INVESTIGATING A PROTECTIVE ROLE FOR PARKIN DOWNSTREAM OF DOPAMINE STRESS IN IN-VITRO CELL MODELS

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Abstract

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the *Substantia nigra pars compacta* (SNc), a region particularly vulnerable to dopamine (DA) metabolite toxicity and oxidative stress. Mutations in the *PRKN* gene, which encodes parkin, an E3 ubiquitin ligase thought to be important for mitophagy, underlie what is known as autosomal recessive, early-onset PD (EOPD). In parkin-linked PD, the loss of DA-producing neurons is relatively selective for SNc and *Locus coeruleus* (LC) neurons, highlighting parkin's crucial role in protecting them. Recent studies from our lab have shown that parkin also plays an antioxidant role, protecting DA-producing neurons from oxidative damage by reducing reactive oxygen species (ROS) and sequestering DA-derived radicals. This protective action leads to parkin's own oxidation and subsequent insolubility. Notably, this shift to an insoluble state has been shown to impair parkin's E3 ligase function, suggesting that parkin's protective role under oxidative stress conditions may be independent of its role in mitophagy. Furthermore, in normal human brain, parkin has been found to associate with neuromelanin (NM), a pigment formed in catecholaminergic neurons via the sequestration of toxic DA radicals, as well as with CD63-positive lysosomal vesicles, suggesting a role for parkin in lysosomal pathways that mitigate cellular damage.

In this study, I aimed to investigate how DA-induced cellular stress affects parkin's oxidation, solubility, and localization within the cell, with a particular interest in its interaction with NM and CD63. To explore this, I used two models of DA-linked stress. The first model involved the direct addition of DA to the culture medium to simulate oxidative stress from DA auto-oxidation. The second model utilized tyrosinase cDNA transfection in M17 neuroblastoma cells to drive intracellular melanin synthesis, creating a controlled system for examining parkin's

interactions with melanin. There, melanin synthesis arises from the formation of DA oxidation products via tyrosinase activity. My results showed that DA treatment led to partial oxidation and insolubilization of parkin, particularly when the antioxidant glutathione was depleted by using buthionine sulfoximine (BSO), which exacerbates oxidative stress. These modifications were not associated with any visible co-localization by wild-type (WT) parkin with CD63 in M17 cells or microscopically visible aggregate formation. Unexpectedly, parkin remained dispersed throughout the cell post DA-treatment. In the tyrosinase model, parkin did not undergo any significant changes in oxidation or solubility with increasing melanin accumulation, nor did parkin visibly co-localize with melanin granules. However, cells expressing parkin showed accelerated melanin formation compared to those without.

Overall, this study explored the behavior of parkin under DA-induced stress conditions in an undifferentiated, human M17 neuroblastoma cell culture system. While parkin does undergo oxidative changes, these results suggested that the extent of these changes—along with parkin's solubility and localization—are highly dependent on the chosen cell model and experimental conditions employed. The cellular environment, as determined by factors, such as the capacity to neutralize endogenous as well as exogenously added DA metabolites, likely plays a role in shaping parkin's response to stress. These findings emphasize the need for further investigation into how parkin behaves in different neural models to better understand its role in protecting dopaminergic neurons, particularly in the context of brain ageing, which will likely inform the pathogenesis of EOPD, and possibly of sporadic PD.

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Abbreviations Used in Text

AADC	Aromatic L-amino Acid Decarboxylase
AIMP2	Aminoacyl-tRNA Synthetase Interacting Multifunctional Protein 2
ALDH	Aldehyde Dehydrogenase
BSO	Buthionine Sulfoximine
cAMP	Cyclic AMP
CCCP	Carbonyl Cyanide m-Chlorophenyl Hydrazone
COMT	Catechol-O-Methyltransferase
DA	Dopamine
DAT	Dopamine Transporter
DBS	Deep Brain Stimulation
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3,4-Dihydroxyphenylacetaldehyde
DOX	Doxycycline
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescent (detection reagent)
EM	Electron Microscopy
EOPD	Early-Onset Parkinson's Disease
ETC	Electron Transport Chain
EV	Empty-Vector
FBS	Fetal Bovine Serum
FBP1	Far Upstream Element-Binding Protein 1
FM	Fontana-Masson (staining)

GPCR	G Protein-Coupled Receptors
GR	Glutathione Reductase
Grx	Glutaredoxin
GSH	(reduced) Glutathione
GSSG	(oxidized) Glutathione
H2O2	Hydrogen Peroxide
HMW	High Molecular Weight
HVA	Homovanillic Acid
IAA	Iodoacetamide
IBR	In-Between-RING
IF	Immunofluorescence
LC	Locus Coeruleus
LC-MS	Liquid Chromatography/Mass Spectrometry
L-DOPA	Levodopa
LRRK2	Leucine-Rich Repeat Kinase-2
LUT	Look-Up Table
LVD	Lentivirus-Doxycycline Inducible (cell line)
LV	Lentivirus
MAO	Monoamine Oxidase
MAO-B	Monoamine Oxidase B
Mfn2	Mitofusin 2
NEM	N-ethylmaleimide
NM	Neuromelanin

NO	Nitric Oxide
PARL	Presenilin-Associated Rhomboid-like Protein
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PINK1	PTEN-Induced Putative Kinase 1
Prx	Peroxiredoxin
RBR	RING-Between-RING
RES	Reactive Electrophilic Species
ROS	Reactive Oxygen Species
r-parkin	Recombinant Parkin
SDS	Sodium Dodecyl Sulfate
SN	Substantia Nigra
SNc	Substantia Nigra Pars Compacta
SNCA	α-Synuclein
S.O.C	Super Optimal Broth with Catabolite Repression
TH	Tyrosine-Hydroxylase
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
TS	Tris-NaCl
TX	Triton X-100
TXS	Triton X-100 in TSS

Ubl	Ubiquitin-like
VDAC1	Voltage-Dependent Anion Channel 1
VMAT2	Vesicular Monoamine Transporter
VTA	Ventral Tegmental Area
WB	Western Blot
WT	Wildtype
γ-GCS	γ-Glutamylcysteine

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1.0 Introduction

1.1 Parkinson's Disease: An Overview

The origins of Parkinson's disease (PD) can be traced back to the pioneering work of James Parkinson in 1817.[1] His seminal essay, titled "An Essay on the Shaking Palsy," marked the first formal recognition of what is now known as Parkinson's disease.[2,3] Since its initial description, the understanding of PD has evolved into it being a complex neurological condition, encompassing a myriad of motor and non-motor symptoms that significantly impact patients' lives.

James Parkinson's description of the shaking palsy in 1817 primarily focused on its characteristic motor signs, such as tremors, slowness of movement, muscle stiffness, and difficulties with balance.[4] However, as our understanding of the disease has deepened over time, it has become evident that Parkinson's encompasses more than just these motor impairments. Individuals with Parkinson's also experience a range of non-motor symptoms, including sleep disturbances, loss of sense of smell, and cognitive issues like memory problems and difficulty with thinking and reasoning.[5]

At the biological level, PD is marked by the loss of dopaminergic neurons in the *Substantia nigra pars compacta* (SNc), a region of the brain that connects to the striatum via the nigrostriatal pathway.[6,7] This neuronal loss leads to a substantial reduction in striatal dopamine (DA) levels, which disrupts signaling in the direct and indirect motor pathways. Under normal conditions, DA facilitates movement by activating D1 receptors in the direct pathway to promote motor activity and by binding to D2 receptors in the indirect pathway, where it inhibits the pathway's activity to prevent excessive suppression of movement.[8,9] In PD, the degeneration of SNc neurons reduces these striatal projections, leading to decreased motor output, ultimately impairing voluntary movement initiation.[8,9]

1.1.1 Pathological Hallmarks of Parkinson's Disease

Dopaminergic neuron loss in the SNc results in a distinct and visible pathological hallmark in PD: the reduction of dark-colored neuromelanin (NM) in the substantia nigra (SN). NM is a pigment primarily found in the SN of the human brain and forms as a byproduct of DA auto-oxidation.[10–13]

Many cases of PD, including both sporadic and familial forms, are also characterized by the presence of inclusions, i.e., Lewy bodies, which are intracellular inclusions primarily composed of misfolded and aggregated alpha-synuclein proteins.[14] As the disease progresses, these inclusions can be typically seen in a progressive manner throughout the central nervous system, affecting various brain regions, such as the olfactory bulb, brainstem, and cerebral cortex.[14,15] The involvement of these areas is believed to contribute to non-motor symptoms of PD, such as anosmia (loss of sense of smell) and cognitive impairment.[16–18]

However, it is important to note that Lewy body pathology is not present in all forms of PD. For instance, parkin-linked PD, an autosomal recessive form of early-onset Parkinson's disease (EOPD), only rarely exhibits Lewy body formation visible in surviving neurons at autopsy.[19–21]

1.1.2 Dopamine Metabolism & Receptor Types

It is well established that DA plays a crucial role in PD, as its deficiency is a key factor underlying the disease's motor symptoms. Understanding DA metabolism is essential to grasp the mechanisms contributing to PD pathology. DA metabolism in dopaminergic cells of the brain

involves a series of enzymatically regulated pathways that ensure proper synthesis, function, and breakdown of DA. DA is synthesized from the amino acid tyrosine, which is taken up by dopaminergic neurons and hydroxylated by the enzyme tyrosine hydroxylase (TH) to form L-DOPA (levodopa). L-DOPA is subsequently decarboxylated by aromatic L-amino acid decarboxylase (AADC) to form DA.[22] Once synthesized, DA is stored in vesicles by the vesicular monoamine transporter-2 (VMAT2) for release into the synaptic cleft upon neuronal firing and membrane fusion.[23,24] After its release, DA interacts with postsynaptic receptors to exert its physiological effects, primarily through dopamine receptors D1 and D2, which mediate various functions such as motor control, reward processing, and mood regulation.[25–27]

Upon its release, DA exerts its effects in the brain through five distinct receptors, classified into two families: the D1 receptor family (D1, D5) and the D2 receptor family (D2, D3, D4).[28,29] These G protein-coupled receptors (GPCRs) regulate various physiological processes, including motor control, cognition, and reward signaling.[28,29] D1 family receptors primarily activate adenylyl cyclase, increasing cyclic AMP (cAMP) levels and promoting neuronal excitability.[28,29] D1 receptors are highly expressed in the striatum and cortex, where they contribute to motor function and executive processes.[30,31] While D5 receptors are expressed at much lower levels in the brain, they have been found in areas such as the hippocampus and hypothalamus.[30,31] In contrast, D2 family receptors inhibit adenylyl cyclase, reducing cAMP levels and modulating neurotransmitter release.[28,29] D2 receptors are abundant in the striatum, SNc, hypothalamus, and ventral tegmental area (VTA), where they influence motor control and reward functions.[30,31] D3 receptors are primarily localized in the *nucleus accumbens*, with additional expression in the SNc and VTA. D4 receptors are distributed across the frontal cortex, amygdala, hippocampus, and hypothalamus.[30,31] All these receptors function within key dopaminergic pathways, including the nigrostriatal,

mesolimbic, mesocortical, and tuberoinfundibular systems.[32] The nigrostriatal pathway, which connects the SNc to the striatum, is particularly relevant to PD, as its degeneration leads to the classical motor deficits.[7,32]

Within this pathway, DA signaling regulates movement through two opposing circuits: the direct and indirect pathways of the basal ganglia.[33] The direct pathway, which facilitates movement, is driven by D1 receptor activation in the striatum, leading to excitatory signals that promote motor activity.[33] In contrast, the indirect pathway, which suppresses movement, is modulated by D2 receptors, where DA binding reduces inhibitory output to downstream structures, refining motor control.[33] By balancing these pathways, DA plays a critical role in coordinated movement, and disruption of this equilibrium due to dopaminergic neurodegeneration contributes to the motor symptoms observed in PD.[33]

Meanwhile, the mesolimbic and mesocortical pathways regulate reward and cognitive functions, with dysregulation observed in conditions such as addiction and schizophrenia.[32] Together, dopamine receptors play a crucial role in maintaining the balance of neural activity required for movement, motivation, and higher-order cognitive functions.

The regulation of DA levels is tightly controlled by its reuptake and metabolism. The dopamine transporter (DAT) plays a crucial role in the reuptake of DA from the synaptic cleft back into the presynaptic neuron.[34,35] Within the neuron, DA can also be metabolized by two primary pathways: enzymatic breakdown by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT).[35] MAO, also present in the mitochondrial outer membrane, catalyzes the oxidative deamination of DA to form the aldehyde, 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further converted by aldehyde dehydrogenase (ALDH) to the less toxic compound 3,4-dihydroxyphenylacetic acid (DOPAC).[36,37] Alternatively, DA can be methylated by

COMT to form 3-methoxytyramine.[37,38] Both DOPAC and 3-methoxytyramine are subsequently metabolized to homovanillic acid (HVA) by COMT and MAO, respectively.[35,39] Once formed, HVA is transported out of the brain into the cerebrospinal fluid and bloodstream, where it is ultimately filtered by the kidneys and excreted in the urine.[35,40] Due to its role in DA metabolism, HVA was among the first biochemical markers investigated for PD, with early studies reporting reduced HVA levels in the CSF of drug-naïve PD patients.[41,42]

1.1.3 Neuromelanin Formation

Neuromelanin (NM) formation involves a complex series of biochemical processes primarily related to the metabolism of DA. Unlike other biological pigments, NM is not synthesized through a specific enzymatic pathway but rather results from the oxidative polymerization of DA and its metabolites.[43,44] NM formation in the midbrain is rather specific to human ageing, with aged non-human primates rarely demonstrating it in post mortem studies.[45]

The first step in NM synthesis involves the re-uptake of DA into dopaminergic neurons. When excess DA accumulates in the cytoplasm and is not stored in vesicles or metabolized, it becomes susceptible to oxidation.[10,43] DA can be oxidized by reactive oxygen species (ROS) or by enzymes like MAO, leading to the formation of highly reactive intermediates, including DA-quinones.[39] These quinones undergo non-enzymatic polymerization, interacting with other DA molecules, proteins, and lipids to form oligomers and eventually the polymeric structure of NM.[44] As NM is formed, the pigment is stored within lysosomes, which are involved in both the sequestration of toxic intermediates and the degradation of cellular components.[44,46] Lysosomes are thought to provide a compartment to sequester the highly reactive dopamine metabolites and iron, preventing their accumulation in the cytoplasm.[44,46,47]

Additionally, the process of NM formation is enhanced by the presence of metal ions, such as iron and copper, which catalyze the oxidation of DA and contribute to the accumulation of the pigment.[44,48] Iron is thought to play a particularly significant role, as NM contains a high concentration of iron, which is tightly bound within the pigment structure.[10,49,50] One key mechanism by which iron contributes to NM formation is through the Fenton reaction, where Fe^{2+} catalyzes the production of highly reactive hydroxyl radicals (•OH) from hydrogen peroxide (H_2O_2).[51] These radicals promote the oxidation of DA and its metabolites, leading to the formation of quinones and semiquinones, which can polymerize into NM.[52] This reaction not only accelerates DA oxidation but also amplifies oxidative stress within dopaminergic neurons, further driving pigment accumulation.[52,53] Given that NM has a high affinity for iron, it may act as a protective reservoir by sequestering excess iron and limiting the availability of free Fe^{2+}, thereby modulating redox activity in the SNc.[52,53]

That being said, the exact role of NM in brain physiology remains somewhat unclear. NM accumulation is generally thought to serve as a protective mechanism for neurons by sequestering excess DA and its toxic intermediates, preventing them from causing cellular damage.[10,43,44,54] However, its accumulation can be a double-edged sword. While it may serve as a protective agent by binding toxic molecules, its buildup over time, especially in the presence of oxidative stress, have also been considered to contribute to the pathogenesis of PD.[43,45,54] For example, when released extracellularly from dying neurons, neuromelanin can trigger a microglial and histiocytic response aimed at clearing the pigment, and this process may inadvertently lead to further neuronal loss in the SNc.[45,55]

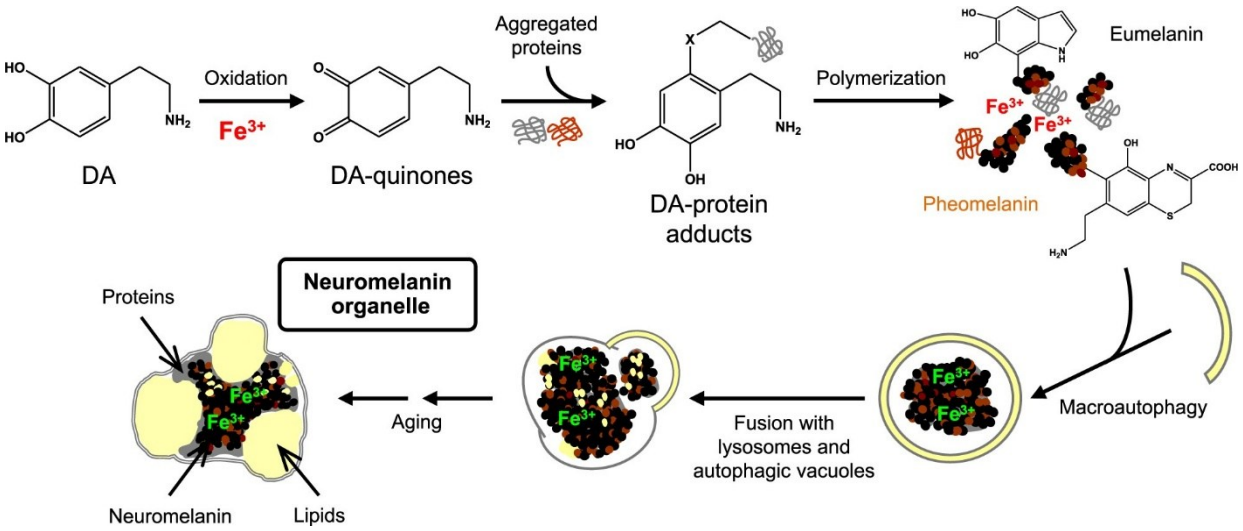

Figure 1.1. Neuromelanin Synthesis

Schematic representation of neuromelanin (NM) formation. Dopamine (DA) undergoes oxidation, facilitated by iron (Fe^{3+}), leading to the formation of DA quinones. These reactive quinones can form adducts with proteins, which subsequently aggregate and polymerize into melanin. NM accumulates within a specialized organelle, where it associates with proteins, lipids, and Fe^{3+}. With aging, NM-containing organelles fuse with lysosomes and autophagic vacuoles.[44] Modified from Sulzer et al. 2018.

1.1.4 (Consequences of) Dopamine Toxicity

To elaborate, DA homeostasis is tightly regulated to ensure proper neurotransmission while minimizing cellular toxicity. As mentioned earlier, under normal physiological conditions, DA is stored in synaptic vesicles by VMAT2 and is efficiently recycled through reuptake by the DAT after synaptic release.[23,24,34,35] This vesicular storage system prevents excess DA from accumulating in the cytoplasm, thereby limiting its exposure to oxidative degradation.[56–58] However, when intracellular DA levels exceed the capacity of vesicular storage—DA can accumulate in the cytosol, where its inherent instability leads to its auto-oxidation.[56,59,60] Especially in the presence of oxygen and metal ions like iron and copper, which speed up this process.[61] This leads to the formation of toxic byproducts, such as DA quinones, that can damage cells.[61,62] DA quinones are highly reactive molecules that can attach to proteins and lipids in cells, disrupting their normal function and causing protein clumping.[63–65] Additionally, when DA is oxidized, it can create hydrogen peroxide (H_2O_2), which, in the presence of iron, turns into harmful hydroxyl radicals.[63] These radicals can damage cell membranes, DNA, and mitochondria, leading to further problems.[64,66,67] Mitochondria, which are responsible for producing energy, are also affected, causing energy shortages and cell death.[64,68] These processes are especially important in diseases like Parkinson's, where DA-producing neurons are particularly vulnerable to oxidative damage due to their high energy demands, extensive arborization and continuous firing pattern for which they rely heavily on mitochondria for energy production.[69,70]

It is for this reason that formation of NM in dopaminergic neurons is thought to be protective, as it sequesters excess DA and its oxidative byproducts, thereby preventing DA toxicity.[10,43,44,54] NM formation effectively removes reactive DA quinones from the cytoplasm, thereby preventing their interaction with cellular components and reducing oxidative stress.[10,43,44,54]

1.1.5 Vulnerability of Dopaminergic Neurons

As can be acknowledged from above, the toxic byproducts of DA auto-oxidation play a significant role in the selective vulnerability of dopaminergic neurons in the SNc. Unlike other dopaminergic populations, such as those in the ventral tegmental area (VTA), SNc neurons are especially vulnerable, not only due to their distinct DA metabolism but also because of additional factors that place a greater physiological burden on them.

Firstly, SNc neurons exhibit unusually high basal activity, characterized by pacemaking firing patterns that require continuous energy production and calcium buffering[71–73]. This persistent activity places a significant burden on mitochondria, which must generate a steady supply of ATP to sustain neuronal function.[71,74] The heightened metabolic demand leads to an increased production of ROS as a byproduct of mitochondrial respiration.[71,75] If not efficiently cleared, ROS can accumulate over time, contributing to oxidative stress and potentially damaging cellular components.[71,75]

In addition to their high metabolic demand, SNc neurons possess extensive and highly branched axonal arbors that project widely into the striatum.[71,73,76] Maintaining these vast axonal networks requires substantial energy and intracellular transport, further straining mitochondrial function. [71,73,76] The complexity of these structures, which play a crucial role in coordinating movement, makes SNc neurons particularly susceptible to degeneration under stress.

Together, these factors—elevated metabolic demand, increased oxidative stress, the energy-intensive maintenance of extensive axonal networks, and the inherent toxicity of DA metabolism—make SNc dopaminergic neurons especially vulnerable to dysfunction and cell

death. This vulnerability is a key consideration, as these neurons are among the primary ones affected in Parkinson's disease.

1.1.6 Current Treatments for Parkinson's Disease

Research indicates that by the time symptoms of PD become apparent, individuals have already experienced a loss of approximately 60% or more of the DA-producing neurons in their SNc.[77] Given this extensive loss of dopaminergic neurons in PD, it is not surprising that many current treatments aim to address DA deficiency in the brain. At present, no available treatments can cure or halt the progression of PD; instead, they focus on alleviating symptoms and improving quality of life.[78,79]

The most effective pharmacological treatment for motor symptoms is a combination of levodopa (L-DOPA) and carbidopa. L-DOPA, a DA precursor capable of crossing the blood-brain barrier, is converted into DA in the brain, thereby compensating for DA loss.[78–80] Carbidopa is co-administered to prevent the premature conversion of L-DOPA to DA in the peripheral nervous system, reducing side effects and increasing its availability in the brain.[78–80] Other pharmacological options include DA agonists, which mimic DA by directly stimulating dopamine receptors.[78–80] These medications are generally less effective than L-DOPA and are often used in early-stage therapy before L-DOPA is introduced.[78–80] Additionally, there are also medications like monoamine oxidase B (MAO-B) inhibitors, which slow the breakdown of DA in the brain, thereby prolonging its effects.[79,80] Despite its efficacy, however, L-DOPA treatment has limitations. Short-term side effects include nausea, vomiting, hypotension, and dizziness.[81] Long-term use is associated with motor fluctuations, where symptom relief becomes less consistent, and dyskinesia, characterized by involuntary, erratic movements, become more common.[80,81]

In cases where medication becomes less effective or causes significant side effects, surgical interventions such as deep brain stimulation (DBS) may be considered.[79,82] DBS is typically recommended for individuals experiencing severe and frequent motor fluctuations and dyskinesia.[82] This procedure involves implanting electrodes into regions such as the subthalamic nucleus to modulate abnormal neural activity, ultimately improving motor function.[82] Notably, DBS enhances the effects of L-DOPA and is most effective in patients who still respond to L-DOPA treatment.[80,82]

Beyond medical and surgical therapies, non-pharmacological approaches play a critical role in symptom management. Routine physical therapy is highly beneficial for maintaining mobility, balance, and overall motor function.[79,83] Regular exercise has been shown to enhance stability, improve motor control, and reduce tremors.[79,83]

1.1.7 Different Forms of Parkinson's Disease

Despite extensive research efforts, the exact causes of typical PD remain elusive. However, scientists have identified various factors that may contribute to its development. Environmental factors, such as exposure to pesticides, air pollution, and heavy metals, have been associated with an increased risk of sporadic PD.[84,85] Sporadic PD, which accounts for approximately 90% of cases, is believed to arise from a combination of genetic predisposition, environmental exposures, and aging.[85,86] Other potential risk factors include traumatic brain injuries, and rural living (possibly due to pesticide exposure).[85,86] While no single environmental factor has been conclusively identified as a direct cause, their cumulative impact over time likely plays a role in disease onset.

Genetic forms of PD account for less than 10% of cases.[87,88] Several genes have been implicated in hereditary PD, with mutations in Parkin, DJ-1, and PTEN-induced putative kinase 1 (PINK1) primarily associated with early-onset, autosomal recessive PD.[87,88] In contrast, mutations in leucine-rich repeat kinase 2 (LRRK2) and α-synuclein (SNCA) are linked to autosomal dominant inheritance.[87,88] Though they account for a smaller fraction of cases, these genetic variations are crucial for understanding the molecular pathways that may be leading to PD. By studying how these mutations affect brain function, we can uncover potential therapeutic targets and possibly develop more effective treatments.

1.1.8 *PRKN*-Linked Parkinson's Disease

Autosomal recessive mutations in parkin encoded by *PRKN* (at the PARK2 locus), are strongly linked to early-onset Parkinson's disease (EOPD).[87–90] Parkin-associated EOPD presents with the typical hallmark motor symptoms of PD, including slowness of movement (bradykinesia), muscle stiffness (rigidity), and tremors at rest.[89] Symptoms usually emerge around the age of 30 years, although cases have been documented in individuals as young as three and as old as 81 years.[89] The condition progresses slowly, with some patients living for more than five decades following diagnosis.[89] Clinical presentations can vary, but hyperreflexia (increased reflex responses) is often observed.[89] Dystonia, particularly affecting the lower limbs, is frequently one of the first signs.[89] Unlike other forms of PD, however, parkin-linked PD is not strongly associated with an increased risk of cognitive decline, which may be due to differences in the underlying pathophysiology compared to other Parkinson's variants.[89,91–93] In parkin-linked PD, there is a specific loss of dopaminergic neurons from the SNc and the *Locus coeruleus* (LC), which distinguishes it from other forms of PD.[93,94] Unlike typical idiopathic PD, where the

neurodegeneration also affects the SNc and is often accompanied by Lewy body pathology, parkin-linked PD does not involve the widespread aggregation of alpha-synuclein or the formation of Lewy bodies.[93,94] This selective neuronal loss suggests that parkin may have a protective role in maintaining the integrity of these specific dopaminergic pathways, but the underlying mechanisms remain to be fully understood.

1.2 Introduction to Parkin

The PARK2 locus, located on chromosome 6q25.2–q27, encodes parkin, an E3 ubiquitin ligase involved in protein quality control, mitochondrial maintenance, and cellular stress responses.[95–97] As one of the largest human genes, PARK2 spans approximately 1.38 Mb and consists of 12 exons.[95,97] The parkin protein (~52 kDa) is primarily expressed in metabolically active tissues, such as the brain, muscles, heart, and testes,[95,98] likely due to its role in maintaining mitochondrial health and facilitating mitochondrial cleanup through mitophagy.[98] In these tissues, parkin regulates protein degradation via the ubiquitin-proteasome system and promotes the clearance of damaged mitochondria.[98]

More than 120 mutations associated with PD have been identified in parkin, distributed across its various domains.[99] Parkin mutations associated with PD affect the protein in various ways. Some mutations disrupt the protein's ability to fold properly, while others interfere with enzyme activity or binding to essential molecules.[99] Certain mutations impact key structural components, while others may alter interactions with partner proteins.[99] These changes can compromise parkin's role in maintaining cellular health.

Structurally, parkin belongs to the RBR (RING-between-RING) family of E3 ubiquitin ligases and is composed of multiple functional domains that enable its precise regulation and interaction with target proteins.[99–101] The N-terminal ubiquitin-like (Ubl) domain (residues 1–76) regulates substrate binding by inhibiting parkin's E3 ligase activity and maintaining its inactive state by blocking RING1.[99,101,102] The Ubl domain releases from the RING1 domain upon activation, allowing parkin to bind substrates and facilitate ubiquitin transfer.[99,101,102] Adjacent to the Ubl domain, the RING0 domain also contributes to maintaining an autoinhibited conformation in the absence of cellular stress by blocking access to RING2.[99,101,102] The RING1 domain contains a zinc-finger motif required for E2 enzyme recruitment, while the In-Between-RING (IBR) domain serves as a structural bridge, stabilizing the protein's conformation.[99,101–103] The C-terminal RING2 domain harbors the catalytic cysteine residue (Cys431) that directly mediates ubiquitin transfer onto substrates.[99,101,102] Parkin's activity is tightly regulated through these domains, ensuring it remains inactive under normal conditions to prevent unnecessary protein degradation.

1.2.1 Parkin as an E3 Ligase

Parkin is primarily activated in response to mitochondrial damage through a mechanism involving PINK1,[99,101,102] a concept largely derived from cell culture studies using the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP).[104–107] Under normal physiological conditions, PINK1 is imported into the mitochondria, where it undergoes cleavage by PARL (presenilin-associated rhomboid-like protein), leading to its rapid degradation.[99,102] However, when mitochondria are damaged—in cases such as membrane depolarization or oxidative stress—instead of being cleaved, PINK1 accumulates on the outer mitochondrial membrane, where it phosphorylates both ubiquitin and parkin at Ser65 within the

Ubl domain.[99,101,102] This phosphorylation event induces a conformational change that releases parkin's autoinhibition, allowing it to bind and ubiquitinate mitochondrial outer membrane proteins such as VDAC1 (voltage-dependent anion channel 1) and Mfn2 (mitofusin 2).[99,101,108] These ubiquitination events serve as signals for parkin to clear the damaged mitochondria through a process called mitophagy. This is especially important in dopaminergic neurons from the SNc, where mitochondria face high energy demands, and their failure can lead to the buildup of harmful ROS, contributing to neurodegeneration.[71,75]

However, parkin's E3 ligase functions are not limited to its interaction with PINK1. In addition to its well-characterized role in mitophagy, parkin has also been implicated in cellular protection against unfolded protein stress, a condition that occurs when misfolded or aggregated proteins accumulate, overwhelming the cell's ability to clear them.[109] Studies have shown that unfolded protein stress leads to increased parkin expression at both the mRNA and protein levels, suggesting a role in protein quality control.[109] Notably, overexpression of wild-type (WT) parkin – but not mutant forms lacking E3 ligase activity – suppresses cell death caused by unfolded protein stress, further supporting its protective function.[109] These findings suggest that parkin contributes to cellular health beyond mitophagy by assisting in the clearance of misfolded proteins.

1.2.2 Postulated E3 Substrates of Parkin

Identifying authentic parkin substrates has been a topic of debate due to the variability in experimental models, differences in detection methods, and the fact that parkin can mediate both degradative and non-degradative ubiquitination.[110–112] While several key criteria are commonly used for evaluation, there is no universal standard. Generally, a protein is considered a parkin substrate if it is ubiquitinated by parkin, accumulates in parkin-deficient models (such as parkin-

null mice), and is found in higher levels in the brains of patients with parkin-linked EOPD, linking it to disease pathology.[110–112] Additionally, the substrate should have functional significance, meaning its dysregulation leads to cellular disruptions. However, not all ubiquitination events lead to degradation, further complicating substrate identification.[110–112]

A few proteins have been widely recognized as 'authentic parkin substrates. Aminoacyl-tRNA synthetase interacting multifunctional protein 2 (AIMP2), involved in protein quality control, is polyubiquitinated by parkin and accumulates in parkin-null mice and PD patients.[110,111] Similarly, far upstream element-binding protein 1 (FBP1), a regulator of glucose metabolism, meets key criteria as it also accumulates in parkin-null models and PD brains.[110–112] PARIS (ZNF746), a transcriptional repressor of PGC-1α, regulates mitochondrial biogenesis and has been shown to accumulate in parkin-deficient conditions, directly linking it to mitochondrial dysfunction in PD.[111,112] Mitofusins (Mfns), which regulate mitochondrial fusion, have also been proposed as parkin substrates. Parkin ubiquitinates these proteins to promote mitophagy. While they are not necessarily upregulated in parkin-null conditions, they tend to accumulate in parkin-null models, suggesting a lack of degradation in the absence of parkin.[112,113]

Other potential substrates include Hsp70, a heat shock protein involved in stress responses and phosphorylated by parkin;[110–112] Cyclin E, a cell cycle regulator upregulated in PD brains and sometimes in EOPD brains;[110–112] and Drp1, a mitochondrial fission protein also phosphorylated by parkin.[111–113] These (and other) proteins have been proposed as parkin substrates due to their roles in cellular processes linked to parkin's function. However, though widely recognized, they do not fully meet the criteria for definitive parkin substrates, making their classification a subject of debate.

1.2.3 Abundance of Cysteine Residues in Parkin

Another unique feature about parkin's structure is its abundance in cysteine residues. Human parkin contains 35 cysteine residues, which make up 7.5% of its total amino acids, much higher than the average of 2.3% found in most human proteins.[114,115] Cysteines are sulfur-containing amino acids that play a key role in the structure and function of proteins.[114,116] Because of the thiol group (-SH) in their side chain, cysteines are highly redox-sensitive.[117–119] In simpler terms, this makes cysteine residues reactive in both oxidation (loss of electrons) and reduction (gain of electrons) reactions. The sulfur atom in this group is nucleophilic, meaning it readily donates electrons to other molecules.[117,120,121] Part of the reason sulfur is classified as a nucleophile is due to its larger atomic radius and a higher electron density.[122,123] These characteristics make sulfur less electronegative (less able to "hold onto" the electrons) and thus share its electrons more easily with other atoms or molecules.[122,123] Therefore, under oxidative conditions, cysteine's thiol group would be particularly reactive because the sulfur atom can donate electrons to form bonds with other reactive molecules. These include ROS such as H_2O_2 or superoxide,[115] which can cause cysteine residues to form disulfide bonds (–S–S–),or undergo other modifications like sulfinic (–SO2H) or sulfonic (–SO3H) acid changes under stronger oxidative stress.[117,120,124,125] Cysteines can also react with electrophilic molecules like quinones or carbonyl compounds, which have areas that "want" electrons, such as metal ions or carbonyl groups in proteins.[125–127]

1.2.4 Parkin's Sensitivity to Oxidation

Given the abundance of cysteine residues within the parkin protein, it is not surprising that several studies have shown parkin to be particularly sensitive to oxidative insults and fluctuations in ROS levels. Oxidative modifications can have dual effects on parkin's E3 ubiquitin ligase

activity, leading to both activation and loss of function, depending on the nature and extent of the modification.[128–130]

For example, one study reported that S-nitrosylation—an oxidative modification in which a nitric oxide (NO) group is added to a cysteine thiol (-SH) group—initially enhances the E3 ubiquitin ligase activity of wild-type (WT) parkin but subsequently leads to its inactivation.[128]

Another study found that under treatment of H_2O_2, a common ROS, cysteine residues in parkin were shown to oxidize, leading to activation of its E3 ligase function. However, under conditions of excessive oxidative stress, these modifications became detrimental, ultimately disrupting parkin's function and impairing its role in mitochondrial quality control.[130]

As these studies demonstrate, oxidative alterations profoundly influence parkin's capacity to regulate protein degradation pathways and mitochondrial dynamics, implicating oxidative stress as a pivotal modulator of parkin function in both health and disease.

1.2.5 Loss of Solubility in Parkin Overtime is due to Cysteine Oxidation

It has been well documented that parkin's high cysteine content makes it particularly susceptible to oxidation, especially in relation to its E3 ligase function. While oxidation has often been linked to parkin dysfunction, recent research from the MGS lab suggests that oxidative modifications may also influence parkin's behavior in ways that affect cellular redox balance. Specifically, our lab has found that parkin undergoes an age-dependent loss of solubility in the human brain, potentially as a protective mechanism against oxidative stress and reactive DA metabolites.[131] Over time, oxidation of parkin's cysteine residues leads to its progressive accumulation in an insoluble form.[131]

In a cellular context, protein insolubility refers to a protein's inability to remain dissolved and properly dispersed in the cytoplasm or membrane environment. When cells are lysed and fractionated, soluble proteins stay in the supernatant after centrifugation, whereas insoluble proteins remain in the pellet and require strong detergents or denaturants (e.g., SDS) for extraction. In the case of parkin, its increasing insolubility appears to stem from covalent crosslinking within the protein, particularly through disulfide bond formation between cysteine residues and other oxidative modifications.[131] These covalent interactions would make parkin more rigid and resistant to dissolving, thus leading to its accumulation in an insoluble state.

To investigate this phenomenon, human brain tissue samples from individuals of various ages—including the cortex, substantia nigra, and red nucleus—were fractionated using detergents of increasing strengths. These included Tris-NaCl (TS), Triton X-100 (TX), 2% SDS (SDS), and a final pellet (P) fraction extracted with 30% SDS buffer. This stepwise extraction allowed for the sequential solubilization of parkin based on its biochemical properties. In younger brains, a substantial portion of parkin was found in the TS and TX fractions, indicating that it remained soluble and did not require strong detergents for extraction.[131] In contrast, in older brains, parkin was predominantly found in the SDS and pellet fractions, which require strong detergents to disrupt stable interactions such as oxidized cysteine crosslinks.[131] This suggests that parkin becomes increasingly insoluble with age, likely due to progressive oxidation.[131]

This phenomenon was specific to parkin, as it was not observed in other PD-related proteins such as α-synuclein or other RBR-domain PD proteins like DJ-1, which remained largely soluble across different ages.[131] Additionally, this effect was restricted to the brain, as parkin from spinal cord and muscle tissue remained primarily in the soluble TS and TX fractions, even in older individuals.[131] Lastly, this phenomenon appeared to be unique to humans, as brain samples from

rats, mice, and monkeys showed no age-related decline in parkin solubility.[131] Overall, these findings suggest that age-related modifications—most likely driven by oxidative stress—play a key role in altering parkin's biochemical state.

1.2.5.1 Confirmation of Parkin Oxidation

Further supporting this, H_2O_2 levels in human brain tissue were found to increase with age, while parkin solubility negatively correlated with H_2O_2 concentrations.[131] Therefore, to directly test whether oxidative stress, particularly cysteine oxidation, drives parkin insolubility, our lab treated recombinant parkin (r-parkin) with H_2O_2 and analyzed its cysteine residues using liquid chromatography–tandem mass spectrometry (LC-MS/MS). A technique called thiol fingerprinting was employed, where reduced cysteines were labeled with iodoacetamide (IAA), while oxidized cysteines were labeled with N-ethylmaleimide (NEM). Initial analysis (prior to H_2O_2 treatment) revealed that most of parkin's cysteines were in a reduced state.[131] However, following H_2O_2 treatment, a significant increase in oxidized cysteines was observed, indicating that oxidation altered parkin's structural state.[131] Notably, the number of oxidized cysteines increased in a dose-dependent manner with higher H_2O_2 concentrations.[131]

Further analysis revealed that oxidation was directly linked to parkin's loss of solubility.[131] Comparing the soluble and insoluble fractions after H_2O_2 treatment showed that oxidized cysteines were predominantly found in the insoluble fraction, while fewer reduced cysteines remained.[131] This indicates that oxidation disrupts parkin's biochemical properties, leading to its progressive aggregation and insolubility over time.

They also performed further studies in which they found that exposing WT mouse brains to H_2O_2 ex vivo significantly reduced soluble parkin levels while increasing its insolubility.[131] At the molecular level, this loss of solubility was attributed to posttranslational oxidation of parkin's

cysteine residues.[131] Collectively, these findings suggest that oxidative modifications at cysteine residues underlie the age-dependent decline in parkin solubility, potentially impairing its neuroprotective functions.

1.2.6 Parkin Cysteine Oxidation Reduces H_2O_2 Levels

It is now well established that parkin undergoes oxidation, primarily at its cysteine residues, in the brain. However, this raises an important question: what effect does this have on ROS, specifically H_2O_2? Through various experiments, our lab had discovered that parkin reduces H_2O_2 levels through a redox mechanism.[131] This process follows a typical redox reaction, where parkin donates electrons to H_2O_2, reducing it while itself becoming oxidized.[131] The key players in this reaction are parkin's thiol (-SH) groups, which serve as electron donors. When parkin is exposed to H_2O_2, its thiols undergo oxidation, and in doing so, they facilitate the reduction of H_2O_2, thereby lowering oxidative stress in the environment.[131]

Because the ability of parkin to carry out its antioxidant function is heavily reliant on the integrity of its cysteine thiol groups, to better understand parkin's antioxidant function, experimental treatments that chemically modify or block these thiols were used. Parkin was pre-treated with NEM, which reacts with the thiol groups, preventing their ability to donate electrons, as well as with IAA, which can also block the thiol groups by forming covalent bonds with them. Both treatments significantly impaired parkin's ability to reduce H_2O_2, confirming that the antioxidant activity of parkin is indeed driven by the reactivity of its thiol groups.[131]

Beyond in-vitro studies, evidence from in-vivo models further supports parkin's role in redox regulation. When comparing H_2O_2 levels in the brains of WT vs parkin-deficient ($prkn^{-/-}$) mice that were put under induced oxidative stress—such as exposure to the neurotoxin MPTP—

prkn$^{-/-}$ mice exhibited significantly elevated H_2O_2 levels compared to WT controls.[131] This indicates that parkin plays a protective role in maintaining redox balance, particularly in response to oxidative challenges.

These findings align with observations in human post-mortem brain samples, where individuals with PRKN gene mutations showed increased cortical H_2O_2 levels.[131] All in all, these findings further support the idea that parkin contributes to ROS regulation in the brain. The heightened oxidative stress observed in parkin-deficient models[131] highlights the importance of parkin's antioxidant function in cellular defense, with potential implications for neurodegenerative diseases when this function is compromised.

1.2.7 Parkin Cysteines Neutralize DA Radicals

DA metabolism, particularly in dopaminergic neurons, generates a variety of reactive species, including DA-derived radicals that can be highly toxic to cells. [61,62,132,133] These radicals are formed through the oxidation of DA, producing reactive metabolites such as DA quinone, aminochrome, and other indole derivatives.[132,133] If not efficiently neutralized, these radicals can cause significant cellular damage. Therefore, maintaining a delicate balance in the brain's redox environment is crucial. Our lab has shown that parkin may be playing an important role in protecting cells by neutralizing these DA radicals through the modification of its cysteine thiol groups.[131]

In experiments conducted by our lab, exposure to DA metabolites, particularly aminochrome, led to the covalent conjugation of parkin with these reactive species.[131] This interaction caused changes in parkin's solubility and the formation of high-molecular-weight

(HMW) species, indicating that DA radicals were effectively bound and sequestered by parkin.[131] The thiol groups within parkin's cysteine residues are key to this protective mechanism. Using advanced LC–MS/MS, the MGS lab identified specific cysteine residues involved in these modifications. Among them, Cys95 was the most frequently modified, highlighting its critical role in parkin's ability to neutralize dopamine radicals.[131] Other cysteines, such as Cys166, Cys169, Cys182, and Cys238, were also modified, albeit to a lesser extent.[131] These findings suggest that, beyond reducing ROS levels, parkin may also contribute to lowering reactive electrophilic species (RES) in the brain by neutralizing DA radicals. By donating electrons and forming covalent bonds with these reactive species, parkin may be preventing oxidative stress propagation.

1.2.8 Parkin Insolubility and the Loss of its E3 Ligase Activity

While parkin is widely recognized for its role as an E3 ubiquitin ligase, evidence indicates that oxidative stress—particularly from DA-derived metabolites—can lead to the functional inactivation of parkin's enzymatic activity. A study by LaVoie et al. (2005) demonstrated that when purified parkin was exposed to DA quinones, it became insoluble and lost its ability to function as an E3 ligase *in vitro*.[134] Specifically, it could no longer ubiquitinate a putative substrate, Pael-R, or itself.[134] This showed that parkin's enzymatic activity was blocked by direct chemical modification, such as thiol oxidation. Importantly, these changes were specific to parkin—other cysteine-rich E3 ligases such as HHARI, as well as Parkinson's disease–linked proteins like DJ-1 and UCH-L1, did not appear to be as sensitive to oxidation.[134]

Additionally, although parkin's E3 ligase activity becomes compromised through oxidative modification and insolubility, parkin still appears to confer protection to cells through a separate function. Cells overexpressing parkin were resistant to DA-induced toxicity, even when its ligase

activity was presumably lost.[134] This implies that parkin has an additional role—independent of its ubiquitin ligase activity—in protecting neurons from stress. Work from our lab supports this idea,[131] proposing that parkin may engage in direct redox interactions with reactive species, including DA metabolites.

Taken together, these findings suggested to us that a rise in oxidative stress can inactivate parkin's E3 ligase activity by driving it into an insoluble state. Yet, parkin still seems to confer cytoprotection through an alternative, non-enzymatic mechanism that may be especially relevant under conditions of DA metabolite-induced stress (and or related redox changes).

1.2.9 Parkin's Involvement in Glutathione Regulation

Given that parkin has been extensively shown to reduce oxidative stress, our lab began to question how this occurs at a mechanistic level. Since dopaminergic neurons in the SNc are particularly vulnerable to oxidative stress due to DA metabolism, our lab was interested in exploring whether parkin plays a role in antioxidant defense systems. One key component of cellular redox regulation is glutathione (GSH), a critical antioxidant that protects against oxidative damage.[135–137] This led to the hypothesis that parkin may be involved in glutathione metabolism, specifically in GSH recycling and redox homeostasis.

1.2.9.1 Glutathione Homeostasis and Its Importance in Neuronal Health

Glutathione is a tripeptide composed of γ-glutamate, cysteine, and glycine residues, and it exists in two forms: reduced GSH and oxidized glutathione (GSSG). The balance between these forms, represented by the GSH:GSSG ratio, is a key indicator of redox homeostasis.[135,136] A higher

GSH:GSSG ratio reflects a more reduced environment, which is protective against oxidative stress, while a lower ratio indicates an oxidative shift, making cells more susceptible to damage.[135]

GSH is a key part of the broader thiol network, a system of sulfur-containing molecules that help maintain cellular redox balance and regulate protein function.[138] The thiol network consists of free thiols (such as GSH and cysteine), protein thiols (cysteine residues within proteins), and redox-regulating enzymes like thioredoxin (Trx) and peroxiredoxin (Prx).[138] These molecules undergo reversible oxidation and reduction, allowing cells to sense and respond to oxidative stress.[138]

Under normal conditions, thiols exist in their reduced (-SH) form, which helps protect proteins and maintain normal cell function.[139] However, when oxidative stress increases, thiols can be oxidized to form disulfide bonds (-S-S-) or attach to GSH (S-glutathionylation). This reversible modification serves as both a protective mechanism and a redox signal. S-glutathionylation prevents the irreversible oxidation of critical cysteine residues while also modulating protein function—some proteins become inactivated, while others are activated in response to oxidative stress.[140,141] When oxidative stress subsides, glutaredoxin (Grx) removes GSH from proteins, restoring them to their active state. This allows proteins to regain their original function, ensuring that the cell can return to homeostasis.[140,141]

The thiol network is closely linked to mitochondrial function through its interaction with NADH and NADPH, two key molecules that influence energy production and antioxidant defenses.[142,143] NADH primarily fuels ATP generation by donating electrons to the electron transport chain (ETC), while NADPH provides reducing power for antioxidant enzymes like glutathione reductase (GR) and thioredoxin reductase (TrxR).[142,143] These enzymes use NADPH to regenerate GSH and Trx, which in turn help neutralize oxidative damage.[142,143]

This continuous cycle of thiol oxidation and reduction, regulated by NADH and NADPH, allows mitochondria to adapt to oxidative stress while maintaining ATP production. Given that dopaminergic neurons rely heavily on mitochondrial function[144], disruptions in the GSH-thiol-NADPH system may contribute to mitochondrial dysfunction and increased oxidative damage in Parkinson's disease (PD). Especially considering that disruptions in GSH homeostasis have been directly linked to neurodegeneration, and GSH depletion is one of the earliest biochemical changes observed in PD.[137] This highlights the importance of understanding how GSH metabolism is regulated and whether parkin contributes to maintaining a favorable redox environment in neurons.

1.2.9.2 Parkin's Role in Glutathione Metabolism

Recent findings indicate that parkin plays a direct role in glutathione homeostasis, independent of its E3 ubiquitin ligase activity.[145] In a study by El Kodsi et al. (2023), parkin-deficient (*prkn*−/−) mice as well as EOPD brain homogenates exhibited an altered GSH:GSSG ratio, suggesting that parkin may regulate glutathione recycling. Interestingly, brains from parkin-deficient mice and *PRKN*-linked PD patients showed an increased GSH:GSSG ratio, which was attributed to upregulated glutathione reductase (GR) activity. This suggests that in the absence of parkin, cells compensate by increasing GR activity to maintain GSH levels, possibly as a response to increased oxidative stress.[145]

Beyond regulating GR activity, parkin itself appears to participate in GSH metabolism directly.[145] In vitro studies demonstrated that parkin could reduce GSSG back to GSH via its cysteine thiols (such as Cys59, Cys95, Cys377), effectively functioning as a redox enzyme.[145] During this process, parkin undergoes S-glutathionylation, a reversible posttranslational modification that may regulate its function in redox balance. These effects were observed in mouse

brain tissue, human brain samples, and multiple cell models (SH-SY5Y, HEK293, and CHO cells), supporting a conserved role for parkin in GSH metabolism across different systems.[145]

Overall, these findings suggest that parkin contributes to redox homeostasis at multiple levels – not only through its previously characterized roles in mitochondrial quality control but also by directly influencing cytosolic antioxidant pathways. Whether parkin's effects on GSH metabolism are a primary function or a compensatory mechanism in response to oxidative stress remains to be fully elucidated. However, the ability of parkin to participate in GSH recycling and undergo reversible S-glutathionylation suggests that its role in cellular redox balance extends beyond proteostasis and mitochondrial health.

1.2.10 Parkin and its Relationship with Neuromelanin

As mentioned earlier, NM is the dark pigment found in DA-producing neurons of the human midbrain. It forms through the oxidation and polymerization of DA, a process that can occur spontaneously over time but is also influenced by cellular factors such as metal ions and oxidative conditions. [10,43,44] While the exact role of NM remains unclear, it is thought to serve as a protective mechanism in the human brain, helping to sequester toxic molecules. [10,43,44,54] With this in mind, and considering parkin's role in reducing oxidative stress and neutralizing DA radicals, our lab also investigated parkin's involvement in NM synthesis.

Aminochrome is a reactive DA oxidation product formed during the metabolism of DA. When incubated in test tubes, it can auto-oxidize, undergo a series of reactions, and polymerize to form NM-like pigment.[131] Our studies have shown that WT r-parkin, when incubated with aminochrome in-vitro, enhanced melanin formation in a protein concentration- and time-

dependent manner.[131] This effect appears to be specific to parkin, as other proteins, such as DJ-1 and BSA, did not influence melanin formation under the same conditions.[131] These findings indicate that parkin may actively participate in NM synthesis, potentially by interacting with DA-derived intermediates, to more effectively neutralize DA radicals. To elaborate, when parkin is introduced, it may be helping stabilize these intermediate compounds or facilitate the polymerization process via its cysteine thiol groups, ultimately leading to the formation of melanin. Given that NM accumulates with age and may sequester potentially toxic DA oxidation products, parkin's role in accelerating its formation could have significant implications for neuronal health. By influencing DA polymerization into melanin, parkin may be helping mitigate oxidative stress.

Moreover, in human brain sections from non-Parkinsonian individuals, the Schlossmacher lab found that parkin is present in NM-rich neurons in the SN.[131] Using parkin monoclonal antibodies, more signal was detected in older individuals, where NM levels were higher. In younger brains, where NM was less abundant, parkin levels were also lower.[131] This further supports the idea that parkin may be aiding NM in sequestering toxic DA metabolites in the human brain.

Finally, further analysis of the same non-Parkinsonian brain sections revealed that parkin was closely associated with CD63, a marker for lysosomes, within NM granules of nigral neurons.[131] In fact, using confocal microscopy, it was observed that both NM and parkin signals were often found surrounded by small circular CD63 signals.[131] This suggests that not only may parkin be helping NM sequester toxic metabolites, but that lysosomal activity could also be involved in this process. However, while these findings point to a possible role for parkin in interacting with lysosomes to mitigate dopamine radical-induced stress, it is important to note that this connection is not yet fully confirmed and warrants further investigation.

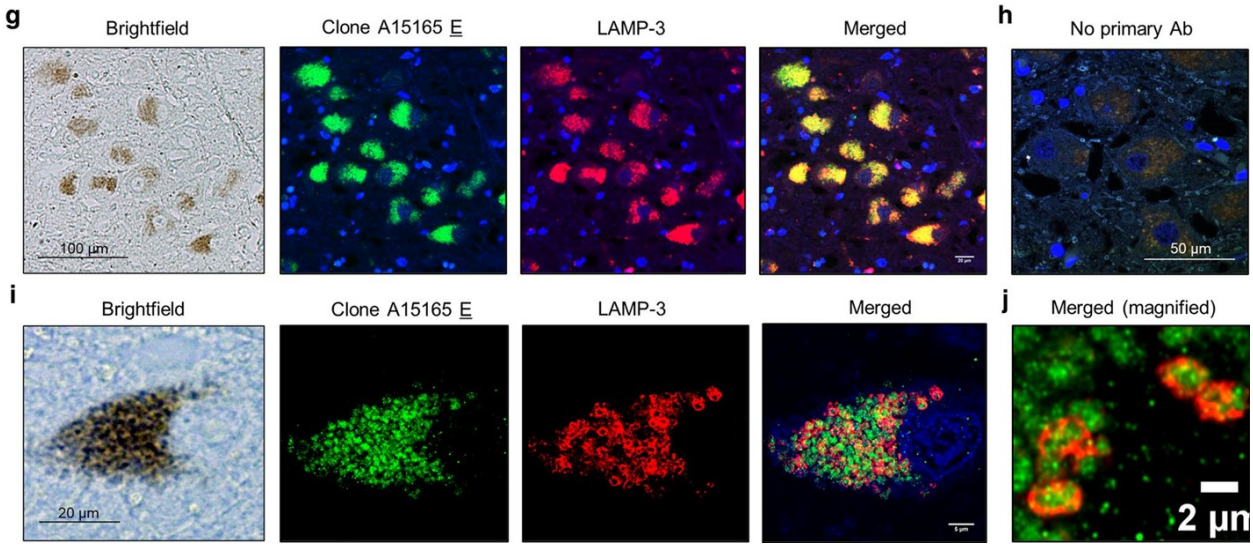

Figure 1.2. Parkin localizes to neuromelanin pigment in SNc neurons of normal human midbrain

(g, i) Brightfield image shows neuromelanin-containing neurons in the substantia nigra pars compacta (SNc). Immunofluorescence staining reveals Parkin (green, Clone A15165 E) colocalizing with the lysosomal marker LAMP-3 (red – also known as CD63) in neuromelanin-rich neurons, as shown in the merged image. (h) Control staining with no primary antibody demonstrates specificity of the immunofluorescence signal.[131] Modified from Tokarew et al. 2021.

2.0 Objectives & Hypotheses

2.1 Summary of Rationale for Thesis Work

The growing recognition of parkin's redox protective capabilities, including its ability to reduce H_2O_2 and neutralize DA-derived radicals, highlights its potential role in protecting dopaminergic neurons from oxidative stress.[131] This is particularly relevant in the context of parkin-linked PD, where it is specifically the DA-synthesizing neurons of the SNc and LC that are selectively vulnerable to oxidative damage due to their high metabolic activity and the byproducts of DA metabolism[71,73]. Together, these observations suggest that parkin's redox protective role is crucial for the survival of these vulnerable neurons throughout adulthood.

For my project, I aimed to understand how DA-induced stress affects parkin as a protein, specifically the toxic DA radicals produced from DA oxidation and the ROS generated during this process. To study this, I will be using in-vitro cell models, which offer the advantage of allowing precise control over experimental conditions and provide a focused environment to study protein-level changes. This approach will enable me to more directly observe how parkin responds to DA stress at the molecular level.

While doing so, I also sought to look into the dynamics between CD63, NM, and parkin to determine whether their relationship is dependent on DA-induced stress. Using M17 neuroblastoma cells, which have a dopaminergic phenotype,[146–148] I decided to employ two different DA stress models. The first model involves directly adding DA to the cell culture media. This is designed to mimic the natural chemical process of DA auto-oxidizing in dopaminergic neurons during DA metabolism. By introducing DA directly to the media, the goal is to simulate oxidative stress in a controlled manner and study parkin’s response. The second model uses tyrosinase, an enzyme that catalyzes melanin formation by converting tyrosine into melanin.

During this process, tyrosinase catalyzes the formation of DA quinones (oxidized DA), ultimately leading to melanin formation within the cell bodies. This model was intended to simulate the formation of neuromelanin in DA neurons and examine how this process impacts parkin.

2.2 Specific Objectives and Hypotheses

Objective & Hypothesis 1

Objective: Investigate the downstream effects of dopamine stress on parkin using an undifferentiated M17 neuroblastoma cell model.

Hypothesis: I postulate that parkin will aggregate into visible foci following exposure to dopamine stress. This aggregation is expected to result from parkin's own oxidation and subsequent insolubility, manifesting as microscopically visible foci.

Objective & Hypothesis 2

Objective: Explore the possible association between parkin and CD63 downstream of dopamine stress in an undifferentiated M17 neuroblastoma cell line.

Hypothesis: I predict that following dopamine stress, there will be increased co-localization between parkin and CD63. This enhanced co-localization would indicate a potential role for CD63 in facilitating parkin's protective function in concert with lysosomes against dopamine-induced stress.

Objective & Hypothesis 3

Objective: Investigate the impact of parkin on neuromelanin production and accumulation in the M17 cell line.

Hypothesis: I hypothesize that parkin will co-localize with neuromelanin in cells and will increase melanin formation and accumulation within M17 cells, as informed by previous in-vitro results.

3.0 Materials & Methods

Cell Lines

Cell Line	Description
BE(2)-M17 (alias: "Parental cells")	(original) M17 neuroblastoma cells
Lvd-BE(2)-M17-PRKN-WT	M17 cells expressing WT parkin upon doxycycline treatment. BE(2)-M17 cells were transduced with a lentivirus expressing doxycycline-inducible WT human parkin. The resulting population is pooled, with individual cells expressing varying levels of untagged, human parkin following induction.
BE(2)-M17-P5	M17 cells stably expressing low levels of WT parkin (no doxycycline required). This is a clonal cell line, with all cells expressing an intermediate level of N-terminally (myc)-tagged, human parkin.
Lv-BE(2)-M17-Empty-Vector	M17 cells stably expressing an empty vector (control for M17 cells stably expressing parkin)

Table 3.1: List of cell lines used in this study. Description of all the cell lines used during experiments.

Antibodies

Antibody	Provider	Species	Clonality	Cat #	Dilution
Primaries					
Anti-parkin	Cell Signaling	Rabbit	Polyclonal	2132S	IF: 1:500 WB: 1:2000
Anti-parkin	Biolegend	Mouse	Monoclonal	(PRK8) 808503	IF: 1:300 WB: 1:1000
Anti-CD63	Santa-Cruz	Mouse	Monoclonal	sc-5275	IF: 1:250
Anti-CD63	Abcam	Rabbit	Monoclonal	ab134045	WB: 1:1000
Anti-tyrosinase	Thermofisher	Mouse	Monoclonal	MA5-14177	IF: 1:500 WB: 1:1000
Anti-tyrosinase	Abcam	Rabbit	Monoclonal	ab170905	IF: 1:500
Anti-TOMM20	Abcam	Rabbit	Monoclonal	ab186735	IF: 1:300

Table 3.2: List of primary antibodies used in this study. Summary of primary antibodies used for immunoblotting and immunofluorescence, including provider, species, clonality, catalog number, and working dilution.

Cell Culture

All BE(2)-M17 human neuroblastoma cells (ATCC CRL-2267) were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12, Wisent Inc., 319-075-CL) supplemented with 10% fetal bovine serum (FBS, Gibco, 12483-020). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. Lvd-BE(2)-M17-PRKN-WT cells were cultured in the same conditions with the addition of 250 µg/mL G418 (Gibco, 10131-035) to select for cells expressing the human *PRKN* cDNA transgene. Cells were passaged every three days at a 1:5 ratio using 75 cm^2 tissue culture flasks. The medium was changed every two days, and cells were discarded after a maximum of 20 passages.

h-Tyrosinase Plasmid Preparation

DH5α E. coli cells (ThermoFisher, 18258012) were transformed with a plasmid containing human tyrosinase cDNA. The cDNA was quantified and assessed for purity using a Nanodrop spectrophotometer with nuclease-free water as a blank. For transformation, 100 µL of thawed DH5α cells were mixed with 1-10 ng of cDNA, incubated on ice for 30 min, heat-shocked at 42°C for 45 sec, and recovered in 0.9 mL of Super Optimal Broth with Catabolite Repression (S.O.C) medium. The suspension was shaken at 225 rpm for 1 hr at 37°C before being plated onto LB-agar containing ampicillin and incubated overnight at 37°C. A single colony was selected and cultured overnight in 4 mL LB + ampicillin at 225 rpm, 37°C. Glycerol stocks were prepared by mixing equal volumes of culture and glycerol, snap-freezing in liquid nitrogen, and storing at -80°C. For

large-scale plasmid preparation, 2-3 mL of the overnight culture was inoculated into 200 mL LB + antibiotic and grown at 225 rpm, 37°C. Bacterial pellets were collected by centrifugation at 4000 x g for 5 min, the supernatant was discarded, and pellets were frozen at -20°C. Plasmid DNA was purified using the Promega PureYield Plasmid Maxiprep Kit (Promega cat # A2392).

Transfection of BE(2)-M17 Cells with Tyrosinase cDNA

BE(2)-M17 cells (both P5 and Lv-Empty-Vector) were seeded in 6- or 12-well plates and allowed to reach 70% confluency in DMEM/F12 medium supplemented with 10% FBS. Cells were then transfected with a human tyrosinase-encoding cDNA plasmid using Lipofectamine 2000 (Invitrogen, 11668027), following the manufacturer's protocol. Plasmid DNA and Lipofectamine 2000 were each diluted in OPTI-MEM and incubated separately for 5 minutes before being combined. The mixture was left at room temperature for 20 minutes to allow complex formation before being added dropwise to the cells. Cells were incubated under standard culture conditions (37°C, 5% CO_2) for 24 hours, after which the transfection medium was replaced with fresh low-glucose DMEM (Gibco, 12320-032) supplemented with 10% FBS. Cells were harvested between 24- and 120-hours post-transfection for downstream analyses.

Immunofluorescence Staining

Coverslip Preparation: 24 hours before seeding, autoclaved and sterile coverslips were coated with Poly-D-Lysine (PDL) (Sigma-Aldrich, P6407-5MG, 5mg in 50mL sterile water) and incubated in a 37˚C incubator. The next day, immediately before plating, the PDL solution was aspirated, and

the coverslips were rinsed 3 times with 1x phosphate-buffered saline (PBS) before being allowed to dry.

Cell Seeding and Fixation: BE(2)-M17 cells were seeded onto the prepared coverslips at 80% confluency in DMEM/F12 cell culture media supplemented with 10% FBS and incubated under standard culture conditions (37°C, 5% CO_2) for 24 hours. After treatments, cells were fixed in cold 4% paraformaldehyde (PFA, diluted from 16% PFA, Electron Microscopy Sciences, 15710) for 10 minutes, followed by 3 washes with PBS.

Permeabilization and Blocking: Fixed cells were permeabilized using 0.2% Triton-X 100 (Sigma-Aldrich, T878-250ML) in 1x PBS for 15 minutes at room temperature. Non-specific binding was blocked by incubating cells in 3% BSA (Sigma-Aldrich, A7906-100G) + 0.1% Tween20 (Sigma-Aldrich, P1379-500ML) in 1x PBS for 30 minutes, again at room temperature.

Primary and Secondary Antibody Staining: Cells were incubated with primary antibodies (see **table 2**) diluted in 1% BSA + 0.1% Tween20 in 1x PBS overnight at 4˚C. The following day, after 3 PBS washes, cells were incubated with fluorophore-conjugated secondary antibodies (in same buffer as for primary antibodies) for 1 hour at room temperature in the dark. Coverslips were washed 3 times with PBS to remove unbound antibodies. The cells were then incubated in NucBlue DAPI stain (1 drop per 1mL of PBS, Invitrogen, R37606) for 10-15 minutes, followed by 3 washes in PBS.

Mounting and Imaging: Coverslips were mounted onto slides using ProLong Glass Antifade Mountant (Invitrogen, P36984) and allowed to set overnight before imaging the next day. Fluorescence images were acquired using either a Leica THUNDER Imager (at the *Cell Biology*

and Image Acquisition Core (RRID: SCR_02184))) or a Zeiss Axio Imager A1, primarily with 40× and 63× objectives, and analyzed using Leica, Zeiss, or Fiji ImageJ software.

Melanin Imaging Using Brightfield Microscopy

Neuromelanin (NM) imaging for Figures 4.7 and 4.8 was performed using a manual imaging method that I developed. This approach utilizes the Zeiss Axio Imager A1 microscope, which is equipped with both brightfield and fluorescence imaging capabilities. First, melanin is imaged in brightfield using a black-and-white setting. Then, without changing the camera, the settings are manually adjusted to switch from brightfield to fluorescence. The brightfield illumination is turned off, and the appropriate fluorescence channels are sequentially activated to capture immunofluorescent staining. Each fluorescence channel is imaged separately, and the resulting images are later merged. To enhance visualization, the look-up table (LUT) of the brightfield image is inverted, converting melanin from black to white and the background from white to black.

Western Blotting

Cell Lysis and Protein Extraction: BE(2)-M17 cells were rinsed in 1x PBS, scraped from wells, and collected in 1.5 mL Eppendorf tubes. Cells were pelleted by centrifugation at 400xg for 4 minutes at 4˚C, the PBS supernatant was discarded, and the cell pellet was stored at -20˚C until lysis. In order to fractionate, cells were first lysed in 2% TritonX-100 in TSS buffer (140 mM NaCl + 50 mM Tris-HCl in ddH_2O) supplemented with protease inhibitors (ThermoFisher, A32963) on ice for 20 minutes. Following centrifugation at 21,000 xg for 30 minutes at 4˚C, the supernatant

was collected as the soluble protein fraction. The remaining pellet was re-lysed in 10% SDS in TSS buffer supplemented with protease inhibitors at room temperature for 20 minutes (to prevent SDS solidification on ice). After centrifugation at 21,000 xg for 30 minutes at 12˚C, the supernatant was collected as the insoluble protein fraction. Total protein concentration for both fractions was determined using a BCA assay (ThermoFisher, PI23227).

SDS-PAGE and Protein Transfer: Equal amounts of protein (10 μg) were mixed with 6x loading buffer (3g SDS, 6mL glycerol, 30mg bromophenol blue, 1.5mL 2.5M Tris-HCl pH 6.8, and 600 mM DTT for reducing conditions) and TSS buffer to make up the final volume. Samples were heated at 95˚C for 5 minutes. The samples were then loaded onto 4-12% gradient SDS-PAGE gels (Invitrogen, NP0336BOX) and separated at 153V for 45–55 minutes. Proteins were transferred onto a membrane using a wet transfer method at 30V for 1 hour.

Blocking and Antibody Incubation: Membranes were stained with Ponceau S (Sigma-Aldrich, P7170-1L) to check for total protein, scanned, and then blocked in 5% milk in TBST for 30 minutes with gentle rocking at room temperature. The membranes were incubated with primary antibodies (diluted in 5% milk in TBST, see **table 2**) overnight at 4˚C with gentle agitation. After three 5-minute washes with TBST the next day, membranes were incubated with secondary antibodies in 5% milk in TBST for 1 hour at room temperature.

Detection: After two 15-minute washes in TBST, membranes were developed using an enhanced chemiluminescent (ECL) detection reagent, (ThermoFisher, 34095) and imaged using a BIO RAD Chemi Doc imaging system.

Masson-Fontana Staining for Melanin Pigment Detection in Cells

The following protocol uses the ab150669 Fontana-Masson Staining Kit, with modifications made from tissue staining to cell-based staining. The Ammoniacal Silver Solution was prepared by mixing ddH_2O (3/4 of the total required volume) with Silver Nitrate Solution (1/4 of the total volume, provided in the kit). Concentrated Ammonium Hydroxide (Sigma-Aldrich, 318612-500ML) was added drop by drop, with gentle swirling after each addition. Initially, the solution turned dark brown, but as Ammonium Hydroxide was added, the solution became transparent with a fine layer of sediment. Once the sediment dissolved, the solution was ready. The freshly prepared solution was placed in a 58–60°C water bath to equilibrate.

Meanwhile, coverslips containing cells were removed from the incubator and rinsed with PBS. Cells were fixed on the coverslips with cold 4% PFA for 10 minutes. After fixation, the coverslips were rinsed three times in PBS, with each wash lasting 5 minutes. Once the Ammoniacal Silver Solution reached the appropriate temperature, the coverslips were incubated in the warmed solution for 30 minutes (as melanin typically stains within 30 minutes).

Following incubation, the coverslips were rinsed in several (5) changes of ddH_2O, followed by a 30-second incubation in 0.2% Gold Chloride Solution (provided in the kit) at room temperature. After this, the coverslips were again rinsed in 5 changes of ddH_2O. The cells were then incubated in 5% Sodium Thiosulfate Solution (from the kit) at room temperature for 2 minutes, followed by 3 changes in regular tap water and then 3 changes in ddH_2O. Next, the coverslips were incubated in Nuclear Fast Red Solution for 2.5-3 minutes (provided in kit). Afterward, the coverslips were rinsed again in 3 changes of regular tap water and then 3 changes of ddH_2O.

To complete the staining procedure, the coverslips were dehydrated by immersing them in three changes of fresh absolute ethanol (Commercial Alcohols, P016EAAN). Finally, the coverslips were mounted in mounting medium onto slides. Once the slides were dry, the edges of the coverslips were sealed with transparent nail polish to prevent slipping.

Dopamine (DA) Treatment of Cells

Lvd-M17-PRKN-WT cells were plated at 75% confluency and allowed to adhere overnight under standard culture conditions (37°C, 5% CO_2). The following day, 50 ng/mL of doxycycline was added to the culture media (DMEM/F12) and incubated for 24 hours. A 100 mM dopamine (DA) stock solution was prepared by dissolving 19 mg of dopamine hydrochloride in 0.067 M potassium phosphate buffer (pH 6). After refreshing the culture medium from DMEM/F12 to OPTI-MEM, the stock solution of 100mM DA was added directly to the media drop-wise in each well to achieve final DA concentrations ranging from 0.25 mM to 2 mM. Cells were incubated under standard culture conditions (37°C, 5% CO_2) for 4 hours, after which they were processed for downstream analyses.

Buthionine Sulfoximine (BSO) Treatment on Cells

Lvd-M17-PRKN-WT cells were plated at 60-65% confluency and allowed to adhere overnight under standard culture conditions (37°C, 5% CO_2). The following day, 50 ng/mL of doxycycline was added to the culture media (DMEM/F12) and incubated for 24 hours. Instead of immediate DA treatment, cells were first pretreated with buthionine sulfoximine (BSO) for 48 hours. A 100 mM BSO stock solution was prepared by dissolving BSO powder in sterile tissue

culture-grade water. The culture media was replaced with OPTI-MEM, and the 100mM stock solution of BSO was added directly to each well drop-wise to achieve a final concentration of 2mM in the wells. After 48 hours, the media was replaced with fresh OPTI-MEM to allow for subsequent DA treatment.

Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP) Treatment of Cells

Lvd-M17-PRKN-WT cells were plated at 75% confluency and allowed to adhere overnight under standard culture conditions (37°C, 5% CO_2). The following day, 50 ng/mL of doxycycline was added to the culture media (DMEM/F12) and incubated for 24 hours. The next day, just prior to the experiment, the culture media was replaced with OPTI-MEM in preparation for treatment with carbonyl cyanide m-chlorophenyl hydrazone (CCCP). To minimize DMSO exposure, CCCP was initially prepared at a high concentration (100 mM) by dissolving CCCP powder (Sigma Aldrich, C2759) in DMSO (Sigma-Aldrich, D2438), allowing for lower volumes to be added to cell culture media. The prepared CCCP was then diluted in OPTI-MEM and added dropwise to the cells to achieve final concentrations ranging from 10–20 μM. Cells were incubated under standard culture conditions (37°C, 5% CO_2) for 15–30 minutes before being processed for downstream analyses.

Dissolving Melanin for Absorbance Plate Read

M17 cells, seeded and transfected in 12-well plates (in duplicate), containing melanin, were scraped from the plates and transferred into 1.5 mL Eppendorf tubes. The cells were lysed using RIPA buffer (supplemented with protease inhibitors), and the supernatant was collected for BCA

assay to determine protein concentration and reserved for future Western blot analysis. The pellet, containing the melanin granules, was resuspended in 50 µL of 5M NaOH with 10% DMSO and incubated at 95°C for 60-90 minutes, allowing the melanin to fully dissolve.

Once the melanin had dissolved, 50 µL of the solution was transferred to a 96-well plate, and absorbance was measured using a plate reader at 405 nm. Each sample was read three times, and the average absorbance value was recorded. To account for differences in cell number and protein content, all absorbance values were adjusted based on their respective protein concentrations, as determined by the BCA assay.

Quantification

Quantitative Analysis of Western Blots

Band and smear intensities were quantified using Fiji (ImageJ) and normalized to their respective Ponceau S stains to control for loading differences. Rectangular regions of equal size were manually drawn around each band or smear to ensure consistency across samples. These regions were aligned evenly across lanes to avoid bias from variations in band shape or background.

For each blot, band intensities in the TXS and SDS extracts visualized by Western blotting were normalized to the mean intensity of bands in the control arms. This normalization approach was used to account for variability across blots.

Qualitative Assessment of Immunofluorescence Images

To examine potential co-localization between parkin and CD63, fluorescent signal overlap was assessed both within individual cells and across treatment conditions. Co-localization between parkin (e.g., red channel) and CD63 (e.g., green) was identified visually as yellow signal where the two channels overlapped.

In each condition, the number of cells showing co-localization was noted. Some cells in the control condition already displayed overlapping parkin and CD63 signals, and while co-localization was still observed after treatment, the number of positive cells did not noticeably increase. Because the degree of co-localization did not appear to change in a treatment-dependent manner, no formal quantification (e.g., overlap or intensity-based analysis) was performed. All images were reviewed across single optical planes as well as in z-stacks to confirm the consistency of signal overlap within the cell volume.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Difference between two datasets were assessed using one-way ANOVA to identify statistical significance. For all samples, a statistical cut-off was set at 0.05. The p-values were represented as *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

4.0 Results

Characterization of Parkin Production in Lvd-BE(2)-M17-PRKN-WT Cell Line

Before proceeding with experiments, it was necessary to confirm that Lvd-BE(2)-M17-PRKN-WT cells produce parkin in response to doxycycline induction and to determine the optimal doxycycline concentration for achieving appropriate wild-type (WT) parkin expression levels. Western blot analysis (Figure 4.1A) and immunofluorescence staining (Figure 4.1B) both confirmed that doxycycline treatment induces parkin expression in Lvd-BE(2)-M17-PRKN-WT cells in a dose-dependent manner, with the immunofluorescence revealing parkin's distribution throughout both the cytosol and nucleus. Based on qualitative observation of immunostained samples, doxycycline treatment resulted in parkin expression in more than 95% of cells. As these cells represent a pooled population, individual cells expressed varying levels of parkin following induction. Based on these findings, all subsequent experiments use a standardized doxycycline concentration of 50 ng/mL to ensure consistent human parkin expression across conditions. This concentration was selected because it produced a level of parkin protein that was comparable to that found in extracts of human brain (by Western blotting).

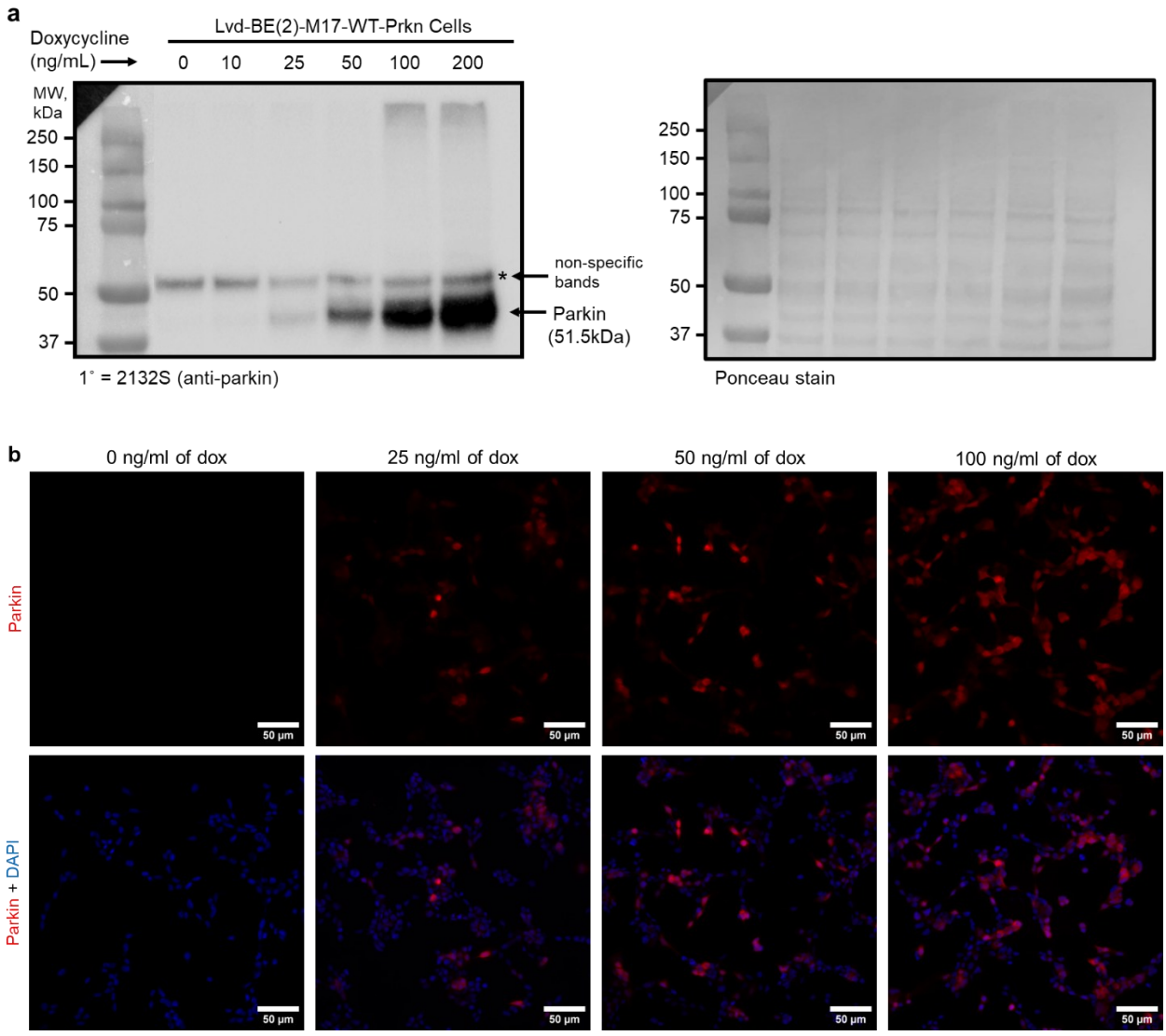

Figure 4.1: Dose-dependent upregulation of parkin expression in Lvd-BE(2)-M17-PRKN-WT cells following doxycycline treatment. Lvd-BE(2)-M17 cells transduced with doxycycline (dox)-inducible wild-type (WT) parkin cDNA show a dox-dependent upregulation of parkin protein expression after 24 hours of dox treatment, as confirmed by both Western blot (A) and immunofluorescence microscopy (B). (A) Western blot analysis was performed using the 2132S anti-Parkin antibody (1:2000 dilution) to detect parkin protein expression. (B) Immunofluorescence microscopy was conducted with the 2132S anti-parkin antibody (1:500 dilution) with nuclei counterstained using DAPI (blue), and images were captured at 40x

magnification using the Leica THUNDER imager. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 50µm.**

Biochemical Effects of Dopamine Addition on Parkin in Lvd-BE(2)-M17-PRKN-WT Cells

Our lab has previously demonstrated by mass spectrometry that oxidative stress leads to the irreversible oxidation of parkin's cysteine residues, which results in its insolubility.[131] To investigate the biochemical effects of dopamine (DA) addition on parkin in Lvd-BE(2)-M17-PRKN-WT cells, particularly regarding potential changes in oxidation and solubility, we employed a serial fractionation method using Western blot analysis. Cells were treated with varying concentrations of DA (0.25–2 mM) for 4 hours, followed by cell lysis using a two-step fractionation approach. First, cells were lysed in a mild detergent buffer containing 2% Triton X-100, which extracts soluble proteins, including parkin in its reduced form. Any smearing observed in this fraction indicates the presence of oxidized cysteine residues in parkin, as oxidized cysteines can promote intermolecular interactions, such as disulfide bond formation, that cause the protein to migrate as high-molecular-weight (HMW) forms on a non-reducing gel. The second step involved lysing the remaining cellular material with a strong detergent buffer containing 10% SDS, which contains insoluble protein, representing parkin that has undergone more extensive oxidation. A 4-hour treatment time was selected, as longer exposure to DA at these concentrations led to excessive cell death (data not shown).

Under these conditions we observed a gradual decrease in parkin solubility as the concentration of DA increases (Figure 4.2A, B), with more prominent smearing (indicating the presence of oxidized, HMW parkin) observed at higher DA concentrations (Figure 4.2A, D). Interestingly, while insoluble parkin is present in DA-treated samples, some insoluble parkin is also found in the non-DA-treated samples (Figure 4.2A, C). These findings suggested that, in this cell model, DA treatment biochemically altered parkin, likely through oxidation at its cysteine residues.

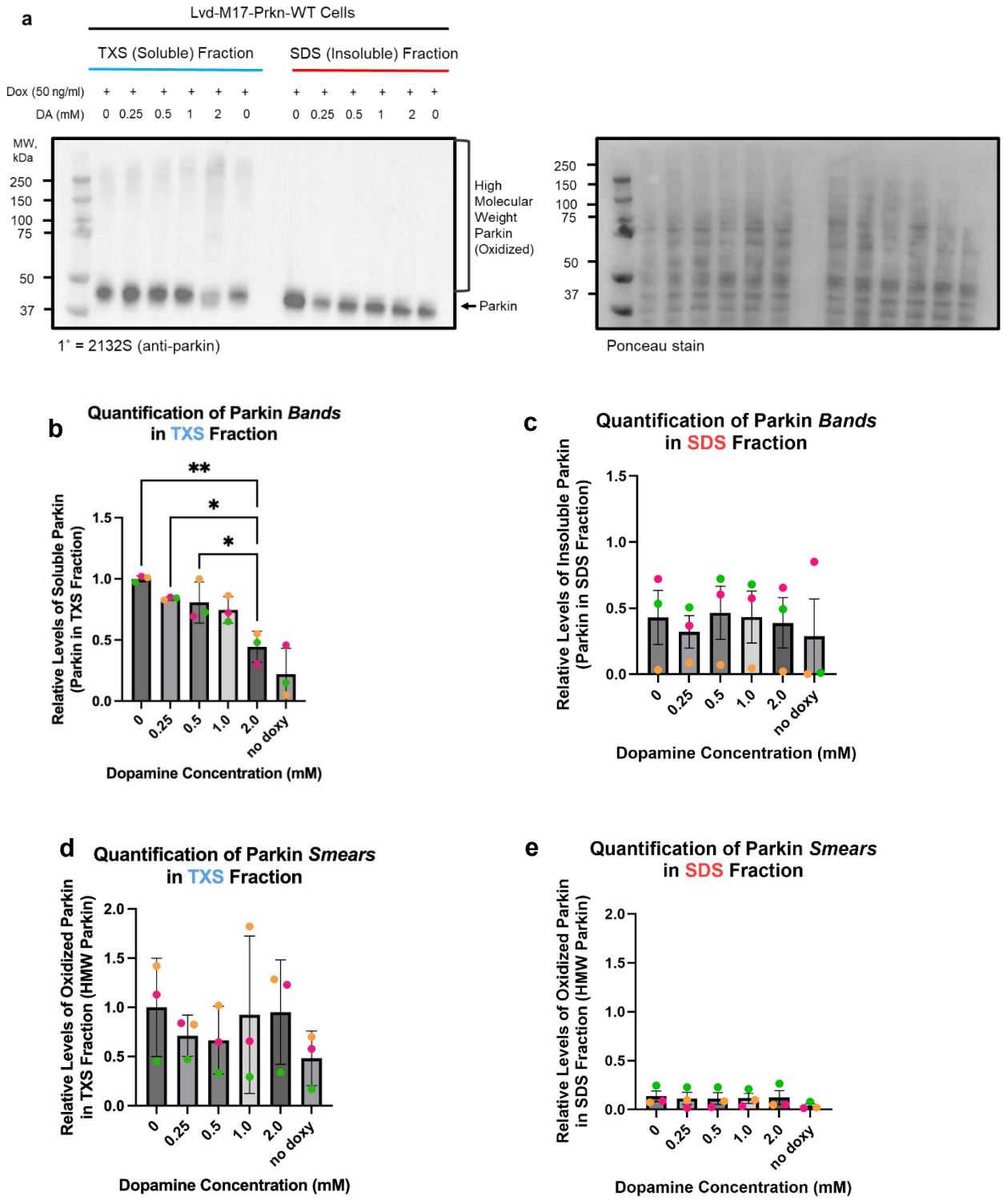

Figure 4.2: Parkin in Lvd-BE(2)-M17-PRKN-WT cells get oxidized post DA treatment. Western blot analysis of Lvd-BE(2)-M17 cells expressing WT parkin (Lvd-BE(2)-M17-WT-

Parkin), induced with 50 ng/mL of doxycycline for 24 hours, and subsequently treated with varying concentrations of dopamine (DA) (0.25–2 mM) for 4 hours. The soluble fraction was extracted using 2% Triton X-100 (TXS) in TSS, while the insoluble fraction was obtained with 10% SDS in TSS. The anti-parkin primary antibody (2123S) was used at a 1:2000 dilution. Panels B-E show the quantification of the TXS and SDS fractions, with the analysis of band intensity and smearing in each fraction at varying dopamine concentrations. n = 3 biological replicates. Individual values were normalized to the mean of the 0 mM (no DA) treatment condition. Specifically, TXS and SDS band intensities were normalized to the mean of the 0 mM TXS *band* values, and TXS and SDS smear intensities were normalized to the mean of the 0 mM TXS *smear* values. Statistical significance across DA treatment groups was assessed using one-way ANOVA followed by Tukey's post hoc test (*$p<0.05$, **$p<0.01$).

Investigation of Parkin's Subcellular Localization Following Dopamine Treatment in Lvd-BE(2)-M17-PRKN-WT Cells

Given that dopamine (DA) treatment induced biochemical changes in parkin in Lvd-BE(2)-M17-PRKN-WT cells (Figure 4.1), I investigated whether such changes were accompanied by alterations in parkin's subcellular localization. Since we have shown that parkin undergoes oxidation and that some of it transitions into an insoluble state following DA treatment, I hypothesized that this could lead to changes in parkin's subcellular location.

Additionally, this experiment provided an opportunity to examine the relationship between parkin and CD63. In human tissue samples, these two proteins are found in close association,[131] but the nature of their interaction remains unclear. Specifically, I aimed to determine whether their colocalization is influenced by DA-induced stress.

To assess this, Lvd-BE(2)-M17-PRKN-WT cells were treated with 2 mM DA for 4 hours, as this concentration produced the most distinct biochemical changes in parkin, including oxidation, as observed in Western blot analysis (Figure 4.2). Imaging analysis (Figure 4.3) revealed that in M17 cells, parkin maintains a diffuse cytosolic distribution following DA treatment, without forming visible foci. Similarly, the distribution of endogenous CD63 remained unchanged (under these microscopic conditions), with no observable shifts in its association with parkin. These findings suggested that under these conditions in M17 cells, DA-induced oxidation did not drive parkin into visible aggregates and it did not generate any visible co-localization with CD63.

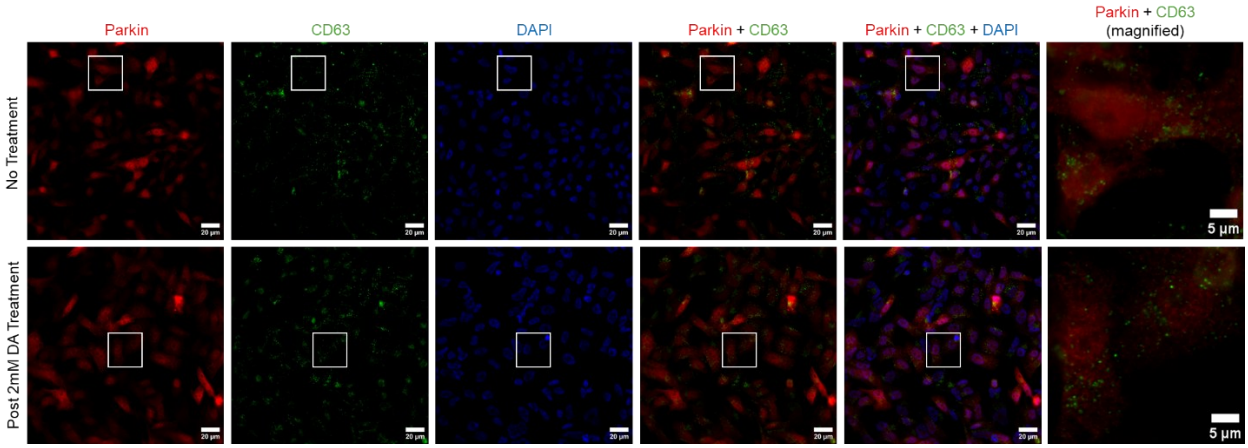

Figure 4.3: Parkin and CD63 localization in Lvd-BE(2)-M17-PRKN-WT Cells is not altered by DA treatment. Immunofluorescence analysis of Lvd-BE(2)-M17-PRKN-WT cells treated with 2 mM dopamine (DA) for 4 hours to examine parkin's subcellular localization and its association with CD63. Cells were induced with 50 ng/mL doxycycline for 24 hours prior to DA treatment. Representative images show parkin (red) and CD63 (green) staining, with nuclei counterstained using DAPI (blue). Merged images illustrate the distribution of parkin and CD63 under DA-treated and untreated conditions. The anti-parkin (2123S) and anti-CD63 (sc-5275) primary antibodies were used at 1:500 and 1:250 dilutions, respectively. Images were captured at 63x magnification, using the Leica THUNDER imager. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 20µm (5µm for magnified images).**

Biochemical Effects of Dopamine Treatment on Parkin in Lvd-BE(2)-M17-PRKN-WT Cells Post Glutathione Reduction

As mentioned earlier in the introduction, parkin expression has an inverse relationship with the redox state of glutathione and plays a direct role in regulating GSH metabolism. Parkin has been shown to participate in glutathione recycling, as well as to undergo S-glutathionylation as an oxidative stress response.[145] Given its involvement in maintaining redox balance, I wanted to determine whether reducing intracellular GSH levels would alter parkin's response to dopamine (DA)-induced oxidative stress.

To achieve this, I pre-treated Lvd-BE(2)-M17-PRKN-WT cells with L-buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis,[149] thereby impairing the cell's ability to neutralize ROS and restore redox homeostasis. Cells were treated with 2mM BSO for 48 hours prior to DA exposure, as previous experiments in our lab showed that this timepoint leads to a significant depletion of GSH. By introducing DA after BSO pre-treatment, I aimed to exacerbate oxidative stress by first having lowered one of the primary antioxidant defenses in the cell, placing a greater burden on parkin's ability to counteract oxidative damage. Since DA oxidation itself generates ROS and quinones, the combination of GSH depletion and DA exposure was designed to create a highly oxidative environment.

Based on Western blot analysis, depleting glutathione before DA treatment significantly reduced parkin's solubility in M17 cells (Figure 4.4). Cells treated with both BSO and DA had noticeably less parkin in the soluble fraction compared to cells treated with DA alone (Figure 4.4A, C). This effect was particularly striking, as insoluble parkin was undetectable when lysed with 10% SDS (Figure 4.4A) but became visible only after using a stronger 20% SDS lysis buffer

(Figure 4.4B). Notably, the highest levels of insoluble parkin were found in cells that had been pre-treated with BSO before DA exposure (Figure 4.4B). These results suggest that in M17 cells, GSH depletion exacerbates parkin insolubility, potentially due to increased oxidative stress.

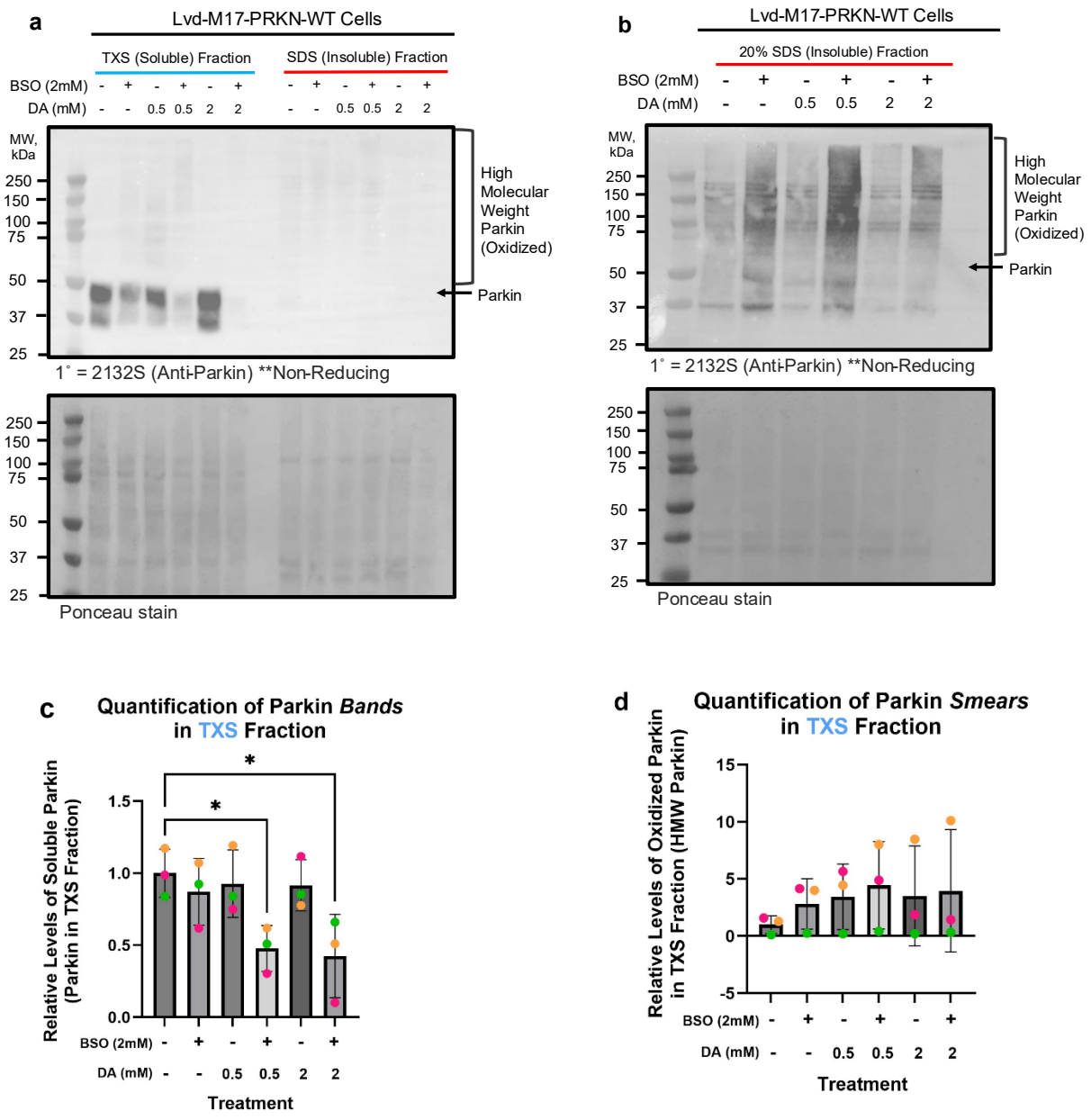

Figure 4.4: Reduction of *de novo* glutathione synthesis prior to dopamine treatment increases parkin's insolubility in Lvd-BE(2)-M17-PRKN-WT cells. Western blot analysis of Lvd-BE(2)-M17 cells expressing WT parkin (Lvd-BE(2)-M17-PRKN-WT), induced with 50 ng/mL of doxycycline for 24 hours and subsequently treated with dopamine (DA, 0.5-2 mM) for 4 hours, with or without pre-treatment with 2 mM BSO for 48 hours. (A) The soluble fraction (TXS) was

extracted using 2% Triton X-100 in TSS, while the insoluble fraction was obtained with 10% SDS in TSS. (B) Samples were further re-lysed in 20% SDS to detect insoluble or detergent-resistant parkin. The anti-parkin primary antibody (2123S) was used at a 1:2000 dilution. Panels C and D show the quantification of parkin bands and smearing in the TXS fraction from panel A, highlighting a reduction in soluble parkin upon BSO pre-treatment. n = 3 biological replicates. Individual values were normalized to the mean of the -DA -BSO treatment condition. Specifically, TXS band intensities were normalized to the mean of the 0 mM TXS *band* values, and TXS smear intensities were normalized to the mean of the 0 mM TXS *smear* values. Statistical significance across treatment groups was assessed using one-way ANOVA followed by Dunnett's post hoc test (*$p<0.05$, **$p<0.01$).

Investigation of Parkin's Subcellular Localization and Association with CD63 Following Glutathione Reduction and Dopamine Treatment in Lvd-BE(2)-M17-Parkin-WT Cells

In line with the findings from Figure 4.4, where dopamine (DA) treatment combined with glutathione depletion notably increased parkin insolubility in Lvd-BE(2)-M17-PRKN-WT cells, I next investigated whether these changes were accompanied by alterations in parkin's subcellular localization and its association with CD63. To do this, I employed immunofluorescence microscopy to visualize parkin distribution and its relationship with CD63 under the same treatment conditions.

As in the Western blot experiments, Lvd-M17-PRKN-WT cells were pretreated with 2 mM BSO for 48 hours, followed by 0.5–2 mM DA treatment for 4 hours. Immunofluorescence analysis revealed that parkin's subcellular localization (Figure 4.5.1) and its localization with respect to CD63 (Figure 4.5.2) remained unchanged following BSO and DA treatment. No visible differences were observed in parkin's distribution within the cell, nor in its association with CD63-positive structures, suggesting that in M17 cells, DA-induced oxidative stress and GSH depletion promoted parkin insolubility without causing detectable changes in its subcellular localization or association with CD63.

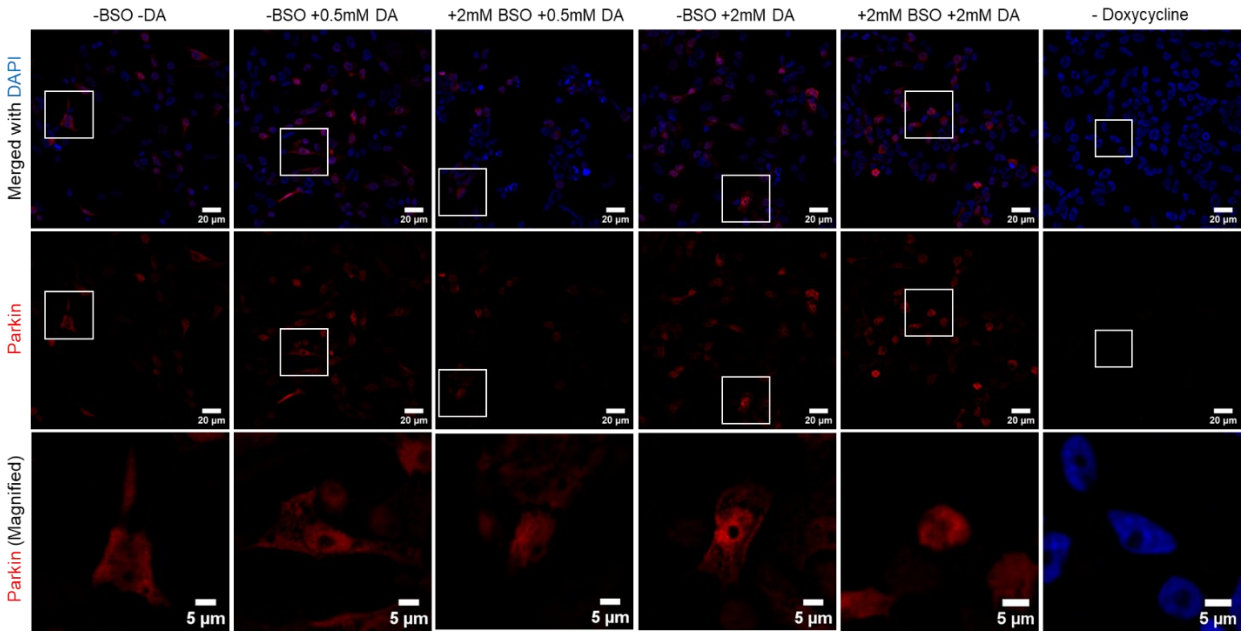

Figure 4.5.1: Parkin localization in Lvd-BE(2)-M17-PRKN-WT cells is not altered by reduction of glutathione prior to dopamine treatment. Immunofluorescence analysis of Lvd-BE(2)-M17-PRKN-WT cells treated with 0.5-2mM dopamine (DA) for 4 hours following pre-treatment with 2mM BSO for 48 hours to examine parkin's subcellular localization under oxidative stress conditions. Cells were induced with 50 ng/mL doxycycline for 24 hours prior to BSO and DA treatment. Representative images show parkin (red) staining, with nuclei counterstained using DAPI (blue). The anti-parkin (2123S) primary antibody was used at a dilution of 1:500. Images were captured at 63x magnification using the Leica THUNDER Imager. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 20µm (5µm for magnified images).**

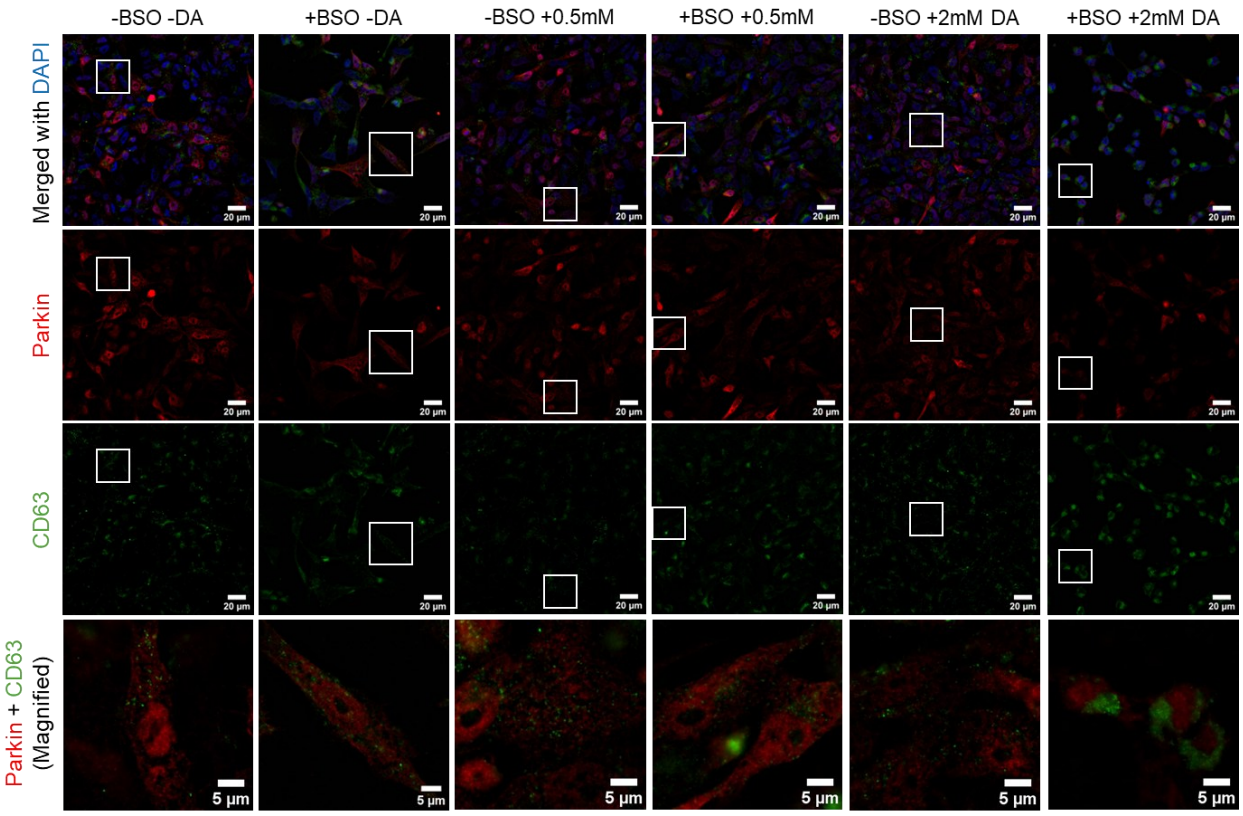

Figure 4.5.2: Reduction of glutathione prior to dopamine treatment does not alter parkin's subcellular distribution with respect to CD63 in Lvd-BE(2)-M17-Parkin-WT cells. Immunofluorescence analysis of Lvd-BE(2)-M17-PRKN-WT cells treated with 0.5-2mM dopamine (DA) for 4 hours following pre-treatment with 2mM BSO for 48 hours to examine parkin's subcellular distribution in relation to CD63 under oxidative stress conditions. Cells were induced with 50 ng/mL doxycycline for 24 hours prior to BSO and DA treatment. Representative images show parkin (red) and CD63 (green) staining, with nuclei counterstained using DAPI (blue). The anti-parkin (2123S) and anti-CD63 (sc-5275) primary antibodies were used at 1:500 and 1:250 dilutions, respectively. Images were captured at 63x magnification using the Leica THUNDER Imager. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 20µm (5µm for magnified images).**

Ruling Out Alternative Causes for Parkin's Unchanged Subcellular Localization Under Dopamine Treatment

Given that dopamine (DA) treatment combined with glutathione depletion increased parkin insolubility in M17 cells (Figure 4.4) without causing visible alterations in its subcellular localization or association with CD63 (Figures 4.5.1 and 4.5.2), I aimed to explore whether there are alternative explanations for the unchanged distribution of parkin under these conditions. Specifically, I employed CCCP treatment to assess the capability of M17 cells to visualize parkin redistribution. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), is a mitochondrial uncoupler known to depolarize mitochondria and induce parkin translocation to mitochondria in other cell types.[150]

Although the primary focus of this thesis is on parkin's antioxidant functions—which is thought to operate independently of its E3 ligase activity—this experiment specifically examines a different aspect of parkin biology. CCCP is a chemical compound that uncouples the mitochondria's normal energy production.[150] Mitochondria generate energy by maintaining an electrical charge across their membrane. CCCP disrupts this process by making the membrane permeable, which causes the mitochondria to lose their electrical charge (also called membrane potential).[150] This loss of charge triggers parkin to move to be translocated to the mitochondria in ex vivo cell culture experiments described by many investigators.[150] Once at the mitochondria, parkin's E3 ligase activity helps to tag damaged mitochondria for removal through a process called mitophagy, which involves a subset of lysosomes.[150] When CCCP is added to other cell types, it generally leads to the rapid translocation of parkin to the mitochondria, where it facilitates the removal of dysfunctional mitochondria.[104–107,150,151] By using CCCP in M17 cells, I sought to test whether these cells also demonstrate the same translocation of parkin under stress conditions.

As shown in Figure 4.6, unexpectedly, CCCP treatment in Lvd-BE(2)-M17-PRKN-WT cells did not induce parkin translocation to the mitochondria. Parkin remained dispersed throughout the cell and showed no signals suggesting a translocation to mitochondria, which were visualized using TOMM20, a mitochondrial protein marker. This result suggests that further experiments are needed to fully understand why parkin does not translocate to the mitochondria in this model—whether this is a characteristic feature of M17 cells or whether something is preventing the translocation process. Nonetheless, these results hinted at a resilience by M17 cells to demonstrate subcellular localization changes in parkin by the range of microscopy employed by us although biochemical changes of the protein had been induced, as shown above.

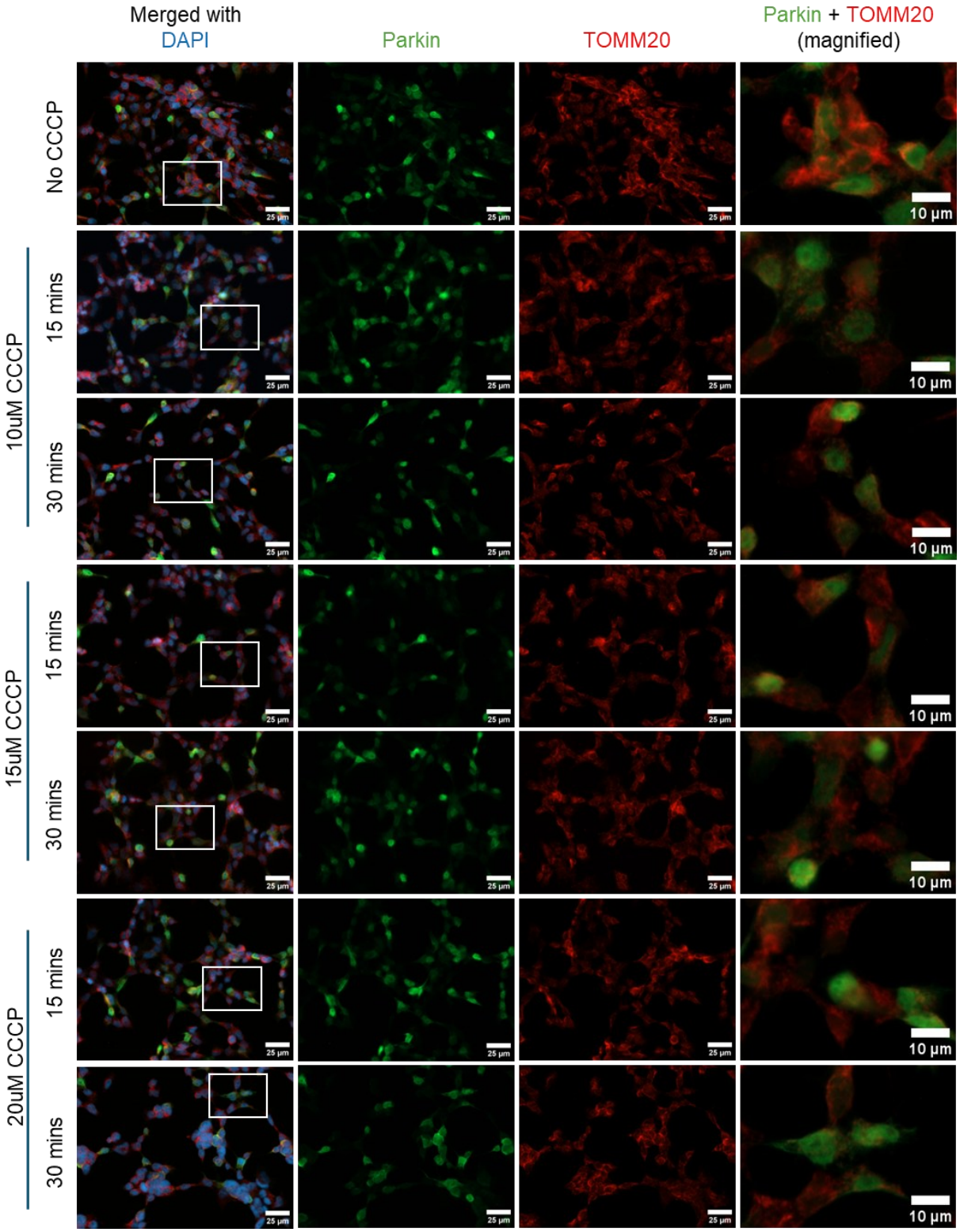

Figure 4.6: CCCP treatment does not change parkin localization in Lvd-BE(2)-M17-PRKN-WT cells. Immunofluorescence analysis of Lvd-BE(2)-M17-PRKN-WT cells treated with 10-

20μM CCCP for 15-30 minutes. Cells were induced with 50 ng/mL doxycycline for 24 hours prior to CCCP treatment. Representative images show parkin (green) and TOMM20 (red) staining, with nuclei counterstained using DAPI (blue). The anti-parkin (PRK8) and anti-TOMM20 (ab186735) primary antibodies were both used at 1:300 dilutions. Images were captured at 40x magnification using the Zeiss Axio Imager A1. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 25μm. (10 μm for magnified images).**

Characterization of Melanin Production in M17-P5 Cells Transfected with Tyrosinase

The following experiments pertain to my second dopamine (DA) stress model, which includes melanin formation stemming from endogenous DA metabolite generation. This is achieved by transfecting M17-P5 cells (which stably express (myc-tagged) WT parkin cDNA without the need for doxycycline) with human tyrosinase cDNA. Tyrosinase is a key enzyme in melanin biosynthesis in the body; it catalyzes the oxidation of tyrosine to L-DOPA and subsequently converts L-DOPA into dopaquinone.[152] Dopaquinone then undergoes a series of oxidation and polymerization steps, eventually forming melanin.[152] This model is designed to mimic neuromelanin accumulation in the brain and is being used to study how parkin responds to melanin accumulation.

However, before investigating parkin in this system, it is essential to confirm and track melanin formation in these cells. Specifically, determining how much melanin accumulates over time is crucial for identifying the optimal time point to analyze parkin.

As shown in Figure 4.7, melanin formation following tyrosinase transfection occurs in a time-dependent manner. Qualitative assessment of tyrosinase staining indicated a transfection efficiency of over 65% in undifferentiated M17 cells. The longer the cells are incubated post-transfection, the more melanin they accumulate. However, after three days, excessive melanin accumulation appears to induce cell death, as heavily pigmented cells exhibit rounding and detachment. This suggests that the optimal time window for studying parkin, particularly in imaging studies, is between days 2 and 3 post tyrosinase transfection.

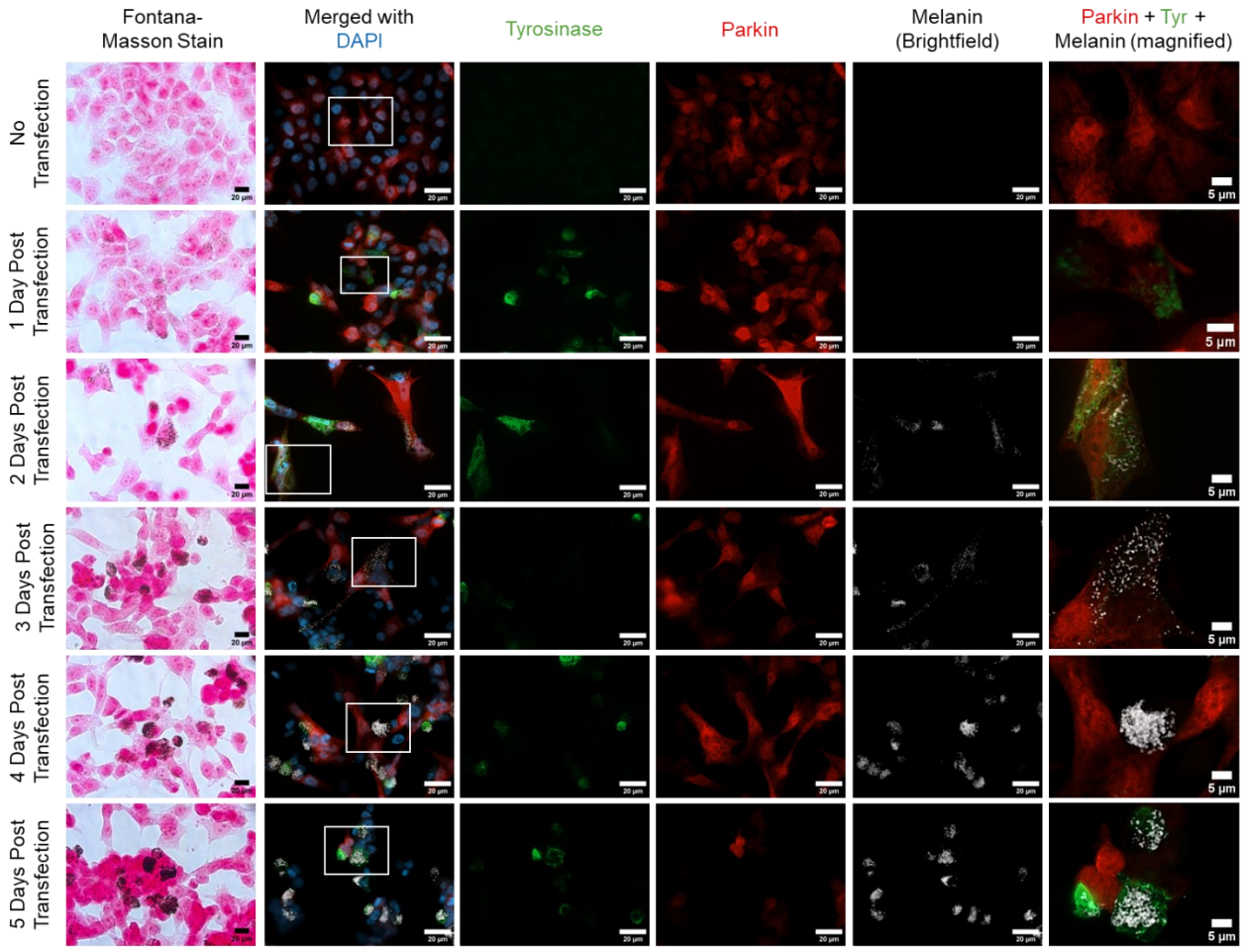

Figure 4.7: Time-dependent melanin accumulation in M17-P5 cells following tyrosinase transfection. Fontana-Masson staining (left) and immunofluorescence staining (right) of M17-P5 cells at 1, 2, 3, 4, and 5 days post-transfection to assess melanin accumulation. Representative Fontana-Masson images (left) highlight melanin deposits, confirming successful pigment formation. Immunofluorescence images (right) show the distribution of tyrosinase (green) and parkin (red) relative to melanin (white), with nuclei counterstained using DAPI (blue). Anti-tyrosinase (MA5-14177) and anti-parkin (2132S) primary antibodies were both used at 1:500 dilutions. Increased melanin accumulation is observed over time, with excessive melanin leading to cellular rounding and detachment by days 4 and 5. Images were captured at 63x magnification for immunofluorescence images and 40x for Fontana-Masson images, all using the Zeiss Axio

Imager A1. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 20µm. (5 µm for magnified images).**

Investigating the Impact of Melanin Accumulation on Parkin's Subcellular Localization in M17-P5 Cells

As shown earlier in Figure 1.2 (from previous findings in our lab), in the *Substantia nigra* (SN) of a normal, non-Parkinsonian aged human brain, parkin is localized to neuromelanin (NM) granules within neurons and is also found to colocalize with CD63.[131] NM is believed to play a role in sequestering harmful DA oxidation products,[10,43,44,54] and its accumulation over time is associated with increased parkin detection in midbrain neurons.[131] Given this, I aimed to investigate whether melanin accumulation in M17-P5 cells, induced by tyrosinase transfection, would alter parkin's subcellular localization or its relationship with CD63. If parkin contributes to NM's function of trapping DA radicals, as has been proposed, its localization might shift in response to melanin accumulation.

To explore this, I examined parkin distribution in M17-P5 cells 2 days post tyrosinase cDNA transfection (to induce melanin accumulation) and also assessed its localization with respect to CD63. Despite the presence of melanin, no visible changes were observed in parkin's localization, nor was there any detectable shift in its association with CD63. These findings suggested, in contrast to our expectations, that at least in this cellular model, parkin's localization was not significantly altered by melanin accumulation.

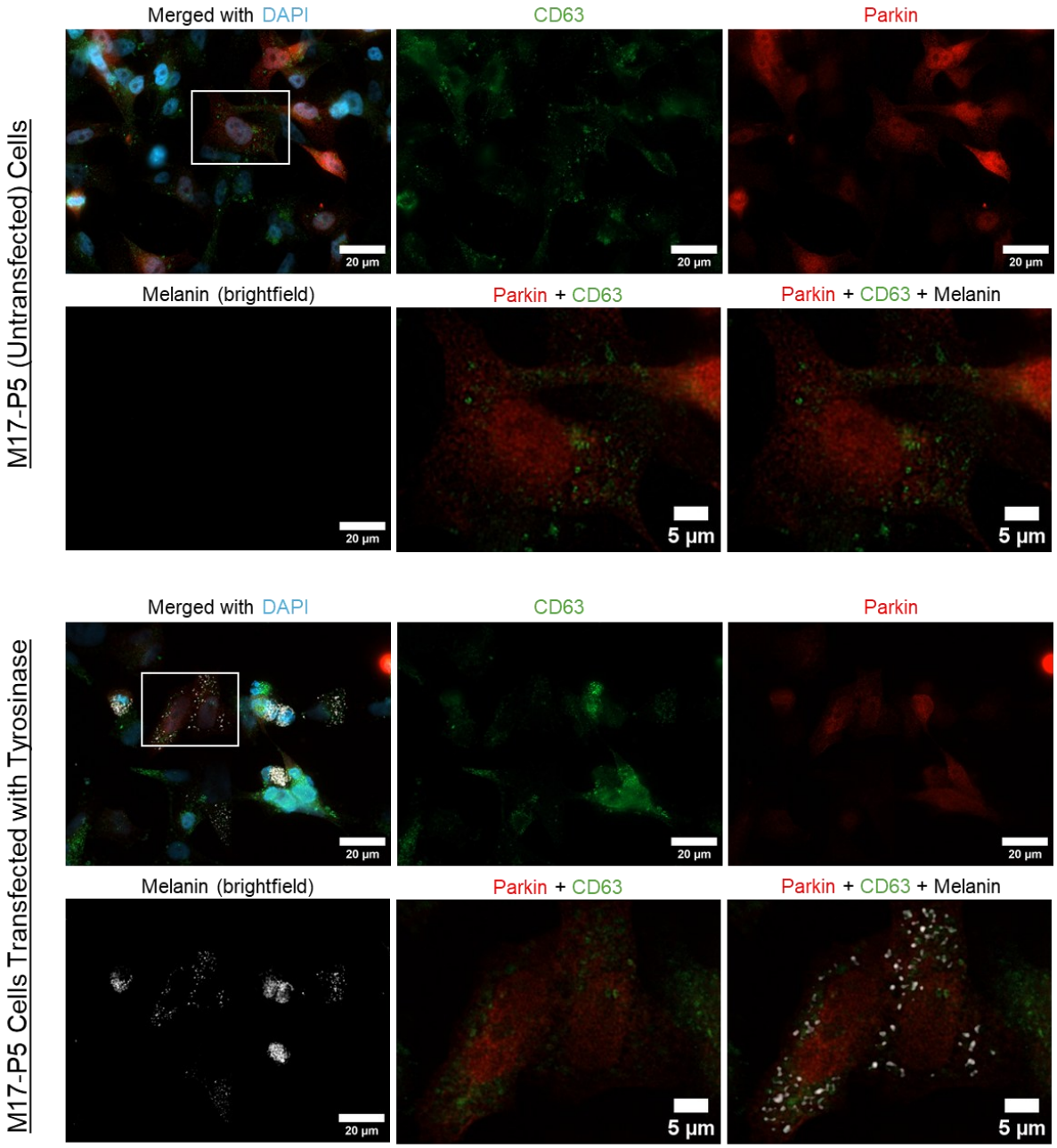

Figure 4.8: Accumulation of melanin in M17-P5 cells does not change subcellular localization of parkin or CD63. Immunofluorescence analysis of M17-P5 cells transfected without (A) or with (B) human tyrosinase to induce melanin accumulation. Cells were fixed and stained for parkin (red) and CD63 (green), with nuclei counterstained using DAPI (blue). Representative images show no visible change in parkin's subcellular localization or its association with CD63 in the

presence of accumulated melanin. Anti-parkin (2132S) and anti-CD63 (sc-5275) primary antibodies were used at 1:500 and 1:250 dilutions, respectively. Images were captured at 63x magnification using the Zeiss Axio Imager A1. n = 3 biological replicates, each analyzed in duplicate. **Scale bar = 20µm (5µm for magnified images).**

Biochemical Effects of Melanin Formation on Parkin in M17-P5 Cells Transfected with Tyrosinase

As shown both in previous studies[131] and some of my own results, parkin solubility decreases under oxidative stress, becoming more insoluble as it undergoes oxidation. Given that tyrosinase uses oxidized dopamine (DA) to form melanin[152], it is possible that DA-induced stress from this process could also affect parkin's solubility. To test this, I applied the same serial fractionation method used in earlier experiments to separate soluble and insoluble parkin, based on the amount of time melanin has accumulated in the cells post-tyrosinase transfection. M17-P5 cells were transfected with tyrosinase cDNA and allowed to incubate for 1-5 days, allowing melanin to accumulate over time. If melanin accumulation affects parkin's solubility, I would expect to observe a decrease in soluble parkin in the TXS fraction, coupled with an increase in insoluble parkin in the SDS fraction. This would suggest that an increase in the presence of melanin causes parkin to become more insoluble.

However, as shown in Figure 4.9, the results did not fully align with the hypothesis that melanin accumulation impacts parkin solubility in the M17 cell line. Although there was a decrease in soluble parkin in the TXS fraction, there was no corresponding increase in insoluble parkin detected in the 10% SDS fraction. Instead, both fractions showed a similar trend, namely parkin levels decreasing in both.

Despite normalizing total protein loading in the Western blot, the observed decrease in parkin levels over time could be due to several factors. Increased cell death at later time points could reduce the overall pool of parkin in the culture, meaning that even with equal total protein loaded, parkin might make up a smaller proportion of the sample. Additionally, melanin-induced stress could be triggering parkin degradation. Overall, these findings suggested that melanin accumulation generated by tyrosinase cDNA transfection in the M17-P5 cell line (which stably

expressed WT parkin cDNA under the CMV promoter) did alter either parkin steady-states, solubility, and/or detectability under these conditions.

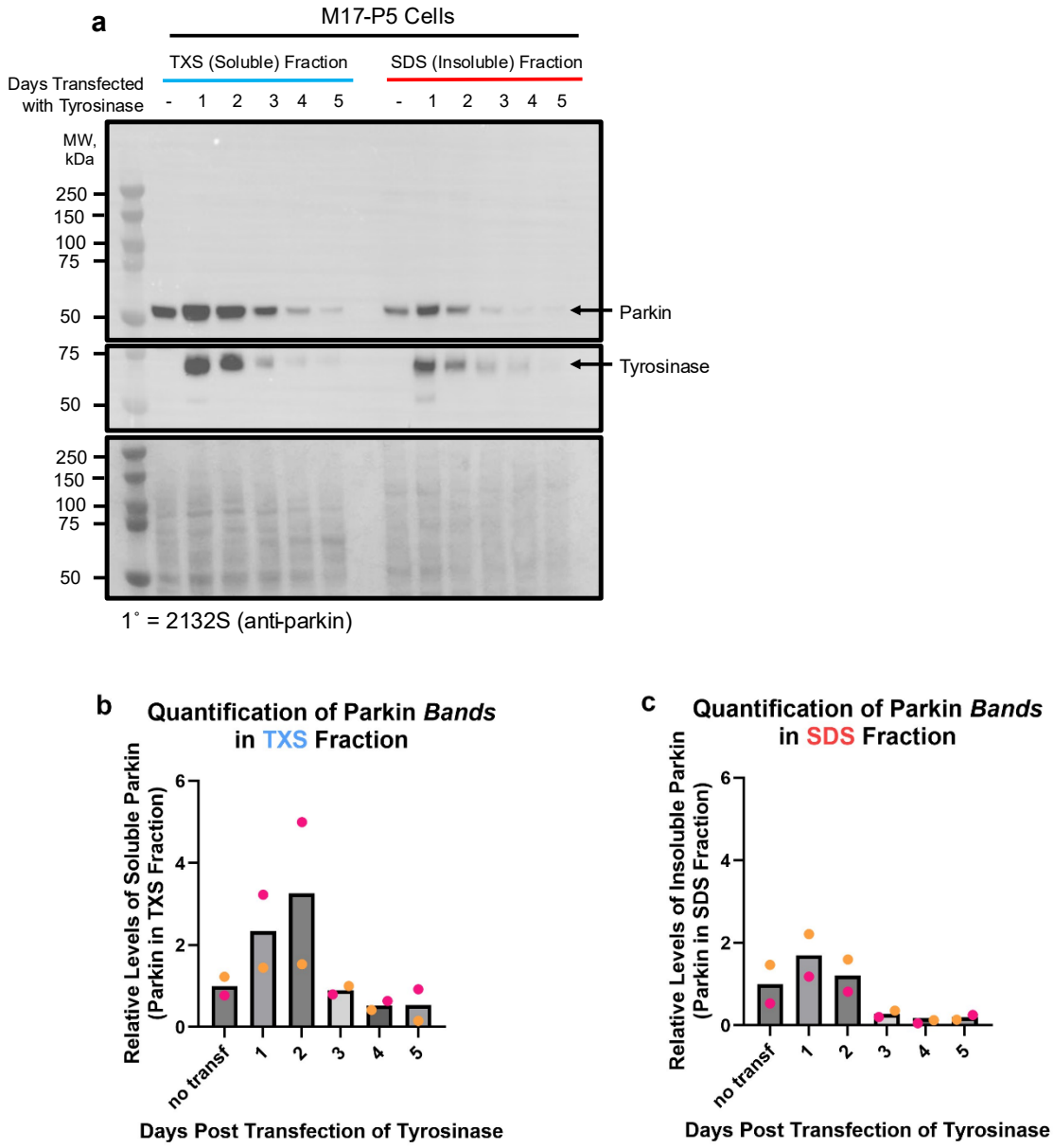

Figure 4.9: Accumulation of melanin in M17-P5 cells does not correlate with an increase in insoluble parkin. Western blot analysis of M17-P5 cells transfected with tyrosinase and allowed to accumulate melanin for 1–5 days. (A) Soluble (TXS) and insoluble (SDS) protein fractions were extracted using 2% Triton X-100 and 10% SDS, respectively, and analyzed for parkin levels. (B, C) Quantification of parkin band intensities in the TXS and SDS fractions over time. The anti-parkin primary antibody (2123S) was used at a 1:2000 dilution. n = 2 biological replicates.

Individual values were normalized to the mean of the *no transf* treatment condition. Specifically, both TXS and SDS band intensities were normalized to the mean of the *no transf* TXS band values.

Examining Whether Presence of Parkin Influences Melanin Accumulation in M17 Cells

Finally, based on previous in vitro findings by our laboratory showing that recombinant parkin (r-parkin) enhanced pigment formation from DA (aminochrome) oxidation,[131] I aimed to investigate whether a similar phenomenon could be observed in M17-P5 cells, where melanin accumulation is driven by tyrosinase activity.

Aminochrome is a reactive byproduct of DA oxidation that can be toxic to cells.[54] It readily undergoes further oxidation and polymerization, leading to the formation of neuromelanin (NM)-like pigment.[54,131] This process is thought to be protective, as converting aminochrome and other reactive DA oxidation products into an insoluble pigment may help limit their reactivity and reduce potential cellular toxicity.[54] Parkin's enhancement of pigment formation suggests that it may play a protective role by accelerating the conversion of aminochrome into a less reactive and insoluble pigment, thus reducing its cytotoxic potential.

To test this in the context of my cell model, I transfected two different M17 cell lines with human tyrosinase. The first was the M17-P5 cell line, which stably expresses parkin, and the second was the M17-empty vector (EV) cell line, which underwent the same treatment but expresses an empty vector instead of parkin. Following transfection, melanin was allowed to accumulate within the cells, and time points were analyzed at 24, 48, and 72 hours post-transfection. Melanin levels were measured by reading the intensity of the pigment at 405 nm on a plate reader. As shown in Figure 4.10, the presence of parkin appears to influence melanin accumulation. Specifically, parkin-expressing cells showed increased melanin accumulation compared to empty-vector cells. These findings build on earlier results, suggesting that parkin promotes melanin formation, possibly as a protective mechanism to convert DA oxidation products into less harmful, insoluble pigments and thus, to reduce oxidative stress.

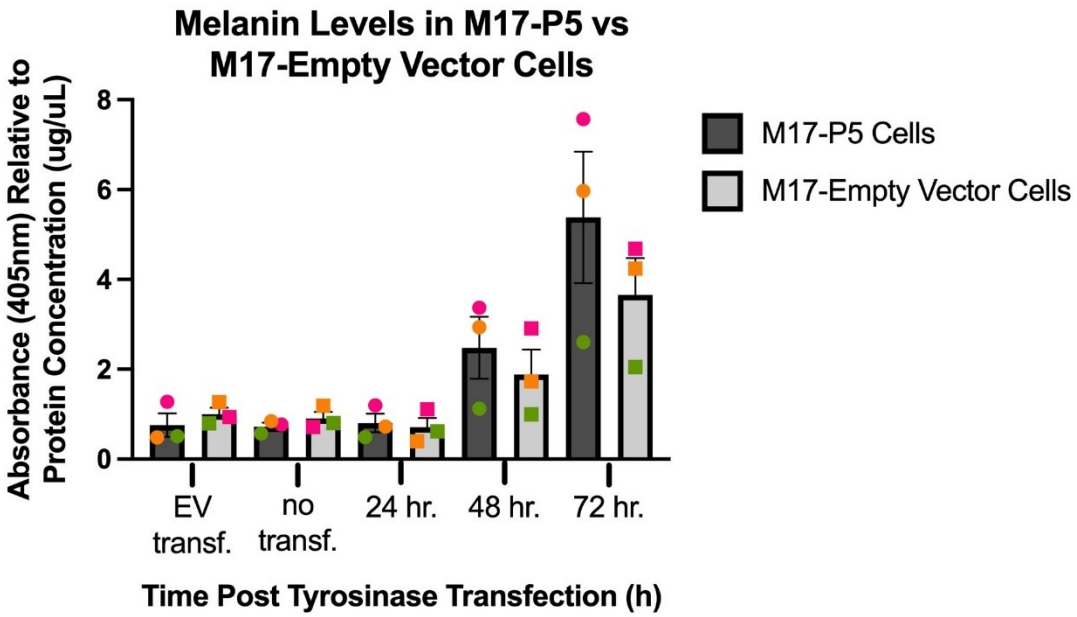

Figure 4.10: Parkin-expressing M17-P5 cells accumulate more melanin than M17-Empty Vector cells. Graph showing melanin accumulation in M17-P5 cells (stably expressing parkin) and M17-Empty Vector cells at 24, 48, and 72 hours post-transfection with human tyrosinase cDNA, with non-transfected and empty-vector transfected cells serving as controls. Melanin levels were measured by reading the intensity at 405 nm using a plate reader. n = 3 biological replicates, with 2 technical replicates in each. All values were normalized to the average of the empty vector transfection (EV transf.) in M17-Empty Vector cells.

5.0 Discussion

This thesis aimed to investigate the effects of dopamine (DA)-induced stress on the parkin protein, using the BE(2)-M17 human dopaminergic neuroblastoma cell line as a model. Given parkin's established ability to mitigate oxidative stress, its function in protecting dopaminergic neurons—particularly those in the *Substantia nigra pars compacta* (SNc)—is of great interest. Parkin-linked PD is characterized by the specific loss of SNc dopaminergic neurons, demonstrating that parkin plays a critical role in protecting these cells.[93,94] Since dopaminergic neurons in the SNc are uniquely exposed to oxidative stress due to their high metabolic activity and DA metabolism,[71,73] understanding how parkin responds to DA-derived stress is critical. If parkin plays a role in counteracting oxidative damage within these neurons, then the absence of a functional parkin protein in individuals with mutations could lead to increased susceptibility to DA stress, contributing to early neurodegeneration.

Based on this premise, I hypothesized that DA-induced stress would lead to parkin oxidation and decreased solubility, potentially altering its localization or interactions with cellular components such as CD63-positive lysosomes and neuromelanin (NM) granules. These hypotheses were grounded in previous work from our lab on *post mortem* tissues.

First, the team had established that parkin undergoes oxidation at its cysteine residues in both human brain tissue and in vitro models, leading to changes in its solubility.[131] Additionally, previous work had shown that wild-type (WT) parkin interacts with DA quinones (reactive DA oxidation products) at its cysteine residues, further supporting its susceptibility to DA-induced modifications.[131] Given this, I expected DA-induced stress to promote similar modifications—oxidized cysteines and reduced solubility. Since oxidized or insoluble forms of parkin might be useful to study in a dynamic ex vivo model system, I also examined whether these modifications

could influence parkin's spatial distribution in the cell, potentially making it appear as distinct structures, such as aggregates or foci, under a microscope. Insolubility of proteins is often linked to aggregation[153,154], where misfolded or damaged proteins cluster together, forming distinct structures that could be identified as larger formations in cellular imaging.

Second, our lab has observed a possible relationship between parkin and NM.[131] NM, which forms by sequestering reactive DA metabolites,[39,44] was found to colocalize with parkin in normal, aged human brain sections, as well as with CD63+ vesicles.[131] Further supporting this association, previous in vitro studies demonstrated that recombinant, WT parkin (but not mutant parkin) enhanced NM-like polymer formation of oxidized DA in a concentration-dependent manner.[131] This suggested that parkin's interaction with NM may serve a protective function, potentially by accelerating the sequestration of reactive DA metabolites to mitigate toxicity.

To investigate these hypotheses, I employed two models of DA stress. The first involved the direct addition of DA to the culture media to simulate DA auto-oxidation, its uptake into the cytosol, thereby mimicking the oxidative stress generated by DA metabolism in neurons. The second model utilized tyrosinase transfection to drive intracellular melanin synthesis, allowing for a controlled system to examine parkin's potential interactions with NM, as observed in human neurons. Based on our previous findings, I expected both models to induce changes in parkin oxidation and solubility, with potential alterations in its localization, particularly in relation to melanin granules and CD63-positive lysosomes. Moreover, in the tyrosinase overexpression model, I anticipated that cells expressing parkin would show increased melanin accumulation compared to those lacking parkin, further supporting its role in promoting NM formation, either directly or indirectly.

Throughout my experiments, I found that DA treatment of M17 cells led to partial oxidation and increased insolubility of parkin (Figure 4.2), which was exacerbated when glutathione was depleted using BSO (Figure 4.4). This suggests that oxidative stress influences parkin's biochemical properties, but under the conditions tested, these changes did not result in parkin forming microscopically visible aggregates or redistributing within the cell, nor did they lead to noticeable colocalization with CD63 (Figures 4.3, 4.5). Instead, parkin remained diffusely localized throughout the cytoplasm. In the second model, where melanin synthesis was induced via tyrosinase transfection, parkin unexpectedly did not exhibit increased oxidation or insolubility as melanin accumulation increased (Figure 4.9). Additionally, despite its known presence in NM-containing neurons in sections of human brain, parkin was not observed to colocalize with melanin granules in this system (Figure 4.8). However, its presence appeared to accelerate melanin formation (Figure 4.10), suggesting that while parkin does not directly interact with melanin under these conditions, it may influence the synthesis process.

5.1 Potential Influence of Antioxidant Capacity in M17 Cells

In this study, to further investigate the role of oxidative stress on parkin solubility and oxidation, I pre-treated M17 cells with BSO, an inhibitor of glutathione synthesis,[149] to deplete cellular thiols. Given that glutathione is a major antioxidant,[135–137] this approach was intended to lower the cell's ability to counteract oxidative stress, making parkin more susceptible to oxidation, as its thiols have shown to be part of a feedback loop with glutathione and its reduction (once oxidized).[145] Interestingly, however, when analyzed by Western blot, I observed that BSO treatment led to more pronounced changes in parkin solubility compared to DA alone (Figure 4.4). This suggests that under normal conditions, M17 cells may have a strong antioxidant capacity that

buffers against DA-induced oxidative stress, potentially masking parkin's expected response. If M17 cells are abundant in GSH or rely heavily on GSH as an antioxidant defence mechanism, then GSH may be the primary factor protecting against DA-induced oxidative stress in this cell model. Given that dopamine o-quinone reacts more rapidly with GSH than undergoing intramolecular cyclization[155], this suggests that in a GSH-rich environment, DA quinones are more likely to be neutralized through conjugation rather than forming secondary oxidative species. This is particularly relevant because the GSH-DA quinone conjugates, while initially reactive, can be stabilized under reductive conditions[156], preventing further oxidative damage. However, when GSH levels are depleted by BSO treatment, DA quinones may persist longer and undergo alternative reactions, such as cyclization into aminochrome or adduct formation with other cellular thiols—including those on parkin itself. The pronounced shift in parkin solubility after BSO treatment suggests that, in this model, GSH may be a dominant mechanism preventing DA quinones from interfering with cellular proteins, including parkin.

Adding another layer to this interpretation, a prior study demonstrated that both dopamine and L-DOPA are unstable in commonly used cell culture media, undergoing rapid oxidation to form hydrogen peroxide and electrophilic species (each capable of depleting free GSH), such as semiquinones and quinones.[157] Importantly, the authors showed that these oxidative reactions occurred in the culture medium itself, rather than being strictly dependent on cellular metabolism.[157] This raises the possibility that some of the oxidative stress observed in cell-based models of DA toxicity may originate extracellularly, even before the neurotransmitter enters cells. In the context of my experiments, this suggests that some of the effects attributed to DA treatment may have involved extracellular oxidation products that exert stress on cells indirectly or non-specifically, which may be further amplified when free GSH is depleted. As such, it is possible

that part of the oxidative stress impacting parkin in my model arises not solely from intracellular DA metabolism, but from reactive species generated directly in the culture medium prior to uptake.

To help minimize these potential interactions, I carried out all DA treatments in OPTI-MEM media without serum, rather than using standard DMEM/F12 with 10% FBS, in an effort to reduce the number of reactive components that DA could interact with in the extracellular space. However, to better assess whether media-based oxidation is influencing my results, future experiments could include measuring H_2O_2 levels in the media after DA treatment, or comparing results between different media conditions. These approaches would help clarify the extent to which extracellular DA oxidation is contributing to the oxidative stress observed in this system and its potential impact on parkin's behavior.

The high antioxidant capacity of M17 cells could have other important implications for interpreting these results. The fact that BSO had a greater impact than DA alone on parkin's solubility could suggest that parkin's function may be directly linked to the thiol network. This aligns with previous findings from our lab, which suggest that parkin and glutathione-related pathways interact, possibly through compensatory mechanisms that regulate oxidative stress.[145] The depletion of thiols by BSO may have disrupted this balance, revealing a stronger effect on parkin's solubility and oxidation than DA alone could induce.

Alternatively, another interpretation is that DA alone did not generate a sufficient level of oxidative stress in M17 cells to trigger parkin's expected response. If parkin's oxidative modifications and solubility changes are threshold-dependent, the high baseline antioxidant capacity of M17 cells might have prevented DA from reaching that threshold. In this case, BSO's effect could reflect the fact that a greater level of redox imbalance was needed before parkin's response became evident.

In line with this theory, it is also possible that the M17 cells used in my experiment do not express sufficient levels of dopamine transporter (DAT), which is responsible for DA uptake into cells. Although studies have shown that BE(2)-M17 cells exhibit characteristics of dopaminergic neurons, including DAT expression[148], I have not directly assessed DAT levels in my specific cell line. Therefore, as this remains unconfirmed in my experiments, it is a factor to be done as a next step. Future studies could address this by using PCR for its mRNA, Western blot analysis to quantify DAT protein levels, or immunofluorescence to visualize DAT distribution within the cells, providing further insight into its expression in M17 cells.

Overall, these findings suggest that the antioxidant capacity of M17 cells may have influenced parkin's response to oxidative stress, potentially masking its expected solubility changes in response to DA alone. The stronger effect of BSO indicates that depleting thiols alters parkin's behavior in a way that DA alone does not, reinforcing the idea that parkin's function may be linked to the cellular redox environment.

5.2 Lack of Parkin Translocation to Mitochondria in M17 Cells Using CCCP Treatment

In this study, treatment with CCCP, a mitochondrial stress inducer, unexpectedly did not lead to translocation of WT parkin to mitochondria in M17 cells. This contrasts with previous observations in other cell types, including HEK293[105,106], HeLa[105,107], and SH-SY5Y neuroblastoma cells[104,151], where CCCP-induced mitochondrial depolarization successfully triggered parkin recruitment within 10 min of exposure. However, a study on cortical neurons similarly reported a lack of parkin translocation, with the authors suggesting that the "unique metabolic properties of neurons likely influence" this pathway.[151] This raises the possibility that M17 cells, as a neuronal model, may exhibit similar resistance to CCCP-induced mitophagy due

to intrinsic neuronal characteristics to protective measures rather than representing a failure of the experimental conditions.

The cell type dependency of parkin translocation underscores the importance of considering which model is most relevant for studying mitochondrial quality control in neurons. Many widely used cell lines, such as HEK293 (kidney-derived)[158] and HeLa (cervical cancer-derived),[159] are limited in their relevance to neurons. Although HEK293 cells do express some neuronal markers, such as neurofilament subunits, they may not recapitulate functional and physiological properties of mature neurons, such as neurotransmitter synthesis.[160,161] Even SH-SY5Y neuroblastoma cells, while of neural origin, exhibit significant differences from primary neurons.[151] If parkin translocation to mitochondria does not robustly occur in certain neuronal model systems, it could suggest that this pathway is not a primary mechanism for mitochondrial quality control in neurons.

In addition, the antioxidant capacity of M17 cells may play a role in their response to mitochondrial stress. Thiol-containing antioxidants, including glutathione, have been shown to directly counteract CCCP-induced mitochondrial depolarization by reversing its uncoupling activity.[162] If M17 cells have high GSH levels, this could buffer against CCCP's effects, preventing mitochondrial stress from reaching the threshold needed to trigger parkin translocation. This parallels their resistance to DA-induced oxidative stress unless GSH is depleted, further suggesting that M17 cells rely heavily on antioxidant defenses to maintain mitochondrial stability.

On the other hand, it is also possible that the experimental conditions used in this study were not optimal for inducing parkin translocation in M17 cells. While CCCP is widely used to trigger mitochondrial depolarization and subsequent mitophagy,[104–107,150,151] the extent and duration of mitochondrial stress required to engage this pathway may vary between cell types.[163]

Factors such as the duration of treatment, CCCP concentration, or antioxidant levels of M17 cells could influence their response. It remains plausible that parkin could still translocate under different conditions, and further optimization may be needed to determine whether M17 cells are truly resistant to CCCP-induced mitophagy or if the appropriate threshold for activation was simply not met.

Under the conditions tested, however, CCCP treatment did not lead to parkin translocation to mitochondria in M17 cells (Figure 4.6), suggesting that either this pathway is less active in this model system or the required conditions were not met. These findings highlight potential differences in how neuronal and non-neuronal cells regulate mitochondrial stress responses, which could be relevant when interpreting results across different cellular models.

5.3 Exploring the Dispersed Localization of Parkin

The lack of parkin translocation to mitochondria following CCCP treatment raises additional questions regarding parkin's dispersed localization observed in my other experiments post DA treatment. In M17 cells, parkin exhibited a largely diffuse distribution, without noticeable re-localization or aggregation. While this could be a characteristic behavior of parkin in this model following DA-induced stress, there are a few factors that could explain this diffuse distribution, which warrant further consideration.

One potential explanation is that the overall abundance of parkin in the cells may have made subtle changes in its localization less noticeable. With a large pool of parkin present, if only a portion of the protein becomes oxidized or insoluble (Figure 4.2 for example), it might not be sufficient to cause a visible shift in its overall distribution. This large amount of parkin could

effectively "mask" any localized changes, leading to the observed dispersed pattern of parkin localization.

Additionally, it is important to consider the limitations of the antibodies used in this study. The antibody might not have been sensitive enough to detect the oxidized or insoluble forms of parkin, especially if these modified forms are present in only a small proportion of the total protein. Previous studies from our lab have demonstrated that certain antibodies can fail to recognize oxidized parkin, which could explain the lack of visible changes in parkin's localization. Moreover, the resolution power of the imaging techniques used in this study may not have been sufficient to detect these subtle alterations in parkin. To address this, further studies using electron microscopy (EM) may be necessary to achieve the necessary resolution for detecting these modifications at the ultrastructural level.

Finally, it is possible that, even if oxidation or insolubility did occur, parkin may not undergo significant re-localization and form aggregates or foci but instead remain dispersed throughout the cell, retaining their general localization despite the modifications. To address these challenges, future experiments could employ more sensitive detection methods, such as super-resolution microscopy, to identify subtle changes in parkin localization. Additionally, manipulating parkin expression levels or using alternative dopaminergic models could help increase the proportion of oxidized parkin, providing deeper insights into its response to oxidative stress.

5.4 Differences Between Neuromelanin and Melanin Formation

A key objective of this thesis was to investigate the relationship between parkin and neuromelanin (NM) in a cellular model. Previous work has demonstrated that parkin is closely

associated with NM in human dopaminergic neurons, where it co-localizes with NM granules and CD63+ vesicles[131]. Additionally, in vitro studies have shown that recombinant parkin enhances melanin formation, suggesting a potential role in modulating NM synthesis[131]. Based on these findings, I hypothesized that, in my experimental model, parkin would similarly associate with melanin granules and potentially enhance pigment formation. However, my results did not fully align with these expected outcomes, raising questions about the differences between NM synthesis in the human brain and melanin formation in my model system.

NM synthesis occurs predominantly in the cytosol of dopaminergic neurons.[43] It is a non-enzymatic process that begins with the oxidation of DA to form DA quinones.[39,44] These quinones then spontaneously polymerize and react with various cellular components – such as proteins and lipids – to form the complex, heterogeneous NM pigment.[44] This process is further influenced by factors such as the presence of metal ions, particularly iron, which catalyzes DA oxidation via Fenton chemistry, accelerating NM formation.[44,48,52] The cytosolic environment, lacking specialized compartments, allows NM formation to proceed through these spontaneous, oxidation-driven reactions.[44]

In contrast, typical human tyrosinase-driven melanin formation occurs in melanocytes throughout the body, such as the skin, where melanin is synthesized within specialized organelles called melanosomes.[152] This process is enzymatically driven, primarily by tyrosinase, which catalyzes the conversion of tyrosine into L-DOPA and then into dopaquinone.[152] Subsequent enzymatic and non-enzymatic reactions lead to the formation of eumelanin or pheomelanin.[152] The vesicular compartmentalization in melanocytes confines the synthesis of melanin. Because melanin is produced within these vesicles, the cellular machinery and pathways involved differ significantly from those in the cytosolic synthesis of NM.[152]

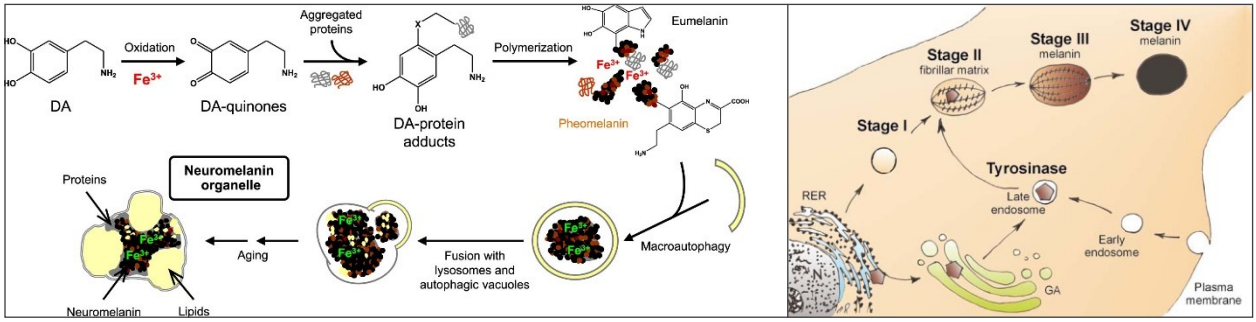

Figure 5.1: Comparison of neuromelanin vs tyrosinase-dependent melanin synthesis pathways.

This figure compares neuromelanin (NM) synthesis from dopamine oxidation (left)[44] with melanin synthesis via tyrosinase (right)[152]. NM formation occurs in the cytosol, where dopamine (DA) undergoes spontaneous oxidation in the presence of redox-active metals like Fe^{3+}, generating DA quinones. These quinones can react with proteins, leading to the formation of DA-protein adducts, which further polymerize into NM. In contrast, tyrosinase-dependent melanin synthesis is confined to intracellular organelles, where melanin is enzymatically formed and progresses through defined maturation stages, from the fibrillar matrix (Stage II) to fully pigmented melanin (Stage IV). This comparison highlights the unregulated, iron-catalyzed cytosolic process of NM formation versus the tightly regulated enzymatic pathway of melanin synthesis within specialized organelles. Modified from Sulzer et al. 2018 and Cichorek et al. 2013.

Given that parkin is normally cytosolic,[105,164] it has immediate access to the cytosolic environment where NM synthesis occurs. However, in the tyrosinase-based model, melanin formation is restricted to vesicles.[152] This spatial separation may preclude parkin from interacting directly with newly formed melanin granules, possibly explaining why the expected co-localization with CD63 or NM-like structures was not observed in my current study.

In summary, while the tyrosinase cDNA transfection model effectively induces bona fide melanin synthesis, it may not fully replicate the complex, cytosolic, non-enzymatic synthesis of NM observed in human dopaminergic neurons. This difference in synthesis pathways may help explain why parkin did not exhibit the anticipated localization patterns. In human neurons, parkin's association with NM and CD63+ vesicles is likely due to its proximity to the cytosolic formation of NM. If parkin is not initially associated with the vesicular compartments where tyrosinase mediates pigment synthesis, it may explain why parkin does not appear directly associated with melanin in this model. Future studies that better replicate the cytosolic conditions of NM synthesis in human neurons could offer further insights into the exact role of parkin in modulating this process. However, under the current experimental conditions – where melanin synthesis is driven by tyrosinase transfection into M17 cells – parkin's localization and solubility do not appear to be altered.

Nonetheless, parkin does seem to enhance melanin formation. Preliminary work by other members of the lab, currently exploring this model in mice, has also shown promising results. Specifically, when murine parkin is endogenously expressed, there appears to be an increase in the degree of melanin pigment. This approach is crucial for confirming the observed effect of melanin formation and may help solidify the link between parkin and melanin synthesis in a more biologically relevant context.

One possible mechanism for this enhancement is that parkin may be reducing ROS levels within the cell, which are known to inhibit tyrosinase activity.[165] Studies have demonstrated that H_2O_2 is a reversible inhibitor of tyrosinase, and that treatment with H_2O_2 in melanoma cells results in decreased levels of tyrosinase mRNA, protein, and activity.[165] Transfection techniques, such as using lipofectamine, can induce cell stress and increase ROS production, which may impair tyrosinase function.[166] Given parkin's antioxidant properties, it could be mitigating these ROS levels, potentially through cysteine residues, thereby allowing tyrosinase to maintain its activity and promote melanin synthesis. Another possibility is that parkin may influence the endocytic pathway,[167] specifically by regulating the movement and function of vesicles that transport melanogenic enzymes like tyrosinase to melanosomes. As demonstrated in Figure 5.1, melanosome formation involves tyrosinase being transported via endosomes to the melanosome.[152,168] If a similar process occurs in the M17 cells after transfection, then parkin may be affecting this pathway. Parkin has been shown to regulate the endocytic pathway by interacting with proteins involved in vesicle formation and membrane fusion.[167] By enhancing this vesicular transport system, parkin could increase the amount of tyrosinase reaching the melanosomes, thereby promoting melanin production.

5.5 Parkin’s Detectability and Stability in the Context of Melanin Formation

As shown previously in Figure 4.8, Western blot analysis using serial fractionation (2% Triton X-100 and 10% SDS lysis buffers) revealed a decrease in detectable parkin levels in both soluble and insoluble fractions of M17-P5 cells transfected with tyrosinase cDNA and incubated for 1–5 days. Notably, the reduction in soluble parkin was not accompanied by a corresponding

increase in the SDS-insoluble fraction, suggesting that parkin was not becoming visibly insoluble under these conditions.

However, given that these are stable cell lines and melanin accumulation increased over time (Figure 4.9), the observed decline in detectable parkin following transient tyrosinase cDNA transfection suggests that parkin could be undergoing modifications that render it undetectable by Western blotting. This could include degradation, conformational changes, or interactions with melanin or other cellular components that interfere with antibody recognition. To further investigate this, future experiments could explore alternative extraction methods using stronger SDS-based buffers (or those containing guanidine hydrochloride or formic acid) to assess whether parkin is present in an even more resistant fraction. Additionally, qPCR analysis of *PRKN* mRNA would help determine whether parkin protein levels are declining due to reduced expression or if the changes are occurring at the post-translational level.

5.6 Limitations of Thesis Study

One of the limitations of this study was the use of transient transfections to express tyrosinase cDNA, rather than utilizing stable cell lines. While transient transfections made it possible to look at both parkin and melanin in the same cell, it could introduce variability in expression levels, which could influence the reproducibility and interpretation of the results. Initially, I worked with a stable SH-SY5Y cell line that expressed tyrosinase in a doxycycline-inducible manner, which was generously provided by the Vila lab. However, when I co-transformed these cells to also express doxycycline-inducible parkin via lentivirus, I encountered an issue whereby the cells selectively expressed either parkin or tyrosinase, but not both

transgenes. This result suggested that there was a competition for doxycycline between the two transgenes' artificial promoter, limiting the ability to express both proteins simultaneously.

Western blot analysis confirmed this issue, as cells with doxycycline treatment showed lower levels of tyrosinase expression compared to the original tyrosinase-expressing cell line. This reduction in tyrosinase expression indicated that the cells were either preferentially expressing parkin or tyrosinase, but not both, as required for my experiments. In an attempt to resolve this, I infected the tyrosinase-expressing SH-SY5Y cells with AAV-parkin, but this strategy also failed to achieve sufficient co-expression. As a result, I shifted to a transient transfection model, using cells that stably express parkin (under a CMV promoter) and transfecting tyrosinase cDNA into them via lipofectamine technique. This approach allowed me to study both parkin cDNA expression and melanin formation within the same cell.

Another limitation of this study is that I did not yet directly show that parkin is protective under the conditions tested. In the human brain (and in virtually all experimental systems examined to date), parkin expression is well-established to confer protection onto neural cells. However, the exact mechanisms through which parkin provides the protection is still unclear. While my experiments did not directly assess this, previous work from several lab members has shown that parkin protects against DA-induced stress in the LVd-BE(2)-M17-PRKN-WT cell line (Figure 5.2). Although these findings were obtained under slightly different conditions, they suggest that parkin may play a similar protective role in my model. Future studies could address this by conducting assays such as for H2O2 (and superoxide) quantification to measure ROS production in the presence or absence of parkin post DA treatment, as well as cell death assays to determine whether cells expressing parkin are more resilient to stress than those without.

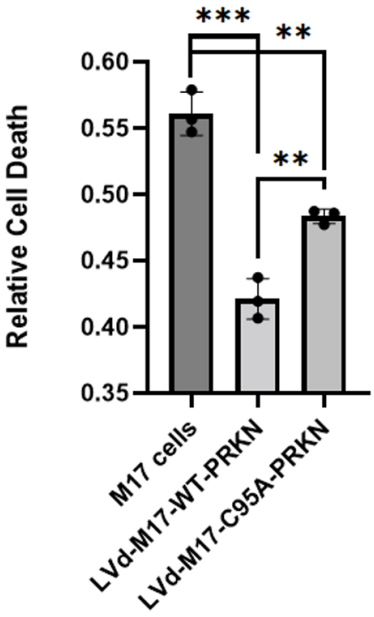

Figure 5.2: Presence of parkin in M17 cells protects cells against dopamine treatment.

Cell cytotoxicity assay for dopamine-treated M17 cells and LVd-M17-WT/C95A-PRKN cells. Cells were exposed to 100 µM dopamine for 12 hours. Data points represent the mean of duplicates ± SEM (n = 3 experiments). Data are from a previous lab member, Mounir El-Hakim (El-Hakim, M. [2022]). *Investigating the Importance of the C95 Residue of Parkin Downstream of Dopamine Stress* [Honour's thesis].[169]

Furthermore, my study did not address how DA stress affects parkin's E3 ligase function. Parkin's E3 ligase activity is an important aspect of its cellular function, particularly in the context of post-mitotic cells. However, due to the focus of this study, I did not specifically assess how DA-induced stress might influence parkin's ability to act as an E3 ligase. That said, research in this area has provided some insights. Previous studies on immunoprecipitated parkin have shown that treatment with 1mM of DA for 10 minutes led to the inactivation of parkin's E3 ligase activity.[170] Additionally, past research from our lab suggests that parkin's protective function could be independent of its E3 ligase activity.[131] For instance, studies with E3 ligase-deficient mutants

(specially C431F mutations) of parkin showed that these mutants were still able to partially protect against DA-induced stress.[131] This suggests that parkin's protective role is not solely dependent on its E3 ligase activity, which is an important aspect to consider. While this was not the main focus of my experiments, future research could look at how DA-induced stress affects parkin's E3 ligase function, either directly or through changes in its downstream substrates, to better understand the full range of mechanisms through which parkin protects cells.

Finally, there are broader limitations to consider when using invitro cell models to study PD-related mechanisms. First, invitro cells do not fully recapitulate the aging processes that occur in the human brain. Aging is a key factor in neurodegeneration, and in vitro models may not account for the complexities of aging in neurons. For example, aging is associated with the accumulation of damaged proteins and impaired mitochondria within neurons, primarily due to oxidative stress.[171] This accumulation can disrupt cellular function and contribute to neurodegenerative processes.

Second, the study does not capture the long-term effects of chronic DA exposure, which is particularly relevant in the context of Parkinson's disease. Chronic exposure to DA metabolites causes damage over time,[62,172] something acute DA stress models may not fully reflect.

Additionally, this study used proliferating, dividing cell lines, rather than differentiated, non-dividing cells. This limits the model's ability to represent more stable, mature cellular states found in post-mitotic neurons. To address this, future studies could incorporate iPSC-derived neurons, which more closely resemble the in vivo cellular environment, allowing for the study of PD mechanisms in differentiated, post-mitotic cells.

Lastly, while DA neurons are central to PD pathology, astrocytes should also be investigated. Recent evidence suggests that parkin likely plays a role in astrocyte function, including their involvement in maintaining neuroinflammatory responses and astrocyte proliferation.[173,174] Since astrocytes are critical for supporting neuronal health and their dysfunction has been implicated in neurodegenerative diseases,[173,174] exploring the role of parkin in astrocytes could offer valuable insights into the broader neurodegenerative process in Parkinson's disease. Therefore, while in vitro models can provide useful insights, they may not fully capture the long-term, age-related, and chronic aspects of DA-induced stress seen in neurodegenerative conditions.

6.0 Conclusion

This study investigated the behavior of parkin under dopamine (DA)-induced stress in undifferentiated M17 neuroblastoma cells, focusing on its oxidation, solubility, and localization. Given parkin's established role in mitigating oxidative stress and interacting with DA quinones, my experiments aimed to further explore parkin's response to DA toxicity and its potential associations with neuromelanin (NM) and CD63. Using two models of DA stress – direct DA addition to the culture media and intracellular melanin synthesis via tyrosinase transfection – I examined how these conditions influenced parkin. In the M17 cell line, DA treatment led to partial oxidation and insolubility of parkin, particularly when glutathione levels were depleted by BSO treatment. However, these changes did not coincide with visible re-localization or aggregation of parkin. In the tyrosinase cDNA-expression model, parkin did not show increased oxidation or insolubility as melanin accumulated, nor did it visibly colocalize with melanin granules, though it appeared to accelerate melanin formation.

A key takeaway from this study is that the extent of parkin's oxidative modifications, solubility changes, and interactions with cellular structures observed in my results appear to be highly dependent on the specific cell model and experimental conditions. Throughout the Discussion section, I considered multiple factors that could contribute to these findings, including antioxidant capacity, DA uptake mechanisms, and differences between NM and melanin formation. Notably, parkin remained largely diffuse even after CCCP treatment, and BSO depletion had a more pronounced effect on parkin than DA exposure alone. These observations suggest that parkin's response to DA stress was likely shaped by the unique cellular environment of M17 cells, including potentially their antioxidant levels, and cannot yet be generalized to parkin's behavior in other neuronal models.

Since parkin is most relevant in the context of DA synthesizing neurons in the *Substantia nigra* and LC—cells that experience high oxidative and dopamine-related stress in ageing humans—future studies should utilize models that better reflect human dopaminergic neurons. This includes primary midbrain neurons, iPSC-derived dopaminergic neurons, or other neuronal lines with a well-defined catecholaminergic phenotype to determine whether these findings extend beyond M17 cells. Additionally, further investigation into the mechanisms regulating parkin's solubility, oxidation, and interactions with NM and lysosomal pathways could provide deeper insight into its role in cellular defense mechanisms in PD.

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