Recessive Parkinsonism, Mitochondria and Translational Regulation

Involvement of DJ-1 in the Oxidative Stress Response

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Cover: Mitotracker staining of a single cell show the detailed morphology of mitochondria. In the background, the pattern of RNA hybridization to a mitochondria revealed enrichment of RNA with DJ-1. Illustration: Jeff Blackinton

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ABSTRACT

Three genes are known to cause recessive forms of Parkinson disease (PD) in humans: parkin, PINK1 and DJ-1. Of these, the rarest is DJ-1; less than fifty known cases worldwide are due to mutations in DJ-1. Though rare, elucidating the function of DJ-1 and other recessive Parkinsonism genes is crucial to understanding pathways involved in pathogenesis. DJ-1 protects cells from oxidative stress in numerous models, an activity likely related to its ability to form a cysteine-sulfinic acid under mildly oxidizing conditions. In the work described in this thesis, the cellular properties and activities of DJ-1 were investigated with the intent of uncovering the underlying mechanism connecting DJ-1 oxidation to cellular protection. Two pathogenic mutants of DJ-1, M26I and L166P, were found to be unstable in cells, thus accounting for their loss-of-function. Both these and stable pathogenic mutants showed increased mitochondrial localization under oxidative stress. We also designed mutations to manipulate the oxidative properties of DJ-1 and observed that formation of a cysteine-sulfinic acid at amino acid cysteine 106 was critical for both mitochondrial localization and protection of mitochondria by DJ-1 as indicated by fragmentation of mitochondrial networks. Fragmentation observed in DJ-1 deficient cells was exacerbated by adding oxidative stressors and counteracted by increasing intracellular levels of the antioxidant glutathione, suggesting the mitochondrial phenotype was driven by misregulation of oxidation responses. Expression of other recessive parkinsonism genes, PINK1 or parkin, fully rescued the phenotype, linking all three genes into a single pathway. Since the three genes must all be expressed in substantia nigra neurons to work in concert, in situ hybridization was performed for PINK1 in human and rodent brain to complement previous observations of DJ-1 and parkin expression. Finally, the RNA interaction properties of DJ-1 were investigated. An interaction between DJ-1 and specific mRNA targets was observed and confirmed using multiple methods. These RNA binding targets included mitochondrial and nuclear encoded components of the oxidative phosphorylation pathway, selenoproteins and other antioxidant proteins including glutathione peroxidases, and components of the PTEN/Akt cell survival pathway. Pathogenic mutants of DJ-1 were deficient in RNA binding activity. This interaction of DJ-1 and RNA was oxidation dependent, as was translational regulation of targets. Increased translation of DJ-1 targets correlated with increased oxidation of DJ-1 in sporadic PD, implicating DJ-1 in the response to PD pathogenesis. This thesis therefore proposes that DJ-1 translationally regulates a localized oxidative stress response that is particularly important in protecting mitochondria.

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List of Papers

This thesis is based on the following papers, which are referred to by their Roman numerals:


III. Blackinton J*, Thomas KJ*, van der Brug M, Beilina A, Miller D, Cookson MR: Increased oxidative stress in DJ-1 deficient cells leads to mitochondrial fission. Manuscript


* Equal contribution


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INTRODUCTION

Parkinson disease

Parkinson disease (PD) is a prevalent neurodegenerative aging disorder defined by the loss of dopaminergic neurons primarily from the substantia nigra pars compacta combined with the presence of cytoplasmic inclusions known as Lewy bodies in remaining nigral neurons\(^1\). Other areas of the brain are commonly affected in PD, including noradrenergic neurons of the locus coeruleus, and other neurons can degenerate or show Lewy body pathology\(^3\).

Dr. James Parkinson first described the clinical symptoms of bradykinesia, resting tremor, rigidity and impaired balance in six patients in his *An Essay on the Shaking Palsy* in 1817\(^4\), though references to these symptoms have peppered human history beginning with ancient Indian\(^5\) and Chinese\(^6\) medical texts. The pathological understanding of the disease was not determined until identification of Lewy bodies in 1912 by Friedrich Lewy.

Since use of the term Parkinson disease requires post-mortem diagnosis of Lewy body pathology, the broader diagnostic category of parkinsonism is often used to describe syndromes with parkinsonian symptoms. A reduction in symptoms in response to levodopa treatment, a precursor to the neurotransmitter dopamine, further delineates parkinsonism from other similar movement disorders. The vast majority of parkinsonism cases are sporadic, also called idiopathic, meaning they have no known cause. These cases are usually confirmed as classical PD with post-mortem analysis. In approximately 5% of cases with parkinsonism a clear familial history of disease can be established\(^7\). Clinically, these cases often have an earlier age of onset often with less Lewy body pathology and more variable symptoms compared to sporadic PD.
Pathogenesis of PD

While this thesis focuses on a narrowly defined subset of parkinsonism, it is important to first examine the etiology of sporadic PD. To a large degree, the pathogenesis of PD is not well understood, though several theories exist. In all likelihood a combination of mechanisms contributes to onset of disease.

PD and proteasome dysfunction

Protein misfolding and misregulation of protein degradation were originally implicated in the pathogenesis of PD due to the observation that Lewy bodies are comprised of aggregated proteins. Proteins are targeted to the proteasome through ligation of multiple ubiquitin peptides by E3 ligases, but Lewy bodies often contain ubiquitin. The α-subunit of the proteasome, a major component of protein degradation, has been observed at lower levels correlating with decreased enzymatic activity of the proteasome in substantia nigra of sporadic PD patients. Inhibiting the proteasome in cultured dopamine neurons induced degeneration and replicated the formation of Lewy body-like inclusions, observations that have been extended to rodent models with varying results.

PD and mitochondria

Mitochondrial dysfunction has been proposed to play an etiological role in PD through a variety of mechanisms. Mitochondria use a chain of protein complexes (complexes I-V) known as the oxidative phosphorylation pathway to maintain an ionic gradient across the innermost of its two membranes to produce ATP, the major energy source for cellular activity. Mitochondrial complex I activity is decreased in the brain of sporadic PD patients, suggesting that the ability to produce energy is impaired. Inhibition of complex I using 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a compound taken up specifically into dopaminergic neurons by the dopamine transporter (DAT) after conversion to its metabolite MPP+ in glial cells, causes the rapid and irreversible onset of parkinsonism in humans. Another inhibitor of mitochondrial complex I, rotenone, drives loss of nigral neurons in a rodent model.

Mitochondria contain their own genome (mtDNA), which encodes several, but not all, subunits of the oxidative phosphorylation complexes as well as ribosomal and tRNA subunits necessary for their translation. Presumably because mitochondria lack histones and other protective mechanisms present in the nucleus, mtDNA is more prone to damage and mutation than nuclear DNA. Since there are hundreds of copies of mtDNA per cell, however, individual mutations are less damaging. Several studies have reported that there are higher proportions of mtDNA with deletions in substantia nigra neurons of sporadic PD cases compared to age matched controls. Mouse models which have an increased rate of mtDNA mutations due to inactivation of the nuclear encoded mtDNA proofreading enzyme (PolgA) show rapid aging and decreased mitochondrial function.
without increases in reactive oxygen species\textsuperscript{32-34} but with an increase in apoptotic markers\textsuperscript{33}.

Apoptosis, a specific form of programmed cell death, has also been implicated in PD pathogenesis. External or cellular stimuli cause Bcl-2 family members to relocalize to the mitochondria and either promote (Bax, Bak) or inhibit (Bcl-2, Bcl-xl) apoptosis\textsuperscript{35}. Promotion of apoptosis involves formation of membrane channels by Bax through which cytochrome c is released, and this is considered the final commitment toward apoptosis. A major pathway regulating cell survival, including entrance into apoptosis, is the PI3K signaling pathway. The product of PI3K, phosphatidyl-inositol, 3,4,5 triphosphate (PIP3) activates Akt, a kinase that promotes cell survival through, among other substrates, inhibition of Bax\textsuperscript{36}. The phosphatase PTEN is a negative regulator of this system through dephosphorylation of PIP3\textsuperscript{37}.

\textit{PD and oxidative stress}

Oxidative stress is caused by high levels of the hydroxyl radical (HO\textsuperscript{\textdegree}) or superoxide anion (O\textsubscript{2}\textsuperscript{\textdegree}) in the cell. These reactive oxygen species (ROS) disrupt proper cellular function through oxidative modification of proteins, nucleic acids, lipids and metabolites. To control these species, many cellular processes are directed at preventing ROS\textsuperscript{26}. Superoxide dismutases react with O\textsubscript{2}\textsuperscript{\textdegree} to form hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), which is then reduced to H\textsubscript{2}O by glutathione peroxidases and catalases. Mice without the mitochondrially localized glutathione peroxidase, GPx4, die before birth\textsuperscript{38}. The necessary co-factor of glutathione peroxidases, glutathione (GSH), is itself a highly abundant antioxidant species, present at around 5 mM in cells. Deficiencies in glutathione synthesis are also lethal to mice\textsuperscript{39}.

Misregulation of oxidation has been linked to PD in many previous studies. Increased oxidative stress has been observed in post-mortem studies of PD brains\textsuperscript{40,41} and results in increased peroxidation of lipids\textsuperscript{42,43} as well as oxidative DNA damage\textsuperscript{44}. This correlates with observations of decreased glutathione in substantia nigra in PD\textsuperscript{45}. Levels of iron and an iron binding protein, ferritin, are increased in PD brains compared to controls\textsuperscript{46-50}. Presumably this negatively affects cells through the propensity for iron to catalyze free radical and ROS production\textsuperscript{51}. The process of oxidative phosphorylation in mitochondria is the major producer of endogenous ROS in the cell\textsuperscript{52}, linking theories of mitochondrial dysfunction and oxidative stress in PD.

\textit{PD and dopamine}

Since the mechanisms suggested to cause PD are general cellular mechanisms active in all cells, most theories of PD pathogenesis are based on the prediction that dopaminergic neurons in substantia nigra pars compacta are particularly sensitive to disruptions in these pathways. One proposed agent for this sensitivity is dopamine itself. Under normal cir-
Cumstances, dopamine synthesized from tyrosine by tyrosine hydroxylase (TH), is packaged into vesicles by vesicular monoamine transporter (VMAT2) and released into the synaptic cleft to bind to dopamine receptors and affect the postsynaptic cell. Reuptake of dopamine to the presynaptic terminal is via the dopamine transporter (DAT), after which dopamine can be either repackaged into vesicles and re-released or broken down through the enzymes catechol-o-methyltransferase (COMT) and monoamine oxidase (MAO).

Failure to properly compartmentalize or degrade dopamine causes increases in cytoplasmic dopamine, which is prone to spontaneous oxidation. Oxidative byproducts can also result from the production and destruction of dopamine through TH and MAO. Neurromelanin deposits, whose black color spawned the name substantia nigra, are thought to be end products of auto-oxidation of dopamine. Dopamine oxidation products can be damaging to the cell in a variety of ways. First, the free radicals produced from this spontaneous oxidation increase intracellular oxidative stress. Second, quinones produced from dopamine oxidation are known to covalently modify cysteine residues, disrupting protein function and protein degradation pathways.

Degeneration of other dopaminergic neurons in the brain is not seen to the same degree that it is in the substantia nigra, including in the ventral tegmental area (VTA), a medial midbrain region. The reasons for this are not completely understood, though gene expression profiling has shown important differences in expression patterns between nigral and VTA dopaminergic neurons, suggesting they are indeed distinct cell populations. Another set of neurons, the tuberoinfundibular dopaminergic neurons, are resistant to moderate doses of MPTP and rotenone that are toxic to nigral, and to a lesser extent VTA, dopaminergic neurons. Nigral and VTA neurons also show dramatically different releases of dopamine in response to nicotine. Mitochondrial mass is lower in substantia nigra dopaminergic neurons and VMAT2 expression is higher in VTA suggesting higher levels of cytosolic dopamine in nigral neurons. These differences all seem to confer increased sensitivity in nigral neurons.

**Hereditary parkinsonism**

Much of the evidence implicating pathogenic pathways in PD has emerged from investigating hereditary forms, where individual causative genes have been identified. The percentage of PD actually related to genetic factors is likely higher than the approximately 5% currently identified not only due to the late onset, but also incomplete penetrance of disease as well as complex relationships between genetics and environmental factors not easily traced through small families. For example, a dominant G2019S mutation in the parkinsonism gene LRRK2 was identified in 30-40% of apparently sporadic North African cases. This illustrates that familial PD can be relevant even in the absence of a family history, meaning that hereditary forms can be disguised as sporadic PD. To date, mutations in five genes are known to cause monogenic forms of parkinsonism (Table 1).
Function of DJ-1 protein

Due to the fact that these single mutations in individual genes drive familial forms of parkinsonism, elucidating and investigating the functions of these genes may offer unique insight into the mechanisms involved in PD pathogenesis.

Table 1. Familial parkinsonism genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Location</th>
<th>Inheritance</th>
<th>Protein</th>
<th>Molecular Weight</th>
<th>Known Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA</td>
<td>PARK1, 4</td>
<td>4q21</td>
<td>AD</td>
<td>α-synuclein</td>
<td>12 kD</td>
<td>A53T, A30P, E46K</td>
</tr>
<tr>
<td>LRRK2</td>
<td>PARK8</td>
<td>12q12</td>
<td>AD</td>
<td>dardarin</td>
<td>260 kD</td>
<td>many</td>
</tr>
<tr>
<td>Parkin</td>
<td>PARK2</td>
<td>6q25.2–q27</td>
<td>AR</td>
<td>Parkin</td>
<td>50 kD</td>
<td>many</td>
</tr>
<tr>
<td>PINK1</td>
<td>PARK6</td>
<td>1p35–p36</td>
<td>AR</td>
<td>PINK1</td>
<td>65 kD</td>
<td>G309D, L347P</td>
</tr>
<tr>
<td>DJ-1</td>
<td>PARK7</td>
<td>1p36</td>
<td>AR</td>
<td>DJ-1</td>
<td>20 kD</td>
<td>L166P, M26I</td>
</tr>
</tbody>
</table>

**Dominant parkinsonism**

Mutations in two genes, α-synuclein and LRRK2, result in familial parkinsonism through dominant inheritance. The mode of inheritance suggests mutations in these proteins cause a gain or enhancement of toxic function. The therapeutic implications of understanding the pathogenic processes caused by mutated forms of these genes is high, as they are relatively abundant and mitigating the destructive effects may prove beneficial to sporadic PD as well.

**α-Synuclein**

The first gene to be linked to parkinsonism was α-synuclein. The gene is unique among parkinsonism genes in that duplication and triplication of the wild type protein also leads to familial parkinsonism, suggesting toxicity of wild type protein is sufficient to trigger disease. This toxicity is dose dependent as duplication cases (three total copies of α-synuclein) present classical PD with early onset while triplication cases (four total copies of α-synuclein) have a much earlier onset in addition to broad Lewy body pathology and dementia.

α-Synuclein is the major component of Lewy bodies. This is likely related to the propensity for α-synuclein to aggregate into oligomers, β-sheet fibrils and Lewy body-like cytoplasmic inclusions. Mutations in α-synuclein enhance oligomer or fibril formation, which is also observed with increased expression of wild type α-synuclein. α-Synuclein in Lewy bodies is often ubiquitinated, potentially implicating a deficient ubiquitin-proteasome pathway in Lewy body formation.
Surprisingly little is known about the normal function of α-synuclein protein, but it can bind lipids and may be involved in vesicle trafficking of dopamine. While one knockout mouse model showed decreases in striatal dopamine, another did not. A pool of α-synuclein localizes to synapses and mitochondria, the latter under conditions of low pH, implying potential recruitment under cellular stress.

LRRK2
Mutations in LRRK2 mainly result in clinically typical PD. Lewy body pathology is quite variable, as broad Lewy pathology, classic nigral pathology and nigral degeneration without Lewy bodies have been observed, in addition to tauopathy more common in Alzheimer’s disease. LRRK2 contains a kinase domain as well as a GTPase domain. While some mutations in LRRK2 have been shown to alter kinase activity, kinase activity is necessary for LRRK2 mediated toxicity. The most common mutation, G2019S, shows increased kinase activity and toxicity in these studies. Although LRRK2 is a dimer that can autophosphorylate, only two substrates, moesin and 4EBP1, have been reported. Similar to α-synuclein, LRRK2 can localize to membranes and vesicular structures, including mitochondria. In addition, LRRK2 is stabilized by the chaperone protein HSP90, which prevents degradation of LRRK2 through the proteasome.

Recessive parkinsonism
The three genes with recessive modes of inheritance, parkin, PINK1 and DJ-1, all lead to onset of parkinsonism around the second to fourth decade of life with slow progression and excellent response to levodopa treatment. Of the approximately ten patients with parkin mutations who have been analyzed, almost all exhibit nigral degeneration and parkinsonian phenotypes in the absence of Lewy body pathology with one case reporting α-synuclein positive inclusions. Pathology from post mortem brain of patients with PINK1 and DJ-1 mutations has not been reported. Though the early onset and lack of Lewy body pathology suggest recessive mutants cause an atypical parkinsonism, they are potentially of interest to sporadic disease because they appear to be driven by a loss of protective protein functions. Understanding the normal functions of these proteins may help to elucidate particular pathways that are essential for neuronal protection, allowing therapeutics that enhance this protection.

Parkin
The first of the recessive parkinsonism genes to be identified was parkin. Mutations in parkin are responsible for approximately 50% of recessive familial parkinsonism. Both protein and mRNA are ubiquitously expressed in the body, including brain.

Parkin is an E3 ubiquitin ligase, which covalently links ubiquitin peptides to proteins as a mechanism of post-translational regulation. Ubiquitin conjugation causes one of two
fates for the modified protein: mono-ubiquitination often promotes signaling activation, while poly-ubiquitination targets proteins for degradation through the proteasome. Both mono\textsuperscript{121,122} and poly-ubiquitination\textsuperscript{123-125} protein fates have been observed for proteins due to interactions with parkin. In cells, parkin can associate with the outer mitochondrial membrane where it protects cells from apoptosis by delaying release of cytochrome c from mitochondria\textsuperscript{126}. Expression of human parkin can rescue \(\alpha\)-synuclein induced toxicity in flies\textsuperscript{127-129}, in primary mouse midbrain neurons\textsuperscript{130} and in rats\textsuperscript{131}.

Parkin knockout mice are grossly normal without major locomotor or aging phenotypes\textsuperscript{132,133}. Defects, however, have been observed at the molecular level, including increases in extracellular dopamine\textsuperscript{134} coupled with decreased dopamine reuptake\textsuperscript{134,135}, decreased mitochondrial respiration, and increased glutathione levels coupled with increased protein carbonyls and lipid peroxidases\textsuperscript{136}. Drosophila lacking parkin have swollen mitochondria and disrupted mitochondrial membrane integrity, resulting in reduced lifespan, locomotor deficits and sterility\textsuperscript{137-139}. In these animals, genes involved in oxidative stress responses are induced\textsuperscript{140}, and increased levels of antioxidant proteins thioredoxin\textsuperscript{141} and glutathione S-transferase\textsuperscript{142} help compensate for parkin deficiency. Overall, parkin links many theories of PD in assisting in the oxidative stress response and mitochondrial maintenance through directing proteosomal degradation and/or regulation of crucial proteins.

**PINK1**

Mutations in PTEN-induced putative kinase (PINK1) constitute a relatively rare form of parkinsonism\textsuperscript{108}, accounting for nearly 10\% of recessive familial cases\textsuperscript{115}. Northern blots suggest broad expression of PINK1 mRNA\textsuperscript{143,144}, while \textit{in situ} hybridization in mouse and rat suggest expression in brain is primarily neuronal\textsuperscript{145}. PINK1 immunoreactivity is observed in both neurons and glia in human brain\textsuperscript{146}, though the questionable quality of PINK1 antibodies used\textsuperscript{147} and differences in species studied leave the question of where PINK1 is expressed in humans unresolved.

PINK1 contains a mitochondrial targeting sequence preceding a kinase domain, and both mitochondrial localization and kinase activity have been confirmed in cells\textsuperscript{143,148,149}. However, the presence of PINK1 in some glial cytoplasmic inclusions\textsuperscript{50} and Lewy bodies in sporadic PD\textsuperscript{46} suggests possible extra-mitochondrial localization of PINK1. Cytoplasmic PINK1 has been observed in overexpression models\textsuperscript{48,151} and PINK1 remains protective without its mitochondrial localization sequence\textsuperscript{52}, suggesting that PINK1 may have extra-mitochondrial roles as well. Fibroblasts from patients with PINK1 mutations show increased lipid peroxidation, superoxide dismutase and glutathione suggesting a higher oxidative burden\textsuperscript{153}.

PINK1 has been reported to phosphorylate the mitochondrial chaperone protein TRAP1/ Hsp75, which is required for PINK1 mediated protection\textsuperscript{154}. It has also been proposed
that the mitochondrial protease HtrA2/Omi is a substrate of PINK1 and that PINK1 mediated protection was dependent on presence of HtrA2/Omi\(^{55}\). A German family with PD has a mutation in HtrA2/Omi\(^{56}\), suggesting a link to parkinsonism, though genetic studies have not shown an association with disease\(^{57}\). PINK1 knockout mice show impaired dopamine release and synaptic plasticity in the absence of degeneration\(^{58}\). In flies, PINK1 knockout mitochondria are elongated and abnormally large, causing reduced lifespan and muscle degeneration\(^{59-62}\).

**Mitochondrial morphology and PINK1 and parkin interactions**

As mentioned above, null mutants of either PINK1 or parkin in *Drosophila* show similar defects, including abnormally large muscle mitochondria leading to muscle degeneration and impaired lifespan. Further investigations revealed that phenotypes in PINK1 knockout flies can be fully rescued by expressing parkin in these animals, but expression of PINK1 does not rescue parkin deficiency\(^{59-62}\). This suggests that PINK1 acts upstream of parkin in a single pathway. This interaction has since been confirmed in mammalian cells, though the output of the pathway limits fission, the opposite effect\(^{63}\).

Mitochondria are dynamically regulated organelles that fuse and divide regularly (Figure 1). This causes dramatically different morphology along a spectrum from large, elongated and tubular mitochondria to small, fragmented and round mitochondria. Two opposing tightly regulated processes known as mitochondrial fusion and fission drive this morphology. A number of proteins are responsible for the fusion and fission processes, including GTPases Mfn1, Mfn2, Opa1 and Drp1. Mfn1, Mfn2 and Opa1 are mitochondrially localized enhancers of fusion, while Drp1 is a cytoplasmic protein that relocates to mitochondria after post-translational modifications to cause mitochondrial fission\(^{64}\). Both fusion and fission events are required to maintain mitochondrial health\(^{65}\), but the induction of fission in particular is tightly linked to apoptosis. Overexpression of fusion proteins can also be toxic to cells\(^{66}\), and neurons in particular may require mitochondrial fission to allow mitochondria into axons\(^{67}\). Increased fission of mitochondria occurs in response to many cell stimuli, including loss of mitochondrial membrane potential, apoptosis signaling mechanisms, iron accumulation and buildup of reactive oxygen species\(^{67}\). Regulation of mitochondrial morphology has been shown to be especially important in neuron survival as mutations in *Opa1* lead to dominant optic atrophy\(^{68,69}\) and mutations in *Mfn2* lead to Charcot-Marie-Tooth disease affecting the peripheral nervous system\(^{70}\). The large and swollen mitochondria in PINK1 and parkin null flies suggest both proteins normally act to enhance mitochondrial fission. Supporting this, overexpression of Drp1 or heterozygous loss of function mutations in Opa1 or Mfn2 rescue mitochondrial phenotypes in flies lacking PINK1 or parkin\(^{71,72}\). In mammalian cells, however,
the phenotypes appear reversed; deficiency of PINK1 causes an increase in mitochondrial fission\(^{163}\). The reason for these different observations is unclear. The pathway mechanistically linking PINK1 to parkin is also undefined. Therefore, although regulation of mitochondrial fusion and fission processes is likely to be important in recessive parkinsonism, the mechanism by which PINK1 and parkin affect mitochondrial dynamics is unclear and may vary between species.

**DJ-1**

The third recessive gene responsible for parkinsonism, DJ-1, is also linked to the fewest number of cases. Three homozygous point mutations and a deletion have been identified to segregate with disease in families while several other heterozygous and less definitively causal mutations have been identified in studies of early onset parkinsonism patients\(^ {109,110,173-177}\). DJ-1 is abundantly expressed in brain and throughout the body, suggesting general cellular functions\(^ {178-180}\). Although DJ-1 mutations are rare, understanding the activity of the wild type protein allows insight into pathways that may prevent the onset of PD.

DJ-1 functions most clearly as an oxidative stress response protein. It protects cells against oxidation damage from complex I inhibitors rotenone and MPP\(^ +\) as well as superoxide generators paraquat and \( \text{H}_2\text{O}_2 \) in cells and in vivo\(^ {181-185}\). Unchallenged, little degeneration is seen in cell and animal models\(^ {185-187}\), though cells or mice deficient in DJ-1 have higher levels of ROS\(^ {188-190}\). Localization of DJ-1 to mitochondria increases under conditions of oxidation in cells\(^ {181}\). A pool of DJ-1 localizes to the mitochondrial matrix as well as the inner membrane space in mice, but no changes in localization were observed with oxidation\(^ {191}\). DJ-1 containing an artificial mitochondrial localization sequence is more protective suggesting this relocalization is important to protection\(^ {192}\). Since DJ-1 contains no clear mitochondrial localization sequence, it is unclear what drives its relocalization. The protection against oxidative stress by DJ-1 can be abolished by mutation of a highly conserved cysteine at amino acid 106 in humans\(^ {185,193}\). In addition, cysteine 106 mutants do not relocalize to mitochondria\(^ {181}\). The 1.1 Å crystal structure of DJ-1 reveals that under mildly oxidizing conditions, cysteine 106 forms a stable cysteine-sulfinic acid\(^ {194,195}\) (Figure 2). This cysteine-sulfinic acid is stabilized by the neighboring glutamic acid (E18), a
relationship that shows absolute evolutionary conservation in all PfPI family members from bacteria to humans\textsuperscript{196}. Due to the constraints of mutational analysis, it remains unclear whether formation of the sulfinic acid or an intrinsic activity of the cysteine amino acid itself is critical to protection.

While DJ-1 clearly responds to oxidative stress and protects cells from oxidative stressors, the details of the underlying mechanism remain elusive. The most direct notion is that DJ-1 itself is a free radical scavenger, since it can exhibit peroxiredoxin-like free radical scavenging activity\textsuperscript{190}. This is, however, unlikely to explain the protective effects of DJ-1 as DJ-1 is less abundant than other thiol containing antioxidants such as glutathione, and the free radical scavenging activity of DJ-1 is at least 1000 fold lower than that of well-defined free radical scavengers like catalase\textsuperscript{190,197}.

DJ-1 encodes a small, dimeric, single domain protein belonging to the PfPI superfamily of proteins\textsuperscript{196,199}. As the superfamily contains proteases and chaperones, the protease and chaperones activity of DJ-1 has been tested. No protease activity has been observed\textsuperscript{194}, but DJ-1 has a weak oxidation dependent chaperone activity directed against α-synuclein \textit{in vitro}\textsuperscript{200,201}. This activity, however, is also unlikely to fully explain the protective effects of DJ-1 since DJ-1 protects against oxidation-induced toxicity in \textit{Drosophila}, which have no
α-synuclein homolog. Although the functions of the clade of proteins most resembling DJ-1 is unknown, sequence alignments separate DJ-1 and its homologues from proteases and chaperone.

DJ-1 also may have subtle effects on metabolism of dopamine. Increased reuptake of dopamine was observed in some DJ-1 knockout mouse models while another showed increased striatal dopamine. DJ-1 knockout dopaminergic neurons are less inhibited compared to wild type neurons in response to dopamine and quinpirole. Another DJ-1 knockout model, however, showed no deficits in dopamine systems. DJ-1 has also been reported to upregulate tyrosine hydroxylase and increase glutathione synthesis in response to oxidation. In addition, DJ-1 has been shown to protect neurons from both glutamate excitotoxicity and hypoxic injury, reducing the size of lesions from induced ischemic stroke through reduction of oxidative stress.

DJ-1 was originally identified in cancer studies where it enhanced Ras mediated transformation of cells, and has since been observed as being increased in proteomic studies in sera from breast cancer patients and lung carcinomas, but downregulated in hepatocellular carcinoma patients. This may be related to the ability of human DJ-1 to negatively regulate PTEN in Drosophila, rescuing PTEN overexpression phenotypes. Furthermore, PI3K rescued eye phenotypes and dopamine neurons in Drosophila DJ-1α knockouts. Downstream effects of PI3K and PTEN regulation could further explain observed decreases in Bax and caspase activation with DJ-1 overexpression and increases in p53 and Bax expression with loss of DJ-1 function.

These various observed effects on regulation of protein levels support a third hypothesis of DJ-1 activity: an interaction with nucleic acid binding proteins, particularly proteins associated with RNA. The rat homolog of DJ-1 was originally identified as RS, a regulatory subunit of an RNA binding complex. Subsequently, a number of reported protein interactors of DJ-1 have been nucleic acid binding proteins, including the RNA helicase Abstrakt and the RNA polymerase binding PSF and p54nrb complex in addition to transcriptional regulators PIASx and DJBP. The proclivity of DJ-1 to interact with RNA interacting proteins suggests it may act to affect proteins levels through translational regulation. This theory is further appealing because it would explain the vast scope of reported cellular activities of DJ-1 with a simple biochemical mechanism.

DJ-1 cannot rescue phenotypes resulting from lack of PINK1 or parkin either in cells or in fly models. This suggests that DJ-1 either works upstream of PINK1/parkin or in an independent pathway. To date DJ-1 has not been reported to affect mitochondria as other recessive parkinsonism proteins do, yet the importance of its relocalization to mitochondria suggests a crucial function at the mitochondrial level. This thesis presents significant new data that explores the role of DJ-1 in mitochondrial function and investigates the RNA binding capability of the DJ-1 protein.
AIMS

- Characterize pathogenic mutants of DJ-1 to understand the loss-of-function mechanisms
- Uncouple cysteine presence from oxidation capability to examine the relative importance of each to DJ-1 mediated protection
- Examine relationships between DJ-1 and mitochondria, including relationships to PINK1 and parkin
- Validate expression of the recessive parkinsonism gene PINK1 in neuronal populations
- Investigate the RNA binding properties of DJ-1
MATERIALS AND METHODS

Animal Tissues
C57BL/6 mice and Sprague-Dawley rats were obtained from Scanbur (Sollentuna, Sweden). Animals were kept under standardized temperature, light and humidity conditions and given food and water ad libitum (paper V, VI). Animal experiments were approved by the Swedish Animal Ethics Committee (Stockholm, Sweden). Adult rat and mouse brains were sacrificed by cervical dislocation, then dissected, frozen on dry ice and stored at -80°C until use. Fourteen 1 µm coronal sections through striatum, substantia nigra and cerebellum were thawed onto slides (Superfrost plus). Slides were stored at -20°C until use.

Human Tissues
Human post mortem brain tissue was provided by Harvard Brain Tissue Resource Center (Belmont, USA) and the Queen Square Brain Bank for Neurological Disorders (London, England), collected with the ethical approval of the London Multicentre Research Ethics Committee and with the informed consent of next-of-kin. Ethical approval for studies of gene expression in archival tissue was obtained from the National Hospital for Neurology and Neurosurgery and Institute of Neurology Joint Research Ethics Committee (London, England).

In Situ Hybridization
Two different oligonucleotides were designed for each targeted gene (Table 2). Additionally we a random probe not matching any sequence in the Genbank database was used. Oligonucleotides were radioactively labeled at the 3’ end with 32P-dATP, purified (QIAquick Nucleotide Removal Kit, Qiagen), added to hybridization solution and incubated overnight at 42°C with prepared tissue slides. Slides were then washed in SSC at 60°C, dipped in water, dehydrated in ethanol and dried. One set of slides was exposed on films for autoradiography and others were dipped in prepared Kodak NTB2 emulsion, dried, and sealed for three weeks at 4°C, then developed and cresyl-violet counterstained. Slides were analyzed under light microscopy and scored using a semi-quantitative scale, – (no expression), +, ++, or +++ (very strong expression). Ratings were replicated independently with similar results by two observers.

Plasmids and Transfections
Expression constructs for human DJ-1 were cloned into pcDNA3.1/GS vector (Invitrogen) containing a C-terminal V5-his tag. Mutations were generated using Quikchange mutagenesis (Stratagene). For untagged mutants, mutagenesis reintroducing the endogenous stop codon at amino acid 189 of DJ-1 was performed and for V5 only mutants, a stop codon was inserted immediately following the V5 tag. Plasmids for mitochondrial
YFP (mito YFP), human Drp1 K38A, Mfn1 and Opa1 were generous gifts from the lab of Dr. Richard Youle (National Institute of Neurological Disease and Stroke, Bethesda, MD). Cells were transfected using Lipofectamine 2000 (Invitrogen). For reporter constructs, forward and reverse primers were designed to amplify the 5' and 3' UTRs of human GPx4 or MAPK8IP1. Sequences were cloned into pEGFP-N1 and pEGFP-C1 respectively (Clontech). Two DJ-1 (GGAAGTAAAGTTACAACACA, GGTCATTTACCTACTCTGAG) and one control (GGCTAGACGCGATAGTATGGA) shRNAs were constructed using the Invitrogen BLOCK-iT Lentiviral system according to manufacturers instructions.

Cell Culture

Parental M17 and PC12 cell lines were purchased from ATCC and cultured in Opti-MEM or DMEM (Invitrogen) as directed plus 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were passed prior to confluency using Trypsin or TrypLE (Invitrogen). To generate clonal M17 cell lines stably expressing DJ-1 and control shRNA plasmids, lentivirus was packaged (Invitrogen; see plasmids) as instructed for two DJ-1 target sequences and one control. After clonal selection, two clones reached 89% and 96% reduction in steady state protein level respectively compared to one nonsense control. Established fibroblast cultures from wild type and DJ-1 knockout mice were generous gifts from Dr. Huaibin Cai and Dr. Jayanth Chandran (National Institutes on Aging, NIH).

Live Cell Imaging

Cells were cultured at a density of 10⁵ cells per well in Opti-MEM with 10% FBS in Lab-Tek chambered coverglass (Nunc) and transfected 24 hours prior to assay with 0.5 μg mito-YFP vector. When necessary, 2 μg of additional constructs or empty vector was co-transfected; in control experiments we have found >90% of mito-YFP positive cells are also positive for co-transfected proteins (data not shown). With drug treatments, mito-YFP was introduced 30 hours prior to assay and toxin added after 6 hours. Paraquat, rotenone, 1-methyl-4-phenylpyridinium (MPP⁺), glutathione ethyl ester (GSH-EE), and 2-oxo-L-thiazolidine-4-carboxylic acid (OTCA), were purchased from Sigma. Immediately prior to assay, media was changed to Opti-MEM without phenol red, and then imaged live on a LSM 510 confocal microscope using a Plan-Apochromat 100x/1.4 objective (Zeiss) under 488 nm excitation. YFP positive cells were selected randomly between fields. A 25-pixel radius region of mitochondria adjacent to the nucleus was photobleached using 488 and 514 nm lasers at 100% power and the recovery of fluorescence observed for 12 seconds with images taken every 250 milliseconds. Recovery was normalized to both background fluorescence and an equivalent non-photobleached region of mitochondria. Mobile fraction values were calculated using the following equation:

Mobile Fraction = ([FRAP₂ - Background]/FRAP₁)*([NSPB₂ - Background]/NSPB₁).
Thirty randomly chosen cells were photobleached per experiment over at least two experiments for a minimum of \( n=60 \) cells per condition.

**Western Blotting**

Protein lysates were heated to 65°C for 15 minutes in loading buffer (Invitrogen) to denature proteins, then loaded onto SDS-PAGE Criterion gels (Bio-Rad) and size separated at 100V. Proteins were then transferred to PVDF membranes overnight at 30V in 10% CAPS/10% MeOH buffer on ice. After blocking for 30 minutes in blocking buffer [5% milk in TBST (1x TBS with 0.1% Tween-20)], Western blotting was performed by incubating with primary antibody at room temperature for one hour, followed by three

<table>
<thead>
<tr>
<th>Table 2. Oligonucleotide sequences used for in situ hybridization</th>
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<tbody>
<tr>
<td><strong>Human</strong></td>
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<td>UCH-L1</td>
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<tr>
<td><strong>Mouse</strong></td>
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<td><strong>Rat</strong></td>
</tr>
<tr>
<td>PINK1</td>
</tr>
<tr>
<td><strong>Random</strong></td>
</tr>
</tbody>
</table>
Function of DJ-1 protein

five minute washes in TBST and incubation with HRP-conjugated secondary antibody. After three final TBST washes, membranes were developed using ECL-plus reagent (GE Healthcare) and exposed to film prior to digital scanning on a Storm 820 phosphoimager (Molecular Dynamics). Quantitation of signal intensities was performed using ImageQuant software. Antibodies used for Western blotting included DJ-1 (Stressgen), V5 (Invitrogen), β-actin (Sigma), GPx4 (Abcam), MAPK8IP1 (Santa Cruz) and MAPK8IP3 (Santa Cruz).

Subcellular Fractionation

Mitochondrial fractions were prepared by Mitochondrial Isolation Kit (Pierce) as directed. These fractions were then stripped of all loosely associated proteins using 20 µM sodium carbonate in HEPES buffer for 30 minutes on ice followed by ultracentrifugation at 60,000g for 30 minutes and Western blotting as indicated.

Immunocytochemistry

Cells were grown on coverslips coated with poly-D-lysine, then transfected with V5-tagged DJ-1 variants. After 48 hours, cells were preincubated with 500 nM Mitotracker CMTMRos (Invitrogen) for 30 min at 37°C, then fixed in 4% paraformaldehyde in Dulbecco’s PBS (DPBS) for 30 min at room temperature. After permeabilization with 0.1% Triton X-100 and quenching with 0.1 M glycine, coverslips were blocked with DPBS containing 10% fetal bovine serum and 0.1% Triton X-100. Coverslips were then incubated with primary monoclonal anti-V5 (diluted 1:200) overnight at 4°C followed by secondary AlexaFluor 488-conjugated goat anti-mouse IgG prior to mounting under ProLong Anti-fade medium (Invitrogen). Slides were examined using a Zeiss LSM510 confocal microscope using independent excitation for both channels.

Two Dimensional Gel Electrophoresis

Protein lysates were prepared using two different methods. For brain tissue, lysates were prepared as if for RNA immunoprecipitation (below). For cultured cells, pellets were resuspended in Urea lysis buffer (8M Urea, 2M Thiourea, 4% CHAPS buffer, 2% IPG Pharmalyte buffer and 60 mM DTT) and incubated 30 minutes at room temperature. Protein samples in either buffer (40 µg in 11 µl) were prepared for loading onto either 7 or 11 cm 4 – 7 linear pH gradient Immobiline DryStrips (GE Healthcare) by addition of 120 µl of rehydration buffer (8 M urea, 2% CHAPS, 2% IPG buffer pH 4-7), 1.5 µl IPG buffer pH 4-7 and 2 µl DeStreak reagent (GE Healthcare). After spreading prepared samples along DryStrips in chambers, 800 µl DryStrip cover fluid (GE Healthcare) was added, then chambers were sealed and samples were separated by isoelectric focusing in an Ettan IPGPhor system (GE Healthcare) for 16,000 Vh. Subsequently, proteins were
Jeoff Blackinton

separated by size using 12.5% Tris HCl SDS PAGE gels (Bio-Rad) and DJ-1 isoforms detected by Western blotting as indicated. 2-D SDS PAGE standards (Bio-Rad) were used to calibrate pl.

RNA Immunoprecipitations

Cell or brain samples were harvested in PLB buffer (0.5% NP-40, 10 mM HEPES, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, RNase OUT and protease inhibitors) and incubated on ice for 10 minutes prior to centrifugation at 14,000xg for 10 minutes. The supernatant was added to either DJ-1 C-16 antibody (Santa Cruz) or non-specific goat IgG antibody (Santa Cruz) bound to Protein G Dynabeads (Invitrogen) or, in the case of transfected 6his tagged proteins, Ni-NTA magnetic beads (Qiagen) in NT2 buffer (0.05% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM MgCl₂). After four washes using NT2 buffer, RNA was extracted by adding DNase for 5 minutes, discarding the supernatant and eluting after 20 minute incubation with Proteinase K. RNA was washed in acid phenol-chloroform, and precipitated with 100% ethanol containing sodium acetate and GlycoBlue (Ambion) overnight. Precipitated RNA was pelleted, washed through 70% ethanol, the concentrations equalized and cDNA generated using the Superscript III kit (Invitrogen).

The CLIP method was performed as reported²²¹. Briefly, cells were UV crosslinked for a total of 3000 mJ to covalently bond protein and nucleic acids, DNase digested to remove DNA, then incubated with either high (1:100), low (1:5000) or no RNase to differentially digest RNA. Subsequently, RNA was immunoprecipitated as above and while still attached to beads, RNA was modified using alkaline phosphatase (Fermentas) followed by ligation of a RNA linker sequence (5’-P-UCGUAUGCGUCUGCGUCUGUCUGU-3’T) (Dharmacon) and end-labeling with ³²P ATP by poly-nucleotide kinase (PNK) (Fermentas). Subsequently, samples were SDS-PAGE size separated and transferred to nitrocellulose membranes. Phosphoimaging and exposure to film of nitrocellulose membranes revealed radioactivity indicating RNA species covalently bound to nitrocellulose bound proteins, with differences between high and low RNase treated samples indicating RNase dependency. To purify RNA, small strips of nitrocellulose membrane corresponding to the highest signal were cut out and treated with proteinase K, then precipitated overnight in ethanol with GlycoBlue (Ambion). Subsequently, a 5’ RNA linker (5’-GUUCAGAGUUACAGGACGAUC) was added to purified RNA and again precipitated overnight. PCR was performed using primers specific to each RNA linker to amplify regions associating with protein. PCR products between 100-200 base pairs were then cut out of an agarose gel and cloned into TA cloning vector (Stratagene) and sequenced.
Expression Arrays and Analysis

Illumina human and mouse oligonucleotide arrays (Illumina, San Diego, CA) were used according to manufacturers instructions, starting with 500 ng of total RNA for each sample. Arrays were read on an Illumina Bead array reader confocal scanner. Differential gene expression values were calculated with the Illumina Custom algorithm within the Illumina BeadStudio software suite.

Quantitative RT-PCR

Primers were designed against DJ-1 RNA targets (Table 3) and validated for comparative use with β-actin primers. SYBR Green PCR master mix (Applied Biosystems) was used as directed and reactions were performed in quadruplicate on an ABI 7900HT Real Time PCR system (Applied Biosystems). Relative abundance was determined using comparative analysis, the logarithmic difference between the cycle value of target and β-actin (2-Δ[Δ]Ct).
RESULTS

The results of papers I-VI are summarized below. Please see the appended papers and manuscripts for full details.

Stability and Localization of Pathogenic Mutants of DJ-1 (Paper I)

The recessive inheritance of DJ-1 parkinsonism suggests disease likely results from a loss of normal DJ-1 function. To investigate the reasons for the loss-of-function, we examined the cellular properties of known human mutations in DJ-1. One mutation, L166P, was previously reported to be highly unstable in cells, suggesting that decreased protein levels are the likely underlying reason for loss of DJ-1 function. Using transient transfections of M17 neuroblastoma cells, we identified a second unstable mutation, M26I, using steady state protein levels as well as cycloheximide chase. Inhibiting the proteosome degradation pathway recovered some M26I DJ-1 protein, although never to wild type DJ-1 levels, suggesting that additional protein degradation pathways may also be involved. Despite its instability and proximity of the mutation to the dimer interface, M26I retained the ability to both homo- and hetero-dimerize. All other pathogenic mutants investigated showed normal steady state protein levels and dimerization. The instability of M26I has been further confirmed in other laboratories. Together, this suggests that two DJ-1 mutations lead to parkinsonism due to reduced abundance of DJ-1 protein but does not explain loss of function in stable mutants.

Wild type DJ-1 had previously been shown to localize to mitochondria under conditions of enhanced oxidative stress and recently this localization has been shown to be important for the protective effects of DJ-1. We investigated this ability as an additional measure of functional deficiencies in the pathogenic mutants. As reported, wild type DJ-1 was largely cytoplasmic in M17 neuroblastoma cells under basal conditions, but relocalized to mitochondria when cells were stressed with sublethal doses of paraquat. The two unstable mutants of DJ-1, M26I and L166P, showed mitochondrial localization under basal conditions and this was increased dramatically under paraquat stress. The stable mutant D149A was cytoplasmic under basal conditions, but localized strongly to mitochondria under conditions of oxidative stress. These results indicate that pathogenic mutants can localize to mitochondria, and do so to a greater degree than wild type DJ-1. That these mutants still fail to protect cells, in particular the stable mutant D149A, suggests that mitochondrial localization is not sufficient for protection. To examine whether mitochondrial recruitment is essential for protection, we further examined oxidation mutants of DJ-1.

Characterization of oxidation mutants (Paper II)

Since DJ-1 protects against oxidative stress and increased cellular oxidation drives formation of the sulfinic acid at cysteine-106, we predicted that sulfinic acid formation was a
Table 3. Quantative real time PCR primers used for analysis of RNA bound to DJ-1.  Hs indicates human specific, Mm indicates mouse specific.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start (F)</th>
<th>Sequence</th>
<th>Start (R)</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BAD (Hs)</td>
<td>41F</td>
<td>TCCGGAGGATGAGTGACAGAGTT</td>
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<td>β-actin (Mm)</td>
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<td>694R</td>
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<td>764R</td>
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crucial event mediating protection by DJ-1. While previous reports suggested mutation of cysteine 106 abolishes DJ-1 mediated protection\textsuperscript{181,193}, presence of the cysteine residue itself could not be uncoupled from a requirement for the post-translational modification. To assess this, we designed three artificial mutations in the glutamic acid (E18) that stabilizes cysteine-106 which we predicted would alter the oxidation properties of DJ-1. We mutated the glutamic acid to an asparagine (E18N), glutamine (E18Q) or aspartic acid (E18D). X-ray crystallography and mass spectrometry of these mutants by collaborators at the University of Nebraska revealed that E18N and E18Q were capable of forming the cysteine-sulfinic acid. While both showed weaker oxidation at high molar ratios of H\textsubscript{2}O\textsubscript{2}, at lower ratios, E18N always was partially oxidized and E18Q was oxidized at levels comparable to wild type DJ-1. E18D, however, could not form the sulfinic acid as the increased hydrogen bonding lengths created could only support a single oxygen atom, forming the readily reducible sulfenic acid. 2-D gel electrophoresis of M17 neuroblastoma cells after oxidation with paraquat showed a consistent pattern with this in vitro analysis. Wild type DJ-1 appeared as two major isoforms while E18N and E18Q showed increased presence of a third, oxidized isoform suggesting increased sulfenic acid formation. In contrast, both E18D and C106A were present mainly in a single unoxidized isofrm. Taken together, the data \textit{in vitro} and in cells suggests that E18N and E18Q increase sulfinic acid formation to levels above those seen in the wild type protein, while E18D and C106A are both unable to form the cysteine-sulfinic acid.

Subsequently, we examined the ability of these oxidation mutants to localize to mitochondria using subcellular fractionation and immunocytochemistry. Both E18N and E18Q were strongly mitochondrial under basal conditions, while wild type, E18D and C106A were largely cytoplasmic (Figure 3). Oxidation with either rotenone or paraquat increased mitochondrial localization of wild type DJ-1, while E18N and E18Q remained mitochondrial. Neither E18D nor C106A showed relocalization to mitochondria, suggesting that cysteine-sulfinic acid formation is crucial for mitochondrial localization.

We also noticed morphological changes in mitochondria after treatment with either rotenone or paraquat. Cells expressing wild type DJ-1 retained the elongated mitochondria typical in untreated cells, as did cell expressing the oxidation-competent mutants E18N and E18Q. In contrast, cells expressing the two oxidation-deficient mutants, E18D and C106A, showed fragmented mitochondria. To further evaluate the functional effects of oxidation mutants, we quantified these mitochondrial phenotypes using DJ-1 knockout fibroblasts in a live cell assay for functional mitochondrial interconnectivity, fluorescence recovery after photobleaching (FRAP). Fluorescent proteins targeted to the mitochondria are photobleached followed by observation of fluorescence recovery in the same region. High levels of recovery indicate high mitochondrial interconnectivity, while low recovery indicates high levels of mitochondrial fragmentation. DJ-1 knockout fibroblasts had a basal deficiency in mitochondrial interconnectivity that was rescued by expressing
human DJ-1, suggesting the increase in mitochondrial fragmentation was due solely to the absence of DJ-1. E18N or E18Q DJ-1 could substitute for wild type DJ-1 to varying extents, but E18D or C106A resulted in no recovery of fragmentation. These results demonstrate that sulfenic acid formation at C106 not only is crucial for mitochondrial localization, but also is required for protection of mitochondrial interconnectivity in cells.

**Mitochondrial morphology and DJ-1 (Paper III)**

To extend our observations of mitochondrial phenotypes accompanying DJ-1 deficiency, we used M17 neuroblastoma cells expressing a short hairpin RNA (shRNA) directed against DJ-1. Two different DJ-1 shRNA cell lines each achieved knockdown of DJ-1 to 85-95 percent of control shRNA cell lines. Both of these clones showed a similar subtle increased fragmentation of mitochondria compared to nonsense shRNA control cell lines (Figure 4). Using FRAP to quantify these effects, we observed decreased functional connectivity of mitochondria.

Since DJ-1 reportedly affects two pathways known to induce mitochondrial fragmentation, increased oxidative stress and apoptosis\(^{367}\), we observed the effects of modulation of these pathways on DJ-1 induced fragmentation. The mitochondrial complex I inhibitors rotenone and MPP\(^+\) as well as the superoxide generator paraquat exacerbated the fragmentation phenotype. Conversely, cell permeable glutathione precursors glutathione ethyl ester (GSH-EE) and 2-oxo-L-thiazolidine-4-carboxylic acid (OTCA) fully rescued the mitochondrial fragmentation, suggesting the phenotype was tightly correlated with the antioxidant capabilities of the cell. For comparison to another reported activity of DJ-1, we modified the PTEN/Akt cell survival pathway. Neither decreasing Akt phosphorylation using LY294002 or decreasing PTEN phosphorylation using 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (DMAT) affected the DJ-1 deficient phenotype. Cumulatively, these results suggest the mitochondrial fragmentation observed was due to increased oxidative stress in the cell, and not modulation of cell survival stimuli.

We further examined whether this phenotype was due to modulation of mitochondrial fusion and/or fission proteins. Overexpression of Opal1 and Mfn1, proteins that increase fusion, rescued the phenotype seen in DJ-1 deficient cells. Similarly, expressing a dominant negative mutant of Drp1 inhibited fission and also fully rescued DJ-1 deficient cells. Increased oxidation from rotenone treatment decreased phosphorylation of Drp1, which is known to negatively regulate Drp1 GTPase activity. These results suggest the increased oxidative stress in DJ-1 deficient cells increases activity of Drp1, linking oxidative stress to increased mitochondrial fission.

PINK1 and parkin also affect mitochondrial fusion and fission machinery and so we examined whether expression of either gene affected the mitochondrial morphology of DJ-1 deficient cells. Both PINK1 and parkin rescued the phenotype fully, quantita-
Figure 3. Oxidation prone mutants of DJ-1 localize to mitochondria basally, while oxidation deficient mutants fail to localize to mitochondria under oxidative stress. M17 neuroblastoma cells transiently transfected with wild type and mutant V5 tagged DJ-1 were treated for 24 hours with either 300 μM paraquat or 100 nM rotenone, incubated with Mitotracker (red), then immunostained for V5 (green) immunoreactivity. Scale bar represents 10 μm.

![Image showing DJ-1 localization to mitochondria under oxidative stress.](image)

**PINK1 is expressed mainly in neurons in human brain (Paper IV)**

Given the relationships between DJ-1 and PINK1 with regard to mitochondrial integrity, we investigated whether PINK1 was expressed in the same brain areas as DJ-1 and whether its expression was changed in sporadic PD. Expression of both DJ-1 and parkin has been characterized in rodent and human brain, but the pattern of PINK1 expression is less clear due to conflicting reports of glial expression of rodent PINK1 mRNA compared to human protein.

In situ hybridization of human, mouse and rat brain tissue revealed a broad expression of PINK1 consistent with expression in neurons. Expression was robust in cortex, substantia nigra and striatum in all species, but regions of strongest expression included dentate nucleus and Purkinje cells in the cerebellum. Only background levels of expression were seen in glia-rich white matter. These results confirm previous reports of primarily neuronal expression of PINK1 in rodent brain. We observed similar expression patterns in human brain, again consistent with neuronal expression, though the possibility of low
Figure 4. Mitochondrial morphology of DJ-1 deficient cells is driven by oxidative stress. (A–C) Typical examples of mitochondria described as fragmented, normal or fused, respectively. (D,G) Mitochondria in DJ-1 deficient cell are morphologically more fragmented than control cells. (E,H) Fragmentation is enhanced by oxidatively stressing the cells using 300 µM paraquat. (F,I) The glutathione precursor OTCA rescues this morphology in DJ-1 deficient cells.

expression of PINK1 in glia cannot be discounted. Similar expression has been observed for DJ-1\textsuperscript{178-180} and parkin\textsuperscript{116-120}, thus demonstrating an overlapping neuronal expression of these genes in brain. No difference was observed in the expression of PINK1 RNA in surviving substantia nigra neurons between PD patients and controls, suggesting PINK1 mRNA expression is not grossly affected in disease.
DJ-1 interacts with specific mRNA targets (Paper V-VI)

DJ-1 has been reported to interact with RNA-protein complexes. Therefore, we investigated the ability of DJ-1 to interact with RNA directly and the effect of that interaction. We isolated RNA associated with DJ-1 from M17 neuroblastoma cells by immunoprecipitation (IP) using a DJ-1 specific antibody and examined associated RNA by analysis on whole genome expression microarrays. Comparing DJ-1 IP samples to non-specific IgG IP samples (negative control), we observed that a number of specific mRNA transcripts were significantly enriched in the DJ-1 samples (Figure 5A). These transcripts included members of the oxidative phosphorylation pathway (both mitochondrial and nuclear encoded) (Table 4), selenoproteins including glutathione peroxidises (Table 5), and members of the PTEN/Akt cell survival pathway (Table 6). The enrichment of these transcripts in an independent series of samples immunoprecipitated with DJ-1 in these cells was confirmed using quantitative real time PCR (qRT-PCR). Furthermore, qRT-PCR was used to confirm the enrichment of the same transcripts in aged human brain (Paper VI). A number of these transcripts were confirmed to be expressed in mouse brain where no difference in mRNA was observed between wild type and DJ-1 knockout mice (Paper VI).

Antibody effects were excluded as a confounding factor by using the DJ-1 antibody to IP from both DJ-1 null and wild type mice. IP from wild type mice showed enrichments of similar mRNA transcripts as the cells when compared to DJ-1 null mice. Using the affinity of a 6his tag for Ni-NTA magnetic beads as an antibody-free technique, transiently transfected pathogenic mutants of DJ-1 lacked the enrichment of mRNA for selenoproteins observed with wild type protein, suggesting they lack RNA binding capacity.

A fourth method, cross-linked immunoprecipitation (CLIP), was used in order to define the interaction of DJ-1 and RNA as it allowed analysis of RNA binding regions (Figure 5B). CLIP analysis confirmed the interaction of DJ-1 with specific mRNA transcripts Akt, SelS and IGF2. We isolated and cloned the region of mRNA bound to DJ-1. This revealed that mRNA sequences bound to DJ-1 are GG/CC rich stretches.

The interaction between DJ-1 and RNA was confirmed to be direct and specific through in vitro pulldown of recombinant DJ-1 at physiological levels (20 nM) using biotin-labeled RNA. DJ-1 interacted with the sequences corresponding to the 5' UTR, but not the 3' UTR, of GPx4.

In order to define the effects of the interaction between DJ-1 and RNA, we selected two mRNA transcripts. GPx4, robustly detected in neuroblastoma microarrays and consistently confirmed in human brain and cells, is a selenoprotein localized to the mitochondria, where it acts to reduce peroxidized lipids. MAPK8IP1 was robustly detected in mouse brain microarrays and more weakly detected in neuroblastoma arrays, though
consistently confirmed in human brain. A similar gene, MAPK8IP3 was also strongly amplified in all experiments. MAPK8IP1 is a regulator of Akt promoting cell survival under glucose deprivation\textsuperscript{226,227}, and has also been implicated in neuronal outgrowth and APP processing through interactions with Fez1 and Kinesin-I\textsuperscript{228,229}.

Reporter constructs containing the 5' and 3' UTRs of GPx4 and MAPK8IP1 were used to show that interaction with DJ-1 resulted in decreased target protein expression. As this is counterintuitive when considering the pro-survival and antioxidant capabilities of DJ-1, we investigated the results of the interaction of DJ-1 and RNA in the context of oxidation. When DJ-1 was oxidized by treating cells with paraquat, RNA was no longer enriched in the IP, implying that DJ-1 released RNA when oxidized. As the consequence of DJ-1 interaction was translational repression, release of mRNA from DJ-1 would enhance translation of the corresponding protein. This suggests a mechanism by which DJ-1 binds to antioxidant and pro-survival transcripts, and upon oxidative challenge, releases them to activate translation in a rapid and localized response to oxidative insults.

Misregulation of MAPK8IP1 in aged wild type and DJ-1 knockout mice provided further evidence in support of this model. Aging also enhanced oxidation of DJ-1, correlating with increased levels of MAPK8IP1 protein only in aged wild type mice. Indirect support of this model was seen when comparing sporadic PD with neurologically normal controls (Paper VI). As has been previously shown\textsuperscript{230,231}, we observed increased oxidation of DJ-1 in cortex cerebri of PD patients compared to controls. Correlating with this was an increase of both GPx4 and MAPK8IP1 protein, but not RNA, suggesting these transcripts were regulated at the post-transcriptional level. The correlation between oxidation of DJ-1 and translational regulation of target transcripts suggests that DJ-1 may play an important role in the cellular response to oxidative stress in sporadic PD.
Figure 5. DJ-1 interacts with RNA. (A) Microarray analysis of DJ-1 IP sample compared to IgG IP samples reveals a number of transcripts specifically interacting with DJ-1 protein. Significantly different transcripts were categorized and to a large extent encoded for mitochondrial proteins (red), antioxidant proteins (blue) and PTEN/Akt cell survival pathway proteins (green). (B) Covalently linked RNA was immunoprecipitated with DJ-1, radioactively labeled and size separated revealing a pattern of radioactivity consistent with other known RNA binding proteins. High RNase digest increased the signal slightly above the molecular weight of DJ-1, while a smear of signal was observed with low or no RNase digest. Little signal was seen for non-specific IgG pulldown.

Table 4. Mitochondrial genes enriched with DJ-1. Fold represents fold increase over non-specific IgG IP. Diff score is a measure of significance from 0-371, with values of 13, 20 and 30 equivalent to p values of 0.05, 0.01 and 0.001 respectively. * confirmed with qRT-PCR, [cross symbol] enriched in mouse brain.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Accession</th>
<th>Definition</th>
<th>Fold Enrichment</th>
<th>Diff Score</th>
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<tbody>
<tr>
<td>MTND4*†</td>
<td>NM_173711.1</td>
<td>NADH dehydrogenase 4</td>
<td>120</td>
<td>356</td>
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<tr>
<td>MTND2*</td>
<td>NM_173709.1</td>
<td>NADH dehydrogenase 2</td>
<td>83</td>
<td>82.8</td>
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<tr>
<td>MTATP6</td>
<td>NM_173702.1</td>
<td>ATP synthase 6</td>
<td>54</td>
<td>371</td>
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<tr>
<td>MTND5*†</td>
<td>NM_173713.1</td>
<td>NADH dehydrogenase 5</td>
<td>46</td>
<td>50.0</td>
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<tr>
<td>MTND1*</td>
<td>NM_173708.1</td>
<td>NADH dehydrogenase 1</td>
<td>39</td>
<td>371</td>
</tr>
<tr>
<td>MTCO2*</td>
<td>NM_173705.1</td>
<td>cytochrome c oxidase II</td>
<td>7.5</td>
<td>109</td>
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<tr>
<td>MTCO1*</td>
<td>NM_173704.1</td>
<td>cytochrome c oxidase I</td>
<td>4.0</td>
<td>359</td>
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</table>
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#### Table 5. Selenoproteins enriched with DJ-1.

<table>
<thead>
<tr>
<th>Symbol</th>
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<th>Diff Score</th>
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<tr>
<td>SEPW1*</td>
<td>NM_003009.2</td>
<td>selenoprotein W, 1</td>
<td>12.1</td>
<td>371</td>
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<tr>
<td>GPX4†</td>
<td>NM_002085.1</td>
<td>glutathione peroxidase 4 (phospholipid hydroperoxidase)</td>
<td>9.2</td>
<td>371</td>
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<tr>
<td>SELH*</td>
<td>NM_170746.1</td>
<td>selenoprotein H</td>
<td>5.3</td>
<td>115</td>
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<tr>
<td>SEPX1*</td>
<td>NM_016332.2</td>
<td>selenoprotein X, 1</td>
<td>3.6</td>
<td>231</td>
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<tr>
<td>SEPHS2†</td>
<td>NM_012248.2</td>
<td>selenophosphate synthetase 2</td>
<td>2.6</td>
<td>213</td>
</tr>
<tr>
<td>SELT*</td>
<td>NM_016275.3</td>
<td>selenoprotein T</td>
<td>2.4</td>
<td>203</td>
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<tr>
<td>GPX3*</td>
<td>NM_002084.2</td>
<td>glutathione peroxidase 3</td>
<td>2.2</td>
<td>79.5</td>
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#### Table 6. PI3K pathway genes enriched with DJ-1.

<table>
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<th>Symbol</th>
<th>Accession</th>
<th>Definition</th>
<th>Fold Enrichment</th>
<th>Diff Score</th>
</tr>
</thead>
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<tr>
<td>JUND*</td>
<td>NM_005354.2</td>
<td>Jun D protooncogene</td>
<td>9.0</td>
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<tr>
<td>RPS6KB2</td>
<td>NM_003952.1</td>
<td>ribosomal protein S6 kinase</td>
<td>5.4</td>
<td>39.8</td>
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<td>PPP2R2C*</td>
<td>NM_181876.1</td>
<td>protein phosphatase 2,</td>
<td>4.0</td>
<td>41.2</td>
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<tr>
<td>BCL2L1†</td>
<td>NM_001191.2</td>
<td>BCL2-like 1, transcript variant 2</td>
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<tr>
<td>RASL10B</td>
<td>NM_033315.2</td>
<td>RAS-like, family 10, member B</td>
<td>3.5</td>
<td>128</td>
</tr>
<tr>
<td>MAPK8IP1†</td>
<td>NM_005456.2</td>
<td>mitogen-activated protein kinase 8, interacting protein 1</td>
<td>3.4</td>
<td>103</td>
</tr>
<tr>
<td>EIF4EBP1</td>
<td>NM_004095.2</td>
<td>eukaryotic translation initiation factor 4E binding protein 1</td>
<td>3.2</td>
<td>58.7</td>
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<tr>
<td>SHC1</td>
<td>NM_003029.3</td>
<td>SHC</td>
<td>3.2</td>
<td>297</td>
</tr>
<tr>
<td>AKT1</td>
<td>NM_005163.1</td>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
<td>2.6</td>
<td>164</td>
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CONCLUSIONS

- Pathogenic mutant M26I causes a loss of function due to decreased stability of the protein
- Sulfinic acid formation at cysteine-106 of DJ-1 is a critical component for both mitochondrial localization and protection by DJ-1
- DJ-1 deficiency induces subtle mitochondrial phenotypes that are driven by increases in oxidative stress and rescued by expression of either PINK1 or parkin, linking all three recessive parkinsonism proteins in a single pathway
- PINK1 mRNA is expressed mainly in neurons and is not changed in sporadic PD
- DJ-1 interacts with specific mRNA transcripts to translationally regulate antioxidant and pro-survival responses to oxidative stress
- Translational regulation of antioxidant and pro-survival responses in sporadic PD coupled with increased oxidation of DJ-1 implies DJ-1 may play a role in the response to sporadic PD pathogenesis
DISCUSSION

Our rationale for studying recessive parkinsonism is to elucidate pathways that protect dopaminergic neurons and prevent the onset of PD. Behind this rationale are three assumptions with ample supportive evidence: the pathways involved in recessive parkinsonism are common to sporadic PD, early onset of disease is indicative of more severe disturbances in these pathways, and that dopaminergic neurons are particularly susceptible to these disturbances. Based on these assumptions, it is crucial to understand the mechanism of neuroprotection of recessive parkinsonism proteins. In this thesis, we have confirmed that in two cases, loss of DJ-1 function is due to mutations that cause destabilization of the protein, though this does not describe the normal, protective function of the wild type protein. DJ-1 is known to form a sulfinic acid at cysteine-106 under oxidative conditions. We have observed two clear consequences of this modification (Figure 6). First, DJ-1 relocates to mitochondria. Mutants unable to form the sulfinic acid never localized to mitochondria while mutants more prone to sulfinic acid formation were always mitochondrial. Second, DJ-1 released RNA. DJ-1 associated with RNA under basal conditions, but not under oxidative conditions.

Figure 6. Observed responses of DJ-1 to oxidation. Sulfinic acid formation (center) has been well documented. This sulfinic acid formation drives both release from RNA (left) and mitochondrial localization (right).
Based on these observations, we propose that DJ-1 functions as a redox sensitive translational regulator of antioxidant and pro-survival mRNA transcripts that has particular importance to mitochondria. The scope of transcripts that DJ-1 appears to regulate suggests it may be an important regulator of oxidative stress responses. Regulating these responses at the RNA level allows both a speed and precision that would be impossible through transcriptional regulation. These responses would be particularly critical in a rapidly changing and highly compartmentalized environment with large energy requirements, like a neuron synthesizing and releasing dopamine in bursts.

The translational regulation of glutathione peroxidases and PTEN/Akt pathway components by DJ-1 is consistent with many of the observed cellular effects of DJ-1, including enhancements of glutathione synthesis and prevention of apoptosis. Rapid enhancement of protein expression of antioxidants and promoters of cell survival would be a logical component of an effective response to increased oxidation. Lacking these DJ-1 dependent responses would likely not affect normal cellular function and merely cause decreases in the threshold of oxidative injury the cell could withstand.

As the fates of mitochondrial transcripts interacting with DJ-1 have not been further investigated, it is less clear how DJ-1 regulates transcripts associated with mitochondrial oxidative phosphorylation. It is also less clear that increased production of oxidative phosphorylation components would be beneficial to the cell under oxidative stress. Decreases in oxidative phosphorylation have been reported in response to $\text{H}_2\text{O}_2$, $\text{HO}^*$ and $\text{O}_2^-$, While enhancing target protein translation would replace damaged oxidative phosphorylation subunits, attempting to increase oxidative phosphorylation would also enhance ROS production. Sulfinic acid formation triggers both the release of RNA and localization to mitochondria. The extent to which the oxidation prone mutants E18N and E18Q localize to mitochondria suggests an active mechanism of localization and a further activity of DJ-1 at the mitochondria. The mechanism of this relocalization has not been defined, as DJ-1 contains no known mitochondrial targeting signals. A mitochondrial activity of DJ-1 is supported by the observation that DJ-1 with an artificial mitochondrial targeting sequence shows enhanced protection. Therefore, it may be that binding of mitochondrial RNA is enhanced under oxidative stress and result in the negative regulation of mitochondrial RNA transcripts though this clearly requires further investigation. Two types of assays would help address this question: investigating the families of transcripts bound by DJ-1 and oxidation mutants under varying oxidation conditions, and examining the activity of the oxidative phosphorylation pathway when the cell is oxidatively challenged.

Even with the uncertainty of how DJ-1 regulates mitochondrial transcripts, we demonstrate that it functionally protects mitochondria from fragmentation. Mitochondria are the major producer of ROS in the cell but also mediators of cell survival via apop-
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totic mechanisms, and both are known to induce mitochondrial fragmentation. Our data suggest the observed phenotype is the result of increased oxidative stress and not modulations of PTEN/Akt signaling, implying translational regulation of antioxidant transcripts are more directly relevant to mitochondrial phenotypes than anti-apoptotic transcripts. Since dopamine metabolism is an additional source of ROS, dopaminergic neurons would presumably be more susceptible due to a higher oxidative stress burden.

We furthermore have provided evidence demonstrated that all three recessive parkinsonism genes, parkin, PINK1 and DJ-1, provide essential protection of mitochondrial networks. It remains unclear whether they act sequentially or in parallel pathways. As shown in this thesis and by others, all three genes are expressed in most neurons in brain, and our results suggest they likely act together in concert to directly mediate mitochondrial interconnectivity.

Putative mechanisms linking these genes are indirect as neither PINK1 nor parkin mRNA is a direct target of DJ-1. One mechanism involves DJ-1 regulation of the PTEN/Akt pathway and PTEN-driven induction of PINK1. Oxidative stress has already been shown to induce expression of parkin mRNA and protein, and it is possible this may also be related to DJ-1 responses. The induction of oxidative stress may drive DJ-1-mediated activation of PTEN which would subsequently upregulate PINK1 expression. This could be tested by measuring induction of PINK1 and parkin expression in response to oxidation in DJ-1 deficient cells compared to controls.

Alternatively, DJ-1 and PINK1/parkin may act in independent but parallel pathways that both affect mitochondrial morphology since experiments examining additive effects of DJ-1 and PINK1/parkin deficits have not been performed. Regardless, the cellular activities of these genes strongly implicate mitochondrial dysfunction and oxidative stress as major pathways in the pathogenesis of PD.

The mitochondrial dysfunction and oxidative stress theories of PD pathogenesis have yielded few therapeutic results thus far. Specific modulation of the activities of recessive parkinsonism proteins, however, offers new opportunities for intervention. While therapeutically targeting DJ-1 would likely be difficult as it is already a highly abundant and active protein, knowledge of the pathways activated by DJ-1 may prove to be more useful targets of therapeutics. In order to do so, the relationship between DJ-1 and RNA must be further refined, including an improved understanding of the RNA recognition sequence and the activity of DJ-1 on mitochondrial genes. In addition, the pathways relating DJ-1 to PINK1 and parkin must be defined, as they offer insight into protection of neurons. Understanding the links between these genes would unify the pathogenesis of recessive parkinsonism into a single mechanism that would make an excellent therapeutic target to prevent the pathogenesis of sporadic PD.
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