation to proline is predicted to interrupt the helix [71,104,105]. The Leu166Pro mutant is rapidly degraded. Interestingly, Leu166Pro tends to form multimeric aggregates [102,101,71]. The Leu166Pro monomer has been recently shown to possess a different conformation from the wild type DJ-1 and the data computational analysis indicates that Leu166 is not located at the subunit surface involved in the dimerization. Thus the different conformation of Leu166Pro monomeric units might affect the protein-protein interaction repertory [99].

It has been shown that L166P and M26I mutants share an increased interaction with TRAF and TNF receptor-associated protein (TTRAP). This binding occurs in the absence of neurotoxic stimuli proving the existence of an altered DJ-1 protein networks in the presence of PD-associated missense mutations. Most importantly, M26I and L166P block the protective activity of TTRAP inducing a TTRAP-dependent pro-apoptotic pathway that involves JNK and p38 MAPKs, a signaling system that plays a central role in dopaminergic cell death and PD pathogenesis [106].

The pathogenic role of other mutants remains to be established. Some of them might be simple polymorphisms that predispose to PD, as demonstrated for the Arg98Gln.

Although DJ-1 mutations account for only a small fraction of early onset PD (1-2%), they are the second most frequent cause of recessive forms of PD, after PARK2-linked families. Indeed, in the more common sporadic form of PD, total DJ-1 levels were significantly lower in the SN region of PD patients compared to health controls [107].

Clinically, DJ-1-dependent PD is characterized by early onset of parkinsonism (the average age is the early 30s), slow disease progression and good initial response to Levodopa.

Psychiatric and behavioural disturbances (including severe anxiety and psychotic episodes) are reported in both original DJ-1 families [108]. Further analysis is necessary to investigate whether this aspect is more frequent in DJ-1 patients than in other PD forms.

DJ-1 in Neurodegeneration

Immunohistochemical staining for DJ-1 stained Tau inclusions found in several neurodegenerative disorders, including Alzeheimer's disease (AD), Progressive Supranuclear Palsy (PSP), Frontotemporal Dementia linked to chromosome 17 (FTDM-17), and Pick's Disease (PiD) [70,109,110]. Furthermore, DJ-1 antibodies readily labelled SNCA glial cytoplasmic inclusions observed in patients with Multisystem Atrophy (MSA) [109] and PD. As with tau and SNCA, a fraction of DJ-1 protein became markedly insoluble in brain from AD, PiD and MSA compared to controls [109,110]. Moore *et al.*, (2005) monitored the level of detergent-soluble DJ-1 in human *post mortem* cingulated cortex tissue. They found a dramatic increase of DJ-1 levels in the detergent-insoluble fraction in PD and Dementia with Lewy Bodies (DLB), indicating that in pathological conditions DJ-1 can undergo a biochemical modification.

These data support the notion that different neurodegenerative diseases might share a common mechanism in which DJ-1 role needs to be elucidated.

1.3 Clinical approaches to Parkinson's disease

Current therapy is largely based on a dopamine replacement strategy, primarily using the dopamine precursor levodopa. However, chronic treatment is associated with the development of motor complications, and the disease is inexorably progressive. Furthermore, disease progression is associated with the emergence of features such as freezing, falling, and dementia which are not adequately controlled with dopaminergic therapies. Indeed, it is now appreciated that these non-dopaminergic features are common and the major source of disability for patients with advanced disease.

Many different therapeutic agents and treatment strategies have been evaluated over the past several years to try and address these unmet medical needs, and many promising approaches are currently being tested in the laboratory and in the clinic.

Clinical trials with intrastriatal transplantation of human embryonic mesencephalic tissue have shown that grafted DA neurons re-innervate the striatum, restore striatal DA release and, in some patients, induce major clinical benefit. Stem cells could provide an unlimited source of DA neurons for transplantation. Recent studies demonstrate that cells with properties of mesencephalic DA neurons can be produced from stem cells of different sources including reprogrammed somatic cells [111].

Although stem cell research is promising, there are many obstacles still to be overcome. The optimal type of stem cell and transplant protocol remain to be defined, cell survival after transplantation is limited, and efficacy in animal models has not yet been established to exceed or even equal that obtainable with fetal nigral transplantation (which so far has failed in clinical trials) [112]. Safety issues must be fully addressed preclinically before clinical trials can be initiated, particularly with respect to the risk of tumor formation and off-medication dyskinesia.

Trophic factors are proteins that support and protect subpopulations of cells. A number have been reported to act on dopaminergic neurons *in vitro* and *in vivo*, making them potential therapeutic candidates for Parkinson's disease. All of these candidate factors protect dopaminergic neurons if given prior to, or with, selective neurotoxins. Fewer trophic factors, primarily Glial-derived neurotrophic factor (GDNF) and its relative, neurturin (NRTN; also known as NTN), have been shown to restore function in damaged DA neurons after the acute effects of neurotoxins have subsided. A major barrier to clinical translation has been delivery. GDNF delivered by intracerebroventricular injection in patients was ineffective, probably because GDNF did not reach the target, the *putamen*, and intraputaminal infusion was ineffective. A randomized clinical trial with gene therapy

for NRTN is underway, in an attempt to overcome these problems with targeting and distribution. Other strategies are available to induce trophic effects in the CNS, but have not yet been the focus of human research. To date, clinical trials have focused on restoration of function (i.e., improvement of parkinsonism). Protection (i.e., slowing or halting disease progression and functional decline) might be a more robust effect of trophic agents.

However, gene therapy offers the potential to provide more diffuse distribution of the therapeutic protein through the brain target. In MPTP monkeys, gene delivery of GDNF was diffusely distributed throughout the striatum, and provided motor benefits, restoration of striatal TH staining, and protection of SNpc dopamine neurons.

1.3.1 GDNF

GDNF supports the development of embryonic dopamine neurons relatively specifically [115] and is particularly important for postnatal survival of mesencephalic dopamine neurons[116]. It is present in the striatum and despite being named glial-derived growth factor, it may reside largely in the striatal medium spiny neurons that receive dopaminergic input from the SN [117]. GDNF levels in various parts of the brain are reported to be no different in parkinsonian than in control patient brains [118]. GDNF mRNA expression was increased in the *putamen* of PD patients [119], there is no evidence that loss of GDNF is responsible for development of PD.

A variety of experiments in rodent models have shown that GDNF directly injected into the SN or *striatum* protects dopaminergic neurons from neurotoxins [118,119,120,121]. Elevating GDNF in the striatum by gene therapy is also protective [122,123,124] Unlike the case with Brain-derived neurotrophic factor (BDNF), however, there is also evidence for restoration of function of injured neurons by GDNF after toxic insults [118,121,]—although restorative actions are generally not as dramatic as the neuroprotective actions [118]. A series of influential studies from Gash, Gerhardt, and co-workers [125] have demonstrated GDNF-induced improvements in bradykinesia, rigidity, and postural instability in monkeys with stable MPTP-induced hemiparkinsonism. In addition to neuroprotective and neurorestorative actions, GDNF also has direct effects on dopamine neurons, modulating excitability via changes in A-type potassium channels [35]. This may be a mechanism by which GDNF acutely increases dopamine release [36,120].

GDNF signals through a two-component receptor complex consisting of the GPI-linked GDNF family receptor alphas (GFRa) and the rearranged during transfection (Ret) receptor tyrosine kinase, a previously identified oncogene-related protein. In vitro studies have implied that GDNF binds to GFRa, which then forms a complex with Ret, resulting in the phosphorylation of a tyrosine residue of Ret.

The GDNF signaling is mediated through the tyrosine kinase properties of Ret. Activation of Ret by GDNF has been shown to result in activation of the Erk and MAP kinase.

Although GDNF was originally characterized as a potent survival factor for midbrain dopaminergic neurons, analysis of KO mice has shown that GFRa1-Ret signaling is not essential for the embryonic development of these neurons, illustrating the complex network of signaling pathways and compensatory mechanisms that regulate neural development. Recently, two groups have reported the generation of different strains of mice with the specific deletion of Ret in dopaminergic neurons [129,130]. Surprisingly, these animals showed normal development and maturation of the nigrostriatal system. A detailed study, including dopaminergic neuron number in SNpc and VTA, fiber density in the *striatum* and *nucleus accumbens*, and dopamine levels, indicated that Ret is not required for the maintenance of midbrain dopaminergic neurons in young adult mice. However, in one of these studies, Kramer and coworkers found that, only in aged animals, Ret ablation leads to a progressive and cell-type specific loss of SNpc dopaminergic neurons and their afferents, thereby indicating that Ret is a critical regulator for the long-term maintenance of the nigrostriatal system. The significance of Ret signaling for the dopaminergic system was additionally studied in knock-in multiple endocrine neoplasia type B (MEN2B) mice by introducing the Ret-MEN2B mutation (Met919Thr), which renders the Ret receptor tyrosine kinase constitutively active. In this work, the authors showed that constitutive Ret activity in mice is sufficient to increase brain dopamine concentration and the number of dopaminergic neurons in SNpc [131, 30].

1.3.2 RET

In 1996, the transmembrane receptor tyrosine kinase Ret was identified as a GDNF receptor. Ret is shared by all of the GFLs as their common signaling receptor.

Mice lacking Ret die a few hours after birth owing to kidney agenesis and show absence of enteric and many parasympathetic neurons [133].

Ret is strongly expressed, both at the mRNA and protein level, in adult dopaminergic neurons of SN and VTA.

Conditional KO mice for Ret receptor show normal development and maturation of nigrostriatal system. In ageing mice ablation of Ret leads to a progressive and cell-type-specific loss of SNpc neurons and their projections into the *striatum*. Ret is specifically required for long-term target innervation. In aged Ret mutant mice, the extent of target innervation loss exceed the degree of cell loss. Mutant mice show also reduced levels of evoked dopamine release but not of total dopamine amounts in the *striatum*. This effect is likely due to the fact that Ret mutants loose neurons during ageing which leaves more time for compensatory mechanisms.

Moreover, Ret ablation resulted in gliosis in the *striatum* and to macroglial activation in the SN.

The absence of RET in midbrain dopaminergic neurons is associated with modest decrements in locomotor activity, whereas most sensorimotor functions seem to be unaffected.

So far there is no evidence that PD can be caused by mutations in the Ret gene itself because analysis of polymorphisms have not shown any association with PD but recently Ret expression level has been studied in PD drug models. Following MPTP treatment Ret was found strongly down-regulated in the *striatum* of adult mice. Indeed, 6-hydroxydopamine and rotenone reduce expression Ret mRNA in the SN and VTA. These studies provide evidence that lack of neurotrophic support, due to down-regulation of Ret receptor, precedes neuronal degeneration.

Recently, Elvidge and collegues have demonstrated a strict correlation existing between the HIF-1a stabilization and a strong down-regulation of the GDNF receptor Ret [134]. This result pointed out to a possible common pathological mechanism shared by different neurodegenerative disorders.

Therefore, down-regulation of Ret indicates that trophic support may be limited not to reduced levels of the trophic factor itself and by the loss of its receptor, leading to a diminished capacity to transduce the actions of neurotrophic factors. This in turn leads to the lack of neurotrophic support and neuronal degeneration.

2. AIMS

Although the exact cause of PD is unknown, familial cases have contributed to the understanding of the molecular pathways involved in the common forms of PD.

DJ-1 mutations are the second most frequent cause of recessive forms of PD after PARK2-linked cases.

Moreover, even in the more common sporadic form of PD, total DJ-1 levels were found significantly lower in the SN region of PD patients compared to healthy controls [107] suggesting that the altered functional DJ-1 level could account not only for the hereditary form of the disease but also be involved in the onset of sporadic cases.

Due to its multifunctional properties, the causative link between the lack of functional DJ-1 and the onset of Parkinson's disease is still matter of debate
Thus, understanding the relationship of DJ-1-mediated neurodegeneration to PD will have a powerful impact on the development of disease-modifying therapies for PD.
The purpose of this thesis is to elucidate these molecular mechanisms by a multi-step approach:

1. developing a cellular model to study the effect of loss of DJ-1 was developed;

2. gene expression profile to find out molecular pathways altered by the loss of DJ-1

3. analysis and validation of pathways altered by DJ-1 loss of function

Results presented in this thesis are part of the following manuscript in preparation:

- **<u>Foti R.</u>,** Zucchelli S., Biagioli M., Roncaglia P., Krmac H., Calligaris R., Gustincich S.
"Parkinson disease associated DJ-1 is required for the expression of GDNF receptor Ret in human neuroblastoma cells"

Indeed, during these years I have been involved in several different projects that are not presented in this thesis but are part of the following pubblications and manuscript:

- Zucchelli S., Vilotti S., Scotto Lavina Z., Calligaris R., Biagioli M., **<u>Foti R</u>**., Casseler C., De Maso L., Pinto M. and Gustincich S.
"Aggresome-forming TTRAP mediates pro-apoptotic properties of Parkinson's Disease-associated DJ-1 missense mutations"
Cell Death and Differetiation Cell. 2009 Mar;16(3):428-38

- Calligaris R., Bellarosa C., **<u>Foti R.</u>,** Roncaglia P. Giraudi P, Krmac H., Tiribelli C. and Gustincich S."
"A transcriptome analysis identifies molecular effectors of unconjugated bilirubin in human neuroblastoma sh-sy5y cells.
Submitted to BMC Genomics

- Deganuto M, Cesaratto L, Bellarosa C , Calligaris R, Vilotti R, Renzone G, Scaloni A, **<u>Foti R.</u>,** Gustincich S,Quadrifoglio F, Tiribelli C , Gianluca Tell G. "A proteomic approach to the bilirubin-induced toxicity in neuronal cells reveals a protective function of DJ-1 protein." Submitted to Proteomics

- Vilotti S., Zucchelli S., Scotto Lavina Z., **<u>Foti R.</u>** and Gustincich S.
"Parkinson's disease associated DJ-1 mutant L166P hampers nucleolar localization of TTRAP and p53 in response to proteasomal impairement"
Manuscript in preparation

3. RESULTS

3.1.1 Generation of inducible stable cell lines expressing RNAi against DJ-1

To mimic the loss-of-function effects as seen in PD patients with DJ-1 mutations, we used DJ-1-specific siRNA constructs to inhibit the synthesis of endogenous DJ-1 in the human SH-SY5Y neuroblastoma cell line. We screened among three different sequences for the best silencing efficiency in transient transfection
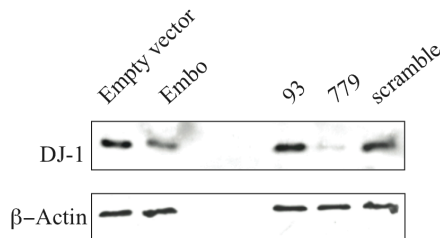

Fig. 4 - Western Blot analysis of DJ-1 expression level in SH-SY5Y after transient transfection of siRNA#Embo, siRNA#93 and siRNA#779 constructs and of scramble sequence.

Since siRNA#93 showed the same DJ-1 protein level than the empty vector, we decided to use siRNA#779 and siRNA#Embo siRNA (siRNA#1 and siRNA#2 respectively) to establish stable cell lines silenced for DJ-1 and the scramble sequence to establish a control cell line.

Several attempts of constitutive expression of very efficient siRNAs against DJ-1 failed for the positive selection of cells that maintained a low but relevant amount of endogenous DJ-1 protein.

Therefore, we decided to take advantage of an inducible RNAi approach, in which the expression of the interfering sequence is controlled by the tetracycline repressor (TetR) system. Inhibition of DJ-1 expression is thus achieved when the inducing agent (tetracycline or doxycycline) is present. This allows the selection of stable clones in absence of interference.

We first generated stable SH-SY5Y cells upon transfection of a plasmid that directs the espression of the TetR protein under a strong constitutive promoter. We screened among 24 clones for the most abundant expression of the Tet-Repressor protein and we selected P17 clone as recipient clone for inducible RNAi sequences.
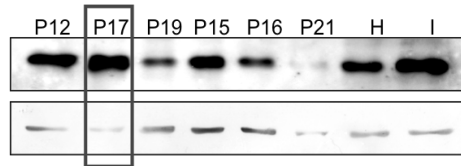

Fig. 5 – Generation of TetR stable cells. SH-SY5Y cells stably transfected with Tet-Repressor (TetR) were generated. Independent monoclonal populations were selected and analyzed for TetR expression. Western Blot analysis for TetR representative clones are shown.

We tested various conditions for the silencing induction in transient transfection and we choose 7 days of induction for the strongest down-regulation of DJ-1 mRNA level but, as expected for its high stability, 10 days of induction for the down-regulation of the protein level.

Tested Conditions				
mRNA analysis			Protein analysis	
Time	Concentration		Time	Concentration
24 hrs	1μg/mL; 2.5 μg/mL; 5 μg/mL; 10 μg/mL		7 days	1μg/mL; 2.5 μg/mL;
48 hrs	1μg/mL; 2.5 μg/mL; 5 μg/mL; 10 μg/mL		**10 days**	**2.5 μg/mL;**
5 days	1μg/mL; 2.5 μg/mL; 5 μg/mL		14 days	2.5 μg/mL;
7 days	**1μg/mL; 2.5 μg/mL;**			

Tab. 2 – Tested conditions for silencing's efficiency.

By a second stable transfection, we were then able to generate RNAi inducible stable cell lines. To monitor the presence of off-target effects, we used both the two different RNAi molecules siRNA#1 and siRNA#2 and, as an additional control, we established inducible cell lines with the scramble sequence.
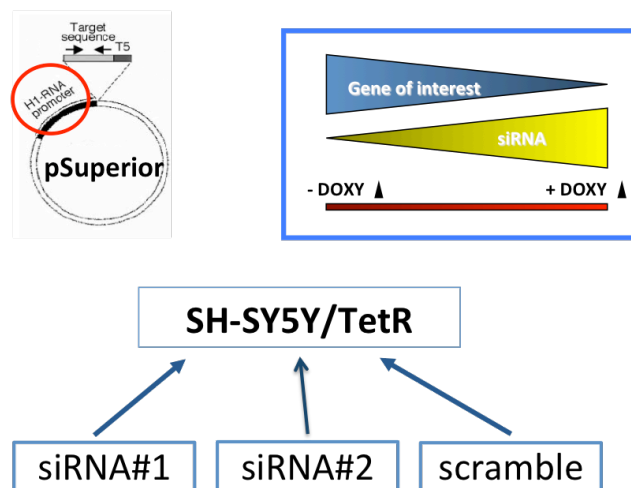

Fig. 6 – Generation of inducible stable cell lines. Schematic representation of inducible expressing system.

All three interference sequences were under the control of TetR. For each of the cell lines we selected 24 single clones in G418, and multiple, independently isolated clones were scored for Doxycycline (Doxy) inducible down-regulation of DJ-1 expression level by quantitative Real Time PCR (qPCR). Scramble clones were used as internal control for endogenous physiological DJ-1 levels. To exclude clonal effects, two independent clones for each cell lines were selected and analyzed. Since, when comparing not-induced and -induced siRNA#1 clones, we observed a partial leakage of the Tet promoter in Dox-untreated cells, all our experiments were carried out comparing DJ-1-interfered clones with scramble controls.

As shown in figure 7, siRNA#1 causes a profound decrease in DJ-1 expression level, achieving a >90% silencing. These results were consistent in the two independent selected clones (B and G). siRNA#2 proved to be only partially effective, with a 50% reduction in DJ-1 levels as compared to scramble clones. These data are in agreement with previously results that used the same sequence in transient transfection experiments [80]. Only one clone was obtained during the selection process for siRNA#2 since most of the clones didn't show any variation in DJ-1 levels.
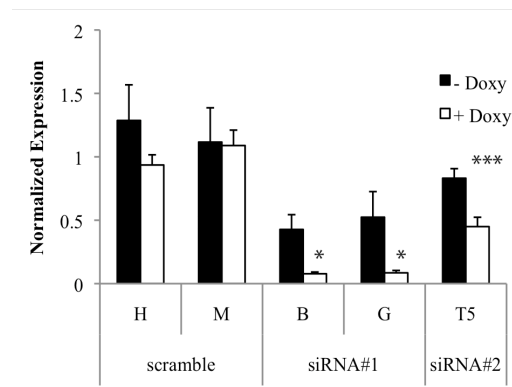

Fig. 7 - qPCR of DJ-1 expression level before and after 10 days of doxycycline-induction. All experiments were performed in triplicate, and error bars indicate SD.

To assess the effects of DJ-1 mRNA silencing on the levels of DJ-1 protein, we monitored the down-regulation of DJ-1 by western blot analysis and by immunofluorescence.
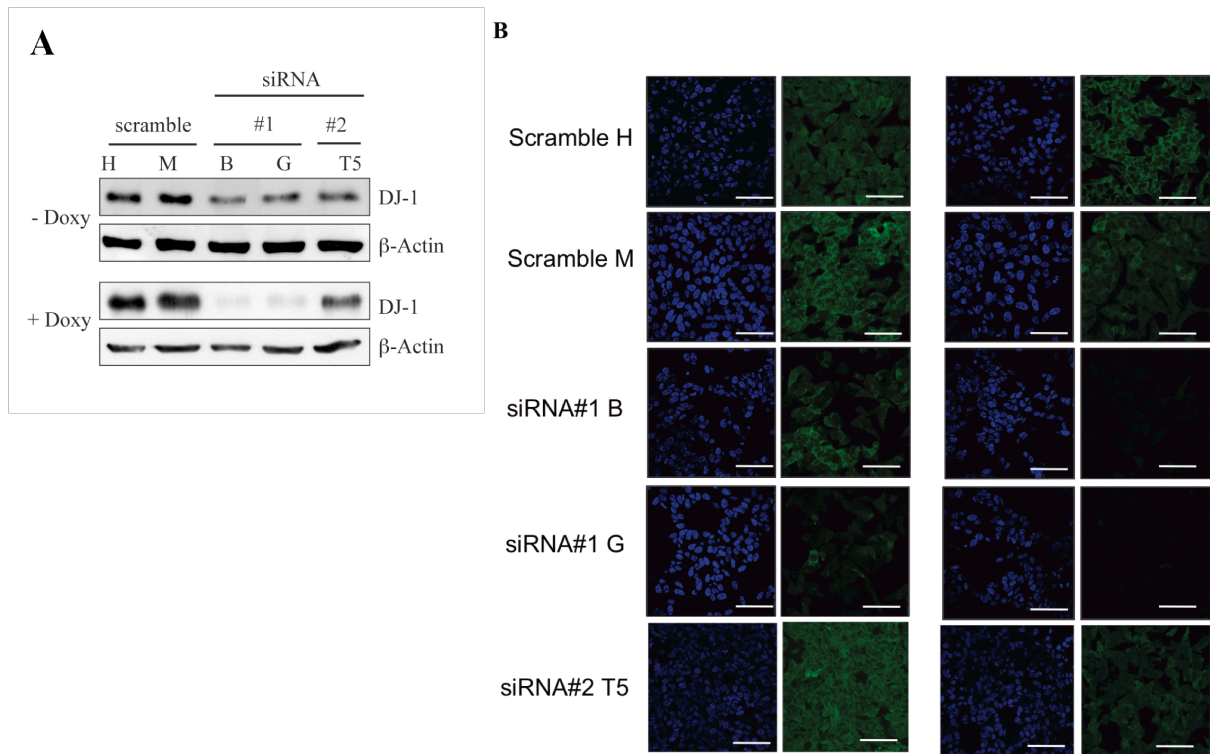
Fig. 8 – Analysis of DJ-1 protein level in absence and in presence of doxycycline-induction. Western Blot (A) and Immunofluorescence (B) analysis of DJ-1 expression level in inducible siRNA clones and scramble clones. Scal bar 50 μm

As shown in figure 8, after induction, DJ-1 protein level were almost undetectable in both siRNA#1 clones compared to scramble while the reduction observed in siRNA#2 clone is only partial.

We then decided to proceed our analysis in both clones siRNA #1 that better represent the pathological condition of a homozygous loss of function.

3.1.2 Silencing kinetics

To monitor the kinetics of silencing, we measured DJ-1 mRNA levels by qPCR at 3, 6 and 10 days of Doxy induction. A 90% reduction of DJ-1 mRNA level was present already after 3 days of induction with Doxy and it persists to the 10th day.

We found that the amount of residual DJ-1 protein was reduced to almost undetectable levels in siRNA#1 as compared to scramble clones only after 10 days of Doxy treatment. Kinetic experiments showed that DJ-1 protein started to decrease at 6 days (70%) and 90% was knocked down after 10 days. These results are compatible with the half-life of the DJ-1 protein as measured in cell culture [80].
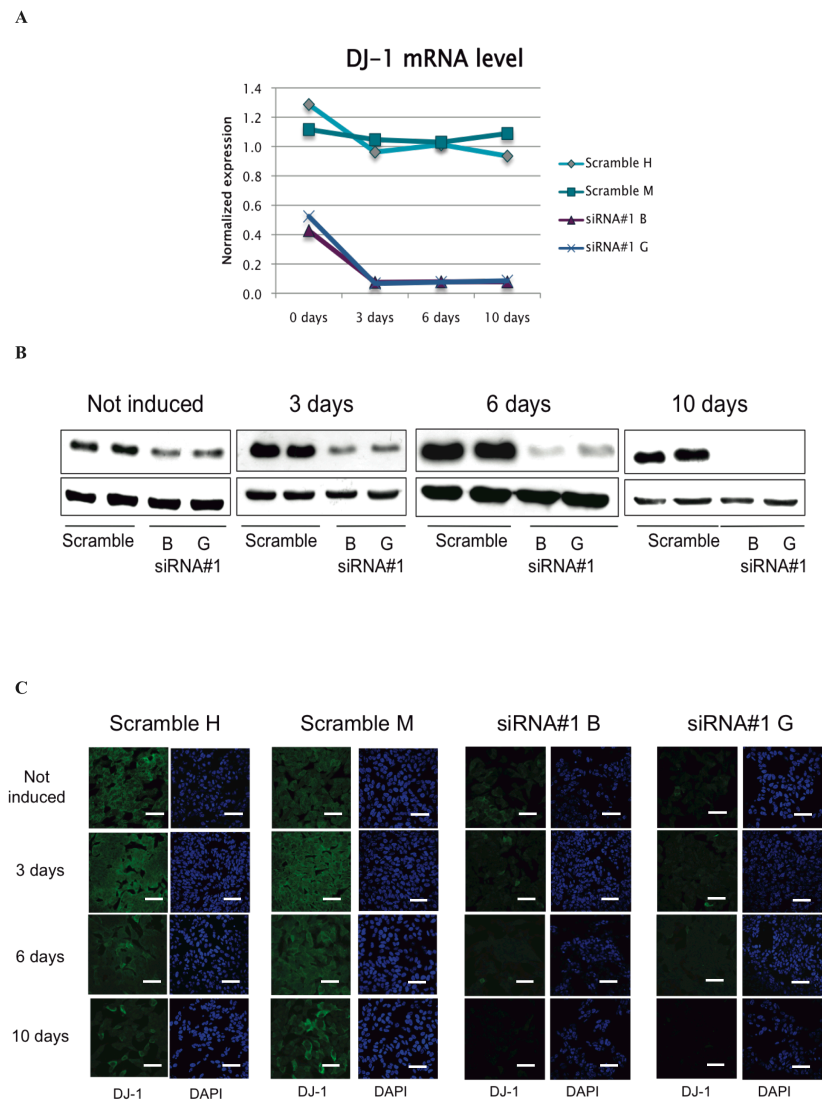

Fig. 9 – Kinetic of silencing in not induced and after 3, 6 and 10 days of induction in siRNA#1 clones and scramble clones; A) qPCR of DJ-1 mRNA level; B) Western Blot analysis of DJ-1 protein level; C) Immunofluorescence analysis of DJ-1 protein. All experiments were performed in triplicate, and error bars indicate SD. Scale bar 20 μm

3.1.3 Cellular Phenotype

Concomitantly with the complete silencing of DJ-1 we observed a modification of intercellular contacts with the formation of mounds of cells and the loss of peripheral cells' monolayer.
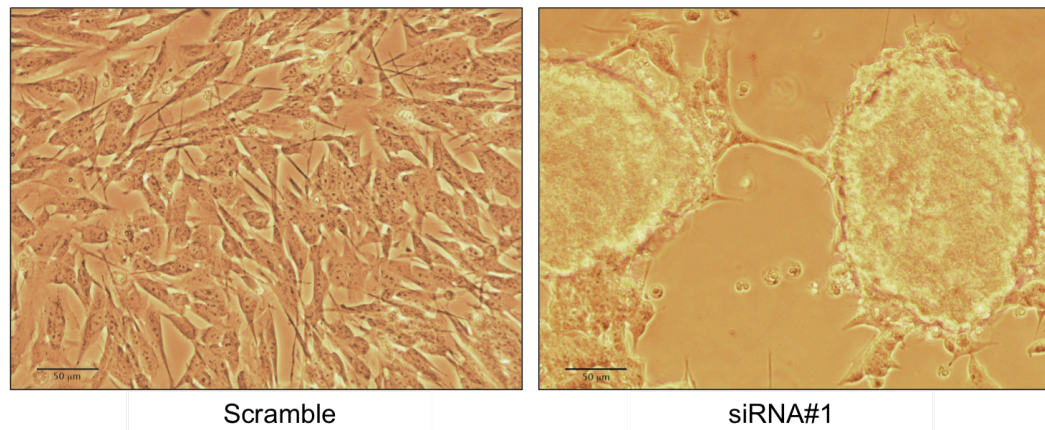
| Scramble | siRNA#1 |

Fig. 10 – Visible phenotype. Rapresentative image of siRNA#1 clones and scramble after 10 days of silencing induction. Scal bar 50 μm.

Interestingly, the spheroids formation was strictly correlated to silencing kinetic. As shown in fig. 11, the first appeareance of spheroids is after six days of silencing induction, perfectly following the kinetic of DJ-1 protein level downregulation.
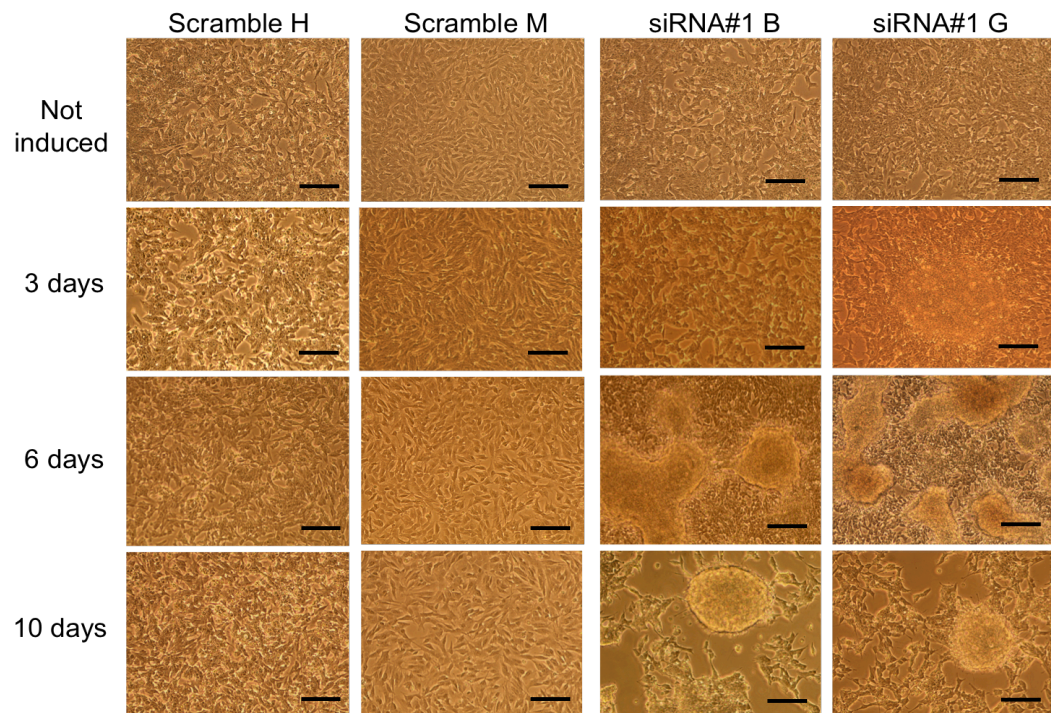
Fig. 11 – Kinetic of silencing. Rapresentative images both of siRNA#1 clones and both scramble clones in not induced and after 3, 6 and 10 days of silencing induction. Scal bar 20 μm

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3.1.4 FACS analysis of the cell cycle

To further characterize the silenced clones, we performed Fluorescent Activated Cell Sorting (FACS) analysis of the cell cycle. We compared the FACS profiles of both scramble clones with profiles of both silenced clones of siRNA#1. We couldn't find any statistically relevant differences between scramble and silenced clones.
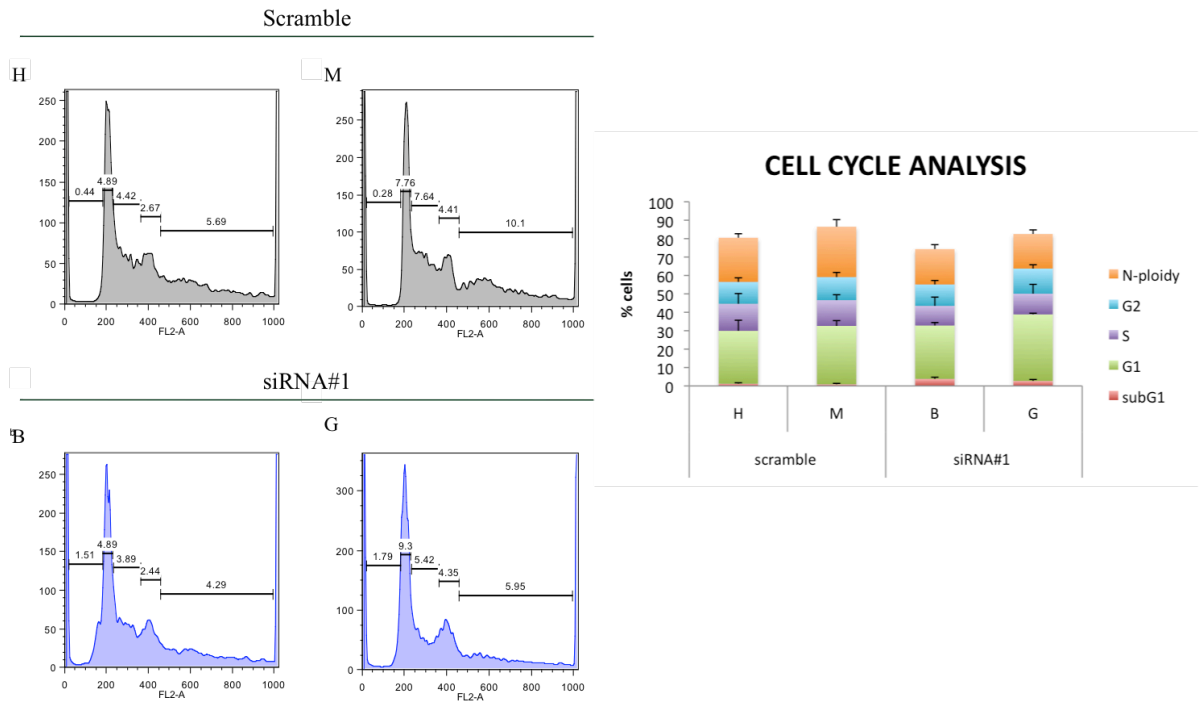
Fig. 12 – FACS analysis of cell cycle of both scramble clones and both siRNA#1 clones after silencing. All experiments were performed in triplicate, and error bars indicate SD.

3.2.1 Affymetrix Gene Chip Analysis

To understand molecular mechanisms altered by the loss of DJ-1 we took advantage of the Affymetrix technology to perform gene expression profile of silenced clones.

We performed gene expression profiling on the two clones containing siRNA#1 (B and G) and two scramble ones (H and M). RNA samples were collected after 10 day of Doxy-treatment, a condition when DJ-1 protein expression is almost completely eliminated. Samples were hybridized on Affymetrix GeneChip Human Genome U133A 2.0. Data obtained from two independent experiments for each line were collected and analyzed.

To ensure that changes warranted further study, statistical analysis was performed with the SAM module (Significance Analysis of Microarrays [159]). Applying a False Discovery Rate (FDR) of 10% we found 166 genes that were differentially regulated in both siRNA#1 clones versus the two scramble ones. with 102 down-regulated and 64 up-regulated transcripts.

By Gene-Ontology analysis, we clustered the down-regulated genes to signal transduction, cell motility, developmental process, cell migration and synaptic transmission biological processes. Up-regulated genes, clustered mainly in biological processes involving chromatin assembly, cytoskeleton organization and biogenesis, cell projection morphogenesis, neurogenesis and regulation of transcription (see Supplementary A. Tab. 1).

A higher stringent analysis (FDR of 0%) was then applied to reduce the number of false positive genes that were differentially regulated in both B and G siRNA#1 clones versus the two scramble ones. This stringent filtering produced a list of 10 genes that were increased and 16 genes that were decreased in cells with siRNA#1 (Tab. 2). As expected, DJ-1 was identified among the down-regulated genes.

Gene name	Gene symbol	Fold change
glutamate receptor, metabotropic 8	GRM8	6,08
cell division cycle 42 (GTP binding protein, 25kDa)	CDC42	4,40
calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	CAMK2B	4,05
latrophilin 3	LPHN3	3,84
AP2 associated kinase 1	AAK1	3,49
polypyrimidine tract binding protein 2	PTBP2	2,95
plakophilin 4	PKP4	2,44
PDZ and LIM domain 3	PDLIM3	2,18
eukaryotic translation initiation factor 2-alpha kinase 1	EIF2AK1	1,97
chromosome 8 open reading frame 33	C8orf33	1,60
electron-transferring-flavoprotein dehydrogenase	ETFDH	-1,45
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	-1,48
dedicator of cytokinesis 9	DOCK9	-1,52
protocadherin alpha 12	PCDHA12	-1,58
integrin alpha FG-GAP repeat containing 1	ITFG1	-1,60
chromosome 5 open reading frame 15	C5orf15	-1,71
ST3 beta-galactoside alpha-2,3-sialyltransferase 6	ST3GAL6	-1,73
mitofusin 1	MFN1	-1,74
septin 6	SEPT6	-1,83
RAB21, member RAS oncogene family	RAB21	-1,94
calcium binding and coiled-coil domain 2	CALCOCO2	-2,01
PDZ and LIM domain 5	PDLIM5	-2,08
integrin, beta 1	ITGB1	-2,24
filamin A, alpha (actin binding protein 280)	FLNA	-2,72
ret proto-oncogene	RET	-3,60
Parkinson disease (autosomal recessive, early onset) 7	**PARK7**	**-7,42**

Tab. 3 - Summary of Affymetrix GeneChip analysis.

3.2.2 Validation of microarray data

To validate microarray data, we performed qPCR analysis on the top-three mostly up-regulated (GRM8, CDC42, CAMKIIB) and down-regulated (ITGB1, FLMNA, RET) genes on clones containing siRNA#1. Changes in gene expression revealed by Affymetrix profiling were all confirmed by qPCR (Fig. 13 a, b). We found that the metabotropic glutamate receptor 8 (GRM8) was up-regulated more than 15-fold compared to scramble clones, while a 7-fold of induction was observed for the GTPase cell division cycle 42 (CDC42) and 2-fold for the calcium/calmodulin-dependent protein kinase (CaM kinase) II beta (CAMKIIB). A 2-fold reduction was indeed confirmed for integrin, beta 1 (ITG-β1), filamin a (FLMNA) and of 9-fold for ret proto-oncogene (RET)
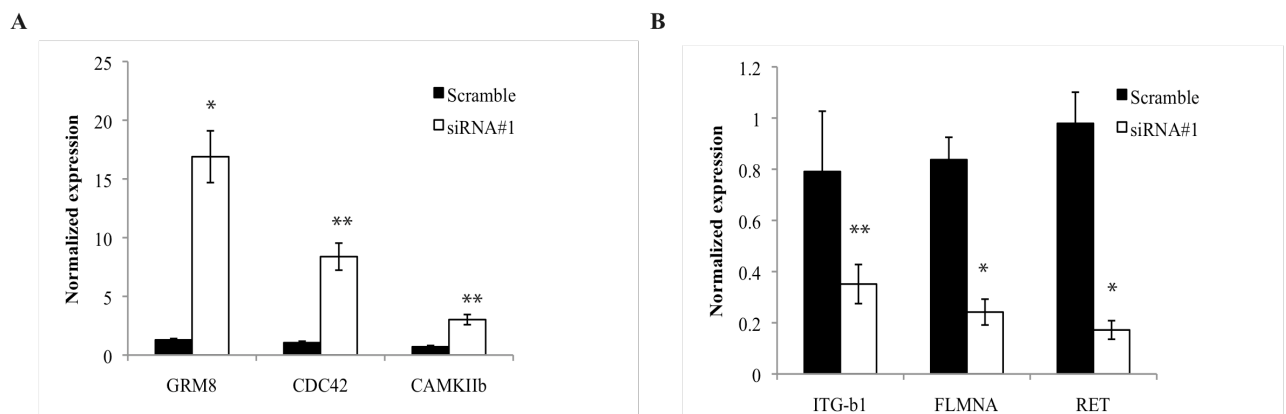

Fig. 13 - qPCR analysis of mRNA expression of both clones siRNA#1 and scramble clones. Validation of the most (A) up- and (B) downregulated genes obtained from Affymetrix analysis. All experiments were performed in triplicate, and Data are presented as means ± SEM.

3.2.3 Analysis of Ret expression

Since conditional genetic ablation of Ret in DA neurons provoked progressive motor dysfunctions and GDNF, the Ret ligand, is a well known neurotrophic factor for DA cells, we decided to focus our attention on the effects of DJ-1 loss on Ret expression.

We first showed by qPCR that Ret expression level was strongly reduced in B and G siRNA#1 versus scramble clones (9-fold).

To ascertain the specificity of DJ-1-mediated Ret down-regulation and to monitor the significance of the DJ-1 dosage, we analyzed Ret mRNA levels also in siRNA#2 clone (Fig. 14a). Importantly, a strong Ret down-regulation (6-fold) was observed even in this clone, proving a causal relationship between DJ-1 and Ret transcripts levels and excluding an off-target effect.

By western blot analysis, Ret protein resulted strongly reduced by loss of DJ-1 (>90% decrease) as shown by the almost complete disappearance of Ret-specific immunostaining (Fig. 14b) in both siRNA#1 clones. Compatible with qPCR results and the partial reduction of Ret mRNA, Ret protein levels in siRNA#2 clone were lowered, although to a lesser extent.

Therefore, we can conclude that in human neuroblastoma cells, loss of DJ-1 expression decreases the levels of Ret, the co-receptor for GDNF.

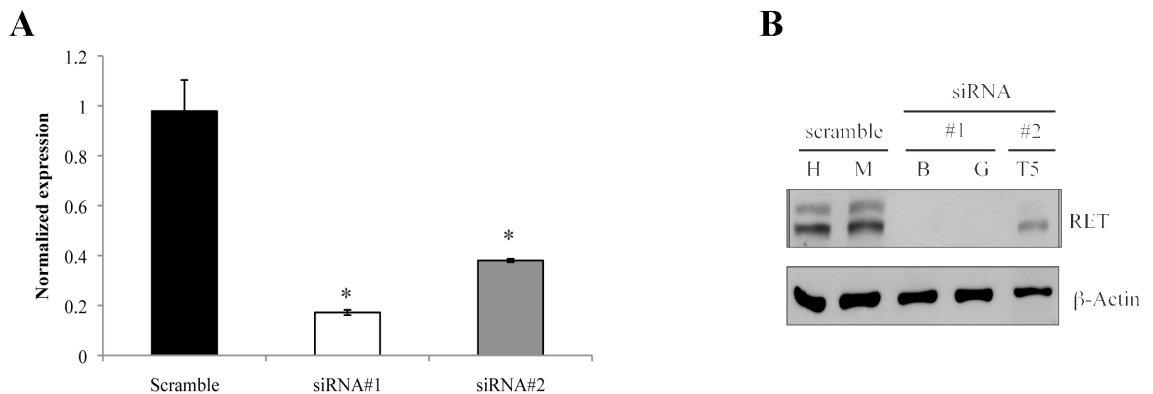**Fig. 14 -** Analysis of RET expression level in both siRNA#1 clones, siRNA#2 clone and scramble clones. A) qPCR analysis of mRNA RET expression level, scramble and siRNA#1 bars indicate average value between two different clones, All experiments were performed in triplicate, and data are presented as means ± SEM; B) Western blot analysis of RET protein level in siRNA#1 clones, siRNA#2 clone and scramble clones

3.2.4 In vivo analysis

We try to validate the down-regulation of RET expression level also in DJ-1 KO mice. We extracted RNA from the ventral midbrain and the striatum of 5 DJ-1 WT, 5 heterozygous (HZ) and 5 KO mice, 3 months and 5 months old, and analyzed RET expression level by qPCR. We did not find significant alteration in KO mice compared to age-matched control.

3.3.1 Loss of DJ-1 stabilizes Hypoxia Inducible Factor 1 alpha (HIF-1a) and triggers hypoxia.

Among the various transcription factors (TF) that regulate Ret transcription, hypoxia inducible factor 1 alpha (HIF-1a) was a good candidate for mediating DJ-1 and Ret interplay. Upon stabilization, HIF-1a has been shown to repress Ret at mRNA level [40].
To verify that HIF-1a was able to down-regulate also Ret protein level in human neuroblastoma, we stabilized HIF-1a treating SH-SY5Y with cobalt chloride ($CoCl_2$) for 6, 12 and 24 hours and measured HIF-1 and Ret protein level by western blot. As soon as after 6 hours of treatment HIF-1a was strongly stabilized and the stabilization correlated with a reduction of Ret protein level. Importantly, after 24 hours of treatment, Ret protein level was undetectable (Fig. 15).
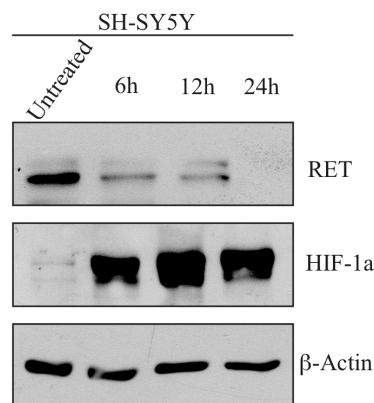
Fig. 15 - Western blot analysis of RET and HIF-1a protein level in SH-SY5Y cells treated with cobalt chloride

Since HIF-1a expression is mainly regulated at the post-transcriptional level, we investigated by western blot HIF-1a protein expression in siRNA#1 clones as compared to scramble ones. Interestingly, HIF-1a protein was stabilized concomitantly to Ret down-regulation in both siRNA#1 clones (Fig. 16).
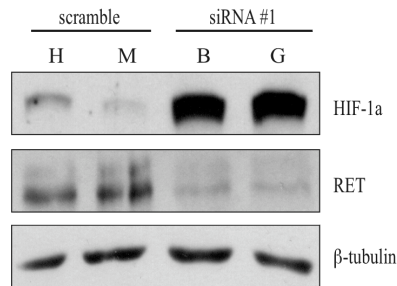
Fig. 16– Western blot analysis of HIF-1a and RET protein level in both scramble and siRNA#1 clones

Since HIF-1a is stabilized in hypoxic conditions, we then asked whether the absence of DJ-1 was sufficient to cause a hypoxic state. Using Hypoxyprobe we were able to verify cellular hypoxia, a strong staining for hypoxic chemical adducts was revealed in both clones silenced for DJ-1 while absent in controls (Fig. 17).
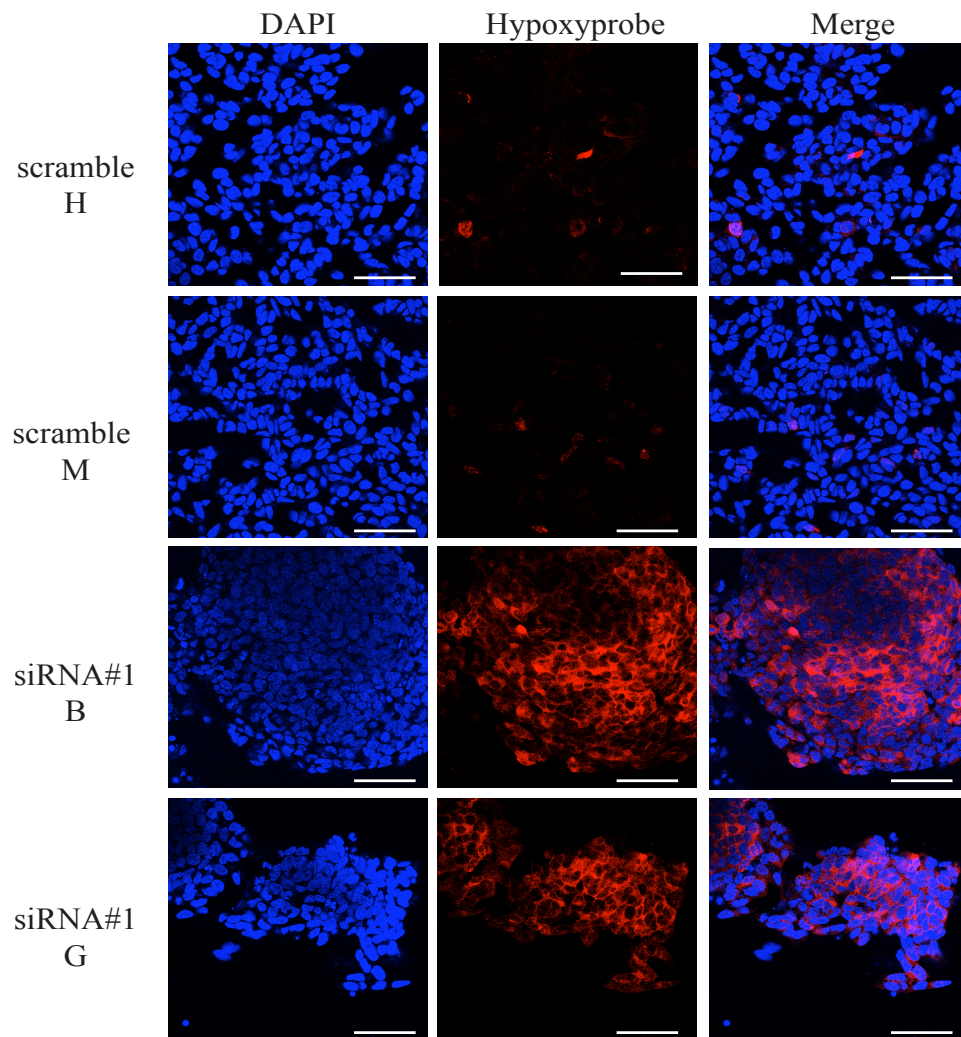
Fig. 17 - Analysis of hypoxia condition in silenced clones. Hypoxyprobe staining of both scramble and siRNA#1 clones. Scale bar 50 μm

Therefore, DJ-1 loss provoked a generalized hypoxic condition in SH-SY5Y neuroblastoma cells.

3.3.2 Loss of DJ-1 induces ROS accumulation.

Since hypoxia increases ROS production [40] and DJ-1 is involved in intracellular redox homeostasis, we asked whether in absence of DJ-1 there was an increased ROS production in our *in vitro* cell model. To this purpose, we took advantage of a fluorescent probe that quantifies ROS production (ROS- probe) as dihydroethidium (DHE) staining for superoxide.

As shown in Fig. 15, after induction of DJ-1 interference, an increase of free radical species was detected compared to controls (Fig. 18).
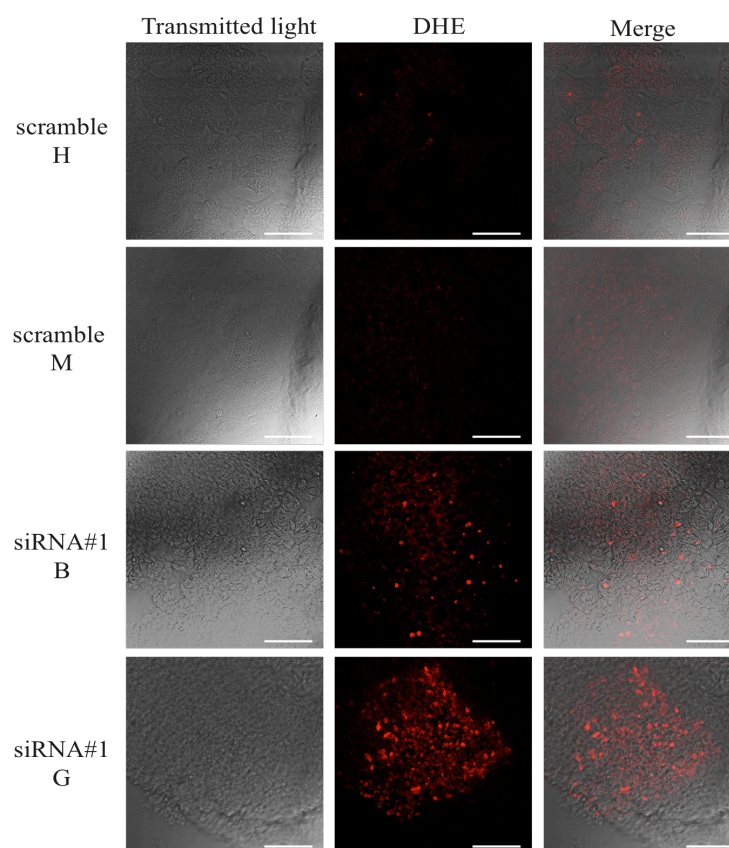
Fig. 18 – Analysis of ROS level in silenced clones. DHE staining of both scramble and siRNA#1 clones. Scale bar 50 μm

Therefore, DJ-1 loss in SH-SY5Y neuroblastoma cells provokes an accumulation of toxic ROS species.

3.3.3 DJ-1 is able to rescue Ret expression. The role of its ROS scavenging activity.

We then re-introduced DJ-1 expression in the inducible siRNA#1 clones to prove that Ret down-regulation was not an off-target effect of this RNAi molecule. Taking advantage that siRNA#1 sequence was designed on the 3' noncoding UTR region, considerable amounts of DJ-1 protein were ectopically expressed upon transfection of 2xflag-DJ-1 in both siRNA#1 and scramble clones.

As shown in Fig. 19, the re-introduction of DJ-1 protein restored Ret expression in siRNA#1 clones. Ret protein levels were slightly less than in the scramble controls, probably due to transfection efficiency.
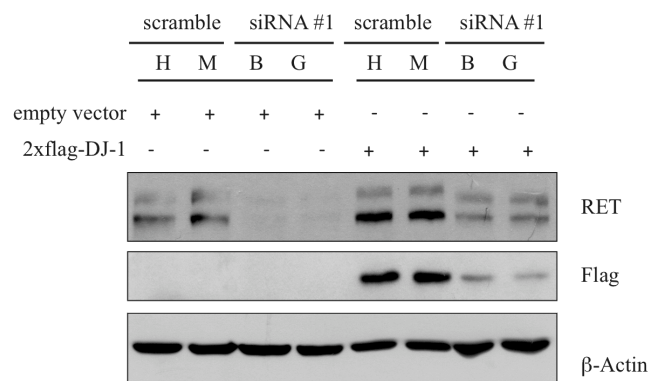
Fig. 19 – DJ-1 rescue in silenced clones. Western blot analysis of RET protein level in siRNA#1 and scramble silenced clones in absence and in presence of Flag-DJ-1.

We analyzed also HIF-1a protein level. Upon DJ-1 transfection we found also a partial reduction in HIF-1a protein level in siRNA#1 clones while no change was found in scramble clones.
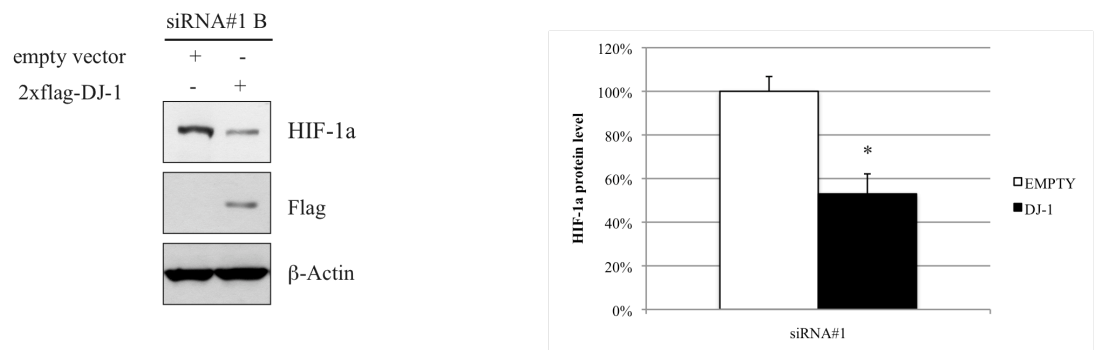
Fig. 20 – Analysis of HIF-1a protein level after DJ-1 rescue. A) Representative panel of Western blot analysis of HIF-1a protein level in absence and in presence of Flag-DJ-1. B) Densitometric analysis of immunoreactivity bands. All experiments were performed in triplicate for both siRNA#1 clones, and data are presented as means ± SEM.

3.3.4 DJ-1 mutants are unable to rescue RET expression level

We than asked whether Ret down-regulation may also play a role in PD cases with DJ-1 missense mutations. To this purpose we transfected the PD-associated L166P mutation (2xflag-L166P) into siRNA#1 clones and control clones. As expected, L166P protein levels were lower than WT DJ-1 for its well known instability. Interestingly, L166P mutants were not able to rescue Ret expression as assayed by western blot. Indeed, since ROS scavenging occurs through oxidation of DJ-1 at the residue C106, we monitored whether this activity was involved in Ret regulation. By transfecting a 2xflag-C106A into siRNA#1 clones, we proved that DJ-1 scavenging activity is required for Ret expression since the ectopic expression of DJ-1 C106A did not have any effect on Ret expression.
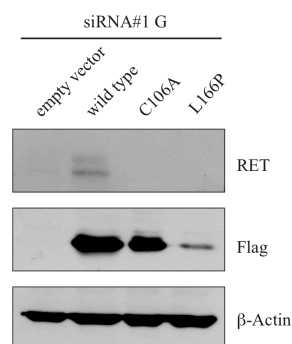
Fig. 21 – RET rescue. Western blot analysis of RET protein level after transfection of DJ-1 wild type, C106A and L166P mutants.

3.3.5 DJ-1 rescue of hypoxia

To further prove that the establishment of hypoxic state was strictly dependent on the loss of DJ-1 and to exclude off target of siRNA#, we performed rescue experiment transfecting DJ-1 in both silenced clones and evaluating hypoxia in transfected cells. We measured Pearson's correlation as colocalization coefficient. As shown in Fig. 23, both scatter plot and Pearson's coefficient indicate a negative correlation bteween the presence of hypoxic condition and the presence of Flag-DJ-1. It's noteworthy that only cells transfected with DJ-1 show negative Person's correlation in contrast with cells transfected with only Ds-Red where persists the hypoxic state as revealed by the positive correlation illustrated by scatter plot and Pearson's coefficient.
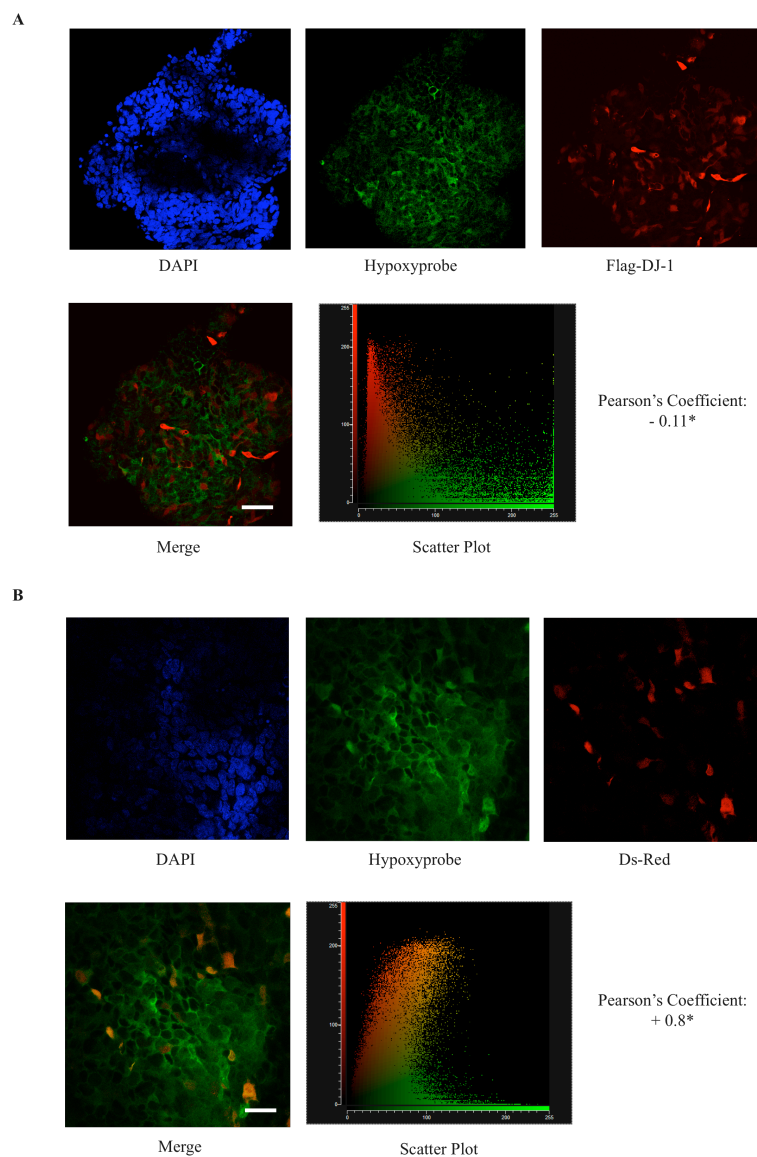
Fig. 23 – Hypoxia rescue. A) Hypoxyprobe and Flag-DJ-1 staining after Flag-DJ-1 transfection. Representative scatter plot of colocalization studies; B) Hypoxyprobe and Ds-Red staining after Ds-Red transfection. Representative scatter plot of colocalization studies. *P < 0.05. Scale bar 20 μm

4. DISCUSSION

Originally identified as an oncogene, DJ-1 is a ubiquitous redox-responsive cytoprotective protein with diverse functions. In addition to cell-autonomous neuroprotective roles, DJ-1 may be up-regulated in reactive astrocytes, in chronic neurodegenerative diseases as well as in stroke. DJ-1 is an important redox-reactive signaling intermediate controlling oxidative stress after ischemia, upon neuroinflammation, and during age-related neurodegenerative processes[41].

In 2003 mutations and deletions of this gene have been associated with inheritable forms of PD [80]. Recently, reduction of DJ-1 expression level was also found in *post-mortem* brain of sporadic PD patients [33].

Missense mutations of DJ-1 found in PD patients may be in homozygous (L166P, M26I and E64D) and heterozygous forms (A104T and D149A) [98]. Among of them, M26I and L166P are the most studied DJ-1 missense mutations.

Although M26I dimer formation and protein levels reported in the literature may vary, differences with WT DJ-1 are small and of unclear biological significance.

On the contrary, L166P is very unstable and its expression level lower than WT [99,100,101,102]. The different conformation of Leu166Pro monomeric units might affect the protein-protein interaction repertory [99]. L166P and M26I mutants share an increased interaction with TRAF and TNF receptor-associated protein (TTRAP). This binding occurs in the absence of neurotoxic stimuli proving the existence of an altered DJ-1 protein networks in the presence of PD-associated missense mutations. Most importantly, M26I and L166P block the protective activity of TTRAP and induce a TTRAP-dependent pro-apoptotic pathway involving JNK and p38 MAPKs, a signaling system that plays a central role in dopaminergic cell death and PD pathogenesis [106].

Moreover, studies performed by Nishinaga and colleagues have shown a low correlation between gene expression profiles performed on DJ-1 knock-down cells and L166P-carryng cells. This work suggests that gain of function can occur in L166P-carrying cells. Gain of function of L166P DJ-1 might include change of transcription factors that bind to DJ-1 [141].

How these mutants abolish or modify DJ-1 functions is a matter of debate, as a common mechanism of action for the various DJ-1 mutants is still lacking.

In order to unveil molecular mechanisms underlying neurodegenerative processes, in vivo and in vitro models without a functional DJ-1 have been developed revealing the protective role of DJ-1 against oxidative stress.

KO mice for DJ-1 showed a progressive decline in selected motor tests, increased striatal dopamine and evoked dopamine overflow in the striatum. However, these models did not completely recapitulate the key symptoms of the disease since they did not show changes in the number of nigral dopaminergic neurons [34,86,36].

Mouse genetic models of the disease have often been limited by the inherent variability of animal experiments, the limited mouse life span, and the difficulties in manipulating whole animals. Instead, genetic *in vitro* cellular models are more readily amenable to molecular dissection of disease mechanisms.

In vitro model of knocked-down DJ-1 have been useful to elucidate its protective role in cellular response to oxidative stress induced by PD related toxins like MPTP and Paraquat. Dopaminergic neurons derived from *in vitro* differentiated [82] DJ-1-deficient embryonic stem cells showed a decreased survival and an increased sensitivity to oxidative stress.

To this purpose human neuroblastoma SH-SY5Y cells have been widely adopted as cellular model to study PD for their high dopamine-β-hydroxylase activity. In particular, experiments from several laboratories using SH-SY5Y cells have clearly demonstrated the protective activity of overexpressed DJ-1 against neuronal apoptosis through various molecular mechanisms (molecular chaperone, regulator of transcription, scavenger of ROS species).

In this study we have been able to generate inducible stable cell lines interfered for DJ-1 expression. The use of an inducible promoter allowed us an unbiased selection of clones in absence of the interference.

We adopted two different RNAi sequences with two different silencing' efficiency. With the siRNA#1 we have been able to obtain inducible stable cell lines in which both DJ-1 mRNA and DJ-1 protein level were almost undetectable.

This model allowed us to mimic more closely the pathological DJ-1 loss of function, since the homozygous mutations or deletions of this gene have been found associated to neurodegeneration.

To our knowledge it's the first time that, using RNAi technology, it was possible to select clones almost completely devoid of DJ-1. Previous attempts have failed for the remaining of residual DJ-1 protein activity.

With the second inducible stable cell line (siRNA#2), we had only a partial reduction of DJ-1 expression level comparable with heterozygous deletion/mutations.

Interestingly silencing kinetics was strictly correlated with an alteration of visible phenotype. Wild type human neuroblastoma cells can form mounds of undifferentiated cells, which then spread differentiated cells into the surrounding area. This growth formation can be referred as the "over-easy formation" and show colonies with a loose peripheral network of cells [43]. Concomitantly with the silencing kinetics, when DJ-1 protein starts to be strongly down-regulated (after 6 days of induction) both silenced clones almost completely lost the peripheral monolayer of cells and preferred to grow as colonies. We confirmed by western blot analysis of neuronal markers a reduction of the differentiated component in both silenced clones compared to scramble ones.

Given the oncogenic role of DJ-1 we asked whether its absence could alter the cell cycle of silenced clones. The absence of DJ-1 did not affect cell cycle of both silenced clones compared to scramble demonstrating that cells lacking DJ-1 were in an active proliferating status.

Taking advantage of the clones showing the strongest down-regulation of DJ-1, we performed gene expression analysis to unveil molecular mechanism altered by the loss of DJ-1 on both silenced clones compared to scramble. Previous gene expression analyses performed in DJ-1 knock-down cells and in PD-derived mutant L166P-carrying cells in other cellular systems have identified genes related to apoptosis, oxidative stress and neurotoxicity confirming the role of DJ-1 as transcriptional regulator [38].

Here we demonstrated that the strong down-regulation of DJ-1 was responsible for the alteration of genes involved mainly in nervous system development and cell adhesion. Among them, we focused our attention on the Glial cell line-Derived Neurotrofic Factor (GDNF) receptor Ret. This result has high relevance for its implication in Parkinson' Disease. GDNF is able to promote neurite outgrowth and sprouting of adult midbrain DA neurons, the neuronal population mainly affected in the disease[116]. In addition to neuroprotective and neurorestorative actions, GDNF also has direct effects on dopamine neurons, modulating excitability via changes in A-type potassium channels [47].

Ret expression level, both transcript and protein, was strongly down-regulated by the absence of DJ-1. Moreover, we found that the observed Ret down-regulation was strictly correlated with the residual quantity of DJ-1, although interference clones expressing 50% of DJ-1 levels showed a modest but consistent decrease of Ret expression level. It's noteworthy that only a strong down-regulation of DJ-1 was able to completely inhibit the expression of Ret receptor and the importance of this result became striking considering that only the homozygous deletion of DJ-1 has been associated to PD onset.

The down-regulation of Ret has strong implications for PD, since it's already well known that mice conditional knock-out for this receptor show a progressive and late degeneration of dopaminergic neurons, main feature of pre-symptomatic PD [40]. Nonetheless, so far there is no evidence that PD can be caused by mutations in the Ret gene itself because analysis of polymorphisms have not shown any association with PD[41] but it's known that PD related toxins like MPTP, Rotenone and 6-hydroxydopamine are able to down-regulate Ret expression level both in vitro and in mice SN and VTA[42]. Down-regulation of Ret could implicate that GDNF neurotrophic support to dopaminergic neurons may be impaired by DJ-1 loss and precede neuronal degeneration.

Interestingly, in wild type human neuroblastoma cells GDNF is secreted in the medium, it stimulates Ret by a cell-autonomous mechanism and, upon retinoic acid treatment, it contributes to SH-SY5Y differentiation [46]. Starting from this observation, the down-

regulation of Ret receptor could account for the reduction of neuronal phenotype in clones lacking of DJ-1 since these cells are unable to respond to the autocrine GDNF stimulus.

However, we have not been able to validate Ret down-regulation in vivo. This could be due to the inherent variability of animal experiments and to the limited mouse life span. However, this may be due to the compensatory mechanisms that account for the increase of other scavenger proteins like GPx, previously found in adult DJ-1$^{(-/-)}$ [54]. On the other hand, it will be interesting to analyze Ret expression levels in older mice. It's noteworthy that DJ-1 KO mice showed slight motor symptoms only after 11 months from the birth. Interestingly, conditional Ret KO mice showed a 40% reduction of TH positive fibers only in 1 year old mice while a 63 % reduction was observed after 2 years [60].

By compiling a list of all transcription factors that can regulate Ret expression, we found the Hypoxia Inducible Factor-1 (HIF-1) is the only transcription factor found to be associated with a down-regulation of Ret transcriptional level[43]. We then demonstrated that the post-transcriptional stabilization of HIF-1 is associated to a strong down-regulation of Ret protein level in human neuroblastoma cells. However, it's not known the exact mechanism by which HIF-1 is able to regulate Ret expression. It will be interesting to analyze the mechanistic link and extend our knowledge of the functions of the hypoxia-related pathways.

Under normal oxygen conditions, HIF-1alpha (HIF-1a) subunit is hydroxylated at two proline residues within the oxygen-dependent degradation domain (ODDD) by prolyl-hydroxylase-domain proteins (PHD), thereby targeting it for proteasomal degradation.
Under hypoxic conditions, PHD are inhibited, thereby preventing hydroxylation of HIF-1a. Inhibiting PHD decreases degradation of HIF-1a by the ubiquitin-proteasome system and leads to rapid HIF-1a accumulation. HIF-1a translocates to the nucleus and dimerize with HIF-1b to initiate transcription of target genes.

Interestingly, we have demonstrated that, in absence of DJ-1, the hypoxia-sensible subunit of HIF-1, HIF-1alpha, is strongly stabilized.
Consequently, we investigated whether the absence of DJ-1 was responsible for the establishment of cellular hypoxia. Using hypoxyprobe, we have been able to demonstrate

for the first time that in absence of DJ-1, human neuroblastoma cells suffered of hypoxic condition.

Although a role of DJ-1 in hypoxia cellular responses has been already postulated[44], here we report for the first time the presence of a hypoxic condition generated by the absence of DJ-1 and not by exogenous stimuli.

Oxygen availability regulates many physiological and patho-physiological processes, including embryonic development, adaptation to high altitudes, wound healing, and inflammation, as well as contibuting to the pathophysiology of cancer and ischemic diseases such as infarction and stroke [64].

As a main consumer of energy, the brain is particularly susceptible to the effects of hypoxia [65]. Mesotelencephalic dopamine (DA) pathways are exquisitely vulnerable to ischemic-anoxic insult. These insults are known to produce long-term derangements in DA signalling. However, it remains unclear whether modest intermittent hypoxia, such as that encountered with repetitive apnoeas in premature infants, contributes to clinically significant impairments in DA signalling [66].

The physiological response to hypoxia includes vasodilatation of brain arteries and veins, which increases cerebral blood flow and delivery of oxygen to the brain. At molecular level, several pathways have recently been shown to mediate hypoxia sensing at the cellular and molecular levels [65]. The induction of Hypoxia Inducible Factor-1 (HIF-1), and the consequent expression of HIF-1 target genes, is the key element of cellular response to hypoxia.

Furthermore, oxidant stress increased under hypoxia and it can activate multiple signalling pathways including HIF-1alpha protein stabilization and gene regulation. Importantly, among genes regulated by HIF-1alpha there's the rate-limiting enzyme for the synthesis of dopamine, tyrosine hydroxylase (TH). Whether the HIF/hypoxia responsiveness of TH might help in understanding the pathogenesis of Parkinson's disease, or be useful in developing new therapies, is still unknown .

Because of its link with oxidative stress, the role of DJ-1 was recently studied in other non-PD-related neuropathological conditions also associated with ROS, such as stroke. It was found that DJ-1 provides protection against excitotoxicity and ischemic brain injury and that the anti-oxidant activity of DJ-1 is essential to its neuroprotective role against ischemia-induced damage. Indeed, it's known strokes predispose patients to dementia

(Internal Medicine, 2003) and PD patients have symptoms not classically associated with movement such as dementia and mood disorders.

We demonstrated an accumulation of free radical species in cells lacking DJ-1. Interestingly, this result confirmed what was observed by Andres-Mateos and colleagues *in vivo* in DJ-1 (-/-) mice. The lack of DJ-1 in the mitochondria induces an increase of mitochondrial H_2O_2 production and reduction of aconitase activity [54]. Moreover, this result points out the essential role of DJ-1 in free radical scavenging.

Importantly, we performed rescue experiment with both wild type and mutant DJ-1. We demonstrated that the re-introduction of fully functional DJ-1 was able to rescue Ret expression level. Conversely, Ret expression level was not rescued up neither by the transfection of DJ-1 PD related mutant (L166P), highly unstable, nor by DJ-1 mutant lacking of its scavenger activity (C106A). C106 residue for the anti-oxidant activity . Indeed, C106 is the most sensitive among all the three cystein residues (i.e. C46, C53 and C106) present in DJ-1 protein to oxidative stress [39]. Indeed, the C106A mutation has been demonstrated to block oxidation-induced mitochondrial localization and protection against 1-methyl-4-phenylpyridinium (MPP^+) toxicity in neuronal cells [58].

These results suggest that the restoring effects exerted by transfected DJ-1 could reside on its scavenger activity. Recent studies have demonstrated that DJ-1 could also be central to other models of neuronal injury where oxidative damage plays a paramount role like stroke. Aleyasin and colleagues have also demonstrated that DJ-1 deficiency sensitizes brains to ischemic damage *in vivo* and that the protective activity depends on cystein 106 residue, shown to be critical in the DJ-1 ability to handle oxidative stress [58].

Interestingly, after transfection of fully functional DJ-1 we observed also a reduction of HIF-1 alpha protein level implying the activation of the oxygen-sensible degradation pathway.

To confirm this hypothesis we wanted to verify whether upon DJ-1 transfection the hypoxic condition was reduced. By immunofluorescence, we demonstrated the negative correlation existing for the hypoxic cells and cells transfected with DJ-1.

These results demonstrate an involvement of DJ-1 in the oxygen signalling machinery. In this contest, it's important to underline the role of mitochondria as integral members of

oxygen-sensing machinery. We could hypothesize that, other than the scavenger activity, DJ-1 could have a supporting role of mitochondrial activity in oxygen sensing. It's known that DJ-1 but not the PD-mutants localize to mitochondria during oxidative stress. Furthermore, it has been demonstrated that resting mitochondrial membrane potential was significantly lower in DJ-1 [(-/-)] astrocytes, as compared to controls [56].

Interestingly, accumulation of clonal, somatic mitochondrial DNA deletions has been observed in the SN during aging and in PD, suggesting that mitochondrial DNA mutations in some instances may pre-dispose to DA neuron death by impairing respiration [63].

Several catalytic subunits of complex I were shown to carry increased levels of protein carbonyls (an oxidative modification of proteins) in the parkinsonian brain, which correlated with reduced electron transfer rates, suggesting that excessive oxidative damage of complex I subunits may lead to complex I misassembly and dysfunction [66]. Interestingly, it appears that the SN is more vulnerable to impairments of complex I activity than other brain regions and peripheral organs, possibly due to the increased levels of reactive oxygen species generated within dopamine neurons as a result of dopamine metabolism and iron [64].

Given the key role of mitochondria in neuronal function, it is not surprising that disturbances in mitochondrial function, transport, dynamics and turnover have emerged as central mechanisms at the convergence of neurotoxin, environmental and genetic approaches to Parkinson's disease

Taken together, these results suggest a new pathological mechanism underlying the DJ-1 loss of function and PD onset. We demonstrated for the first time that lack of function of DJ-1 is responsible for the impairment in the oxygen sensing machinery and for an increase of free radical species. These phenomena may thus have strong implications in the GDNF neurotrophic signalling pathway and therefore could account for the neurodegeneration of dopaminergic neurons, a clear hallmark of PD.

5. MATERIALS AND METHODS

5.1 Constructs

Expression vectors encoding for FLAG-DJ-1 WT, C106A and pcDNA3-FLAG empty were previously described [106]. Two different oligonucleotide sequences were selected for the knock-down DJ-1 expression using siRNA Target Finder software (Invitrogen). The hairpin-encoding oligonucleotides were cloned into the p*Superior* vector (Invitrogen). siRNA constructs: siRNA#1 (5' - GGTCATTACACCTACTCTG - 3'), siRNA#2 (5' - TGGAGACGGTCATCCCTGT - 3'), scramble (5' - TGGAGACGGAGATCCCTGT - 3')

5.2 Cell culture and transfections

Human neuroblastoma SH-SY5Y cells (*ATCC*) were maintained in culture as suggested by vendors. SH-SY5Y cells were transfected with LipofectAmine 2000 (Invitrogen), according to manufacturer's instructions.

We generated SH-SY5Y inducible stable cell lines by transfecting linearized constructs with LipofectAmine 2000 and selecting for positive clones in the presence of 300 µg/ml of G418 (Invitrogen) and 3 µg/ml of Blasticidin (InvivoGen). Individual clones were confirmed by western blot analysis, immunofluorescence and quantitative real time PCR. Silencing induction was performed by adding 2.5 µg/ml of Doxycicline Hyclate (Sigma-Aldrich) every 48h.

5.3 DJ-1 KO mice

Generation of DJ-1 (-/-) was previously described [84]. mRNA from total ventral midbrain and striatum of DJ-1$^{-/-}$, DJ-1 WT and HZ 3 and 5 months old mice was collected and analyzed by qPCR.

5.4 RNA isolation, reverse transcription and qPCR

Total RNA was isolated using the TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Single strand cDNA was obtained from 1 µg of purified RNA using the iSCRIPT^{TM} cDNA Synhesis Kit (Bio-Rad) according to manufacturer's instructions. Quantitative RT-PCR (qPCR) was performed using SYBR-Green PCR Master Mix (Applied Biosystem) and an iCycler IQ Real time PCR System (Bio-Rad). Expression of DJ-1, RET, CDC42, GRM8, FLMNA, CAMK-IIb, and ITG-b1 was analyzed using specific oligonucleotides (supplementary material).

5.5 Microarray processing and data analysis

Total RNA was extracted as described above and purified using the RNeasy mini kit (Qiagen). The quality of total RNA was assessed using a bioanalyzer (Agilent 2100; Agilent Technologies) and RNA was quantified by using a ND-1000 Nanodrop spectrophotometer. 10 μg of each total RNA sample was labelled according to the standard one-cycle amplification and labelling protocol developed by Affymetrix (Santa Clara, CA). Labelled cRNA was hybridized on Affymetrix GeneChip Human U133A 2.0 Arrays containing over 14,500 transcripts. Hybridized GeneChips were stained, washed (GeneChip Fluidic Station 450) and scanned (GeneChip Scanner 3000 7G). Cell intensity values and probe detection calls were computed from the raw array data using the Affymetrix GeneChip Operating Software (GCOS). Further data processing was performed in the R computing environment using packages from the BioConductor software project (http://www.bioconductor.org/). Robust Multi-Array Average (RMA) normalization was applied [157]. Normalized data were then filtered based on the Affymetrix detection call, so that only probes that had a Present call in at least one of the arrays were retained. Data were then imported in the MultiExperiment Viewer (MeV) software [158], and statistical analysis was performed with the SAM (Significance Analysis of Microarrays) module [159] to detect significantly differentially expressed genes. Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) with Accession Number GSE17204. Differentially expressed genes were then specifically examined, based on their Gene Ontology annotation [160] and through the use of Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com) and DAVID Bioinformatics Resources [161].

5.6 Western blot analysis

Cells were lysed in 2X SDS sample buffer, boiled and analyzed by western blot. Proteins were then separated by molecular weight by SDS/PAGE through polyacrilamide gels ranging from 8% to 10%. Proteins were electrophoretically transferred to nitrocellulose membranes and blocked using 5% nonfat dry milk in TBS with 0.1% Tween 20.

The following antibodies were used: anti-DJ-1 1:1000 (purified from immunized rabbits) and anti-DJ-1 1:1000 (Stressgene), anti-Flag 1:2000 (Sigma), anti-Ret 1:400 (SantaCruz), anti-Hif-1a 1:400 (SantaCruz), anti-β-actin 1:5000 (Sigma).

5.7 Immunocytochemistry and immunohistochemistry

For immunofluorescence experiments, SH-SY5Y cells were fixed in 4% paraformaldehyde and indirect immunofluorescence was performed following standard protocols (supplementary material). We used a anti-DJ-1 1:100 (purified from immunized rabbits) and anti-DJ-1 1:100 (Stressgene), Anti-HypoxyprobeTM-1 antibody (Chemicon International Corp.), anti-Flag 1:1000 (Sigma) For detection, Alexa Fluor-488 or -594 (Invitrogen) labeled anti-mouse or anti-rabbit antibodies were used. Nuclei were visualized with DAPI (1mg/ml). All images were collected using a confocal microscope (LEICA TCS SP2).

5.8 ROS measurements

Dihydroethidium (DHE) is a specific dye for $O_2\cdot^-$ detection. The blue fluorescent dye HE is oxidized by $O_2\cdot^-$ to ethidium, which stains the nucleus a bright fluorescent red. SH-SY5Y cells (4×10^5 cells/60-mm Petri dishes) were rinsed and 10 mM DHE was loaded. After 20-min incubation at 37 °C in serum-free medium, cells were examined under a confocal microscope.

5.9 Colocalization analysis

Scatter Plot and Pearson's Coefficient of colocalization have been obtained with ImageJ software from 10 different fields each experiment. All experiment have been performed in triplicate and in two different clones.

5.10 Statistical analysis

All experiments were repeated in triplicate or more. For stably transfected cells, at least two independent clones were used for each cell line in all experiments. Data represent the mean ±SEM. When necessary, each group was compared individually with reference control group using Student's t-test (Microsoft Excel software).

SUPPLEMENTARY

gene_name	gene_symbol	FC
NEL-like 1 (chicken)	NELL1	5.00
cell division cycle 42 (GTP binding protein, 25kDa)	CDC42	4.40
calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	CAMK2B	4.05
transmembrane protein 16C	TMEM16C	4.03
glutamate receptor, metabotropic 8	GRM8	3.91
latrophilin 3	LPHN3	3.84
stathmin-like 4	STMN4	3.50
AP2 associated kinase 1	AAK1	3.49
v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	ERBB4	3.44
GNAS complex locus	GNAS	3.24
jun oncogene	JUN	3.15
zinc finger protein 804A	ZNF804A	3.09
sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	SPOCK3	3.07
polypyrimidine tract binding protein 2	PTBP2	2.95
sorbin and SH3 domain containing 2	SORBS2	2.92
solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	2.92
nucleosome assembly protein 1-like 3	NAP1L3	2.87
jumonji domain containing 6	JMJD6	2.83
roundabout, axon guidance receptor, homolog 2 (Drosophila)	ROBO2	2.77
achaete-scute complex homolog 1 (Drosophila)	ASCL1	2.71
basic helix-loop-helix domain containing, class B, 2	BHLHB2	2.66
plexin C1	PLXNC1	2.62
RUN and FYVE domain containing 3	RUFY3	2.62
dehydrogenase/reductase (SDR family) member 2	DHRS2	2.59
ATP-binding cassette, sub-family A (ABC1), member 12	ABCA12	2.58
fibronectin leucine rich transmembrane protein 3	FLRT3	2.58
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	ST6GALNAC5	2.54
DKFZP564C152 protein	DKFZP564C152	2.49
plakophilin 4	PKP4	2.44
tripartite motif-containing 29	TRIM29	2.42
histone cluster 1, H2bg	HIST1H2BG	2.42
potassium voltage-gated channel, Shab-related subfamily, member 1	KCNB1	2.41
receptor accessory protein 1	REEP1	2.32
synaptotagmin XVII	SYT17	2.31
regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	RCBTB2	2.28
brain-specific angiogenesis inhibitor 3	BAI3	2.24
metastasis suppressor 1	MTSS1	2.21
ankyrin 2, neuronal	ANK2	2.20
ADP-ribosylation factor-like 4C	ARL4C	2.20
neuropilin 1	NRP1	2.19
regulator of G-protein signalling 4	RGS4	2.19
PDZ and LIM domain 3	PDLIM3	2.18
histone cluster 2, H2be	HIST2H2BE	2.17
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	2.16
phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	PIK3R1	2.16
diacylglycerol kinase, iota	DGKI	2.15
eyes absent homolog 1 (Drosophila)	EYA1	2.13
leucine rich repeat neuronal 3	LRRN3	2.12
solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	2.11
solute carrier family 18 (vesicular monoamine), member 2	SLC18A2	2.11
glutamate receptor, ionotropic, AMPA 2	GRIA2	2.09

guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	GNAO1	2.08
p21 (CDKN1A)-activated kinase 3	PAK3	2.08
histone cluster 1, H2ag	HIST1H2AG	2.07
regulator of G-protein signalling 7	RGS7	2.06
40S ribosomal protein S20 pseudogene	LOC440992	2.06
PDZ and LIM domain 7 (enigma)	PDLIM7	2.05
endothelial PAS domain protein 1	EPAS1	2.05
solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	SLC7A5	2.03
UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	B3GALT2	2.02
tropomyosin 1 (alpha)	TPM1	2.01
mitofusin 1	MFN1	0.50
chromosome 4 open reading frame 31	C4orf31	0.50
ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	0.50
calcium binding and coiled-coil domain 2	CALCOCO2	0.50
related RAS viral (r-ras) oncogene homolog	RRAS	0.49
SH3 domain and tetratricopeptide repeats 1	SH3TC1	0.49
NAD(P)H dehydrogenase, quinone 1	NQO1	0.49
frizzled homolog 7 (Drosophila)	FZD7	0.49
odz, odd Oz/ten-m homolog 4 (Drosophila)	ODZ4	0.49
ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	ATP7A	0.49
SNF1-like kinase 2	SNF1LK2	0.48
pre-B-cell leukemia homeobox 1	PBX1	0.48
glutaredoxin (thioltransferase)	GLRX	0.48
carbohydrate (chondroitin 4) sulfotransferase 11	CHST11	0.48
low density lipoprotein receptor-related protein 10	LRP10	0.48
interferon-induced protein 44	IFI44	0.48
annexin A2 pseudogene 2	ANXA2P2	0.48
peroxidasin homolog (Drosophila)	PXDN	0.47
arylsulfatase family, member J	ARSJ	0.47
actin, alpha 2, smooth muscle, aorta	ACTA2	0.47
G protein-coupled receptor 177	GPR177	0.47
neurogenin 2	NEUROG2	0.47
SMAD family member 3	SMAD3	0.46
chromosome 20 open reading frame 103	C20orf103	0.46
sprouty homolog 2 (Drosophila)	SPRY2	0.46
vimentin	VIM	0.46
pleckstrin and Sec7 domain containing 3	PSD3	0.45
Niemann-Pick disease, type C2	NPC2	0.45
integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	0.45
lectin, galactoside-binding, soluble, 3	LGALS3	0.44
annexin A2	ANXA2	0.43
collagen, type IV, alpha 1	COL4A1	0.43
phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	PYGL	0.43
guanine nucleotide binding protein (G protein), gamma 11	GNG11	0.43
H2A histone family, member Y	H2AFY	0.42
guanine nucleotide binding protein (G protein), gamma 12	GNG12	0.41
septin 6	SEPT6	0.41
AHNAK nucleoprotein (desmoyokin)	AHNAK	0.41
PTPRF interacting protein, binding protein 1 (liprin beta 1)	PPFIBP1	0.41
BCL2-associated athanogene 3	BAG3	0.41
NADPH oxidase 3	NOX3	0.40
NAD(P)H dehydrogenase, quinone 1	NQO1	0.40
nidogen 1	NID1	0.40
transcription factor 7-like 2 (T-cell specific, HMG-box)	TCF7L2	0.40
limb bud and heart development homolog (mouse)	LBH	0.40

integrin, beta 5	ITGB5	0.39
PDZ and LIM domain 5	PDLIM5	0.39
low density lipoprotein receptor-related protein 4	LRP4	0.39
major histocompatibility complex, class I, C	HLA-C	0.39
target of myb1 (chicken)-like 1	TOM1L1	0.38
dermatopontin	DPT	0.38
platelet-derived growth factor receptor, alpha polypeptide	PDGFRA	0.37
catenin (cadherin-associated protein), delta 1	CTNND1	0.37
chromosome 10 open reading frame 56	C10orf56	0.37
heparan sulfate (glucosamine) 3-O-sulfotransferase 2	HS3ST2	0.36
cytokine-like 1	CYTL1	0.36
leucine-rich repeat-containing G protein-coupled receptor 5	LGR5	0.35
tensin 1	TNS1	0.35
glutamate receptor, metabotropic 7	GRM7	0.35
brain and acute leukemia, cytoplasmic	BAALC	0.33
filamin A, alpha (actin binding protein 280)	FLNA	0.33
inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	ID4	0.33
cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminate monooxygenase)	CMAH	0.32
cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	0.32
neuron navigator 2	NAV2	0.32
peptidylprolyl isomerase C (cyclophilin C)	PPIC	0.32
G protein-coupled receptor 37 (endothelin receptor type B-like)	GPR37	0.31
plasminogen activator, tissue	PLAT	0.30
protease, serine, 12 (neurotrypsin, motopsin)	PRSS12	0.30
family with sequence similarity 59, member A	FAM59A	0.30
synaptosomal-associated protein, 23kDa	SNAP23	0.30
5-hydroxytryptamine (serotonin) receptor 2B	HTR2B	0.29
sushi-repeat-containing protein, X-linked	SRPX	0.29
Notch homolog 2 (Drosophila)	NOTCH2	0.29
neuropeptide Y	NPY	0.28
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	KDELR3	0.28
WD repeat domain, phosphoinositide interacting 1	WIPI1	0.28
ret proto-oncogene	RET	0.28
tensin 3	TNS3	0.27
sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	SPOCK1	0.27
PTPRF interacting protein, binding protein 2 (liprin beta 2)	PPFIBP2	0.24
trophoblast glycoprotein	TPBG	0.24
popeye domain containing 2	POPDC2	0.23
pleiomorphic adenoma gene-like 1	PLAGL1	0.23
glutamate decarboxylase 1 (brain, 67kDa)	GAD1	0.22
collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	0.21
dihydropyrimidinase-like 3	DPYSL3	0.21
complement component 7	C7	0.20
integral membrane protein 2A	ITM2A	0.20
sprouty homolog 1, antagonist of FGF signaling (Drosophila)	SPRY1	0.19
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	0.19
pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	PTN	0.19
reelin	RELN	0.16
tissue factor pathway inhibitor 2	TFPI2	0.15
Parkinson disease (autosomal recessive, early onset) 7	PARK7	0.13
regulator of G-protein signalling 13	RGS13	0.10
versican	VCAN	0.08
dickkopf homolog 2 (Xenopus laevis)	DKK2	0.04

qPCR SPECIFIC OLIGONUCLEOTIDES

Human DJ-1

Fwd
5'- GAGACGGTCATCCCTGTAG – 3'
Rev
5'- CATCTTCAAGGCTGGCATC – 3'

Human Grm8

Fwd
5' – GACCCTGATCTCCTTTCCAACATC – 3'
Rev
5' – TCTCCATTAGCACACTTCACATCC – 3'

Human CAMKIIb

Fwd
5' – ACTCAACAAGAAAGCAGATG – 3'
Rev
5' – ATCCTCTATGGTGGTATTGG – 3'

Human CDC42

Fwd
5' – GCACTTACACAGAGAGGTC – 3'
Rev
5' – AGTAGTGGGACAGGAAGC – 3'

Human FLMNA

Fwd
5' – GACGGCTCCTGTGATGTG – 3'
Rev
5' – TCTGCTGGCTTGTTGACG – 3'

Human RET

Fwd
5' – GCCACCGACCAGCAGACC – 3'
Rev
5' – CGCCACACTCCTCACACTCC – 3'

Mouse RET

Fwd
5'- GGCACCTTCTACCACTTCC – 3'
Rev
5'- CCTCCAGCACATACTTCTCC – 3'

HOUSEKEEPING GENES

Human β-Actin

Fwd
5' – CGCCGCCAGCTCACCATG – 3'
Rev
5' – CACGATGGAGGGGAAGACGG – 3'

Human GAPDH

Fwd
5' – TCTCTGCTCCTCCTGTTC – 3'
Rev
5' – GCCCAATACGACCAAATCC – 3'

Mouse β-Actin

Fwd
5' – CCTTCTTGGGTATGGAATCCTGTG – 3'
Rev
5' – CAGCACTGTGTTGGCATAGAGG – 3'

Mouse GAPDH

Fwd
5' – GCAGTGGCAAAGTGGAGATT – 3'
Rev
5' – GCAGAAGGGGCGGAGATGAT – 3'

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