Role of DJ-1 in the Activation of AKT Via Binding and Inhibition of PHLDA3 Under Oxidative Stress

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the selective loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). PD affects ~1% of the population over 65, as demonstrated by characteristic symptoms such as tremor, rigidity, and bradykinesia. While the majority of PD cases are idiopathic, some cases are familial, including those caused by homozygous loss-of-function mutations in DJ-1 (PARK7), which lead to early onset PD. Although the physiological role of DJ-1 is not fully understood, DJ-1's neuroprotective role against oxidative stress is well documented. DJ-1 is required for AKT-mediated neuroprotective effects, however the mechanism by which DJ-1 affects membrane localization/activation of AKT is unknown and is likely a critical aspect of DJ-1 function. In this thesis we explore the mechanism through which DJ-1 confers neuroprotection through AKT membrane recruitment, particularly in the case of oxidative stress insult. We demonstrate here that DJ-1 interacts with PHLDA3, a negative regulator of AKT, and loss of DJ-1 leads to hypersensitivity of neurons to PHLDA3-mediated death. Additionally, we demonstrate that in the absence of DJ-1, PHLDA3 localization at the membrane is increased, and overexpression of PHLDA3 causes reduced AKT phosphorylation in DJ-1 KO MEFs in response to oxidative stress. Taken together, these studies provide a potential novel mechanism by which DJ-1 regulates the activity of AKT, a critical neuronal survival pathway. Elucidation of these mechanisms may provide insight into the design of neuroprotective therapies for PD.

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6-OHDA	6-HydroxyDopamine
α-syn	α-synuclein
ANOVA	Analysis of Variance
AR	Androgen Receptor
bbFGF	bovine basic Fibroblast Growth Factor
CS	Citrate Synthase
DA	Dopaminergic
DBS	Deep Brain Stimulation
DIV	Days In Vitro
DMC	Dorsomedial Clusters
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal Growth Factor
GFP	Green Fluorescent Protein
GI	Gastrointestinal
GSH	Glutathione
GSK-3β	Glycogen Synthase Kinase 3 Beta
GST	Glutathione S-transferase
H_2O_2	Hydrogen Peroxide
HSP	Heat Shock Protein
KD	Knockdown
KEAP1	Kelch-like ECH-Associated Protein 1
KO	Knock-out (also -/-)
L-dopa	Levodopa
LB	Lewy Body
LC	Locus Coereulus
LCNEC	Large Cell NeuroEndocrine Carcinoma
LOH	Loss of Heterozygosity
LPOA	Lipid-Protein Overlay Assay
MAO-B	Monoamine Oxidase B
Mdm2	Murine Double Minute 2
MEF	Murine Embryonic Fibroblasts
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTORC2	mammalian Target Of Rapamycin Complex 2 (also PDK2)
Myr	Myristolated
NQO1	NAD(P)H dehydrogenase, Quinone 1
NRF2	NFE2L2, Nuclear Factor (Erythroid-derived 2)-Like 2
pAKT	phosphorylated AKT
panNET	pancreatic NeuroEndocrine Tumor
PD	Parkinson's Disease

PDGF	Platelet-Derived Growth Factor
PDK1	3-Phosphoinositide Dependent protein Kinase-1
PDK2	3-Phosphoinositide Dependent protein Kinase-2
PFA	Paraformaldehyde
PH	Pleckstrin Homology
PHLDA3	Pleckstrin Homolog Domain family A3
pI	Isoelectric Point
PI3K	PhosphatIdylinositol 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B (also AKT)
PKC	Protein Kinase C
PON2	Paraoxonase-2
PTEN	Phosphatase and TENsin homolog
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse transcription polymerase chain reaction
SH2	Src Homology 2
SNc	Substantia nigra pars compacta
STN	Subthalamic Nucleus
TH	Tyrosine Hydroxylase
UPDRS	Unified Parkinson Disease Rating Scale
UPS	Ubiquitin Proteasome System
UV	UltraViolet
VHL	Von Hippel Lindau
VTA	Ventral Tegmental Area
WT	Wild-type (also +/+)

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INTRODUCTION

1. PARKINSON'S DISEASE

1.1. History

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, primarily characterized by the loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNc). First to medically describe the disease was James Parkinson, who in 1817 wrote his monograph entitled 'An Essay on the Shaking Palsy' based on 6 patients he observed (Parkinson, 1817). Charcot further refined the disease differentiating it from other tremulous diseases, and identified what are known as the cardinal features of PD to this day: tremor, rigidity, bradykinesia (slowness of movement), and postural instability, terming it "Parkinson's Disease" (Goetz, 1986).

1.2. Clinical Features

Affecting ~1% of the population over 65 years of age (Lang and Lozano, 1998), PD displays a mixed clinical phenotype due to its complex multifactorial nature. As a result, official PD diagnoses can only be confirmed post-mortem through autopsy.

Despite variations in presentation, the previously mentioned tremor, rigidity, bradykinesia and postural instability, comprise the four principal motor components of PD. Due to gradual onset, it is common for the earliest symptoms to go unnoticed or misinterpreted in the early stages of the disease. Non-motor symptoms such as anosmia (loss of smell) may only be noticed once formally tested, and disturbed sleep patterns may only be brought to light if the patient's partner is questioned (Doty et al., 1988; Haehner et al., 2011; Iranzo et al., 2005). Commonly misinterpreted, symptoms such as slowness of movement may be attributed to the aging process. As such, a lag of 2-3 years between the first

symptoms and diagnosis is not uncommon. Once diagnosed, patients and families commonly start recalling relevant signs and symptoms dating back many years.

In the late stages of the disease, a patient's face becomes masked and expressionless, speech becomes monotonous and slightly slurred, and all dexterous movement is carried out slowly and awkwardly. Patients develop simian posture and experience gait disturbances resulting in a shuffle-like walk (UPDRS; Hoehn and Yahr, 1967; Goetz et al., 2004). Depression is a common feature and the risk of dementia also exists with the greatest risk factor being age (Blonder and Slevin, 2011; Collier et al., 2011). Parkinson's disease is not a fatal condition, however advanced symptoms lead to incidents that result in death, with pneumonia being the most common (Iwasaki et al., 1990).

1.3. Pathology

As a disorder of the nigrostriatal pathway, pathologic examination of PD patient brains exhibit selective loss of DA neurons in the SNc, as well as the presence of proteinaceous cytoplasmic inclusions, termed Lewy Bodies (LBs). Referred to as the hallmark of PD, degeneration in the SNc and therefore decreased dopaminergic striatal innervation, results in decreased motor cortex activity manifesting as the previously mentioned motor symptoms. It is important to note that while PD is characterized by loss within the nigrostriatal pathway, cell loss can also be seen in the locus coeruleus (LC) and ventral tegmental area (VTA), possibly accounting for PD symptoms such as sleep disturbance (Uhl et al., 1985; Zweig et al., 1993; McCarter et al., 2012).

The finding of α-synuclein (α-syn) deposits throughout the brain and brainstem, in various degrees of severity that coincide with PD progression, have led to the Braak

hypothesis which suggests pathology occurs much earlier and only when this pathology reaches a certain degree do clinical symptoms manifest. Additionally, it is believed that the olfactory bulb and gastrointestinal (GI) tract may provide entry points to pathogens, which would account for reported anosmia and GI disturbances seen decades prior to the clinical manifestation of PD (Braak et al., 2003a; Braak et al., 2003b; Braak et al., 2006).

1.3.1 Oxidative Stress in the Pathogenesis of PD

While production of reactive oxygen species (ROS), most namely via the electron transport chain within the mitochondria, is a natural byproduct and is even thought to participate in cell signaling, the production of ROS beyond the antioxidant capacity of the cell results in irreversible damage and cell death (Simon et al., 2000). Mitochondrial dysfunction is likely to increase ROS, rendering cells more vulnerable to additional stress, which in turn can further damage mitochondria. This cycle of mitochondrial dysfunction and increased ROS is suspected to play a role in the pathogenesis of PD (reviewed by Henchliffe and Beal, 2008; Hauser and Hastings, 2013).

Autopsy of PD brains have revealed defects in mitochondrial complex 1 leading to decreased activity (Schapira et al., 1990), along with the presence of mitochondrial genome mutations (Tanaka et al., 1996). Additional post-mortem studies demonstrated increased markers of oxidative stress (oxidative damage to lipids, proteins and DNA) within the SNc of PD patients (Dexter et al., 1989). Also, decreased levels of GSH (anti-oxidant pathway component) have also been consistently observed within the SNc of PD patients (Perry et al., 1986). Furthermore, pesticides and dopaminergic neurotoxins such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which exert their toxicity via inhibition

of complex 1, replicate features of PD in humans and animal models (Langston et al., 1983; Betarbet et al., 2000; Cicchetti et al., 2005). Lastly, some genes linked to PD have functions in the regulation of ROS and/or mitochondrial health, strengthening the notion for a role of mitochondrial function and ROS in PD (Lin and Beal, 2006).

1.4. Treatment

PD is currently an incurable disease with no treatments available to halt or even slow the progression of the disease. Symptomatic therapies do exist, generally targeted to increasing overall dopamine levels, to compensate for the loss of DA-producing cells. To date Levodopa (L-dopa) combined with peripheral decarboxylase inhibitors (benserazide, carbidopa) provide the most effective therapy looked upon as the 'gold standard', aiming to increase levels of dopamine precursor (Rinne et al., 1972). MAO-B inhibitors (selegiline, rasagiline) inhibit MAO-B, an enzyme involved in the breakdown of dopamine, and allow for decreased metabolic degradation of dopamine and an increased persistence of dopamine in the brain (Chrisp et al., 1991). Dopamine agonists take a different approach to PD treatment by bypassing DA synthesis completely, and directly stimulating a subset of postsynaptic DA receptors (Gopinathan et al., 1981). Unlike L-dopa, which is converted to dopamine, agonists are able to behave like dopamine without the need for dopamine itself. While these treatments do not cure the disease they do significantly improve the patients quality of life and functional capacity.

In times when the disease presents itself with motor complications that are as so severe and/or drug intolerances that lead to an unacceptable quality of life, surgical procedures such as deep brain stimulation (DBS) can be performed. Briefly, a

neurostimulator is surgically implanted (most commonly in the STN) providing electrical stimuli to specific regions of the brain, to correct the disrupted nigrostriatal pathway circuit.

1.5. Genetic Causes (PD genes)

PD is primarily an idiopathic disorder (unknown origin) with less than 10% of all cases having familial inheritance. Investigations into familial PD genes and studies of their functions has provided insight into the pathogenesis of PD. Familial PD genes can be broken down into 2 categories: autosomal dominant - including *α-synuclein* and *LRRK2*; and autosomal recessive – including *Parkin*, *PINK1*, and *DJ-1*. DJ-1 is the focus of this thesis and will be further discussed.

2. DJ-1

2.1. Discovery

DJ-1/PARK7 was first discovered as a novel oncogene transforming NIH3T3 cells in cooperation with ras (Nagakubo et al., 1997). Following this studies emerged implicating DJ-1 in androgen receptor (AR) regulation (Wagenfield et al., 1998; Takahashi et al., 2001; Niki et al., 2003) as well as RNA-binding function (Hod et al., 1999). In 2003, Bonifati *et al* identified DJ-1 as an autosomal recessive PD gene, and two mutations associated with early-onset PD were identified: 1) Dutch family who revealed a 14kb genomic deletion within the DJ-1 gene, and 2) Italian family displaying a missense mutation in the DJ-1 gene (L166P) (Bonifati et al., 2003).

2.2. Structure

DJ-1, the product of DJ-1/PARK7 gene located on chromosome 1 (1p36.2-36.3), is an 189 amino acid protein comprised of 7 β-strands and 9 α-helices (Taira et al., 2001; Wilson et al., 2003). The DJ-1 amino acid sequence is highly conserved amongst prokaryotes and eukaryotes, and due to its homology with heat shock protein chaperones as well as ThiJ/PfpI proteases, is a member of the DJ-1/ThiJ/PfpI superfamily (commonly referred to as DJ-1 superfamily) (Lee et al., 2003). Existing as a dimer, DJ-1 adopts a helix-strand-helix sandwich structure which exhibits structural resemblance to bacterial protease PH1704 and chaperone protein Hsp31 when comparing crystal structures (Lee et al., 2003; Tao and Tong 2003). DJ-1 contains three cysteine residues at amino acids 46, 53, and 106 (C46, C53, and C106 respectively). These cysteine residues undergo oxidation in response to oxidative stress. Of the three cysteine residues, C106 is highly susceptible to oxidative stress, and oxidation of C106 is necessary for DJ-1 to exert its full functions.

Pathogenic mutations have been shown to result in loss of DJ-1 function. Deletion of exons 1-5, as described in the Dutch family, results in complete lack of DJ-1 expression, whereas pathogenic point mutation L166P disrupts DJ-1 dimer formation by preventing the normal folding of DJ-1, resulting in its destabilization and subsequent degradation via the UPS system (Bonifati et al., 2003; Miller et al., 2003; Olzmann et al., 2004).

2.3. Expression

Expressed ubiquitously and abundantly in almost all tissues, DJ-1 expression can be detected in both neuronal and non-neuronal cells, with expression highest in the testes, kidney, and brain (Nagakubo et al., 1997; Bandopadhyay et al., 2004). Brain regions such as

the basal ganglia, substantia nigra, and motor cortex- all of which are involved in motor function- exhibit DJ-1 expression (Bader et al., 2005; Bandopadhyay et al., 2004; Shang et al., 2004). Within the brain, both neurons and glia, namely astrocytes, have reported DJ-1 expression (Bonifati et al., 2003; Rizzu et al., 2004).

DJ-1 is not a major constituent of PD hallmark LB, however patients of sporadic PD exhibit overexpression of DJ-1 in reactive astrocytes, in addition to a dramatic increase of insoluble DJ-1 and excessively oxidized DJ-1 in PD brain tissue (Bandopadhyay et al., 2004; Choi et al., 2006; Yanagida et al., 2009).

2.4. DJ-1 Functions

DJ-1 is a multifunctional protein implicated in numerous biological processes including fertilization, cancer, apoptosis and mitochondrial regulation (Niki et al., 2003; Yoshida et al., 2003; Hao et al., 2010; Nagakubo et al., 1997; Junn et al., 2005). To be discussed are the roles of DJ-1 in: chaperone/protease activity, oxidative stress response, transcriptional regulation, and finally DJ-1 mediated signaling pathways.

2.4.1. DJ-1 Chaperone and Protease Activity

In order to control protein quality, cells exposed to stressful environmental conditions respond by overexpressing a highly conserved set of proteins called Heat Shock Proteins (HSPs). HSPs may function as molecular chaperones or as proteases (Parsell and Lindquist, 1993). DJ-1 exhibits both chaperone and protease activity.

Chaperone Activity

Human DJ-1 and Hsp31 both belong to the ThiJ/PfpI family of proteins, in which family members share a conserved ThiJ domain, and are associated with several functions including chaperone activity. Hsp31, an *E. coli* ThiJ domain protein which functions as a molecular chaperone and whose expression is induced by heat shock, was found to have structural, and thus possible a similar functional, resemblance to DJ-1 (Lee et al., 2003). DJ-1, but not its L166P mutant, was reported to suppress the heat-induced aggregation of both citrate synthase (CS) and glutathione S-transferase (GST), two well characterized chaperone assays (Lee et al., 2003; Shendelman et al., 2004). Furthermore, DJ-1 was found to inhibit the formation of α–syn aggregates (Shendelman et al., 2004). The aggregation of α–syn has been implicated in familial and sporadic forms of PD with evidence suggesting that these mutations generate toxic abnormal oligomeric protein aggregates, as well as α–syn fibrils being the main constituent of LBs (Spillantini et al., 1998; Trojanowski and Lee, 2003). Interestingly, mutation of cysteine 53 to an alanine completely abrogated DJ-1 chaperone activity, suggesting that DJ-1 chaperone function requires C53 (Shendelman et al., 2004). While Shendelman *et al* reported C106 was not required for DJ-1 chaperone activity, Zhou *et al* later reported that the oxidation state of C106 regulates DJ-1's ability to act as a chaperone. Only oxidized DJ-1, in which C106 has been oxidized to sulfinic acid (SO_2H), was able to prevent fibrillation of α–syn inhibiting its aggregation, while both unoxidized (native) DJ-1 and more highly oxidized forms (SO_3H) of DJ-1 displayed attenuated chaperone activity towards α–syn (Zhou et al., 2006).

Protease Activity

DJ-1 not only is structurally similar to chaperone Hsp31, which possesses protease activity, but is highly homologous to bacterial protease PH1704 as well (Lee et al., 2003). Due to its lack of a catalytic triad (Cys-His-Glu/Asp) and the presence of a C-terminal α-helix (H8), which according to crystal structure, appears to block substrate access to putative catalytic site C106, DJ-1 protease function had been long argued (Tao and Tong, 2003). Olzmann *et al* reported that although low, DJ-1 possesses intrinsic proteolytic activity, and this activity was completely abolished by DJ-1 mutant C106A. Familial DJ-1 mutant L166P also abolished DJ-1 proteolytic function (Olzmann et al., 2004).

2.4.2. Anti-Oxidative Function

2.4.2i. Acidic Shift

DJ-1 was first implicated in the oxidative stress reaction when Mitsumoto & Nakagawa demonstrated DJ-1's ability to act as a hydroperoxide-responsive protein with a pI shift from 6.2 to 5.8 under oxidative stress (Mitsumoto and Nakagawa, 2001). Also, evidence *in vivo* showed an accumulation of DJ-1 acidic forms in PD brains (Bandopadyay et al., 2004; Choi et al., 2006). Further studies have since demonstrated that oxidative stress induces a modification of DJ-1 at C106, leading to the formation of cysteine-sulfinic acid and translocation of DJ-1 from the cytosol to the mitochondria (Canet-Aviles et al., 2004). The formation of cysteine-sulfinic acid is a well-known reversible post-translational modification of proteins, and was confirmed in oxidized DJ-1 using mass spectrometry. In terms of oxidation, DJ-1 with C106A mutation does not undergo a shift in isoelectric point following oxidative stress, in M17 human neuroblastoma cells, unlike WT and C53A

expressing cells. The mutant C106A, but not C53A, has not only been shown to prevent DJ-1 oxidation, but the mitochondrial translocation of DJ-1 as well, following paraquat treatment. Additionally, cells expressing the C106A mutant exhibit increased sensitivity to MPP^+ compared to those of control cells (Canet-Aviles et al., 2004).

2.4.2ii. ROS Scavenging

DJ-1 also has a role as an antioxidant protein, both scavenging and quenching ROS through self-oxidation as a mechanism to overcome oxidative stress. *In vitro*, when reacted with H_2O_2, human recombinant DJ-1 significantly decreased H_2O_2 levels compared to control, conferring ROS scavenging activity (Taira et al., 2004; Andres-Mateos et al., 2007). Overexpression of DJ-1 in both *Drosophila* and SH-SY5Y cells displayed reduced concentration of H_2O_2 compared to control flies and cells, respectively (Taira et al., 2004; Yang et al., 2005). DJ-1 mutant C53A displayed only 20% of the activity of WT DJ-1, resulting in significantly less quenching activity (Taira et al., 2004). Conversely, knockdown (KD) of DJ-1 rendered flies with higher levels of ROS staining compared to control flies (Yang et al., 2005), and many studies have also noted higher levels of ROS in isolated mitochondria from DJ-1 KO mice compared to WT control (Andres-Mateos et al., 2007; Irrcher et al., 2010; Billa et al., 2013). Interestingly, one study suggests that DJ-1 confers this ROS scavenging activity by acting as an atypical peroxiredoxin-like peroxidase, scavenging H_2O_2 similar to peroxiredoxins, with data suggesting that likely C106 is the target for oxidation (Andres-Mateos et al., 2007).

2.4.2iii. Cytoprotection

Many studies indicate a protective role for DJ-1 in both neuronal and non-neuronal models of oxidative stress-induced cell death. Early studies have shown KD of DJ-1 by siRNA enhanced susceptibility of cell lines such as SH-SY5Y, NIH3T3, and Neuro2a to H_2O_2, as well as neurotoxins 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA) but not to the general kinase inhibitor staurosporine (Yokota et al., 2003; Taira et al., 2004). Overexpression of WT DJ-1 provided protection against H_2O_2-induced cell death, compared to control cells. DJ-1 mutants such as L166P abrogated this protection (Yokota et al., 2003).

Inactivation of *Drosophila* DJ-1A leads to photoreceptor loss and rough eye phenotype when driven in the eye, age-dependent reduction in tyrosine hydroxylase (TH) positive neurons in dorsomedial clusters (DMC) when driven in dopaminergic neurons, as well as an age-dependent reduction of DA levels in DJ-1A KD brains when compared to controls (Kim et al., 2005a; Yang et al., 2005). When challenged with H_2O_2, DJ-1A KD *Drosophila* display hypersensitivity to oxidative stress resulting in a higher rate of mortality (Yang et al., 2005; Lavara-Culebras et al., 2007).

These protective effects of DJ-1 can also be seen in primary neurons as well as *in vivo*. DJ-1 KO cortical neurons display increased sensitivity to oxidative stress, and these effects can be dramatically rescued through overexpression of WT DJ-1, however not DJ-1 mutant L166P (Kim et al., 2005b). Similar to aforementioned cell lines, DJ-1 null primary neuronal cells are not hypersensitive to non-oxidative insults, such as camptothecin and staurosporine. *In vivo*, DJ-1 null mice display higher sensitivity to MPTP, with a higher degree of loss of TH+ neurons in the SNc as well as more striatal denervation versus control

mice (Kim et al., 2005b). Much of this hypersensitivity can be rescued by overexpression of DJ-1 by adenoviral expression, strengthening the importance of DJ-1 protective function.

2.4.3. Transcriptional Regulation

DJ-1 has been shown to respond to oxidative stress through the regulation of many transcription factors acting as a co-activator or co-repressor.

2.4.3i. NRF2

A well-documented example of DJ-1 exerting transcriptional regulation is via its interaction with Keap1, an inhibitor of Nrf2 (Itoh et al., 1999). Nrf2 is a master transcription factor that regulates the expression of many antioxidant pathway genes in response to oxidative stress (Itoh et al., 1997; Venugopal and Jaiswal, 1996; Sykotis and Bohman, 2010). It has been shown that under basal conditions Nrf2 is maintained in the cytosol where it remains bound to Keap1, resulting in its degradation (Itoh et al., 1999). However under oxidative stress DJ-1 sequesters Keap, allowing for Nrf2 translocation to the nucleus where it is able to activate antioxidant genes such as NQO1 (Clements et al., 2006).

2.4.3ii. p53

p53 is a tumor suppressor protein that exerts its function primarily through transcriptional regulation of an array of pro-apoptotic target genes involved in senescence, cell cycle arrest, and apoptosis. Upon cell stress p53 is phosphorylated at multiple sites, activating and stabilizing the protein. Interestingly, postmortem PD brains display increased levels of p53 as well as phosphorylated p53 within the substantia nigra (Nair et al., 2006; de la Monte at al., 1998). Numerous studies indicate the ability of PD gene DJ-1 to bind directly

to p53 both *in vitro* and *in vivo*, and have reported DJ-1 to regulate p53 activity in multiple pathways:

Shinbo *et al*., provided one of the first studies to describe a relation between DJ-1 and transcriptional activator p53. Reporting that DJ-1 positively regulates p53 through its interaction with Topors/p53BP3 (Shinbo et al., 2005), these findings have since been contrasted by multiple studies.

DJ-1 has since been shown to inhibit Bax expression through inhibition of p53 transcriptional activity, repressing apoptosis (Bretaud et al., 2007; Fan et al., 2008a). Overexpression of DJ-1 results in decreased Bax expression as well as inhibited caspase activation in A549 cells (which contain p53), but not in p53-null H1299 cells. Conversely, KD of DJ-1 leads to increased Bax expression accompanied by enhanced cell death in response to UV stress, in A549 cells but not H1299. DJ-1 displayed the ability to inhibit p53 transactivation on the Bax promoter, thereby downregulating Bax expression in a p53-dependent manner (Fan et al., 2008a). Furthermore, it has been reported that a post-translational modification, sumoylation, of DJ-1 on K130 is necessary for DJ-1 to repress p53 transcriptional activity. A mutant DJ-1 that cannot be sumoylated (K130R) fails to localize to the nucleus and prevents the repression of p53 transcriptional, thus losing its anti-apoptotic activity (Shinbo et al., 2006; Fan et al., 2008b).

A recent study has shown that DJ-1 binds to the DNA-binding domain of p53 (p53-DBD), and that this interaction is dependent on the oxidative status of DJ-1 (Kato et al., 2013). C106 of DJ-1 is required for DJ-1 binding to p53 under oxidative stress conditions, and mutations at this site (C106S) failed to replicate this effect. In addition, oxidative stress enhanced DJ-1 binding to p53, and this interaction is necessary for DJ-1 to inhibit p53

transcriptional activity by sequestering p53 from promoters, preventing p53 promoter recognition (Kato et al., 2013).

2.4.4. DJ-1 Mediated Signaling Pathways

DJ-1 mediates many signaling pathways such as the ASK1, ERK, and AKT signaling pathways in order to exert its neuroprotective effects. Of importance to this dissertation is the relationship between DJ-1 and the AKT signaling pathway, and will be discussed next.

3. AKT

AKT is a serine/threonine kinase that promotes cell survival and growth through the phosphorylation of downstream substrates (Vivanco and Sawyers, 2002; Sen et al., 2003). AKT was first discovered as an oncogene within the mouse leukemia retrovirus AKT8 and shortly thereafter published as a novel kinase homolog of PKA and PKC, with the name PKB (Staal et al., 1977; Bellacosa et al., 1991; Jones et al., 1991).

3.1. Structure

AKT is present in three isoforms, all of which are structurally similar containing: an N-terminal pleckstrin homology (PH) domain, central kinase domain, and a regulatory domain at the C-terminal (Figure 1). While structurally similar, all three differ in their phosphorylation sites, with AKT1 being phosphorylated within the kinase domain at T308 and regulatory domain at S473, whereas AKT2 at T309 and S474, respectively. AKT3 (PKBγ) resembles AKT1 and AKT2 however it lacks 23 amino acids in its C-terminal regulatory domain and is phosphorylated at sites at T305 and S472, within its kinase domain

(Altomare et al., 1995; Nakatani et al., 1999; Brodbeck et al., 1999). All 3 isoforms localize primarily to the cytosol (90%), but differ in expression level. AKT1 is widely expressed with the highest levels in the brain, thymus, heart, and lung. AKT2 follows a similar expression pattern whereas AKT3 is more restricted with higher levels in the brain and testes, and lower levels in the heart, spleen, lung and skeletal muscle (Nakatani et al., 1999; Altomare et al., 1995; Brodbeck et al., 1999; Owada et al., 1997).

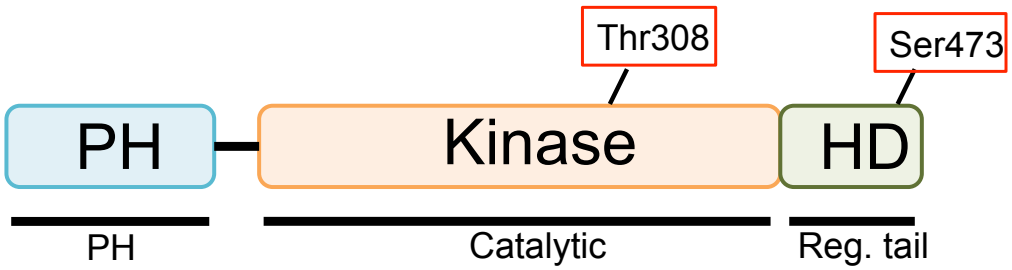

Thr308
Ser473
PH
Kinase
HD
PH
Catalytic
Reg. tail

Figure 1: Structural domains of AKT1. N-terminal PH domain, central kinase domain, and a regulatory domain at the C-terminal.

3.2. AKT Activation

AKT1 (hereby referred to as AKT) activation is a multi-step processes tightly regulated by a number of well-characterized proteins. To begin, membrane-spanning cell surface receptors receptor tyrosine kinases (RTKs) are activated by binding of ligands (such as growth factors PDGF, EGF, bbFGF, and insulin) to the extracellular domain of the RTK, inducing dimerization of the receptor and autophosphorylation of its cytoplasmic tyrosine residues. Some of these residues function as docking sites for the SH2 domain within the regulatory subunit of PI3K. Once activated, Class 1a PI3Ks act to phosphorylate membrane phospholipid PIP2 at the 3' position of its inositol ring generating the secondary messenger PIP3, an effector of many downstream targets of the PI3K pathway. Generation of PIP3 allows for PH-domain containing proteins such as PDK1 and AKT to bind to the membrane. PH domains regulate protein interactions with phosphoinositides, making them important for signal transduction. AKT is maintained in its inactive state in the cytosol by interaction of its PH and kinase domains, however once PIP3 is generated, AKT translocates from the cytosol to the membrane. AKT directly binds to PIP3 via its N-terminal PH-domain, causing a conformational change in which phosphorylation sites become exposed. PDK1 also binds to PIP3, via its C-terminal PH domain, creating a microdomain in which PDK1 is able to phosphorylate AKT at Thr308, producing partial activation of AKT. Full activation of AKT is achieved by PDK2 (also referred to as mTORC2) which is also activated through RTK signaling and further phosphorylates AKT at S473 within the C-terminal regulatory domain, achieving full activation (Figure 2). Once activated, AKT becomes independent of lipids and dissociates from PIP3 binding, translocating to the nucleus in order to transduce downstream survival signals through phosphorylation of its substrates. Mutation of AKT PH domain, as

well as point mutations within phosphorylation sites S473 and Thr308 (T308A, S473A, respectively) have been shown to significantly inhibit AKT activation, demonstrating the importance of PIP3 binding and phosphorylation for full activation (Kandel and Hay, 1999; Yang et al., 2004; Schlessigner, 2000).

Recapitulated, AKT activation is a PI3K-dependent process that is regulated by a two-part mechanism that requires translocation of AKT to the membrane, followed by phosphorylation of residues Thr308 and S473. Absence of either of these processes renders AKT inactive, preventing AKT-mediated survival signals.

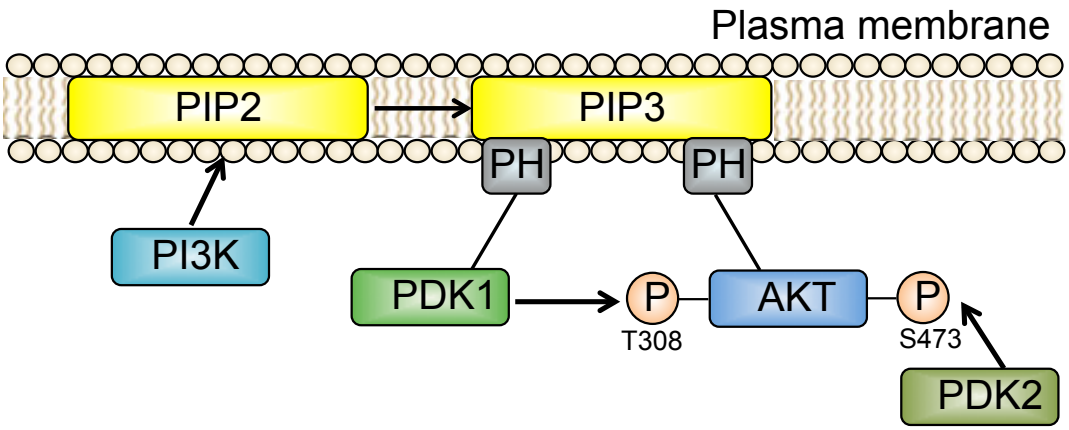

Plasma membrane
PIP2
PIP3
PH
PH
PI3K
PDK1
P
T308
AKT
P
S473
PDK2

Figure 2: AKT Activation. AKT activation is a PI3K-dependent process that is regulated by a dual mechanism that requires translocation of AKT to the membrane, followed by biphosphorylation of residues Thr308 and Ser473.

3.3. AKT/p53

AKT and tumor suppressor p53 are key players in the survival pathway by regulating the anti-apoptotic and pro-apoptotic signals transduced within a cell, respectively. As such, it is no surprise that there is considerable crosstalk between the two in order to regulate cell death.

3.3.1. PTEN

Many studies support the notion that p53 negatively regulates AKT activity through induction of p53 target genes, such as *PTEN* (phosphatase and tensin homolog) (Stambolic et al., 2001). PTEN is a 3-phosphoinositide-specific lipid phosphatase that antagonizes the PI3K/AKT pathway by dephosphorylating PIP3, which is essential for AKT activation (Maehama and Dixon, 1998). Directly upstream of the PTEN gene is a p53 binding element, and following p53 induction a subsequent increase in both mRNA and protein levels of PTEN is observed further confirming its role as a p53 target gene. Additionally, PTEN is required for p53-mediated apoptosis in immortalized MEFs (Stambolic et al., 2001). Conversely, PTEN-/- fibroblasts have been shown to display hyperphosphorylation of AKT along with noted resistance to various apoptotic stimuli (Kim et al., 2005a).

3.3.2. p53/Mdm2 Auto-Regulatory Feedback Loop

Not only has crosstalk between AKT and p53 been noted, but there is also the presence of auto-regulatory feedback loops. Once activated, AKT kinase transduces anti-apoptotic signals through phosphorylation of downstream substrates, such as Mdm2 (murine double minute 2). Mdm2 is an E3 ubiquitin ligase that triggers p53 degradation. AKT has

been shown to phosphorylate Mdm2 on S166 and S186, which enables translocation of Mdm2 from the cytoplasm into the nucleus (Mayo and Donner, 2001; Gottlieb 2002). In the nucleus, Mdm2 forms a complex with p53, binding to its N-terminal transactivation domain and inhibiting p53 expression (Momand et al 1992; Oliner et al., 1993). Once formed, the Mdm2/p53 complex translocates back to the cytoplasm where the C-terminal RING finger motif of Mdm2 ubiquitinylates p53 on several lysine residues, targeting it for subsequent degradation.

Interestingly, Mdm2 is a p53 target gene (Barak, 1993). As such, p53 positively regulates Mdm2 inducing gene expression, while Mdm2 negatively regulates p53 by inactivating and degrading it, creating a negative feedback loop tightly regulating cell survival and death (Wu et al., 1993).

3.4. DJ-1/AKT link

DJ-1 was previously mentioned to mediate signaling pathways such as AKT. The first link between DJ-1 and AKT emerged from a *Drosophila* genetic screen performed by Kim *et al* in which they identified DJ-1 as a negative regulator of PTEN (Kim et al., 2005a). Overexpression of dPTEN in the *Drosophila* eye using the GAL4/UAS system eye reduced eye size by 80% due to reduction in both cell size and proliferation, compared to control. Co-expression of human DJ-1 was able to almost fully rescue the disrupted eye phenotype seen in the overexpressing dPTEN eye, functioning as an antagonist of PTEN function (Kim et al., 2005a). This link was further supported by evidence that degeneration seen in the eye of DJ-1A RNAi *Drosophila* (rough eye phenotype and loss of photoreceptors in some ommatidia) was significantly enhanced by co-expression of PTEN, resulting in reduced eye size, necrotic

spots, collapsed and fused ommatidia, and almost a complete loss of photoreceptor neurons (Yang et al., 2005). Furthermore, co-expression of DJ-1 was able to rescue PTEN-induced death in NIH3T3 cells leading to almost a complete restoration of cell viability (Kim et al., 2005a).

As these studies implicated a role for DJ-1 in the suppression of PTEN, researchers investigated the effect of DJ-1 on downstream targets of PTEN, such as AKT. It was found that knockdown of DJ-1 lead to a reduction in endogenous AKT phosphorylation in cell lines as well as in *Drosophila* brains, while overexpression of DJ-1 resulted in an increase of endogenous AKT phosphorylation, in addition to upregulation of cyclin D1 and GSK-3-β phosphorylation- downstream targets of AKT (Kim et al., 2005a, Yang et al., 2005).

Importantly, Aleyasin *et al* further explored the relevance of these effects in neuronal cells: both *in vitro* and *in vivo*, AKT phosphorylation was reduced in the absence of DJ-1 following oxidative stress (Aleyasin et al., 2010). DJ-1 KO cortical neurons displayed significantly lower levels of phosphorylated AKT (pAKT) (S473) following H_2O_2 treatment compared to DJ-1 WT. *In vivo*, pAKT was significantly reduced in the SNc of DJ-1 KO mice following MPTP treatment compared to WT control. Studies investigating the neuroprotective function of DJ-1 suggest that DJ-1 acts as an upstream regulator of AKT. While exogenous expression of AKT in WT cortical neurons confers protection against H_2O_2- induced neuronal death, AKT overexpression in DJ-1 KO cortical neurons fails to provide the same protection. In addition, WT mice that received adenoviral injections of AKT exhibited significant protection of nigrostriatal neurons when treated with MPTP compared to DJ-1 KO mice, demonstrated by an increase in TH+ neurons in the SNc, as well as higher striatal density in the dopaminergic terminals of the striatum. These findings

suggest that AKT requires DJ-1 to exert its neuroprotective function, both *in vitro* and *in vivo*.

The relationship between DJ-1 and AKT has also shown that not only does AKT require DJ-1, but DJ-1 requires AKT to exert its neuroprotective effects, at least in part. Inhibition of AKT via pharmacological inhibitor LY294002 in DJ-1 overexpressing cortical neurons abolished the neuroprotective activity of DJ-1 following H_2O_2, resulting in reduced neuronal survival.

Interestingly, while overexpression of AKT confers neuroprotection in WT but not DJ-1 KO cells, myristolated AKT (membrane anchored, constitutively active AKT) provides protection to both WT and DJ-1 KO cells. This link led researchers to investigate whether DJ-1 is capable of modulating AKT localization, thereby preventing AKT phosphorylation and subsequent activation. Indeed, it was found that in the absence of DJ-1 in both MEFs and cortical neurons, AKT levels in the membranous fraction were lower following H_2O_2 treatment compared to WT cells. These findings demonstrate that in both a neuronal and non-neuronal context, AKT requires DJ-1 to localize to the membrane following oxidative stress. The method by which DJ-1 modulates this translocation however remains to be elucidated.

4. PHLDA3

Pleckstrin homolog domain family A3 (*PHLDA3*) is a p53 target gene residing in human chromosome 1q31, transcribing a 127a.a. protein composed primarily of a PH domain (Frank et al., 1999; Tawase et al., 2008). While PHLDA1 and PHLDA2 are involved in insulin-like growth and placental growth, respectively, PHLDA3 KO animals (also named

Tih1 KO) are viable, fertile, lack gross abnormalities, and display no placental overgrowth (Frank et al., 2002). PHLDA3 is expressed in a wide range of fetal and adult tissues, with lowest expression in the liver and spleen, and is most prominent in lung and skeletal muscle, demonstrating non-overlapping function between family members (Frank et al., 1999).

4.1. Structure

PHLDA3 has been identified as a direct target gene of p53 (Kawase et al., 2009). PHLDA3 contains a putative p53-response element (p53RE) located upstream of the PHLDA3 gene which is conserved amongst species, and full activation of the *PHLDA3* promoter requires phosphorylation of p53 on Ser15, regulating PHLDA3 expression (Kawase et al., 2009).

4.2. Function

The function of PHLDA3 has not been fully elucidated and research still remains limited, however recent studies have emerged indicating PHLDA3's key role in both the AKT signaling pathway (Kawase et al., 2009), as well as cancer (Ohki et al., 2014).

4.2.1. PHLDA3/AKT

The role of PHLDA3 in the AKT pathway has been furthered explored. Studies demonstrated that PHLDA3 inhibits AKT activity by preventing AKT translocation to the membrane via its PH domain, inducing apoptosis (Kawase et al., 2009). Due to its PH domain, PHLDA3 is able to bind to PIP when expressed, localizing at the cellular membrane (Kawase et al., 2009). Expression of WT-PHLDA3 induced caspase-dependent cell death in

many cell lines. Overexpression of a mutant PHLDA3 (MT-PHLDA3), containing a small deletion in the PH domain which renders the PH domain non-functional, repressed apoptosis - suggesting a functional PH domain is necessary for PHLDA3-induced apoptosis (Kawase et al., 2009). The overexpression of WT-PHLDA3 in cell lines exhibited a significant reduction in AKT phosphorylation and impeded GFP-PH-AKT translocation to the membrane (Kawase et al., 2009). Consistent with this line of evidence, PHLDA3 KD conversely displayed increased pAKT, stronger GFP-PH-AKT localization at the membrane, and increased Mdm2 phosphorylation, compared to control (Kawase et al., 2009). When PHLDA3 was knocked down in MM468 cells (cells which do not possess functional PTEN), overexpression of p53 caused lower levels of pAKT and a decrease in p53-dependent apoptosis, implicating a role for PHLDA3 in AKT repression by p53 independent of PTEN (Kawase et al., 2009). Interestingly, overexpression of MT-PHLDA3 did not inhibit GFP-PH-AKT translocation to the membrane, and myristolated AKT decreased WT-PHLDA3-induced apoptotic cell death compared to control (Kawase et al., 2009). The mechanism by which PHLDA3 impedes AKT activity was found not to be by means of physical interaction, nor by modifying the amount of PIP3 present for AKT to bind within the cell, however instead PHLDA3 was found to competitively bind to PIP3, preventing AKT binding and subsequent activation (Figure 3) (Kawase et al., 2009).

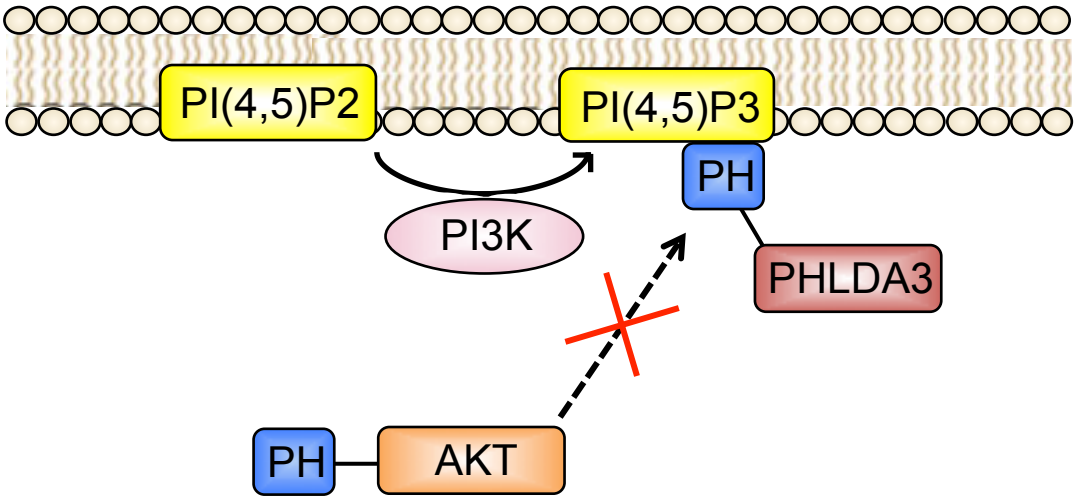

PI(4,5)P2
PI(4,5)P3
PH
PI3K
PHLDA3
PH
AKT

Figure 3: Inhibition of AKT by PHLDA3. PHLDA3 inhibits AKT activity by preventing AKT translocation to the membrane via its PH domain.

4.3. PHLDA3 & Cancer

Proteins within the PI3K/AKT pathway have been largely implicated in tumor suppression, and PHLDA3's involvement has been noted (Kawase et al., 2000; Ohki et al., 2014). Loss of PHLDA3 gene is frequent in human lung endocrine tumors as well as panNETs (pancreatic neuroendocrine tumors) (Kawase et al., 2009; Ohki et al., 2014), and is correlated to poor prognosis as a result of advanced staging (Ohki et al., 2014). This LOH (loss of heterozygosity) is accompanied by a high frequency of PHLDA3 promoter methylation, implicating a two-hit inactivation of the PHLDA3 gene (Ohki et al., 2014). LCNECs (large cell neuroendocrine carcinoma) display decreased PHLDA3 expression in tumors along with hyperphosphorylation of AKT. PHLDA3 KO mice develop hyperplastic islets that are the result of enhanced proliferation (Ohki et al., 2014), confirming PHLDA3's essential role in the inhibition and downstream effects of AKT.

RATIONALE & HYPOTHESIS

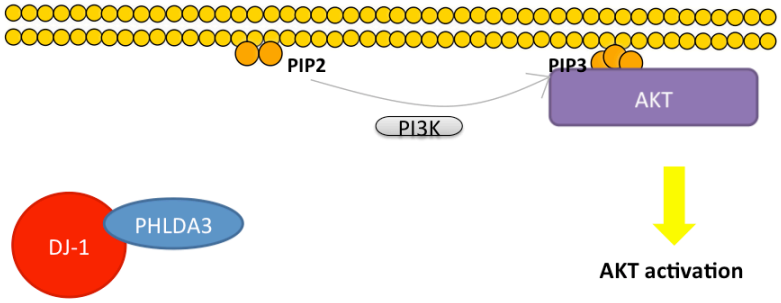
Rationale:

In collaboration with Dr. Dan Figeys (University of Ottawa, Systems Biology), we embarked on an unbiased screen to examine potential interactors of DJ-1. Interestingly, PHLDA3 was revealed as a very exciting and novel candidate. Given the ability of PHLDA3 to competitively bind to PIP3 and prevent AKT binding and activation, combined with the necessity of DJ-1 for AKT to exert its full neuroprotective effects under oxidative stress (specifically in order for AKT to translocate to the membrane), we propose the following:

Hypothesis:

DJ-1 binds to PHLDA3 sequestering it from PIP3 binding, allowing AKT to bind to PIP3 at the lipid membrane, exerting its neuroprotective function.

(A) ***In the presence of DJ-1, under oxidative stress:***



(B) ***In the absence of DJ-1, under oxidative stress:***

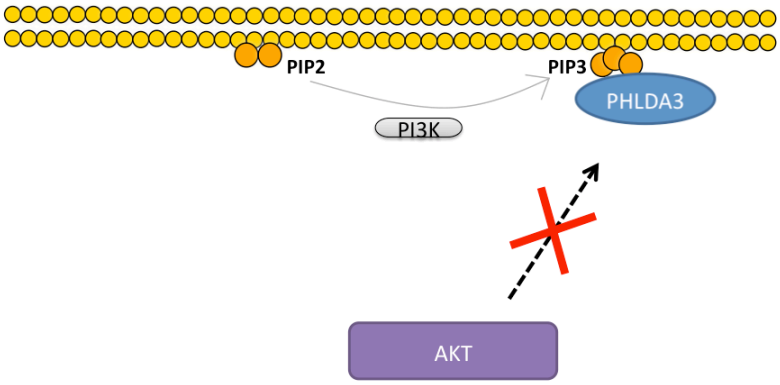


Figure 4: Proposed model. Proposed model of DJ-1/PHLDA3 interaction allowing for AKT binding in the presence (A) and absence (B) of DJ-1, under oxidative stress.

OBJECTIVES

To test this hypothesis, we will-

<u>Aim 1</u>: Confirm the interaction between PHLDA3 and DJ-1. Our initial screen suggested an interaction between PHLDA3 and DJ-1, we will confirm this interaction through IP analyses.

<u>Aim 2</u>: Determine functional relevance of PHLDA3 in neuronal survival under oxidative stress. PHLDA3 has yet to be investigated in neuronal cells, we will examine the potential relevance of PHLDA3 in regulating neuronal loss.

<u>Aim 3</u>: Determine if PHLDA3 is directly targeted and sequestered from the membrane by DJ-1.

a) Determine whether PHLDA3 affects AKT signaling in the presence/absence of DJ1

b) Determine whether PHLDA3 affects AKT membrane localization in the presence/absence of DJ-1

MATERIALS AND METHODS

Cell Culture & Cortical Neuronal Dissection

Murine Embryonic Fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% Antibiotic. Cortical neuronal cultures were prepared by extracting embryos at 14-15 days gestation followed by dissection and incubation of cortices with 0.50 mg/ml trypsin in Hank's balanced salt solution (HBSS), shaking for 25 min 37˚C. Neurobasal medium containing 0.2 mg/ml trypsin inhibitor and 0.2mg/ml DNaseI was added to cell solution, triturated, and centrifuged at 1000rpm, 4°C for 5 minutes. Cell pellet was resuspended in Neurobasal Medium containing 0.2 mg/ml trypsin inhibitor and 0.25 mg/ml DNaseI, and once again centrifuged at 1000rpm, 4°C for 5 minutes. Pelleted cells were resuspended in Complete Neurobasal Medium (Supplemented with B-27, N-2, Pen-Strep and L-Glutamine), and plated onto poly-D-lysine (PDL) coated plates. DJ-1 genotype was determined from C57Bl/6 embryos by obtaining a small piece of brain stem from each individual embryo and digesting the samples in order to perform PCR. DJ-1 WT and KO embryos were selected for experiments.

For pAKT analysis, WT and DJ-1 KO MEFs were transfected with Myc and Myc-PHLDA3 using Lipofectamine 2000 transfection reagent. 24 hours after transfection, cells were treated with 500μM H_2O_2 for 15 minutes. Cells were then washed with 1X PBS, harvested, and spun down at 1000rpm at 4°C for 5 minutes. Cell pellet was lysed using lysis buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA, 0.2% Triton X-100, 1mM DTT and protease inhibitor), and centrifuged at 13,500rpm at 4°C for 20 minutes. Supernatant was used for SDS-polyacrylamide gel (SDS-PAGE) and western blotting. Note: Due to use of

phospho-antibodies, both blocking of membrane and incubation of secondary antibody were completed in 5% BSA.

Co-Immunoprecipitation

For co-immunoprecipitation experiments, MEFs were transfected with GST, GST-DJ-1, and Myc-PHLDA3 using Lipofectamine 2000 transfection agent. 24 hours post-transfection, cells were washed with 1X phosphate buffered saline (PBS), and centrifuged at 1000rpm for 5 minutes at 4°C. Cell pellet was lysed in lysis buffer (5M NaCl, 1M Tris pH 6.8, Triton X-100, 1mM DTT, and Protease Inhibitor), and centrifuged at 13,500 rpm at 4°C for 20 minutes. Supernatant was incubated with 30ul of glutathione sepharose beads with agitation for 3 hours at 4°C. Precipitated complexes were washed 3X with washing buffer (50mM Tris pH 6.8, 300mM NaCl, 1mM EDTA, 0.5%Triton X-100, 1mM DTT), and eluded by boiling in 2X SDS loading buffer.

Protein Quantification & Western Blotting

Protein quantification was assessed by Bradford assay according to the manufacturer's protocol. Protein samples were mixed with 2X SDS loading buffer and boiled at 95°C for 10 minutes. Proteins were then separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then blocked with 5% milk for 1 hour at room temperature, and incubated with primary antibody diluted in 5% BSA overnight to probe target protein. Membranes were washed 3X 5 minutes with 1X PBS-T, and incubated with secondary antibody diluted in 5% milk for 1 hour. Membranes were then washed 3X 10 minutes with PBS-T and protein levels were detected using Pierce™ ECL Western Blotting

Substrate.

Antibodies

The rabbit polyclonal anti-Myc was obtained from abcam and used at a dilution of 1:5000. Rabbit polyclonal anti-pAKT (S473) and -AKT antibodies were purchased from Cell Signaling and used at a dilution of 1:2500 and 1:2000, respectively. Mouse monoclonal antibody against β-actin was obtained from Sigma and used at a dilution of 1:10,000. Peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Thermo Scientific and used at a 1:5000 dilution.

Survival Assays

For survival assays, cortical neuronal cultures were plated into 24-well plates and transfected with Myc-PHLDA3 and GFP at a 3:1 ratio using Lipofectamine 2000. CD1 cortical neuronal cultures were transfected at 3 days in vitro (DIV), while WT and DJ-1 KO cortical cultures derived from C57Bl/6 mice were transfected at 4 DIV. 24 hours post-transfection, cells were treated with 30μM H_2O_2 for 3 hours. Neurons were fixed with 4% PFA, stained with Hoescht, and survival was assessed by evaluating the nuclear integrity of GFP-positive cells (Survival%= Alive cells/ Total number of cells (GFP positive)).

Subcellular Localization

MEFs were grown in 24-well plates on glass cover slips, transfected with DsRed, DsRed-wtPHLDA3, DsRed-mtPHLDA3, and GFP-PH-AKT using Lipofectamine 2000, and left to express for 36 hours (plasmids kindly supplied by Dr. Reiko Ohki). Cultures were then

subjected to 500μM H_2O_2 for 5 minutes, and washed with 1X PBS. Cells were fixed using 4% PFA and subcellular localization was assessed using Zeiss AxioImager M2 microscope, and images were captured using Axiovision software. CD1 cortical neurons followed the aformentioned protocol, however were transfected with Lipofectamine3000 at 3 DIV, and were subjected to 100μM H_2O_2 for 5 minutes.

Statistical Analysis

Data was analyzed as indicated for each experiment, expressed as mean ± standard error of mean. Statistical significance was determined by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-hoc analysis (Prism) where appropriate.

RESULTS

Identification of PHLDA3 as a DJ-1 Interacting Protein

In an attempt to better understand DJ-1 mechanisms, we in collaboration with Dr. Dan Figeys embarked on an unbiased mass spectrometry screen to examine for DJ-1 interactors. Using DJ-1 as a bait protein, potential interacting partners of DJ-1 were immunoprecipitated and subsequently identified using mass spectrometry (Ewing et al., 2007). From this collaboration, PHLDA3 was identified as a novel potential DJ-1 interacting protein. We confirmed the interaction of PHLDA3 and DJ-1 in WT MEFs by expressing Myc-PHLDA3 along with GST-DJ-1 or GST alone as control, and immunoprecipitating GST-DJ-1 using glutathione sepharose beads (Figure 5). We observed that Myc-PHLDA3 was co-immunoprecipitated with GST-DJ-1, but not GST alone.

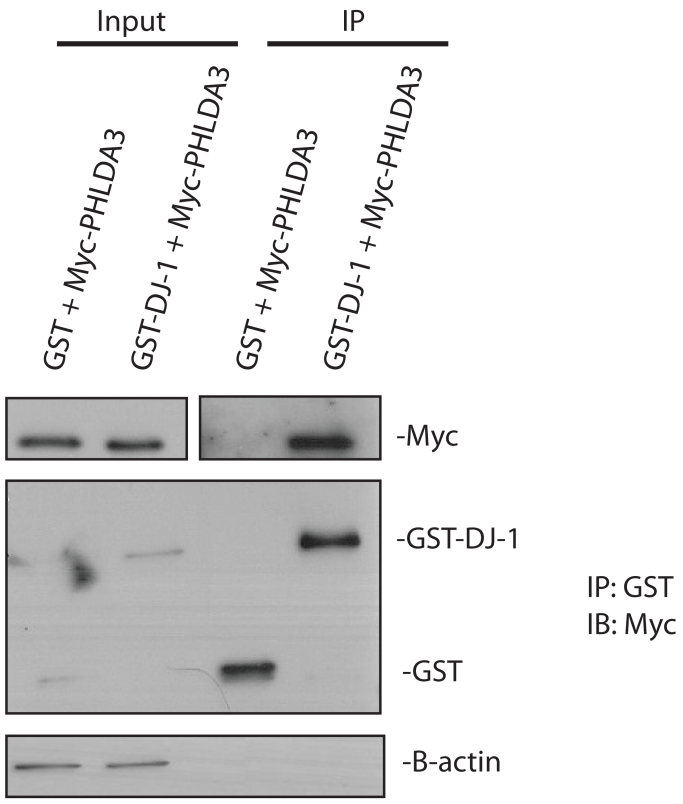

Input
IP
GST + Myc-PHLDA3
GST-DJ-1 + Myc-PHLDA3
GST + Myc-PHLDA3
GST-DJ-1 + Myc-PHLDA3
-Myc
-GST-DJ-1
-GST
-B-actin
IP: GST
IB: Myc

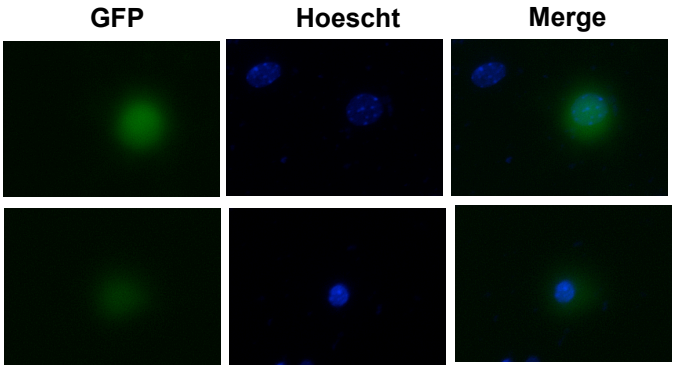
Figure 5: DJ-1 and PHLDA3 interact. WT MEFs expressing Myc-PHLDA3 and GST-DJ-1 or GST as control, were lysed and GST-DJ-1 was immunoprecipitated with glutathione sepharose beads and analyzed by western blot using Myc antibody.

PHLDA3 Decreases Neuronal Survival in Cortical Neurons

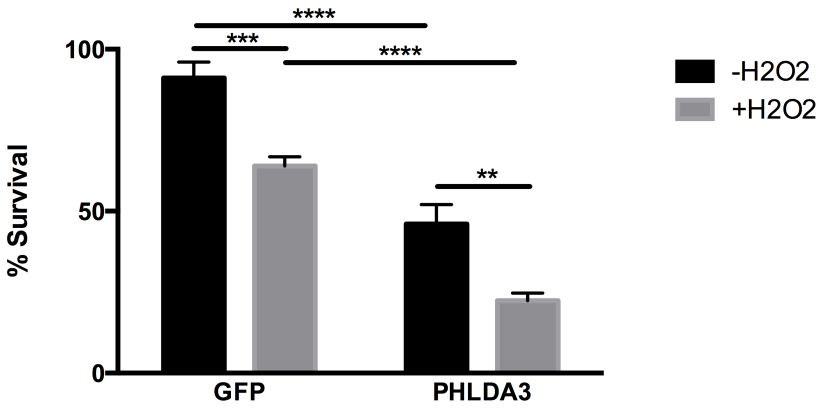
We next evaluated the functional role of PHLDA3 in neuronal survival following oxidative stress. To test this, neurons harvested from WT CD1 embryos were transfected with GFP, or Myc-tagged PHLDA3 along with GFP as a marker of transfection, and 24 hours post-transfection were treated with 30μM H_2O_2 for 3hrs. As demonstrated in Figure 6A, overexpression of PHLDA3 resulted in a significant decrease in neuronal survival compared to GFP control. Furthermore, H_2O_2-induced death in cortical neurons was further enhanced by overexpression of PHLDA3.

To determine whether the effect of PHLDA3 we had seen on neuronal survival could be modulated by DJ-1, we assessed whether PHLDA3-induced neuronal death could be counteracted or exacerbated by concurrent DJ-1 deficiency. To test this, neurons harvested from DJ-$1^{+/+}$ and DJ-$1^{-/-}$ embryos were transfected with GFP, or PHLDA3 with GFP at 4 DIV, and were treated 24 hours after transfection with 30μM H_2O_2 for 3hrs. The results of this experiment indicate that overexpression of PHLDA3 caused significantly more death in DJ-1 KO neurons compared to WT, and in the presence of H_2O_2 this effect is exacerbated (Figure 6B). Additionally, overexpression of PHLDA3 displayed a greater effect in DJ-1 KO than that of WT neurons, compared to GFP control.

(A)


GFP
Hoescht
Merge

(B)


****
***
****
**
100
50
0
% Survival
GFP
PHLDA3
-H2O2
+H2O2

(C)

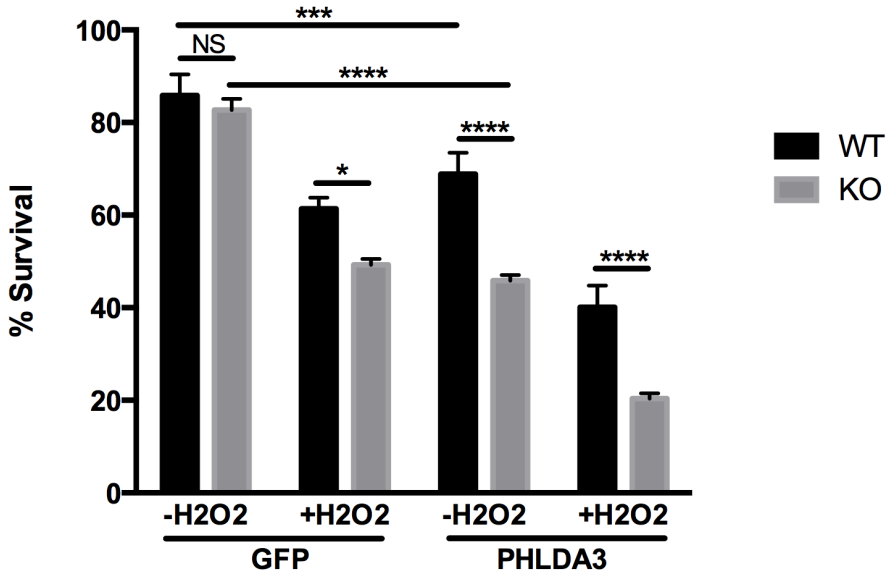
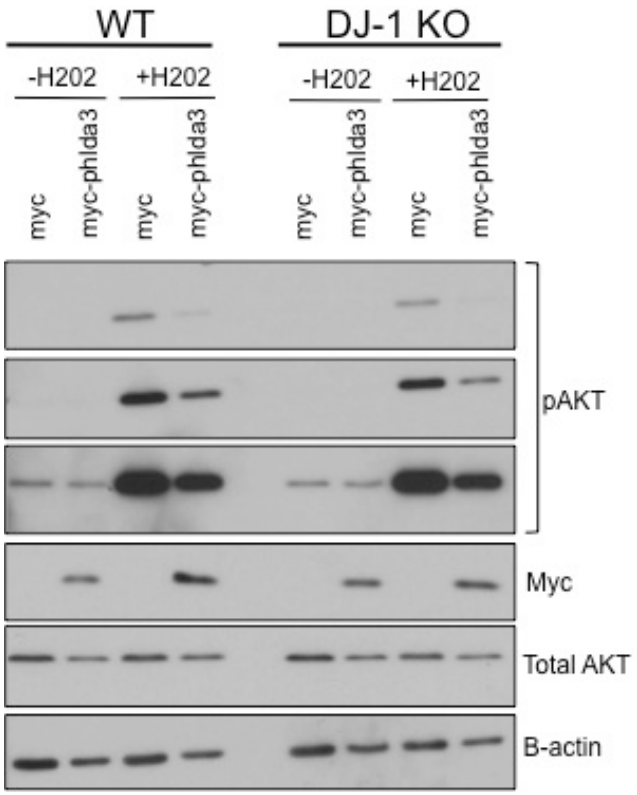

***
NS
****
*
****
****
100
80
60
40
20
0
% Survival
-H2O2
+H2O2
-H2O2
+H2O2
GFP
PHLDA3
WT
KO

Figure 6: PHLDA3 decreases neuronal survival in cortical neurons. (A) Representative pictures of alive (Upper) and dead (Lower) neurons. Neuronal survival measured by identifying GFP positive cells and determining their nuclear integrity by Hoechst stain. (B-C) Cortical neurons from WT CD1 (B) or DJ-$1^{+/+}$ and DJ-$1^{-/-}$ C57Bl/6 (C) embryos were harvested, plated, and transfected with GFP, or PHLDA3 with GFP. Cells were treated with H_2O_2 (30μM) or vehicle control (-H_2O_2) for 3 hours. Neuronal survival was measured by identifying GFP-positive cells and determining their nuclear integrity by Hoescht stain. Data is presented as mean ± S.E.M. *, P<0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001; NS, no significant difference.

PHLDA3 Causes Reduced AKT Phosphorylation in Response to Oxidative Stress in the Absence of DJ-1

The observations that DJ-1 deficiency sensitizes neurons to PHLDA3-mediated neuronal death suggests a protective role for DJ-1 in PHLDA3-induced neuronal death. PHLDA3 has been shown to inhibit AKT phosphorylation in cell lines and loss of DJ-1 results in reduced AKT signaling (Kawase et al., 2009; Aleyasin et al., 2010). As such, we next evaluated whether PHLDA3 affects AKT pro-survival signaling in a DJ-1-dependent manner. To determine this we transfected both WT and DJ-1 KO MEFs with Myc or Myc-PHLDA3, and treated with H_2O_2 (500μM, 15 minutes). As indicated in Figure 7A and quantified in Figure 7B, overexpression of PHLDA3 resulted in a significant reduction of pAKT following H_2O_2 in DJ-1 KO MEFs, compared to WT. DJ-1 KO MEFs displayed reduced levels of AKT phosphorylation following H_2O_2 compared to WT MEFs, and this decrease in pAKT was further enhanced by PHLDA3 overexpression.

(A)


WT
DJ-1 KO
-H202
+H202
-H202
+H202
myc
myc-phlda3
myc
myc-phlda3
myc
myc-phlda3
myc
myc-phlda3
pAKT
Myc
Total AKT
B-actin

(B)

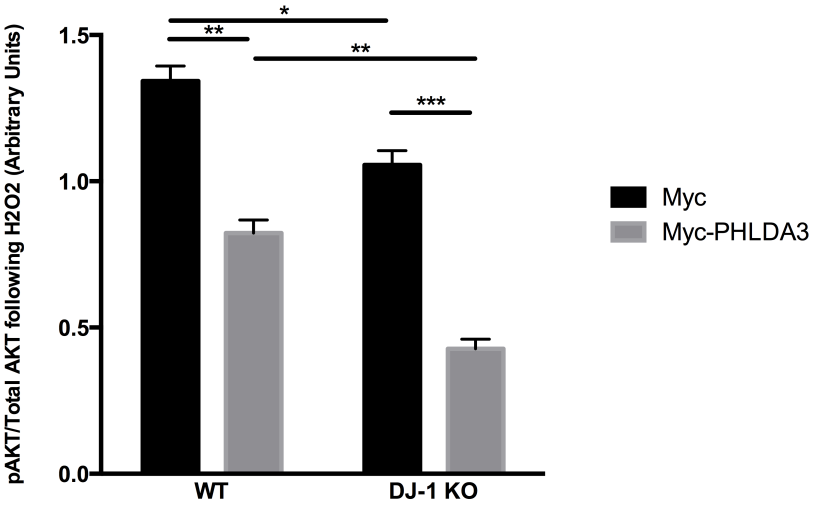
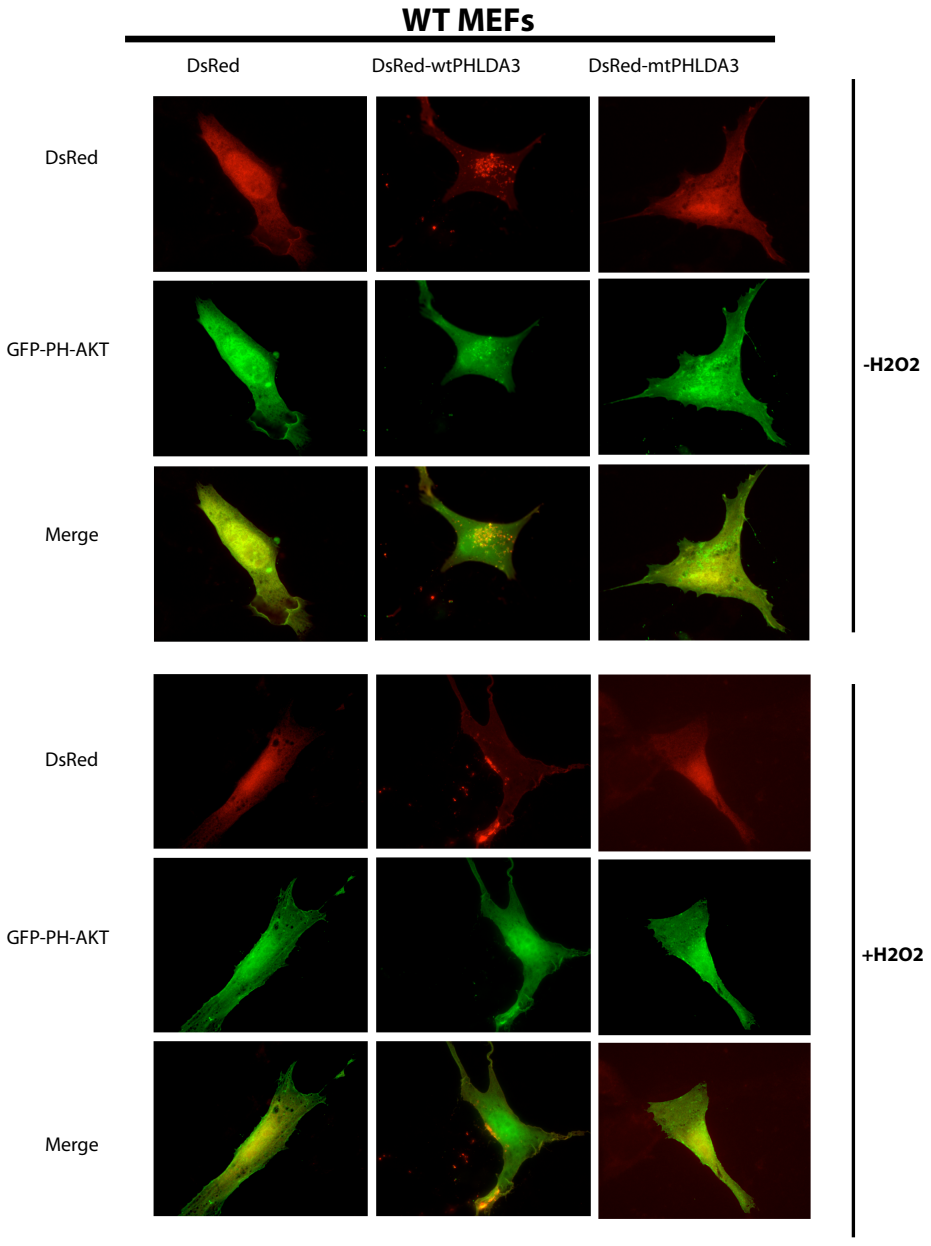

pAKT/Total AKT following H2O2 (Arbitrary Units)
1.5
1.0
0.5
0.0
*
**
**
***
WT
DJ-1 KO
Myc
Myc-PHLDA3

Figure 7: PHLDA3 causes reduced AKT phosphorylation in response to oxidative stress in the absence of DJ-1. (A) DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEFs were transfected with Myc or Myc-PHLDA3, and treated with H_2O_2 (500μM) or vehicle control (-H_2O_2) for 15 minutes. Extracts were probed for pAKT (S473), total AKT, Myc, and β–actin by western blot. pAKT is presented at three levels of exposure. (B) Quantification of (A) from 2 independent experiments. Data are presented as mean optical density relative to total AKT. Data is presented as mean ± S.E.M. *, $P<0.05$; ** $P< 0.01$; *** $P< 0.001$.

DJ-1 Deficiency Leads to Increased PHLDA3 Translocation to Membrane in MEFs

AKT is required to bind PIP3 at the membrane in order to be phosphorylated and fully activated (Kandel and Hay, 1999). PHLDA3 has been shown to interfere with AKT translocation to the membrane and as such we wanted to determine whether PHLDA3 modulates AKT translocation to the plasma membrane in a DJ-1 dependent manner. To evaluate this, we transfected WT and DJ-1 KO MEFs with GFP-PH-AKT (GFP-tagged PH domain of AKT which has been shown to mimic AKT localization) together with DsRed, DsRed-wtPHLDA3, or DsRed-mtPHLDA3 which has a defective PH domain and as such is unable to bind PIP3 at the membrane. Cells were treated with H_2O_2 (500μM, 5 minutes) 36 hours after transfection, fixed with 4% PFA, and subcellular localization was analyzed. As shown in Figure 8A, WT PHLDA3 is expressed as punctate within the cell in the absence of H_2O_2. Following H_2O_2 treatment, WT, but not mutant, PHLDA3 exhibited localization at the membrane. Unlike WT MEFs, DJ-1 KO MEFs display WT PHLDA3 localization at the membrane in the absence of H_2O_2 (Figure 8B). Following H_2O_2, localization of PHLDA3 at the membrane in DJ-1 KO MEFs appears to significantly increase, displaying membranous localization much higher than any other condition. Mutant PHLDA3 did not have the same effect.

(A)


WT MEFs
DsRed
DsRed-wtPHLDA3
DsRed-mtPHLDA3
DsRed
GFP-PH-AKT
Merge
-H2O2
DsRed
GFP-PH-AKT
Merge
+H2O2

(B)

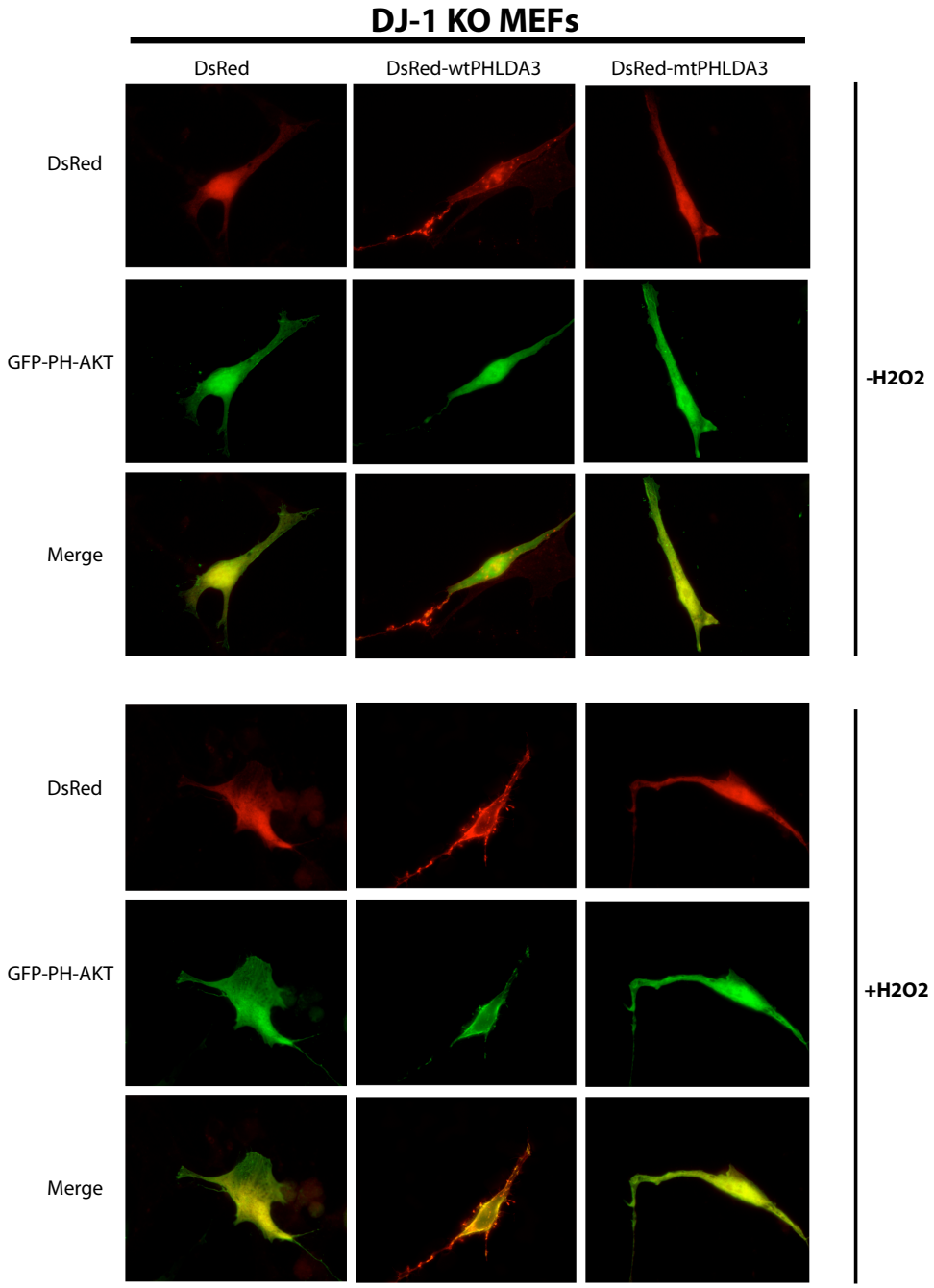

DJ-1 KO MEFs
DsRed
DsRed-wtPHLDA3
DsRed-mtPHLDA3
DsRed
GFP-PH-AKT
Merge
-H2O2
DsRed
GFP-PH-AKT
Merge
+H2O2

Figure 8: DJ-1 regulates PHLDA3 translocation in MEFs. DJ-$1^{+/+}$ (A) and DJ-$1^{-/-}$ (B) MEFs were transfected with GFP-PH-AKT together with DsRed, DsRed-wtPHLDA3 or DsRed-mtPHLDA3, with and without H_2O_2 (500μM, 5 min). Cells were fixed and subcellular localization was analyzed. Images are representative of at least 90 cells and 3 independent experiments.

Next we wanted to look at the implications of PHLDA3 on AKT translocation in neurons. To test this, cortical neurons were harvested from WT CD1 embryos at 14-15 days GA, plated, and transfected with GFP-PH-AKT, DsRed, DsRed-wtPHLDA3 and DsRed-mtPHLDA3 three days after plating. Neurons were then treated with 100μM H_2O_2 for 5 minutes, and subcellular localization was analyzed. As visualized in Figure 9, in the absence of H_2O_2, overexpressed AKT extends from the cell body and somewhat throughout the processes of the neuron. Expression of WT PHLDA3 seems to overlap with AKT localization. Following H_2O_2 treatment, AKT localization to neuronal processes increased. Interestingly, H_2O_2-treated neurons expressing WT PHLDA3 displayed AKT localization restricted to the cell body. MT PHLDA3 expression did not have the same effect on AKT localization and itself was restricted to the cell body.

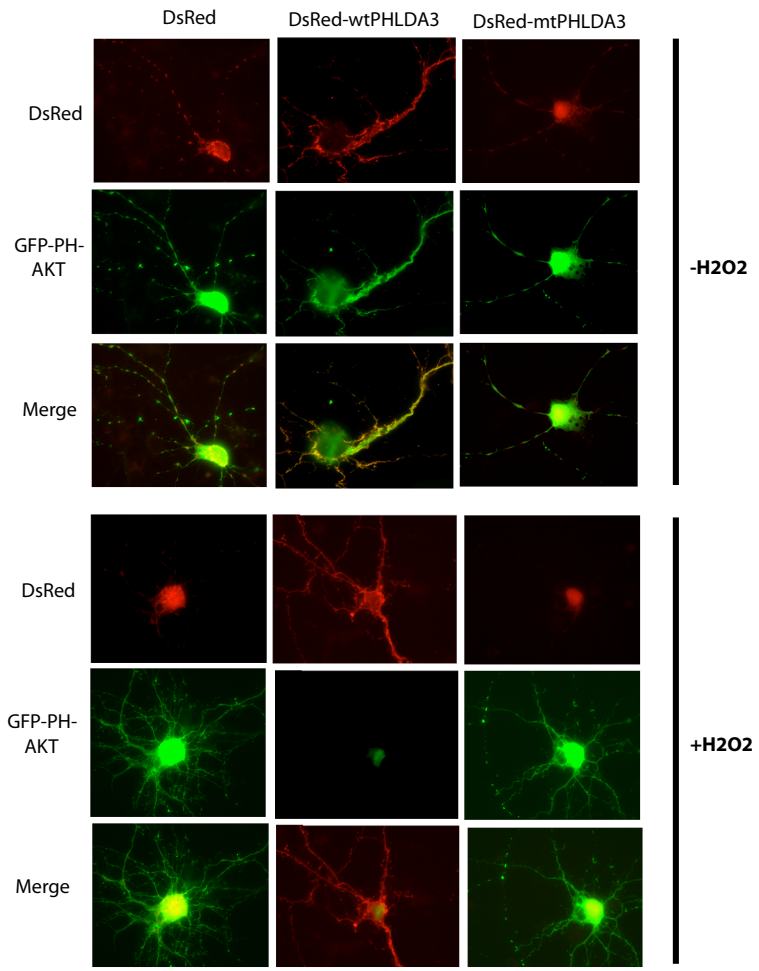

DsRed
DsRed-wtPHLDA3
DsRed-mtPHLDA3
DsRed
GFP-PH-AKT
Merge
-H2O2
DsRed
GFP-PH-AKT
Merge
+H2O2

Figure 9: PHLDA3 interferes with AKT localization in cortical neurons. CD1 neurons were harvested, plated, and transfected with GFP-PH-AKT together with DsRed, DsRed-wtPHLDA3 or DsRed-mtPHLDA3. Cells were treated with H_2O_2 (100μM, 5 min) or vehicle control (-H_2O_2), and analyzed for subcellular localization. Images are representative of at least 50 cells and 3 independent experiments.

DISCUSSION

Parkinson's disease was reported to affect 4.1-4.6 million people in 2005 (Dorsey et al., 2007). As the most common neurodegenerative movement disorder, the inability of available therapies to halt, slow, or most desirable- reverse the disease, is unacceptable. Currently, therapies are only capable of providing symptomatic relief, highlighting the importance of elucidating the molecular mechanisms implicated in PD. With further understanding of these mechanisms, much needed new therapeutic approaches can be developed. The identification/discovery of familial linked genes provided researchers with an incredible tool to delineate such pathways and mechanisms. The autosomal recessive PD-linked gene DJ-1 was discovered by Bonifati *et al* in 2003, with homozygous loss-of-function mutations in DJ-1 leading to early onset PD (Bonifati et al., 2003). The mechanism by which loss of DJ-1 leads to PD is unknown, and delineating this link may provide clues into the pathogenesis of the wider PD population.

Although the physiological role of DJ-1 is not fully understood, DJ-1's neuroprotective role against oxidative stress is well documented. DJ-1 deficient neurons are sensitive to oxidative stress, and this sensitivity can be reversed by WT DJ-1 expression (Kim et al., 2005b). Several hypotheses as to the role of DJ-1 in neuronal survival following oxidative stress have been postulated- such as chaperone activity (Lee et al., 2003; Shendelman et al., 2004), scavenging of ROS (Taira et al., 2004; Andres-Mateos et al., 2007), transcription regulation (Itoh et al., 1999; Clements et al., 2006), as well as protection against mitochondrial damage (Junn et al., 2005). Alternatively DJ-1 has also been shown to modulate key signaling pathways, such as the AKT survival pathway: a central player in neuronal survival (Kim et al., 2005a; Yang et al., 2005; Aleyasin et al., 2010).

DJ-1 requires AKT activation to promote cellular survival, demonstrating that AKT is important to the mechanism of protection conferred by DJ-1. In the absence of DJ-1, AKT phosphorylation is reduced following oxidative stress. Key to this thesis, DJ-1 modulates AKT translocation to membranous fractions following oxidative stress, displaying the necessity of DJ-1 for activation of the AKT pathway in response to oxidative injury in neurons (Aleyasin et al., 2010). The mechanism by which DJ-1 impacts AKT recruitment to the membrane is unknown and is likely a critical aspect of DJ-1 function.

In this thesis we provide a potential novel mechanism by which DJ-1 exerts its neuroprotective function through the AKT pathway via direct interaction with PHLDA3.

Co-Immunoprecipitation of PHLDA3 and DJ-1

Mass spectrometry screening is an extremely valuable tool used to map protein-protein interactions, allowing for better understanding of protein function and role in disease (Ewing et al., 2007). In order to better understand DJ-1 function we embarked on an unbiased screen in order to identify potential interactors of DJ-1, and identified PHLDA3 as a potential interactor (in collaboration with Dr. Dan Figeys).

In this study, we have demonstrated that DJ-1 and PHLDA3 co-immunoprecipitate, confirming preliminary studies performed by Dr. Figeys. GST-DJ-1 positively interacted with Myc-PHLDA3, demonstrating PHLDA3's ability to bind DJ-1. This supports our hypothesis that DJ-1 and PHLDA3 physically interact, possibly sequestering PHLDA3 from binding at the membrane. Other potential interactors obtained from this screen have also confirmed interaction with DJ-1. PON2 binds DJ-1, protecting neurons against MPP+ induced neuronal death (Mohammed et al., 2014a). VHL, another candidate protein derived

from the screen, interacts with DJ-1, inhibiting VHL ubiquitination activity and protecting neurons against oxidative damage (Mohammed et al., 2014b). These findings give validity to the mass spectrometry screen and the presence of true DJ-1 interactors, and also reinforce the protective role of DJ-1. Another member of the AKT pathway, PTEN, has recently been shown to physically interact with DJ-1 (Kim et al., 2009). In addition, under oxidative stress DJ-1's binding to PTEN is enhanced. It would be interesting in future studies to determine whether or not the binding of PHLDA3 and DJ-1 is enhanced under oxidative stress.

Future experiments are required to determine whether PHLDA3 and DJ-1 interact endogenously. As PHLDA3 is a fairly novel protein to be explored in the field, the lack of successful PHLDA3 antibody was a running issue throughout the course of this project and limited experiments to PHLDA3 overexpression. While groups such as Kawase *et al* were successful in identifying endogenous PHLDA3 in human cells (Kawase et al., 2009), we were unable to locate a successful PHLDA3 antibody.

It would also be very interesting to identify the site at which DJ-1 and PHLDA3 interact. As PHLDA3 is a PH-domain only protein, with its PH domain occupying the majority with only short flanking N- and C- terminals, it is likely that DJ-1 binds within the PH domain. However, the possibility exists that DJ-1 binds within the PH domain however not to a site that would interfere with lipid binding, possibly affecting PHLDA3 function in a manner other than the preventing its direct binding to PIP3. This would be an intriguing avenue to explore.

Overexpression of PHLDA3 induces death in cortical neurons & hypersensitizes neurons to oxidative stress

PHLDA3 has been identified as a p53 target gene, inducing apoptosis in cell lines (Kawase et al., 2009). The role of PHLDA3 has yet to be explored in a neuronal context, and furthermore has not been investigated under conditions of oxidative stress. We demonstrate here that overexpression of PHLDA3 induces neuronal death in CD1 cortical neurons, consistent with previous findings (Kawase et al., 2009). Furthermore, we showed that this overexpression leads to hypersensitivity of neurons to H_2O_2, implicating a role for PHLDA3 in the oxidative stress response.

Several lines of evidence indicate AKT pro-survival pathway responds to oxidative stress and exerts a neuroprotective function. P53 negatively regulates AKT signaling pathway through induction of p53 target genes, which decrease AKT activation (Stambolic et al., 2001; Barak et al., 1993). In line with our observations, PTEN, another p53 target gene and negative regulator of AKT, also induces cell death upon overexpression, and its down-regulation results in protection (Kim et al., 2005a; Stambolic et al., 1998; Sun et al., 1999). Furthermore, pharmacological inhibitors of AKT such as LY294002 and Wortmannin, decrease viability and increases susceptibility to death induced by H_2O_2, similar to PHLDA3 (Taylor et al., 2004; Aleyasin et al., 2010; Hou et al., 2012).

Previously described, PHLDA3 induces apoptosis through competitive binding to PIP3, inhibiting AKT binding and subsequent downstream pro-survival signals (Kawase et al., 2009). As such, experiments investigating whether MT-PHLDA3 (containing a non-functional PH domain) abrogates PHLDA3's apoptotic function, increasing survival in neurons, would be of interest.

In addition, investigation into the effects of PHLDA3 downregulation on cell survival would be of importance. During the duration of these studies confirmation of PHLDA3 knockdown proved to be difficult due to absence of successful antibody, however methods such as RT-PCR could be employed for future studies.

Loss of DJ-1 sensitizes neurons to PHLDA3-induced neuronal death following H_2O_2

Next, we investigated PHLDA3 in the context of DJ-1, during oxidative stress. DJ-1 exerts an important role in the AKT pathway and is necessary for AKT-mediated neuroprotective effects (Kim et al., 2005a; Yang et al., 2005; Aleyasin et al 2010). As such, we wanted to determine if the PHLDA3-induced neuronal death we previously observed could be modulated by the presence/absence of DJ-1. We observed a sensitivity of DJ-1 KO cortical neurons to H_2O_2, consistent with many studies (Kim et al., 2005b; Aleyasin et al., 2010). In support of our hypothesis, we demonstrated that loss of DJ-1 results in hypersensitivity of cortical neurons to PHLDA3 overexpression. This effect was exacerbated following H_2O_2 treatment.

The AKT pathway is an integral part of the mechanism of protection by which DJ-1 exerts its neuroprotective effects. As PHLDA3 is a negative regulator of AKT (Kawase et al., 2009), our findings were consistent with other studies regarding the inhibition of the PI3K/AKT pathway. Neuroprotective activity of DJ-1 is significantly reduced upon suppression of AKT via PI3K inhibitor LY294002 (Aleyasin et al., 2010). Transfection of DN-AKT (dominant negative), another means of AKT suppression, also displayed diminished DJ-1 neuroprotective function (Aleyasin et al., 2010). Furthermore, knockdown

of DJ-1 in PTEN overexpressing cells resulted in enhanced cell death (Yang et al., 2005; Kim et al., 2005a), similar to PHLDA3.

We demonstrated here that in the absence of DJ-1, PHLDA3 induced significant neuronal death following H_2O_2. Future studies to investigate whether the overexpression of DJ-1 in PHLDA3 KO cortical neurons is capable of increasing cell survival, compared to PHLDA3 WT control, would be of importance, as it would determine the necessity of PHLDA3 for DJ-1 to exert its neuroprotective function.

In addition, future studies regarding the transcriptional activity of p53 would be of interest. DJ-1 has been shown to bind p53 directly, sequestering p53 from promoters and inhibiting p53 transcriptional activity of target genes (Bretaud et al., 2007; Fan et al., 2008a). As a p53 target gene, it is possible that under oxidative stress DJ-1 binds p53 sequestering it from the PHLDA3 promoter decreasing PHLDA3 expression, and therefore exerting its neuroprotective effects.

Phosphorylation of AKT is reduced following oxidative stress in DJ-1 KO PHLDA3-overexpressing cells

AKT exerts neuroprotective effects following its phosphorylation and subsequent activation (Kandel and Hay, 1999; Yang et al., 2004). We demonstrated, that in the absence of DJ-1, PHLDA3 overexpression induced lower pAKT levels following H_2O_2 treatment compared to WT MEFs, consistent with our previous survival data.

In support of our findings, many cellular models have demonstrated an inverse relationship between AKT phosphorylation and PHLDA3. PHLDA3 overexpression results in decreased pAKT, whereas knockdown consistently demonstrates an increase in pAKT

levels (Kawase et al., 2009; Lee et al., 2014; Lee et al., 2015; Ohki et al., 2014). In addition, DJ-1 has been shown to modulate AKT phosphorylation with loss of DJ-1 resulting in lower pAKT following oxidative stress (Yang et al., 2005; Aleyasin et al., 2009; Kim et al., 2005a), supporting our hypothesis. Furthermore, lymphoblasts from patients with L166P DJ-1 mutation have also reported lower pAKT following oxidative stress, strengthening the importance of functional DJ-1.

Within the PI3K/AKT pathway, PTEN, another negative regulator of AKT, has shown DJ-1 dependent AKT phosphorylation with loss of DJ-1 in PTEN expressing cells resulting in decreased pAKT (Kim et al., 2005a). Overlapping downstream targets of AKT such as GSK-3β have also been associated with both DJ-1 and PHLDA3 (Ohki et al., 2014; Kim et al., 2005a).

Interestingly, recent reports have also noted a decrease in AKT downstream substrate mdm2 following overexpression of PHLDA3 (Ohki et al., 2014; Lee et al., 2015). It has been reported that PHLDA3 represses AKT-mediated mdm2 phosphorylation, leading to diminished mdm2-p53 interaction and decreased destabilization of p53, resulting in p53 accumulation (Lee et al., 2015). This finding is of interest as it implicates PHLDA3 in a positive feed forward loop and suggests the possibility of DJ-1 as a negative regulator. This is also of great interest as p53 accumulation can be seen in the SNc of patients of sporadic PD (Nair et al., 2006).

PHLDA3 localization in the absence of DJ-1 following H_2O_2

Lastly, using constructs given to us by Dr. Ohki we were able to investigate DJ-1's effect on PHLDA3 localization. PHLDA3 was previously shown to be membrane bound

however these findings had yet to be investigated in the context of neurons, under oxidative stress, and in the presence/absence of DJ-1. As such, we found that loss of DJ-1 led to higher localization of WT PHLDA3 at the membrane in MEFs, and that this localization was exacerbated in the presence of H_2O_2. Kawase *et al* have previously shown competitive binding of PHLDA3 to the membrane, displacing AKT and localizing it in the cytosol (Kawase et al., 2009). While we did not observe the same displacement of GFP-PH-AKT, we did see differential WT PHLDA3 localization. As well, preliminary studies into DJ-1 WT C57Bl/6 cortical neurons demonstrated more specific GFP-PH-AKT subcellular localization than CD1 cortical neurons, with GFP-PH-AKT more strictly restricted to neuronal membranes rather than a diffused ubiquitous appearance. Cell line specific/animal line can differ in terms of results and we believe future investigations into PHLDA3 localization would perhaps be better in a C57Bl/6 background.

Our findings are also supported by the reported necessity of DJ-1 for AKT to localize to the plasma membrane following oxidative stress (Aleyasin et al., 2010).
In order to further confirm PHLDA3/AKT localization in the presence and absence of DJ-1, subcellular fractionation can be performed differentiating membrane from cytosol. In addition, assessment of the direct competitive binding of PHLDA3 to PIP3 in the presence and absence of DJ-1 are necessary and could be achieved through methods such as Lipid-Protein Overlay Assay (LPOA).

Interestingly, a recent study has emerged reporting a pathway by which DJ-1 and AKT are recruited, providing protection against oxidative stress (Tanti et al., 2014). SGN2A has been reported to interact with both DJ-1 as well as AKT, binding in separate domains acting as a scaffold protein. No interaction occurs between the two in the absence of SG2NA,

however all three have been reported to co-localize to the plasma membrane, and this recruitment is enhanced following H_2O_2 (Tanti et al., 2014). DJ-1 mutants associated with familial PD were not recruited by SGN2A and did not provide protective effects (Tanti et al., 2014). This study in co-operation with our findings, support a role for DJ-1 in the modulation of AKT localization under oxidative stress.

As noted in this thesis, PHLDA3 binds to PIP3 in order to inhibit AKT binding (Kawase et al., 2009), thereby inhibiting the AKT pathway. However PHLDA3 only has moderate affinity and poor selectivity for PIP3, and is able to bind several PIP's (Saxena et al., 2002; Kawase et al., 2009). This in combination with the presence of numerous PH-domain containing proteins begs the question as to what other PH-domain containing proteins PHLDA3 targets, and if DJ-1 regulates these processes as well.

Investigations into the mechanism regulating PHLDA3 PH binding would also be of great value. While it is a necessity for PIP3 binding to occur in order to inhibit AKT, not all PH domains that bind PIP3 have AKT-inhibiting activity (Varnai et al., 2005). PHLDA3 for example had no effect on cell adhesion and spreading, another PIP3 regulated process (Varnai et al., 2005; Kawase et al., 2009). Additionally, the possibility of protein-protein interactions influencing the binding of PHLDA3 PH domain exists. AKT for example, is phosphorylated on Thr34 of its PH domain by PKCζ, which prevents AKT recruitment to the membrane (Powell et al., 2003). If PHLDA3 used a similar mechanism in order to prevent PH binding, such mechanism could possibly compensate for loss of functional DJ-1 as seen in familial PD. Research into these alternative mechanisms will be important.

Conclusion

In conclusion, we have demonstrated a role for DJ-1 in the activation of AKT via binding and inhibition of PHLDA3 under oxidative stress, thereby conferring neuroprotection. Taken together, these studies provides further strength to the notion that the DJ-1/AKT signaling axis may be important in regulating neuronal function or death, and describe a novel mechanism by which DJ-1 regulates the activity of AKT, a critical neuronal survival pathway. Elucidation of these mechanisms may provide insight into the design of neuroprotective therapies for PD.

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