Parkin cooperates with GDNF/Ret signaling during the development and maintenance of the dopaminergic system in mice

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Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

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To whom it may concern:

Prakash Nidadavolu, who was born on 18th of November 1984 and has matriculation number 6646283, is submitting his doctoral dissertation in English. The title of his thesis is: **'Parkin cooperates with GDNF/Ret signaling during the development and maintenance of the dopaminergic system in mice'.**

I hereby certify as a native speaker and molecular biologist that the English language used in this thesis is sufficiently correct for submission.

Yours truly,

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Abbreviations

3-MT	3-methoxytyramine
6-OHDA	6-hydroxydopamine
A/P	Anterior-posterior axes
AADC	Aromatic L-amino acid decarboxylase
ADH	Alcohol dehydrogenase
AHA	Azidohomoalanine
ALDH	Aldehyde dehydrogenase
AR-PD	Autosomal recessive-Parkinson's disease
ARTN	Artemin
ATC	Anaplastic thyroid carcinoma
BDNF	Brain-derived neurotrophic factor
CAKUT	Congenital anomalies of the kidneys and the lower urinary tract
CDNF	Cerebral dopamine neurotrophic factor
CGA	Chromogranin A
CHIP	C-terminus of HSC70 interacting protein
CHX	Cycloheximide
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CSF	Cerebrospinal fluid
D/V	Dorsoventral axes
DA	Dopaminergic
DAT	Dopamine transporter
DBS	Deep brain stimulation
DCB	DAT-Cre BACmid
DCC	Deleted in colorectal cancer
DDC	DOPA decarboxylase
DJ-1	Daisuke-Junko-1
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
DR	Dopamine receptor
Drp1	Dynamin-related protein 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
En	Engrailed
ENS	Enteric nervous system
EOPD	Early-onset Parkinson's disease
EPDA	European Parkinson's disease association
EPM	Elevated plus maze
EPS15	EGFR pathway substrate number 15
ER	Endoplasmic reticulum
FGF8	Fibroblast growth factor 8
FMTC	Familial medullary thyroid carcinoma
FTC	Follicular thyroid carcinoma
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
Gbx2	Gastrulation brain homeobox 2
GCH	GTP-cyclohydrolase I
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family of ligands
GFR	GDNF family of receptors
GPI	Glycosylphosphatidylinositol
GWAS	Genome-wide association studies
HHARI	Human homolog of Drosophila ariadne
HIF-1α	Hypoxia-inducible factor-1α
HOIP	HOIL-1-interacting protein
HSCR	Hirschsprung's disease
Hsp70	Heat shock protein 70
HVA	Homovanillic acid
IBR	In-between-RING
IMM	Inner mitochondrial membrane
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
K	Lysine
KRAB	Krüppel-associated box
LB	Lewy body
LC	Locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine
Lmx	LIM homeobox 6
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO	Monoamine oxidase
MCI	Mild cognitive impairment
MEFs	Mouse embryonic fibroblasts
MEN2	Multiple endocrine neoplasia type 2
MFB	Medial forebrain bundle
Mfn	Mitofusin
MHB	Midbrain-hindbrain boundary
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MSNs	Medium spiny neurons
MTC	Medullary thyroid carcinoma
MTS	Mitochondrial targeting sequence
NC	Neural crest
NCAM	Neural cell adhesion molecule
NCD	Natural cell death

NE	Norepinephrine
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
nRCCs	Nuclear-encoded respiratory chain components
NRF1	Nuclear respiratory factor 1
NRTN	Neurturin
NT	Neurotrophin
OMM	Outer mitochondrial membrane
OP	Open field
OPA1	Optic atrophy 1
Otx2	Orthodentical homeobox 2
P	Postnatal day
Pael-R	Parkin-associated endothelin receptor-like receptor
PARIS	Parkin interacting substrate
PARL	Presenilin-associated rhomboid-like protease
PARP	Poly ADP-ribose polymerase
Pax	Paired box genes
PD	Parkinson's disease
PDI	Protein disulfide isomerase
PGC1α	PPAR-γ coactivator-1α
PI3K	Phosphatidylinositol-3-kinase
PINK1	PTEN-induced putative kinase 1
Pitx3	Pituitary homeobox 3
PKC	Protein kinase C
PLCγ	Phospholipase C γ
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PRAS40	Proline-rich Akt substrate of 40 kDA
PSF	PTB-associated splicing factor
PSPN	Persephin
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog
PTPN11	Protein tyrosine phosphatase non-receptor type 11
PV+	Parvalbumin-positive
RBR	Ring-between-Ring
REP	Repressor element of parkin
Ret	Rearranged during transfection
Rheb	Ras homologue enriched in brain
RING	Really-interesting-new-gene
ROS	Reactive oxygen species
rpS6	Ribosomal protein S6
RTKs	Receptor tyrosine kinases
SHANK3	SH3 and multiple Ankyrin repeat domains protein 3
Shh	Sonic hedgehog
SN	Substantia nigra
SNP	Single-nucleotide polymorphism
SNpc	Substantia nigra pars compacta
SQSTM1	Sequestosome-1
ST	Striatum
STAT3	Signal transducer and activator of transcription 3
STN	Subthalamic nucleus
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
TIM	Translocase of the inner membrane
TM	Transmembrane
TOM	Translocase of the outer membrane
TRAF	TNF receptor-associated factor
TrkA	Tyrosine receptor kinase A
TSC2	Tuberous sclerosis complex 2
Ub	Ubiquitin
UB	Ureteric bud
UBL	Ubiquitin-like domain
UPD	Unique parkin domain
UPR	Unfolded protein response
VDAC	Voltage dependent anion channel
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
Y	Tyrosine

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Abstract

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the progressive loss of nigrostriatal dopaminergic (DA) neurons. While the incidence of PD is increasing, the etiology of cell type-specific degeneration of DA neurons still remains obscure. Mutations in parkin are the most common cause of recessively inherited early onset PD. Recently, we showed that aged mice lacking parkin and the GDNF receptor, Ret in the DA system evince an enhanced degeneration of nigrostriatal DA neurons compared to mice that only lack Ret. In addition, overexpression of parkin rescued the Ret deficiency phenotype in the mouse DA system, suggesting a functional cross talk of parkin and Ret in the maintenance of the nigrostriatal DA system.

The present study investigated whether deletion of parkin could normalize the enlarged nigrostriatal DA system in MEN2B mice, which comprise a point mutation (M918T, MEN2B) in the kinase domain resulting in constitutive activation of Ret. For this purpose parkin deficient MEN2B mice were generated and as a first result it was found that deletion of parkin normalized the nigrostriatal DA neurons but not elevated striatal dopamine levels in adult MEN2B mice. DA neurons undergo biphasic natural cell death (NCD) postnatally, like any neuronal population. In MEN2B mice, soon after the second NCD event, the augmented DA system appears. Further analysis revealed that parkin deletion normalizes the augmented DA system right after the second NCD event in MEN2B mice hinting at an important role of parkin in the developmental context of the DA system. Biochemical analysis of parkin deficient mice and parkin deficient MEN2B mice revealed that parkin loss negatively regulates Ret/MEN2B protein levels in the DA system. Cancerous cells are well known to switch their energy source by activating glycolysis and inhibiting complex I activity of mitochondria. Nigrostriatal DA neurons of MEN2B mice showed reduced complex I activity, however, deletion of parkin fully restored mitochondrial function in MEN2B mice, most likely by reducing MEN2B protein levels. Investigating the underlying molecular mechanisms of parkin and Ret cross talk *in vitro* underscored the *in vivo* findings and further revealed that parkin may regulate Ret/MEN2B protein levels at the translational level. Finally, parkin and Ret converge on common signaling pathways, where parkin overexpression complements Ret signaling and deletion of parkin normalizes constitutively active Ret (MEN2B) signaling. Further, knockdown of parkin demolishes Ret mediated downstream signaling *in vitro*.

In conclusion, these findings reveal a role of parkin in regulating trophic signaling to promote the survival of nigrostriatal DA neurons in the context of PD, and to an extent this complex functional cross talk of parkin and Ret could also be pivotal in cancer. Convergence of parkin

and Ret function over mitochondrial function and signaling pathways could be an ideal target to develop novel therapies against both life threatening diseases.

Zusammenfassung

Morbus Parkinson (Parkinson's disease, PD), die zweithäufigste neurodegenerative Erkrankung, ist durch einen progredienten Verlust nigrostriataler dopaminerger Neurone (DA) charakterisiert. Während die Inzidenz von PD ansteigt, ist die Ätiologie der zelltypspezifischen Degeneration der DA Neurone noch immer unklar. Die häufigste Ursache der rezessiven Form der PD mit juvenilem Krankheitsbeginn stellen Mutationen im Parkin-Gen dar. Kürzlich konnten wir zeigen, dass ältere Mäuse, die sowohl für Parkin als auch für den GDNF Rezeptor, Ret, defizient sind, eine erhöhte Degeneration nigrostriataler Neurone im DA System aufweisen im Vergleich zu Mäusen, die nur für Ret defizient sind. Darüber hinaus konnte durch Überexpression von Parkin der Ret Phänotyp in der Maus im DA System normalisiert werden, was nahelegt, dass ein funktioneller Austausch zwischen Parkin und Ret für die Sicherung des Bestehens des nigrostriatalen DA Systems stattfindet.

In der vorliegenden Arbeit wurde untersucht, ob eine Parkin-Deletion zu einer Normalisierung des vergrößerten nigrostriatalen Systems von MEN2B Mäusen beiträgt, in denen Ret konstitutiv aktiv ist aufgrund von einer Punktmutation (M918T, MEN2B) in der Kinasedomäne. Zu diesem Zweck wurden Parkin-defiziente MEN2B-Mäuse generiert. Als ein erstes Ergebnis wurde festgestellt, dass die Deletion von Parkin die nigrostriatalen DA-Neuronen normalisierte, aber den striatalen Dopamin Spiegel bei erwachsenen MEN2B-Mäusen nicht erhöhte. DA Neuronen unterliegen postnatal biphasischem natürlichem Zelltod (natural cell death, NCD), wie jede neuronale Population. Bei MEN2B-Mäusen erscheint das erweiterte DA-System kurz nach dem zweiten NCD-Event. Weitere Analysen zeigten, dass die Parkin-Deletion das erweiterte DA-System nach dem zweiten NCD-Ereignis in MEN2B-Mäusen normalisiert, was auf eine wichtige Rolle von Parkin bei der Entwicklung des DA-Systems hindeutet. Die biochemische Analyse von Parkin-defizienten Mäusen und Parkin-defizienten MEN2B-Mäusen zeigte, dass Parkin-Verlust die Ret / MEN2B-Proteingehalte im DA-System negativ reguliert. Von Krebszellen ist bekannt, dass sie ihre Energieversorgung auf eine Aktivierung der Glykolyse und Hemmung der Komplex I-Aktivität von Mitochondrien umschalten. Nigrostriatale DA-Neuronen von MEN2B-Mäusen zeigten eine verminderte Komplex-I-Aktivität, die jedoch durch die Deletion von Parkin völlig wieder hergestellt wurde, vermutlich durch die Verringerung des MEN2B-Proteingehalts. Die Untersuchung der zugrundeliegenden molekularen Mechanismen der Parkin Interaktion mit Ret *in vitro* unterstrich die *in vivo* Befunde und zeigte ferner, dass Parkin die Ret / MEN2B-Proteinkonzentrationen auf Translationsniveau regulieren könnte. Schließlich laufen Parkin und Ret auf gemeinsamen Signalwegen zusammen, wobei Parkin Überexpression die Ret Signalweiterleitung komplettiert, Parkin Deletion konstitutiv aktive Ret (MEN2B)

Signalweiterleitung normalisiert. Weiterhin wird *in vitro* die Ret vermittelte Signalweiterleitung durch einen *knock down* von Parkin inhibiert.

Zusammengefasst zeigen diese Ergebnisse, dass Parkin bei der Regulation trophischer Signale zur Sicherung des Erhalts nigrostriataler DA-Neurone im Zusammenhang mit Parkinson eine Rolle spielt. Bis zu einem gewissen Ausmaß könnte diese komplexe funktionelle Interaktion von Parkin und Ret auch bei Krebs entscheidend sein. Die Konvergenz der Parkin- und Ret-Funktion bei mitochondrialen Funktionen und Signalwegen könnte ein ideales Ziel sein, um neuartige Therapien gegen diese beiden lebensbedrohlichen Krankheiten zu entwickeln.

1. Introduction

1.1 Brain Ageing

The ageing process influences a large number of factors as changes occur across multiple organs, including the brain. These changes include molecular ageing, intercellular and intracellular ageing, tissue ageing, resulting in alterations in the organ which ultimately lead to changes in cognition and behavior ***(Peters 2006)***. Physical changes during the ageing process include a decline in weight of the brain with respect to age, at a rate of about 5% per decade after 40 years and the actual rate of decline in brain volume seems to increase rapidly over the age of 70. This decline in brain volume has been attributed to a decline in neuronal volume rather than neuronal numbers which could be related to sex based on the area's most affected in men and women ***(Murphy et al. 1996; Peters 2006).*** Further, volume loss is also accompanied by increased ventricular volume and other cerebrospinal fluid (CSF) spaces and changes in the number, organization of synapses and the dendritic arborization ***(Anderton 2002; Barnes 2003)***. Changes in the brain areas do not occur to the same extent in all brain regions, as volume of the prefrontal cortex and striatum (ST) are most affected, followed by temporal lobe, cerebellar vermis, cerebellar hemispheres and hippocampus that are reduced over the age of 60 based on the magnetic resonance imaging (MRI) studies ***(Scahill et al. 2003; Peters 2006)***. Alterations in the dopamine neurotransmission are also linked to ageing, as decrease in dopamine release with increasing age, around 10% per decade from early adulthood. This leads to reduced binding of dopamine to its receptors and results in decline of dopaminergic (DA) pathways between the frontal cortex and ST, which ultimately leads to decline in cognitive and motor performance over age ***(Mukherjee et al. 2002; Nyberg & Bäckman 2004; Peters 2006; González-Hernández 2010).***

Other factors that are implicated at the cellular level of ageing brain include higher production of superoxide anion radicals, hydroxyl radicals, nitric oxide and peroxynitrite which are major source of free radicals that exceeds the endogenous antioxidant reserves ***(FLOYD & HENSLEY 2002; Volchegorskii et al. 2004)***. Further, calcium dysregulation ***(Toescu et al. 2004)***, abnormal accumulation of damaged proteins and organelles ***(Trojanowski & Mattson 2003; Mattson & Magnus 2006)*** as well as nucleic acids ***(Lu et al. 2004)*** and impaired cellular energy metabolism are also included ***(AMES & LIU 2004; Melov 2004)***. Oxidative damage to mitochondrial DNA and electron transport chain proteins lead to cellular energy deficits in aged tissue as mitochondria are one of the major producers of reactive oxygen species (ROS) ***(Gibson et al. 2010)***. General physiological alterations of brain ageing are caused by cells lack of responsiveness to stress, which could potentially lead to

various neurological disorders and this can be amended by exercise and nutritional interventions ***(Stranahan & Mattson 2012)***.

1.1.1 Neurodegeneration: an exacerbation of the ageing process

One of the strongest risk factors for the development of most neurodegenerative disorders is ageing. The current dogma holds that the combinations of unknown environmental and genetic factors exacerbate the fundamental cellular and molecular changes of normal ageing in selective neuronal populations, hastening to cell death. In this perspective, increased oxidative and nitrative stress and the resultant damage interfere with biomolecule function, and promote ***(Hwang 2013)*** protein aggregation events, as seen for α-synuclein in Parkinson's disease (PD), which are also observed during normal ageing ***(Gray et al. 2003; Kanaan et al. 2008; Hindle 2010)***, albeit to a lesser extent. However, these changes are greatly exacerbated during pathophysiological conditions. Neurons vulnerable to age related degeneration are protected by multiple neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) for DA neurons. Neurotrophic signaling is another factor that is affected in normal physiological process of ageing and is exacerbated in neurodegenerative conditions ***(Mattson et al. 2004; Budni et al. 2015; Farrand et al. 2015)***. Neuroprotective signaling exerted by neurotrophic factors involve receptor tyrosine kinases (RTKs) and are important for suppression of oxidative and metabolic stress, excitotoxicity, calcium overload, protein and DNA damage ***(Mattson 2003; Mattson & Magnus 2006)***. A significant number of studies have stated that dietary restrictions guard against neurodegeneration. A low calorie diet ***(Maswood et al. 2004; Mattson & Magnus 2006)*** and physical exercise ***(Tillerson et al. 2001; Cotman & Berchtold 2002; Cohen et al. 2003)*** were proven to increase the expression of endogenous neurotrophic factors like GDNF and BDNF and consequent activation of neuroprotective signaling pathways that promote survival of neurons.

1.2 Parkinson's disease

1.2.1 History

The disease was first medically described by the English doctor James Parkinson as Shaking Palsy in 1817 based on his observations from 6 patients ***(Parkinson 2002)***. However, rest tremor and festination were described by Sylvius de la Boë in 1680 and Sauvages in 1768 respectively and early descriptions of the disorder, dates back to 1000 BC based on the traditional Indian texts, under the name of "Kampavata" ***(Corti et al. 2011)*** and ancient Chinese sources ***(Manyam 1990; Goetz 2011)***. Bradykinesia was later distinguished as a separate cardinal feature of PD from the descriptions of Jean-Martin Charcot in 1872. He also differentiated PD from other tremor based neurological disorders and classified them

into Parkinsonism-plus syndromes and was the first to use the term “Parkinson’s disease”. Anatomical alterations and damage to the substantia nigra (SN) in PD was first proposed by Edouard Brissaud and further studies of the midbrain in relevance to the disease were pursued by others ***(Pearce 1998; Goetz 2011)***. Together, PD is classified as a movement disorder characterized by four cardinal symptoms: resting tremor, muscular rigidity, bradykinesia and postural and gait impairment. Although, not all patients with PD have dementia, around 40% of all patients develop the condition at later stages of the disease ***(Dodel et al. 2008; Lokk & Delbari 2012; Meireles & Massano 2012)***.

1.2.2 Epidemiology

PD is the second most prevalent neurodegenerative disorder after Alzheimer’s disease, with an estimated worldwide population of 6.3 million affected, according to the European Parkinson’s disease Association (EPDA). Studies based on age, gender and the geographic location reveal that the incidence of PD rises with age. Meta-analysis of worldwide population showed for every 100,000 people, 41 are affected at the age of 40–49; 107 at the age of 50–59; 173 at the age of 55–64; 428 at the age of 60–69; 425 at the age of 65–74; 1087 at the age of 70–79 and 1903 people older than 80 were affected for every 100,000 people ***(Pringsheim et al. 2014)***. Gender based differences in the prevalence of PD are generally higher in males and are significantly higher between the age groups, 60–69 and 70–79 in males compared to females at similar age. Furthermore, an increased premature mortality rate correlating with earlier onset of the disease was also reported in male patients compared to females, due to increase in dopamine levels as a consequence of high estrogen activity in females ***(Haaxma et al. 2007; Hirsch et al. 2016)***. Based on the geographic location, higher prevalence of the disease was found in Europe, North America and South America compared with African, Asian and Arabic countries. The risk of PD is expected to increase by more than 50% by 2030 ***(Kalia & Lang 2015)***.

1.2.3 Etiology and pathology

The etiology of PD remains ambiguous and it is believed that the risk of developing PD is due to the contribution of both environmental and genetic factors affecting numerous fundamental cellular processes. PD is classified into two major classes, which are sporadic and familial forms. Sporadic PD is due to complex factors where both environment and susceptibility genes play a role ***(Dawson & Dawson 2010)*** and is a consequence of exposure to toxins or traumatic head injuries, while familial forms of the disease follow a Mendelian pattern of inheritance due to mutations in one or more genes. According to the meta-analysis studies of genetic variants, single nucleotide polymorphisms between the control cohorts and PD patients of all existing European-ancestry PD Genome-wide association studies (GWAS), there are 28 gene loci including the previously known, that are

shown to associate as independent risk factors of the disease in the human genome ***(Nalls et al. 2014; Kalia & Lang 2015)***.

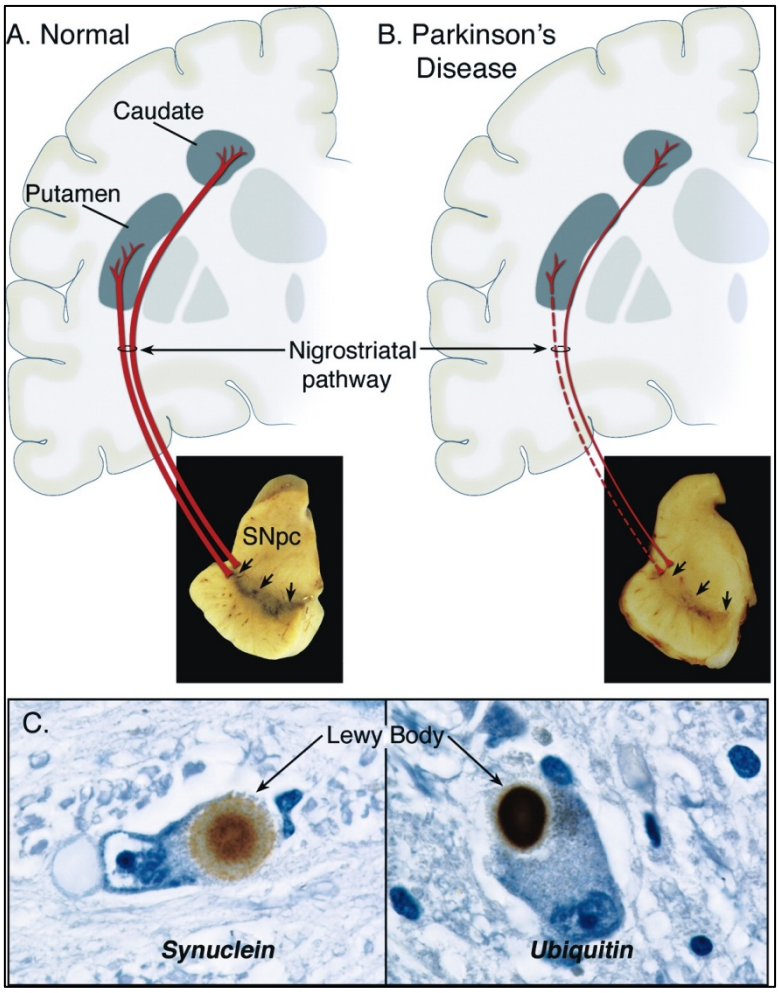


Figure 1.1 Pathology of Parkinson's disease

Scheme depicting the DA neurons of SNpc (black arrows) with neuromelanin pigmentation, innervating (thick solid red lines) into dorsolateral putamen and caudate nucleus (striatum) under normal conditions **(A)**; Scheme depicting the pathogenic conditions (PD), where DA neurons of SNpc lack neuromelanin pigmentation, as a sign of pronounced degeneration of cell bodies in SNpc and their innervations in the ST (dotted lines represent more profound and solid red lines for modest degeneration) **(B)**; Photomicrographs depicting neuronal inclusions, also known as Lewy body (black arrows) in SNpc DA neurons, immunolabeled with α-synuclein and ubiquitin **(C)**; Information and image courtesy ***(Dauer & Przedborski 2003)***.

Both sporadic (accounts for 90%) and familial (accounts for 10%) forms of PD present with similar clinical and pathological hallmarks of PD. These include the profound loss of midbrain DA neurons and their innervations, which in turn leads to insufficient amounts of dopamine in the ST resulting in motor impairment ***(Antony et al. 2013)***. Among the other DA populations, the substantia nigra pars compacta (SNpc) DA neurons of ventrolateral tier innervate into dorsal ST, known as nigrostriatal pathway. These nigrostriatal neurons are primarily prone to

degeneration in PD which is progressive in nature (Figure 1.1). Along with motor symptoms, those arise primarily due to lack of dopamine, PD patients also present with non-motor symptoms including autonomic dysfunction (constipation), rapid eye movement, sleep disorder, impaired olfaction and other psychiatric symptoms (anxiety and depression), pain, fatigue and mild cognitive impairment (MCI) ***(Kalia & Lang 2015).*** In fact, non-motor symptoms are more frequent and precede the severe motor symptoms during the course of the disease and as the disease progresses, patients present with more complications like postural instability with frequent falls, freezing gait and psychosis (Figure 1.2).

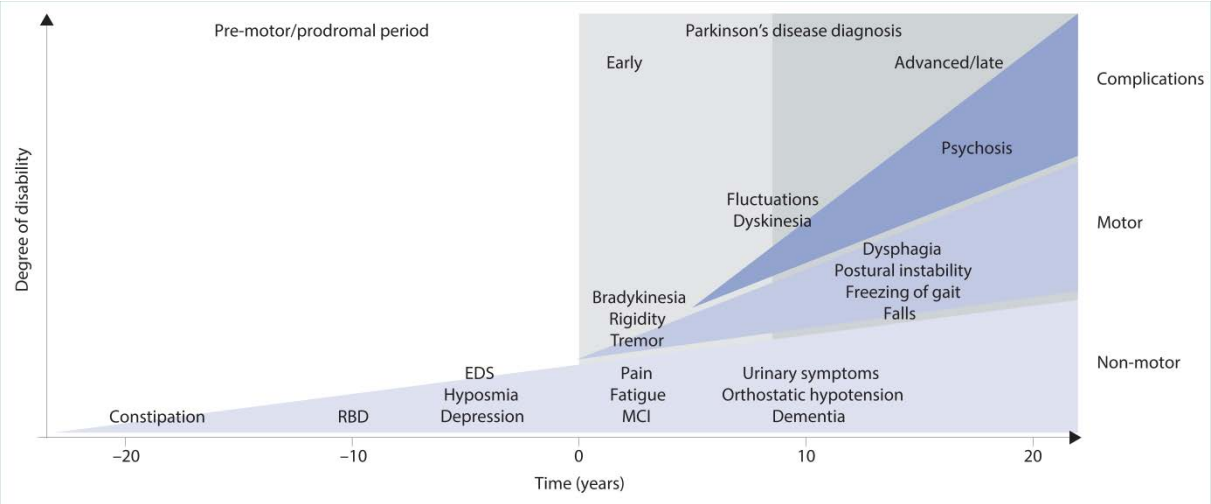


Figure 1.2 Progression during the course of Parkinson's disease

Frequency of the pre-motor and motor symptoms illustrated during the course of Parkinson's disease. Where time zero indicates the time of diagnosis of the disease, based on the early motor symptoms that are only evident after almost 80% of the dopamine depleted in the ST and around 60% of the SNpc DA neurons were already lost ***(Dauer & Przedborski 2003)***. However, early diagnosis preceding the motor symptoms and during the prodromal period of 20 years will provide a time window for disease modifying therapies, prior to degeneration of SNpc DA neurons. RBD=Rapid eye movement sleep behavior disorder; EDS=Excessive daytime sleepiness; MCI=Mild cognitive impairment. Information and image courtesy ***(Kalia & Lang 2015)***.

Another hallmark of PD is Lewy pathology, which is the presence of intracytoplasmic proteinaceous inclusions positive for α-synuclein, ubiquitin, neurofilaments and other proteins in cell bodies called Lewy bodies (LB) and processes called Lewy neurites (Figure 1.1) ***(Goedert et al. 2013)***. Extensive analysis of healthy and diseased post-mortem human brains have shown that Lewy pathology in different regions of the central nervous system (CNS) emerges in a predictable fashion and a staging procedure was described by Braak and colleagues, based on the topography of these changes ***(Braak et al. 2003)***.

1.2.4 Diagnosis and treatment strategies

Criteria for clinical diagnosis of PD, is a two-step process. The first step is based on the onset of motor disabilities (bradykinesia with rest tremor or rigidity), as the central feature to define Parkinsonism. The second step is to determine if the previous criteria is attributable to PD ***(Postuma et al. 2015)***. However, as clinical symptoms are only evident after most (60%) of the DA neuronal population is lost ***(Dauer & Przedborski 2003)***, increasing recognition is also given to premotor symptoms and a separate criteria is incorporated for the prodromal PD diagnosis ***(Postuma et al. 2015)***.

Although, there is no cure for this debilitating disease, in the late 1950s, a seminal discovery showed that dopamine replacement by delivering its natural precursor levodopa could reverse Parkinsonism symptoms in the reserpine-model ***(Carlsson et al. 1957; Carlsson et al. 1958)***. Since these first discoveries, levodopa is the primary medication used to ease Parkinsonism symptoms in patients. Despite the symptomatic benefit of levodopa, long term use decreases the effectiveness overtime due to further loss of DA neurons, and is associated with complications like dyskinesias and motor fluctuations. Deep brain stimulation (DBS) of subthalamic nucleus (STN) is a surgical procedure considered as an effective treatment for late or advanced PD patients whose motor symptoms are disabling and cannot be adequately controlled with medication ***(Health Quality Ontario 2005; Martinez-Ramirez et al. 2015)***. Even though, the combination of DBS and DA drugs, well improve the patient quality of life, these treatment strategies cannot halt or reverse the disease progression.

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the brain, whose synthesis is catalyzed by glutamic acid decarboxylase (GAD). Tyrosine hydroxylase (TH), a rate limiting enzyme for dopamine synthesis along with GTP-cyclohydrolase I (GCH) and aromatic L-amino acid decarboxylase (AADC), mediates dopamine synthesis in DA neurons. Clinical studies were performed to restore the GABAergic balance by delivering viral vectors encoding for GAD to STN and also to restore dopamine synthesis by delivering viral vectors encoding for AADC, TH and GCH bilaterally into the putamen under the name of Prosavin in patients. Despite safe delivery of viral vectors into brain with successful long term expression of transgenes with no adverse effects, outcomes from the clinical trials were not completely satisfying. Moreover, both approaches were symptomatic treatments and could be helpful for patients with more advanced disease ***(Kirik et al. 2017; Blits & Petry 2017)***. Therefore, current research should target more towards disease modifying strategies that stop or interfere with the course of disease. For example, cell replacement therapies in conjunction with neurotrophic factors are highly pertinent. Target derived neurotrophic factors are known to facilitate the functional integration of neural transplants into the local circuitry which is paramount in regulating extrapyramidal movement in patients. Alternatively, neurotrophic

factors delivered in gene therapy particularly during early stages of the disease could be an appropriate strategy.

1.2.5 Genes involved in PD

Environmental factors are an important risk for developing PD, but are not solely responsible. Indeed many studies have been carried out on the pathophysiological features of PD, but recent advances in identifying genetic causes of PD have led to the elucidation of many mechanistic details resulting from a complicated interplay of both genetic and environmental factors. Although large-scale GWAS have proposed 28 gene loci as potential independent risk factors of PD ***(Nalls et al. 2014)***, only a subgroup of those have gained the status of *PARK* loci (Table 1.1) ***(Lubbe & Morris 2014)*** to date and they are expected to increase. Mutations in genes linked to familial monogenic forms of PD are proposed to mediate autosomal dominant *(SNCA, UCHL1, LRRK2, VPS35* and *EIF4G1)* and autosomal recessive (*PARKIN, PINK1, DJ-1, ATP13A2, PLA2G6* and *FBX07*) forms of Parkinsonism and encode proteins of seemingly diverse functions. Although mutations in these genes account for only a small fraction (10%) in causing the disease, there is enough evidence stating that same genes are affected in most common sporadic forms of PD such as LRRK2 ***(Gilks et al. 2005; Lesage et al. 2007)*** and parkin ***(Dawson & Dawson 2010; Dawson & Dawson 2014)***. Furthermore, genes encoding for parkin, phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and DJ-1, those are implicated in familial forms of PD and are crucial in some aspects of mitochondrial function. Even more compelling observations come from neurotoxin models such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) ***(Mizuno et al. 1988)*** and rotenone ***(Brown et al. 2006)***. These have been confirmed to cause Parkinsonism-like symptoms by specifically inhibiting complex I activity of the mitochondria. These tantalizing observations from PD genetics and environmental toxins suggest that the underlying pathogenic mechanisms in both familial and sporadic PD could be similar. Moreover, they all point to altered mitochondrial function as a key aspect of PD etiology.

1.2.5.1 Parkin

Mutations in parkin are the most common causes of early-onset PD (EOPD), estimated to account for at least 50% in families with autosomal recessive-PD (AR-PD) ***(Lücking et al. 2000)*** and are also implicated in (15%) sporadic PD ***(Periquet et al. 2003)***. Parkin is an E3 ubiquitin (Ub) ligase. It is ubiquitously expressed and belongs to a Ring-between-Ring (RBR) family of ligases that use an auto-inhibitory mechanism that was first identified for parkin, which modulates ubiquitination activity ***(Chaugule et al. 2011)***. Ubiquitination (transfer of the small 76 amino acid protein, Ub, through a series of enzymes) depends on the type of Ub

(covalently binds to Lysine (K) residues of protein substrates) linkage such as K27, K29, K48, and K63 determined by E2 Ub-conjugating enzymes or E3 ligases.

Table 1.1 Defined loci and genes for PD, including Mendelian genes

Locus	Chromosomal position	Gene	Inheritance	Disorder	Mutations
PARK1	4q21–22	*SNCA*	AD	EOPD	A30P, E46K, H50Q, G51D, A53T; genomic duplications/triplications
PARK2	6q25.2–27	*Parkin*	AR	EOPD	~ 170 mutations, including point mutations and exonic rearrangements
PARK3	2p13	Unknown	AD	Classical PD	Unconfirmed
PARK5	4p14	*UCHL1*	AD	Classical PD	Unreplicated mutations in single sibling pair
PARK6	1p35–36	*PINK1*	AR	EOPD	~ 50 point mutations; rare large deletions
PARK7	1p36	*DJ-1*	AR	EOPD	~15 point mutations and large deletions
PARK8	12q12	*LRRK2*	AD	Classical PD	~ 7 pathogenic (including G2019S) of ~80 missense mutations
PARK9	1p36	*ATP13A2*	AR	Kufor-Rakeb syndrome	~ 5 point mutations
PARK10	1p32	Unknown	Risk factor	Classical PD	Unconfirmed
PARK11	2q36–37	Unknown (not *GIGYF2*)	AD	LOPD	7 missense variants
PARK12	Xq21–25	Unknown	Risk factor	Classical PD	Unconfirmed
PARK13	2p12	*HTRA2*	AD or Risk factor	Classical PD	2 missense variants
PARK14	22q13.1	*PLA2G6*	AR	Early onset dystonia-parkinsonism	2 missense mutations
PARK15	22q12–13	*FBXO7*	AR	Early onset parkinsonism-pyramidal syndrome	3 point mutations
PARK16	1q32	Unknown	Risk factor	Classical PD	Unconfirmed
PARK17	16q11.2	*VPS35*	AD	Classical PD	~ 3 missense variants
PARK18	3q27.1	*EIF4G1*	AD	Classical PD	Rare mutations

AD autosomal dominant, *AR* autosomal recessive, *EOPD* early onset PD, *LOPD* late onset PD

Table 1.1 List of genes which gained *PARK* status and are potential risk factors of PD with their chromosomal positioning and the mode of inheritance is depicted. Risk factor indicates the association with the risk of disease onset, whereas further validation is needed to clearly state the mode of inheritance. Image modified from ***(Lubbe & Morris 2014)***.

Although parkin was primarily considered as a classical RING domain E3 ligase, those catalyze the direct transfer of Ub's from the E2 enzyme to substrate proteins with no physical interaction with Ub. However, later studies have shown that parkin functions as a

RING/HECT hybrid in which the catalytic cysteine residue (Cys431) in its RING2 domain (Figure 1.3) transiently binds to Ub forming a thioester linkage before ligating onto a substrate, similar to other RBR family members human homolog of *Drosophila* ariadne (HHARI) and HOIL-1-interacting protein (HOIP) ***(Spratt et al. 2014; Truban et al. 2016)***. Taken together, parkin uses a hybrid mechanism combining both RING and HECT E3 ligase functions to catalyze different modes of mono- and polyubiquitination of structurally and functionally distinct substrates in a context specific manner. Polyubiquitinated substrates like FBP1 ***(Ko et al. 2006)***, PARIS ***(Shin et al. 2011)***, parkin-associated endothelin receptor-like receptor (Pael-R) ***(Dutta et al. 2014)***, cyclin E ***(Ikeuchi et al. 2009)*** and MIRO ***(Birsa et al. 2014)*** are marked for degradation by the proteasomal system, while monoubiquitinated substrates such as Septin-5 or CDCrel-1 ***(Zhang et al. 2000)***, synphilin I ***(Lim et al. 2005)***, dopamine transporter (DAT) ***(Jiang et al. 2004)*** and EPS15 ***(Fallon et al. 2006)*** are involved in signaling. Monoubiquitination by parkin has been specifically implicated in the regulation of receptor endocytosis and trafficking. Loss of parkin function leads to accumulation of its polyubiquitination substrates and disruption in the signaling of its monoubiquitination substrates.

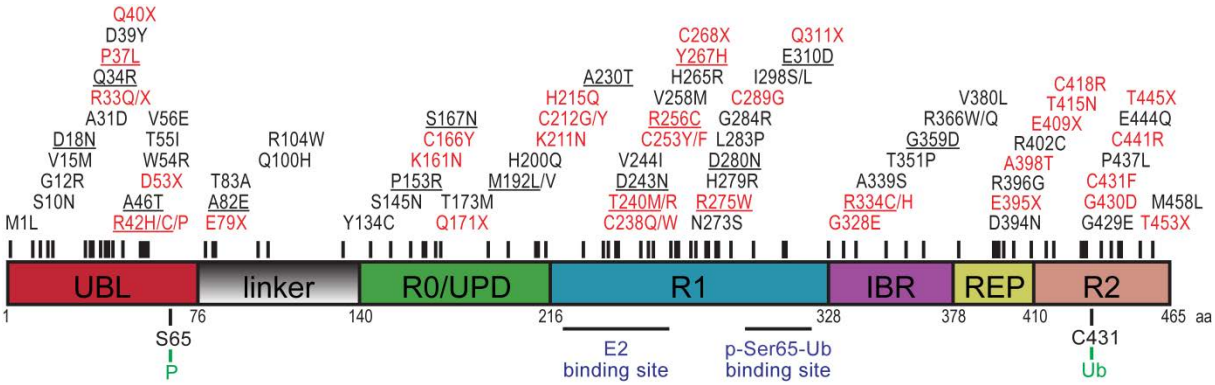


Figure 1.3 Domain structures of parkin and its pathogenic mutations in PD

The *PARKIN* gene spans more than 500 kilobases, has 12 exons and encodes a 465 amino acid protein. Parkin protein contains a ubiquitin-like domain (UBL) at its amino-terminal, flexible linker, really-interesting-new-gene (RING) 0/unique parkin domain (R0/UPD), RING1 (R1), in-between-RING (IBR), repressor element of parkin (REP) and RING2 (R2) at its carboxyl-terminal. E2 co-enzyme and pSer65-Ub binding sites as well as Ser65 phosphorylation and Cys431 catalytic sites are depicted. Mutations in parkin are the primary cause of EOPD and till date 150 pathogenic mutations have been identified including missense and nonsense mutations. Mutations depicted in red are experimentally confirmed loss of function mutations, whereas the functionality of other variants depicted in black remains obscure. Underlined mutations are common variants with allele frequencies greater than 1:10,000. Information and image courtesy ***(Truban et al. 2016).***

1.2.5.1A Mitochondrial targets of parkin

Mitochondria are interconnected and form dynamic networks that appear as long tubules. The morphology of mitochondria is modified through constant fission and fusion processes. Mitofusins 1/2 (Mfn 1/2) together with optic atrophy 1 (OPA1) regulate the outer and inner membrane fusion events of mitochondria respectively in a sequential manner ***(Song et al. 2009)***, while cytosolic dynamin-related protein 1 (drp1) mediates mitochondrial fission. Recent findings suggest a role of parkin in mitochondrial fusion by transcriptionally upregulating OPA1 through NF–κB signaling ***(Müller-Rischart et al. 2013)***. Further, parkin (by activation through PINK1) leads to ubiquitination and ultimate degradation of mitochondrial fusion protein, mitofusin ***(Poole et al. 2010)***. However, the role of parkin in mitochondrial fission is more controversial, as parkin was shown to subject drp1 to proteasomal degradation ***(H. Wang et al. 2011)***, while other reports suggest that parkin promotes mitochondrial fission by positively regulating drp1 ***(Deng et al. 2008)***. Sub-cellular trafficking of mitochondria to various compartments like axons and dendrites is well understood ***(Scarffe et al. 2014)***. Miro, an outer mitochondrial membrane protein facilitates the transport of mitochondria through its binding to kinesin-1 via an adaptor protein Milton. Parkin, along with PINK1 was shown to influence mitochondrial mobility via phosphorylation and ubiquitination of its substrate Miro, targeting it for proteasomal degradation and thereby arrests mitochondrial motility ***(X. Wang et al. 2011; Liu et al. 2012)***. In addition, parkin dependent ubiquitination and degradation of phosphorylated Miro sequesters or quarantines damaged mitochondrion prior to their clearance ***(X. Wang et al. 2011)***.

Numerous studies have shown that parkin/PINK1 signaling is essential for mitochondrial clearance through mitophagy. PINK1 has been shown to recruit parkin to damaged mitochondria where it subsequently ubiquitinates the autophagic adaptor protein p62 or SQSTM1 (sequestosome-1) and voltage dependent anion channel1 (VDAC1) both of which are necessary for the final clearance of damaged mitochondria ***(Geisler et al. 2010)***. Mediating mitochondrial autophagy (mitophagy) is no longer viewed as primary function of parkin. Recent findings described that parkin restores mitochondrial defects under moderate stress conditions through NF-κB signaling and protects from stress-induced cell death. However, parkin, along with PINK1 promotes elimination of irreversibly damaged mitochondria through mitophagy, indicating that parkin triggers distinct pathways in mediating mitochondrial health and quality control based on the severity of mitochondrial damage ***(Müller-Rischart et al. 2013)***. In addition to fusion, fission and mitophagy, parkin seems to play a role in mitochondrial biogenesis as well. ***(Shin et al. 2011)*** showed for the first time that parkin interacts with a Krüppel-associated box (KRAB domain) containing transcriptional repressor, parkin interacting protein (PARIS). PARIS is a zinc-finger protein, represses the

expression of peroxisome proliferator-γ (PPARγ) coactivator-1α (PGC1α) and nuclear respiratory factor 1 (NRF1), both of which are involved in mitochondrial biogenesis (Figure 1.4). Parkin, ubiquitinates PARIS leading to proteasomal degradation and hence de-represses PGC1α and NRF1.

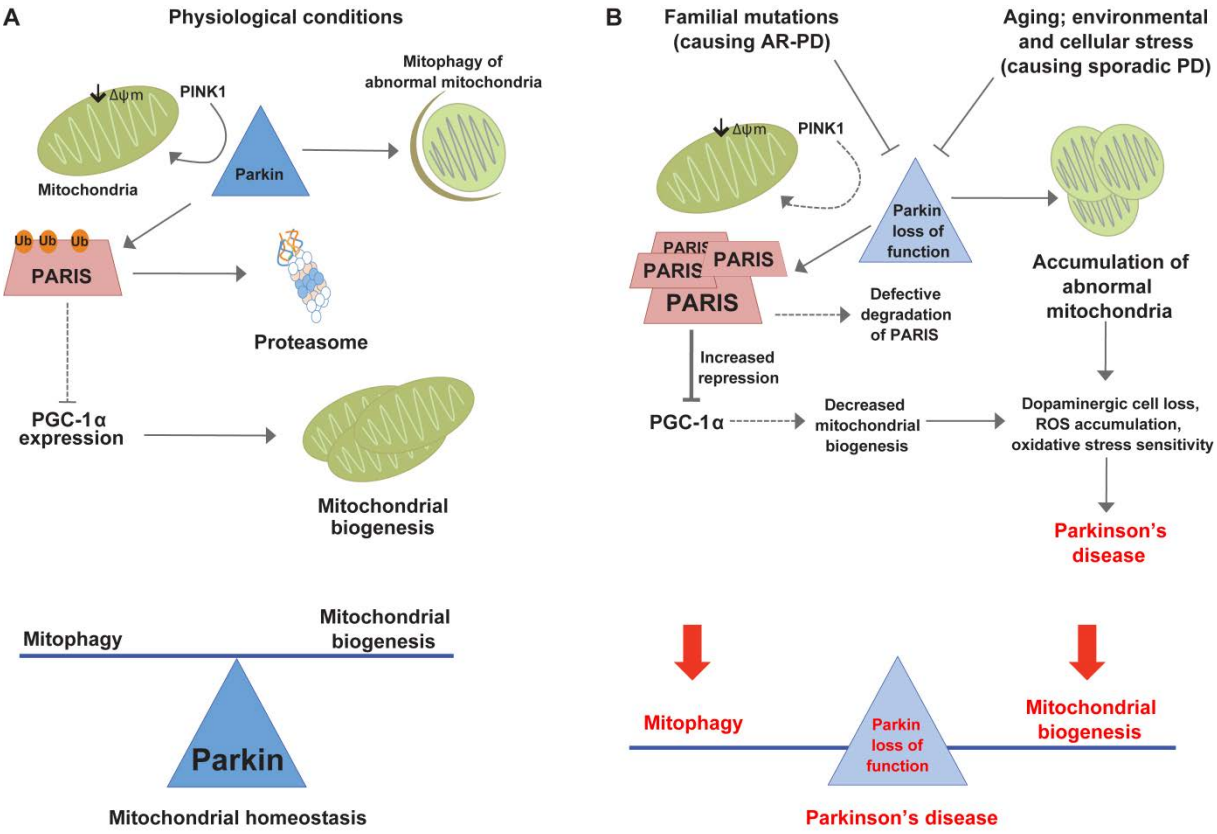


Figure 1.4 Role of parkin in mitochondrial biogenesis

Under physiological conditions alterations in mitochondrial membrane potential ($\Delta\Psi m$) initiate parkin mediated mitophagy, where PINK1 phosphorylates and recruits parkin to mark defective mitochondria for degradation. Further, parkin ubiquitinates and promotes degradation of PARIS, a transcriptional repressor leading to PGC1α expression that enables mitochondrial biogenesis **(A)**; Parkin loss of function resulting from various stressors leads to accumulation of defective mitochondria and accumulation of parkin substrates such as PARIS, increases repression on PGC1α expression, blocking mitochondrial biogenesis contributing to DA cell loss and ultimately leading to PD. Parkin mediates mitochondrial homeostasis via balancing degradation and biogenesis under normal conditions, whereas, parkin loss of function perturbs mitochondrial homeostasis, leading to pathogenic conditions. Information and image courtesy ***(Castillo-Quan 2011)***.

As suggested by the evolving body of evidence, parkin is an integral regulator of multiple aspects of mitochondrial quality control, ranging from mitochondrial dynamics and mitophagy to biogenesis. Any disruption of parkin function either due to mutations or environmental stressors will affect the above processes, resulting in decreased expression of proteins

involved in mitochondrial function ***(Palacino et al. 2004)***, impaired energy generation as well as reduced proton gradient thus, rendering the cell highly vulnerable to any kind of stress.

1.2.5.1B Non-mitochondrial targets of parkin

Parkin has been shown to have neuroprotective activity against a wide range of stressors (extrinsic and intrinsic) in both *in vitro* and various *in vivo* models and it has been shown to mediate this neuroprotection through the activation of NF-κB (nuclear factor-κB) signaling. NF-κB is a transcription factor which regulates various biological processes such as apoptosis and differentiation. The well elucidated classical NF-κB pathway involves the association of IκB with NF-κB, hence inhibiting NF-κB signaling. Upon stress stimuli, IκB kinase (IKK) complex phosphorylates IκB, marking it for proteasomal degradation via polyubiquitination mediated by parkin ***(Henn et al. 2007)***. Recent studies have shown that parkin regulates IKK signaling through proteasome-independent ubiquitination, apart from classical proteasomal dependent degradation of IκB. Parkin mediated K63-linked ubiquitination of the IKK complex and other upstream regulators such as TRAF2 (tumor necrosis factor (TNF) receptor-associated factor) and TRAF6 eventually leads to activation and translocation of NF-κB to nucleus, where it promotes transcription of cell survival genes ***(Krappmann & Scheidereit 2005)***. Perturbation in calcium homeostasis, accumulation of unfolded secretory proteins and energy deprivation are common causes of endoplasmic reticulum (ER) stress that have been shown to contribute to the pathogenesis of PD ***(Lindholm et al. 2006; Wang & Takahashi 2007)***. Pael-R is a multipass G-protein-coupled transmembrane protein and a putative parkin substrate. Misfolded Pael-R tends to aggregate and its presence in LB suggests a role in PD pathogenesis. The C-terminus of HSC70 interacting protein (CHIP) upregulates and sequesters heat shock protein (Hsp70) and facilitates parkin-mediated ubiquitination and degradation of unfolded and insoluble Pael-R, wherein CHIP serves as a cofactor of parkin and suppresses Pael-R induced ER stress. Further, parkin loss of function causes ER stress with accumulation of cytotoxic fibrils and protein aggregates in cells ***(Imai & Takahashi 2004)***. Growth factor-mediated endocytosis and signaling of epidermal growth factor receptor (EGFR) was shown to be regulated by parkin through its E3 ligase function. EGFR pathway substrate number 15 (EPS15), enhances the internalization of EGFR, as EPS15 mediates the association of EGFR with clathrin-coated pits through binding to its adaptor protein AP-2. However, parkin-mediated monoubiquitination inhibits the ability of EPS15 to interact and facilitate the binding of EGFR to clathrin-coated pits and increases the timeframe for downstream signaling activation, suggesting parkin as a negative regulator of receptor endocytosis ***(Fallon et al. 2006)*** (Figure 1.5).

Interestingly, parkin null mice do not exhibit the phenotype of nigrostriatal DA cell loss ***(Goldberg et al. 2003; Itier et al. 2003)***. But, parkin loss seems to confer susceptibility to inflammation and neurodegeneration induced by other neuronal stressors ***(Frank-Cannon et al. 2008)***. Further, parkin overexpression is proven to be neuroprotective in MPTP mouse model of PD ***(Yasuda et al. 2011)***.

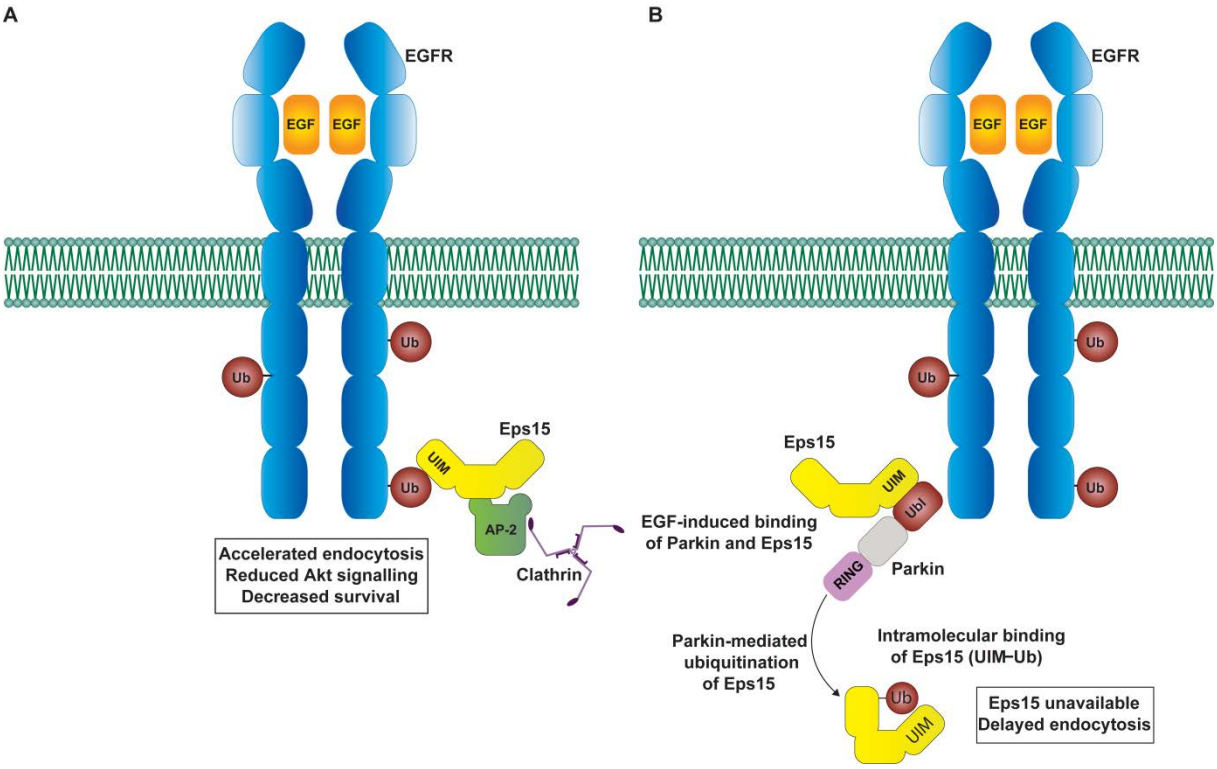


Figure 1.5 Role of parkin in receptor endocytosis

Upon growth factor (EGF) binding, in the absence of parkin, EPS15 is continuously available for facilitating the binding of ubiquitinated EGFR to clathrin coated pits, accelerating receptor endocytosis and decreasing the time-window for downstream signaling which may not be good for neuronal survival **(A)**; The presence of parkin inhibits EPS15 association with ubiquitinated EGFR through monoubiquitination, leading to intramolecular UIM-Ub binding within EPS15, delaying receptor endocytosis and increasing the time-window for downstream signaling, which promotes neuronal survival. Information and image courtesy ***(Husnjak & Dikic 2006)***.

1.2.5.2 PINK1

Mutations in PINK1 are the second most common cause of EOPD in AR-PD families, and heterozygous PINK1 mutations are implicated in sporadic PD ***(Valente et al. 2004)***. PINK1 is a highly conserved Ser/Thr kinase which triggers the alarm during mitochondrial impairment and promotes degradation of damaged mitochondria. It was initially identified in an expression analysis of genes upregulated in response to tumor growth suppressor **p**hosphatase and **ten**sin homolog deleted on chromosome 10 (PTEN), hence the name PINK1 (PTEN-induced putative kinase 1). PINK1 consists of mitochondrial targeting

sequence (MTS) and a transmembrane domain (TM) at its N-terminal region and is localized to both the cytosol and imported into mitochondria ***(Truban et al. 2016)***. PINK1 remains as a full-length 63 kDa protein within the cytosol, and upon mitochondrial import via translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) it undergoes proteolytic cleavage resulting in an approximately 52 kDa fragment. Consecutive proteolytic processing of PINK1 is mediated by mitochondrial processing peptidase (MPP), which cleaves MTS ***(Greene et al. 2012)*** and the inner mitochondrial membrane (IMM), presenilin-associated rhomboid-like protease (PARL) cleaves TM in the matrix ***(Deas et al. 2011)***.

Selective import of PINK1 into healthy mitochondria, rapid proteolytic cleavage and subsequent degradation through proteasome system forms a regulatory switch to maintain healthy pool of mitochondria in the cell. However, alterations in the mitochondrial membrane potential blocks this process, resulting in the accumulation of full-length PINK1 along with TOM machinery forming a multimeric protein complex on the outer mitochondrial membrane (OMM) indicating mitochondrial impairment. Parkin is well known for promoting the degradation of damaged mitochondria through polyubiquitination of damaged mitochondrial surface proteins that recruit the autophagic machinery. However, recent studies clearly stated that mitochondrial depolarization activates PINK1, and is stabilized on the OMM with its kinase domain facing the cytosol as PINK1 kinase activity is a pre-requisite for the activation and translocation of cytosolic parkin on to depolarized mitochondria. OMM stabilized PINK1, phosphorylates its substrates on the OMM like Mfn2, voltage-dependent anion channel (VDAC) or Miro which may act as a receptor to dock parkin at the depolarized mitochondria ***(Kondapalli et al. 2012; Scarffe et al. 2014)***. PINK1 was shown to phosphorylate Ser65 of the Ub moiety which binds to the RING1 domain, triggering the conformational change and release of auto-inhibitory state of parkin. Further, PINK1 phosphorylates Ser65 position of parkin in the N-terminal of UBL domain leading to activation of E3 ligase activity resulting in translocation and polyubiquitination of OMM proteins of the depolarized mitochondria thereby refining the damaged mitochondria ***(Kazlauskaite et al. 2014; Shaw 2014)***. Although, PINK1 and parkin deficiency exhibit similar morphological deficits of mitochondria including muscle degeneration and impaired locomotor activity, parkin overexpression could rescue PINK1 deficiency phenotypes but not vice versa, indicating that PINK1 acts upstream of parkin ***(Truban et al. 2016)***. Interestingly, PINK1 deficient mice do not exhibit alterations in the nigrostriatal DA neurons ***(Kitada et al. 2007)***.

1.2.5.3 DJ-1

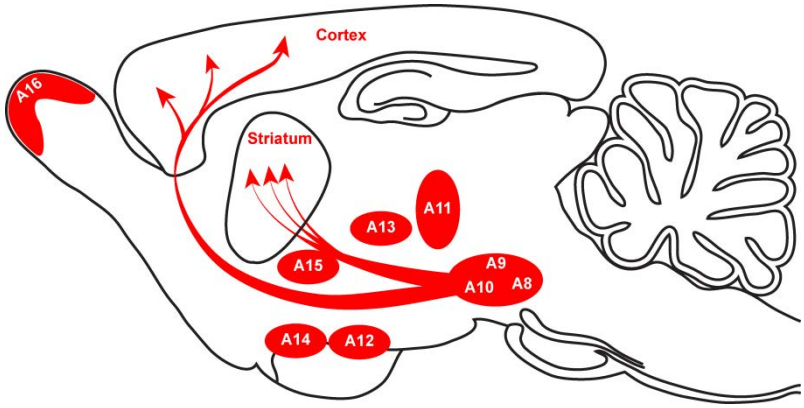
Extremely rare mutations in Daisuke-Junko-1 (DJ-1) are less common causes of EOPD compared to parkin and PINK1 ***(Kalia & Lang 2015)***. Expression of DJ-1 is ubiquitous in most cell-types including the brain and was first identified as a novel oncogene ***(Nagakubo***

et al. 1997). DJ-1 is a small 189 amino acid protein that is highly conserved among the species and exists as a dimer. It is a multifactorial protein which acts as a transcriptional regulator ***(Shinbo et al. 2005; Zhong et al. 2006; Clements et al. 2006)***, antioxidant ***(Görner et al. 2007)***, molecular chaperone ***(Bandyopadhyay & Cookson 2004; Deeg et al. 2010)***, and is also involved in mitochondrial homeostasis ***(Canet-Avilés et al. 2004; Hayashi et al. 2009)***. As loss of DJ-1 function is implicated in many diseases like ARPD, familial amyloid polyneuropathy (FAP), Type II diabetes, stroke and chronic obstructive pulmonary disorder (COPD), whereas, gain of function is associated with cancer ***(Ariga et al. 2013)***. Being a transcriptional modulator, DJ-1 positively regulates gene expression of TH, the rate limiting enzyme for dopamine synthesis by sequestering a transcriptional repressor of the human TH gene promoter, polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF) ***(Zhong et al. 2006)***. Tumor suppressor p53 activity is regulated by DJ-1 through its direct association with the DNA-binding region of p53 to inhibit its transcriptional activity to suppress p53-induced Bax-mediated apoptosis ***(Fan et al. 2008)***. ROS induced oxidative modification of sulfhydryl (R-SH) groups in cysteine residues to sulfenic (R-SOH) and sulfinic acids ($R\text{-}SO_2H$) are reversible, whereas modification to sulfonic acid ($R\text{-}SO_3H$) is irreversible. Among the three cysteine residues at 46, 53 and 106 positions in DJ-1, Cys106 is more prone to oxidative damage. Under physiological circumstances DJ-1 localizes to cytosol and nucleus, however it translocates into mitochondria upon oxidation of the Cys106 residue in response to oxidative stimuli and mediates protection against stressors ***(Canet-Avilés et al. 2004)***. DJ-1 was also shown to confer neuronal protection by directly binding to PTEN, a negative regulator of PI3K/Akt signaling, to inhibit its enzymatic activity thereby promoting PI3K/Akt signaling pathways during stress conditions ***(Raymond H. Kim et al. 2005; Kim et al. 2009)***. Further, DJ-1 was shown to genetically interact with ERK signaling to control the development of eye and wing in *Drosophila* ***(Aron et al. 2010)***. Although DJ-1 protects DA cultures from rotenone-induced death by enhancing the ERK-dependent mitophagy ***(Gao et al. 2012)***, DJ-1 deficiency does not result in DA degeneration in mice similar to parkin and PINK1 deficient mice as mentioned earlier ***(Yamaguchi & Shen 2007; Chandran et al. 2008)***. Interestingly, inactivation of ARPD genes- *Parkin*, *PINK1* and *DJ-1* at the same time (triple knock out) seems to have no effect on the nigral DA neurons in mice of all ages ***(Kitada et al. 2009)***. However, loss of function in any of these proteins (parkin, PINK1, DJ-1) leads to EOPD with an average onset of below 40 years, in humans ***(Kalia & Lang 2015)***.

1.3 Midbrain DA system

Dopamine is a catecholamine neural transmitter in the nervous system that was first described during the 1950's. Besides its function as a neurotransmitter, it also acts as a

precursor in the synthesis of another neurotransmitter, norepinephrine (NE), from which epinephrine is synthesized ***(Carlsson et al. 1957; CARLSSON 1959)***. Dopamine was initially assumed only to be an intermediate in tyrosine degradation ***(Blaschko 1942)*** or as a precursor of the neurotransmitters NE and epinephrine and it was only later recognized as an independent chemical transmitter ***(Hornykiewicz 2002; Carlsson 2001; Meiser et al. 2013)***. DA neurons and their basic organization in the brain ***(CARLSSON et al. 1962)*** were first studied in the early 1960's using the formaldehyde histofluorescence method that was developed by Falck, Hillarp and coworkers ***(FALCK et al. 1962)***. There are nine distinct population of DA neurons, A8–A16 (Figure 1.6) those are anatomically and functionally heterogeneous and localized to diencephalon, mesencephalon and to the olfactory bulb ***(Chinta & Andersen 2005; Björklund & Dunnett 2007)***. Although the full degree of conservation between the mouse and human midbrain DA system is not completely clear, the basic anatomical organization seems well conserved (Figure 1.6; 1.7) ***(Blaess & Ang 2015)***.

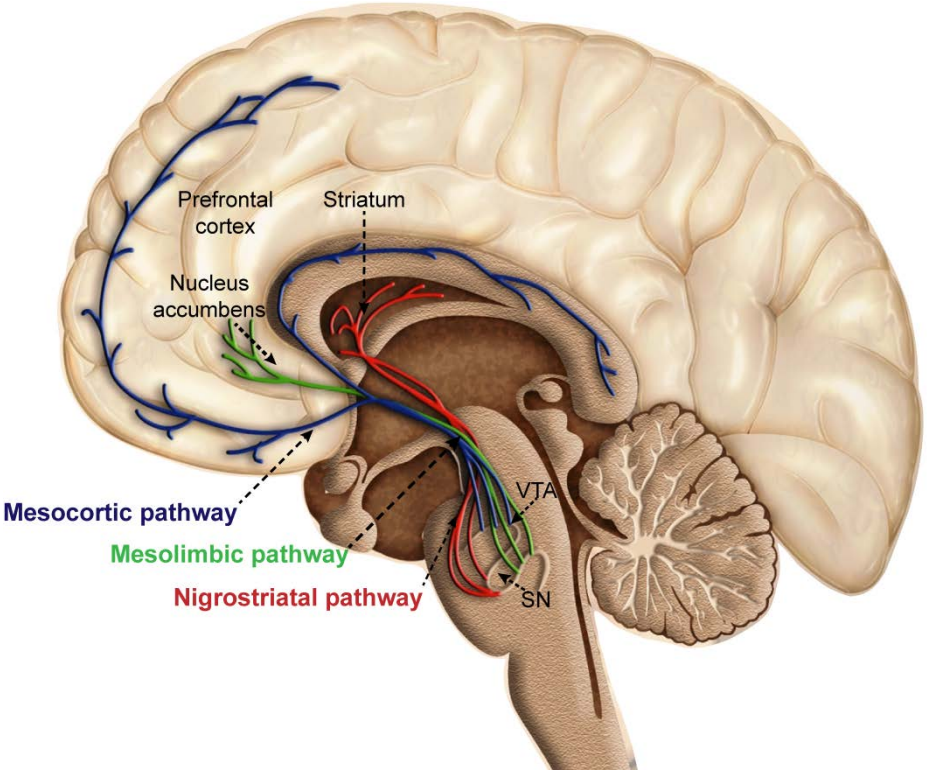


1.6 Distribution of DA neuronal population in rodents

Schematic view of sagittal adult mouse brain depicting the localization and distribution of DA cell bodies and their projections. Total nine distinct DA neuronal populations are innervated from the mesencephalon to the olfactory bulb as illustrated by arrows. A8 (Retrorubral field in mouse), A9 (SNpc), A10 (VTA), A11 (caudal diencephalic periaqueductal gray), A12 (arcuate nucleus), A13 (zona incerta in the ventral thalamus), A14 (periventricular nucleus), A15 (preoptic area and rostral hypothalamus), A16 (dendritic periglomerular). Image modified from ***(Björklund & Dunnett 2007; Kauer & Malenka 2007)***.

DA neurons constitute less than 1% of the total neuronal population of the brain and approximately 75% of total DA neurons are located in the ventral midbrain region and regulate several aspects of basic brain function ***(Arias-Carrión et al. 2010; Blaess & Ang 2015)***. These midbrain DA neurons were sub-grouped based on their localization, namely retrorubral field (RRF) (A8), SN (A9) and ventral tegmental area (VTA) (A10) and there anatomical organization and projection patterns are much more complex than the DA

neurons located in other regions of the brain ***(Björklund & Dunnett 2007)***. DA innervations originating from the SN (A9), VTA (A10) and hypothalamus (A12, A14 and A15) interconnect many areas of the brain and were categorized into four major paths based on their distinct anatomical and functional components. The four pathways include mesocortical (from VTA to the prefrontal, cingulate and entorhinal cortices), mesolimbic (from VTA to the nucleus accumbens), nigrostriatal (from SN to the striatum) and tuberoinfundibular (from hypothalamus (arcuate and periventricular nuclei) to the pituitary gland) ***(Rasheed & Alghasham 2012; Hou et al. 2014)*** (Figure 1.6).



1.7 Human midbrain DA system

Schematic representation of midbrain DA neurons in SN and VTA and there axonal projections. SNpc DA neurons project into striatum (putamen and caudate nucleus) forming nigrostriatal pathway (Red). VTA DA neurons innervate into prefrontal cortex (dorsal and ventral), nucleus accumbens and ventral striatum together forming mesocortical pathway (blue) and mesolimbic pathway (green). Image modified from ***(Arias-Carrión et al. 2014)***.

As SN and VTA DA neurons partly project into forebrain region they were initially thought to be a homogeneous population based on their use of dopamine as its neural transmitter, however, later studies shed light on the heterogeneity of these populations based on their target innervation patterns ***(Menegas et al. 2015; Khan et al. 2017)***. In contrast, A9 and A10 DA neurons are closely related with <1% of differentially expressed genes based on the transcriptome analysis ***(Grimm et al. 2004)***.

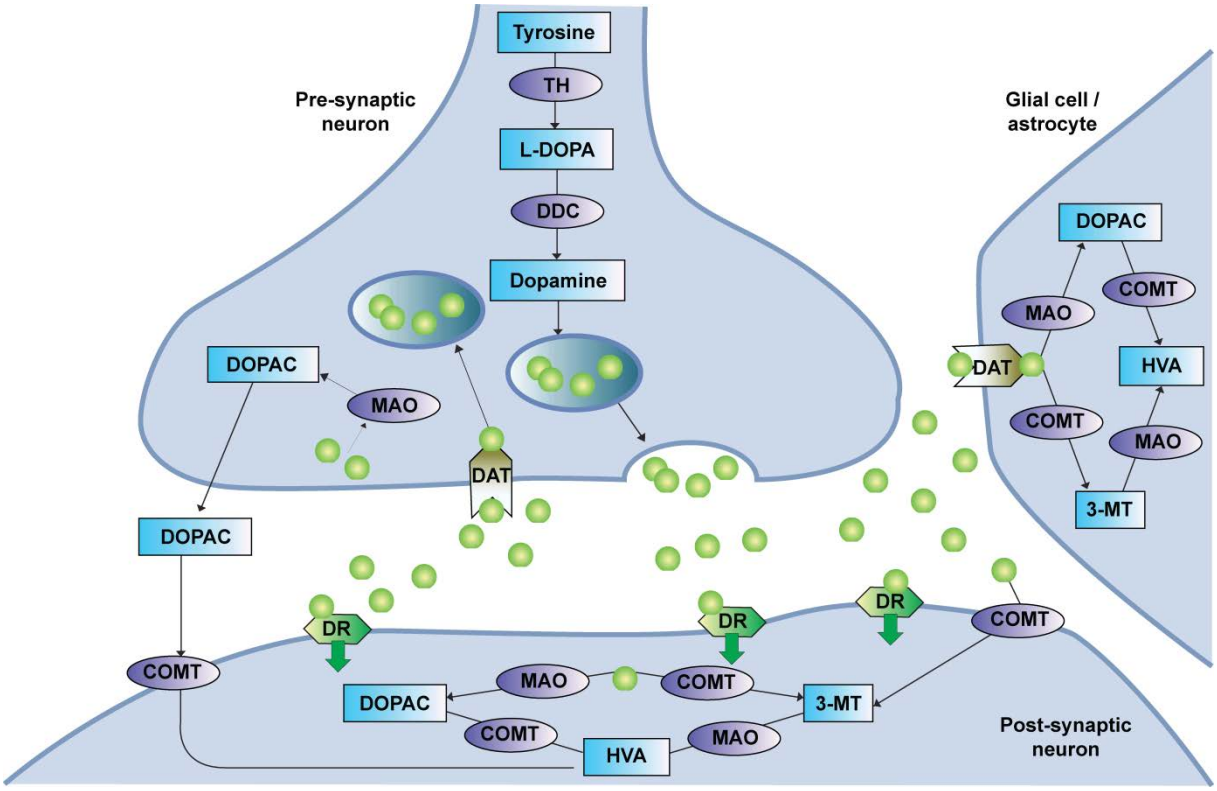
Since midbrain DA neurons regulate a broad spectrum of behaviors including the control of extrapyramidal movement, emotion, motivation, reward processing, sleep, mood, attention, learning and working memory ***(Schultz 2007a; Schultz 2007b; Roeper 2013)***, much attention has gone to this particular cluster of DA cell groups. Including PD, their dysfunction is also prominently implicated in neuropsychiatric disorders such as attention deficit hyperactivity disorder, schizophrenia, obsessive-compulsive disorder, depression and addiction ***(Arias-Carrión et al. 2010; Poulin et al. 2014; Hou et al. 2014; Blaess & Ang 2015).***

1.3.1 Dopamine metabolism

The structure of dopamine consists of a catechol ring and an amine side chain that is synthesized from tyrosine, which is a conditionally essential amino acid as it is synthesized from hydroxylation of an essential amino acid phenylalanine by phenylalanine hydroxylase. Classical dopamine synthesis in the CNS involves a two-step enzymatic reaction within the cytosol or neurites of DA neurons, where the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) (precursor of dopamine) that is catalyzed by an enzyme TH is the first step. TH utilizes endogenous source of tyrosine, molecular oxygen (O_2), and BH_4 (tetrahydrobiopterin) as its cofactor synthesized from GCH and catalyzes the addition of a hydroxyl group to the *meta* position of tyrosine to form L-DOPA and the by-products H_2O and dihydrobiopterin. This step is considered to be rate-limiting as it uses endogenous sources for the synthesis of L-DOPA and regulation of TH activity represents prime means of dopamine concentration in neurons ***(Kumer & Vrana 1996)***. High levels of endogenous tyrosine could saturate TH, due to its K_m (Michaelis constant) for tyrosine in the µM range. Regulation of TH activity is highly intricate, being the initial step for the biosynthesis of 3 different neurotransmitters serving distinct functions in various cell or tissue types. For example, dopamine serves differentially in SN and VTA DA neurons, including a small fraction of neurons in the hypothalamus. Similarly, neurons in locus coeruleus (LC) and peripheral sympathetic neurons use norepinephrine and adrenergic neurons in CNS and adrenergic secretory cells in the adrenal medulla use epinephrine as neurotransmitters ***(Kumer & Vrana 1996)***. Further, DOPA decarboxylase (DDC) or AADC catalyzes the removal of the carboxyl group from L-DOPA to form dopamine, and is final step in the cascade in DA neurons. This conversion is highly efficient due to the low K_m and a high V_{max} of DDC compared to L-DOPA.

However, for other neuronal populations that use NE and epinephrine as neurotransmitters, dopamine β-hydroxylase (DBH) utilizes copper as a cofactor to catalyze the reaction to form norepinephrine. Subsequently, phenylethanolamine *N*-methyltransferase (PNMT) catalyzes

the final reaction of forming epinephrine (Figure 1.8) ***(Kuhar MJ, Couceyro PR 1999; Meiser et al. 2013)***.



1.8 Dopamine biosynthesis and degradation

Enzymatic processes involved in the biosynthesis of dopamine, the neurotransmitter for DA neurons. Shown are action potential evoked release of dopamine into synaptic cleft and reuptake of excess dopamine through DAT and other surrounding glial cells, subsequent degradation of intracellular dopamine in glial cells, cytosolic (vesicle leaked) dopamine in DA neurons and also in the post synaptic neurons. DR= dopamine receptors (D1R or D2R), Image courtesy ***(Quaak et al. 2009)***.

Newly synthesized dopamine is readily sequestered into specialized storage vesicles by vesicular monoamine transporter 2 (VMAT2) to prevent or minimize oxidative stress due to dopamine oxidation within the DA neurons. Within the vesicles, dopamine is stabilized from oxidation-prone conditions due to the slightly acidic environment, which protects from enzymatic degradation by monoamine oxidases (MAO's) and facilitates regulated release and rapid replenishment. Amphetamine, reserpine and other similar compounds can inhibit VMAT2, interfering with vesicular sequestration of newly synthesized dopamine and thereby perturbing the dopamine homeostasis by collapsing the proton gradient necessary for dopamine transport ***(Sulzer & Rayport 1990; Chaudhry et al. 2008)***.

Upon excitation of DA neurons, by the process of exocytosis, vesicles target to the active zone of the presynaptic cleft and release their soluble contents (dopamine) into the synapse through docking and rapid fusion with the synaptic membrane. Excess dopamine in the

synaptic cleft is either captured by surrounding glial cells and metabolized or taken back to the presynaptic terminals for reuptake. Reuptake of dopamine into neurons is mediated by DAT, which transports dopamine from the extracellular (synaptic cleft) to the intracellular space and sequestered into storage vesicles by VMAT2 or is metabolized by MAO's ***(Giros & Caron 1993)***. The importance of the reuptake process was to terminate the dopamine induced signal transduction and was illustrated using DAT-deficient mice, as these mice exhibit hyperactive behavior due to accumulation of dopamine in the synapse ***(Giros et al. 1996; Gainetdinov et al. 1999)***.

Intracellularly accumulated dopamine as a consequence of leakage from the vesicles or synthesized dopamine that is not efficiently packed in to the vesicles is metabolized to avoid dopamine oxidation that is capable of producing toxic ROS and reactive semiquinones and quinones ***(Chinta & Andersen 2005)***. Dopamine is converted into 3,4-dihydroxyphenylacet-aldehyde (DOPAL) by oxidative deamination and the reaction is catalyzed by MAO. DOPAL is predominantly oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) by either alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH). Resultant DOPAC diffuses into the extracellular space and is converted to homovanillic acid (HVA) by catechol-O-methyl transferase (COMT) outside of the DA neurons, as no activity of COMT is observed in nigrostriatal neurons ***(Myöhänen et al. 2010)***. Dopamine that is captured by glial cells within the synaptic cleft is instantly metabolized by COMT to 3-methoxytyramine (3-MT) and is further converted to HVA, an end product of dopamine metabolism, and the reaction is catalyzed by MAO-B along with ALDH (Figure 1.8) ***(Meiser et al. 2013)***.

1.3.2 Development of DA neurons

The ontogeny of the midbrain DA system is a complex multistep process. It begins with early events such as specification, migration and differentiation. Following the early developmental events, DA neurons innervate into the target positions and form specific patterns of connectivity with the post synaptic population. Early during development, the neural plate is subdivided into various domains through the anterior-posterior (A/P) and dorsoventral (D/V) axes. As it progresses, floor plate, basal plate, alar plate and roof plate arise from D/V patterning, whereas, A/P patterning segregates forebrain, midbrain and hindbrain territories ***(Smits et al. 2006)***. The molecular and anatomical borders of the midbrain are maintained by the expression of certain markers such as orthodentical homeobox 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2), which, in turn, induce the expression of their downstream targets which are important for DA progenitor cells ***(Orme et al. 2009)***. Progenitors of the midbrain DA neurons are born at the midbrain-hindbrain (MHB) boundary also known as isthmus, which is determined by the overlapping expression of fibroblast growth factor 8 (FGF8) and the morphogen sonic hedgehog (Shh) ***(Smidt & Burbach 2007)***. Many factors play a crucial

role in the induction of the progenitor pool of cells to develop and mature into DA neurons. These factors include the early expression of paired box genes (Pax), pax2 and pax5 that are important for the development of MHB via induction of FGF8 expression (important in the development of MHB and cerebellum), through its direct binding to the transcriptional factor engrailed 2 (En2) ***(Li Song & Joyner 2000)***. WNT signaling contributes to the establishment of MHB through activating the engrailed genes En1 and En2. Although they are not essential for specification of DA neurons, these factors function later to maintain the survival of progenitors. Further, mice deficient for En1 and En2 exhibit early morphological defects (at E9) and loss of DA neurons in a gene-dose dependent manner ***(Liu & Joyner 2001; Simon et al. 2001)***. Specific hierarchical signals from the floor plate (Shh signaling) and roof plate facilitate the segregation of midbrain and diencephalon into dorsal and ventral regions and further Shh expression induces the ventralizing effect of progenitor cells. Shh together with FGF8 signaling and other transcription factors like LIM homeobox6 (Lmx)1a/b, OTX1/2 and NKX6.1 influence the early differentiation into the midbrain DA neurons ***(Smidt & Burbach 2007; Orme et al. 2009)***. In addition to Shh, transforming growth factor β (TGF-β) is required for the induction and survival of ventrally located midbrain DA neurons ***(Farkas et al. 2003)***. TGF-β co-ordinates the support of GDNF signaling through the recruitment of the glycosyl-phosphatidylinositol (GPI) anchored GDNF family receptor 1α (GFR1α) to the plasma membrane to induce neurotrophic effects. However, GDNF is still capable of mediating the downstream signaling through its binding to soluble GFR1α in the absence of TGF-β ***(Peterziel et al. 2002)***. Nurr1, a transcription factor, plays a crucial role in partly determining the fate of DA neurons through mediating the expression of several DA markers that are required for the synthesis, storage-release and reuptake of dopamine, including TH ***(Sakurada et al. 1999; Schimmel et al. 1999)***, VMAT2 ***(Hermanson et al. 2003)***, and DAT ***(Sacchetti et al. 2001)***. Further Nurr1 induces the expression of the GDNF receptor, Ret which mediates neurotrophic signaling to the young DA neurons ***(Wallén A et al. 2001; Galleguillos et al. 2010)***. In addition, Lmx1b induces the expression of pituitary homeobox 3 (Pitx3) in the ventralized neurons to promote the final differentiation or maintenance of DA neurons ***(Smidt et al. 2000)***. Pitx3 expression is restricted specifically to the midbrain DA neurons and further, the expression pattern seems to be differentially regulated in specific subsets of midbrain DA neurons namely SN and VTA ***(Smidt et al. 1997; Smidt et al. 2004)***.

1.3.2.1 Migration

The differentiating DA neurons form a mantle layer of the ventral midbrain to apparently form anatomically distinct clusters of RRF, SN and VTA. Interaction with radial glia is required for the migration of DA neurons. Two pathways were proposed for the terminal migration of DA neurons, the first one being the radial migration from the ventral midline and the second

being the combination of radial migration followed by tangential migration ***(Smidt & Burbach 2007; Blaess & Ang 2015)***. An extracellular matrix molecule, Reelin was shown to be important for radial neuronal migration in other brain regions including cortex and cerebellum ***(Zhao & Frotscher 2010)***. Further studies show that lack of Reelin signaling affects the speed and path of the tangential migration of DA neurons in the midbrain, with SN neurons being particularly sensitive. These neurons fail to migrate laterally and cluster just lateral to the VTA neurons ***(Nishikawa et al. 2003)***.

1.3.2.2 Axonal outgrowth and plasticity

Midbrain DA neurons upon complete maturation, undergo axonal pathfinding and synaptogenesis. These events are well studied in rats and they seem to happen at E13 in rats and E11 in mice. Axonal outgrowth of midbrain DA neurons is guided through various intrinsic and extrinsic (repulsive and attractive) cues. The molecular cues that are necessary for these processes include ephrins, netrins, semaphorins, and slits. Ephrins are of two types, either class A or B, which are GPI-anchored or transmembrane forms, respectively, and show affinity towards Eph receptors to promote axon guidance. Netrins, which exist as secretory or GPI-anchored either bind with deleted in colorectal cancer (DCC) and function as attractive cues to promote axon growth or bind to Unc5 in complex with DCC or alone and function as axon repulsive cues. DCC was shown to be crucial for the axonal outgrowth of midbrain DA neurons due to the expression pattern of both DCC and netrin1 in DA neurons and its targets, ST and prefrontal cortex in a complementary form ***(Hegarty et al. 2013)***. Secretory or transmembrane semaphorins bind to plexins or in combination with neuropilins and slits are secretory proteins that bind to Robo receptors to act as axon guidance cues ***(Smidt & Burbach 2007; Van den Heuvel & Pasterkamp 2008)***. Based on these spatially and temporally distributed attractive and repulsive cues, nigrostriatal, mesocortical and mesolimbic circuits are formed by initially extending the dorsal trajectory in the midbrain region. Further, these axons deflect ventro-rostrally, reorienting growth towards the forebrain in response to the repulsive cues in the brain stem. They then extend through the diencephalon towards the telencephalon in response to attractive cues of the medial forebrain bundle (MFB) ***(Nakamura et al. 2000; Gates et al. 2004)***. These projections invade the ventrolateral part of the telencephalon and initially reach nucleus accumbens and to the caudate putamen, where the external capsule forms a barrier for the axons of most midbrain DA neurons and only few proceed further towards the cortex to form target innervations and to establish functional synapses. During development, axonal targeting seems slightly nonspecific, as fibers originating from embryonic SNpc and VTA neurons do not exhibit specific preference for the dorsal (caudate putamen) or ventral (nucleus accumbens) ST. However, the specificity is achieved through selective elimination (axonal

pruning) of non-specific projections to form the nigrostriatal, mesocortical and mesolimbic pathways during late embryonic or early postnatal development ***(Smidt & Burbach 2007; Van den Heuvel & Pasterkamp 2008)***. DA neurons of SN are topographically mapped by the time of birth and only synaptogenesis span during the pre and postnatal period. Single nigrostriatal DA neuron can exert a strong influence over a large number of striatal neurons through dopamine transmission due to their widely spread and highly dense axonal arborization in the ST. Indeed, this was suggested to be the key aspect in PD, as degeneration of a single SNpc neuron can affect multiple neurons in the ST ***(Matsuda et al. 2009)***.

Neurotrophic factors do play a vital role in the development and maintenance of DA neurons. As discussed earlier, TGF-β induces the DA phenotype together with other factors like Shh, another growth factor that belongs to the TGF-β superfamily, GDNF promotes the survival of DA neurons post development ***(Hegarty et al. 2013; Hegarty et al. 2014)***. SN obtains significantly high number of DA neurons developmentally, than those are seen during adulthood in both mice and rats ***(Burke 2003)***. During synaptogenesis, SN DA neurons undergo natural cell death (NCD) which is apoptotic in nature and is critical to only maintain those DA neurons that properly establish their synaptic connections. NCD is biphasic, with an initial major peak at postnatal day (P) 2 and a second minor peak at P14 with the process largely subsiding by p20 ***(Janec & Burke 1993; Oo & Burke 1997)***. However, this programmed cell death seems to rely on target-derived neurotrophic factor (GDNF) both *in vitro* and *in vivo*, as only the DA neurons accessible to GDNF could survive these events. These findings were validated by axon-sparing target lesions, DA terminal destruction, axotomy of MFB, and by intrastriatal injection of neutralizing antibody against GDNF that led to augmented cell death of SN DA neurons. In contrast to these findings, overexpression of anti-apoptotic protein Bcl-2 or GDNF overexpression in the striatum of mice could protect SN DA neurons against this programmed cell death ***(Jackson-Lewis et al. 2000; Burke 2003; Kholodilov et al. 2004)***. Together, these findings substantiate the role of neurotrophic factors in the development and maintenance of SN DA neurons.

1.4 Neurotrophic factors

Growth factors including neurotrophins or neurotrophic factors are a group of small secretory proteins that promote neurite outgrowth, neuronal differentiation (biochemical/morphological) and are vital for maintaining the integrity of neurons post-development. In addition to autocrine and paracrine (adjacent cells) mode of action, growth factors are primarily derived from the target of the nerve terminals either from distinct cell types of peripheral tissue or neurons of CNS. Upon binding to their respective receptors, growth factors are retrogradely transported to the cell bodies to promote growth and maintenance of the neurons. The

neurotrophic hypothesis states that programmed cell death occurs prominently in the early pool of developing neuronal populations (post-mitotic and excess in number), particularly during synaptogenesis after the axons reach their target positions. During this period, 20-80% of discrete neuronal populations with long projections die abruptly. Further, a series of studies proved that, fate of the individual neuronal type during this period is solely regulated by a particular neurotrophic factor secreted in limiting amounts in the target field for a specific neuronal type according to the original hypothesis. However, the refined neurotrophic hypothesis states the possibility that more than one neurotrophic factor can regulate the survival of certain neuronal populations. This process seems to be fundamental to match the size and requirements of the relative number of innervating neurons and their target fields, abrogating the excess and nonspecific innervations. Supporting this hypothesis, the overall amount of neurotrophic factors and their corresponding receptors directly correlates to the spatial and temporal balance between neuronal survival and death ***(Hamburger & Levi-Montalcini 1949; Levi-Montalcini & Angeletti 1968; Davies 1996; Huang & Reichardt 2001; Davies 2003)***.

The above hypothesis was successfully demonstrated with nerve growth factor (NGF), which acts through tyrosine receptor kinase A (TrkA) and is well characterized among the neurotrophin family of neurotrophic factors, since its discovery ***(LEVI-MONTALCINI & HAMBURGER 1951; Cohen et al. 1954)***. Although, NGF is a primary neurotrophic factor acting on sympathetic and sensory neurons in the peripheral nervous system, NGF also mediates the development and maintenance of basal forebrain cholinergic neurons in the CNS that express TrkA ***(Dreyfus 1989)***.

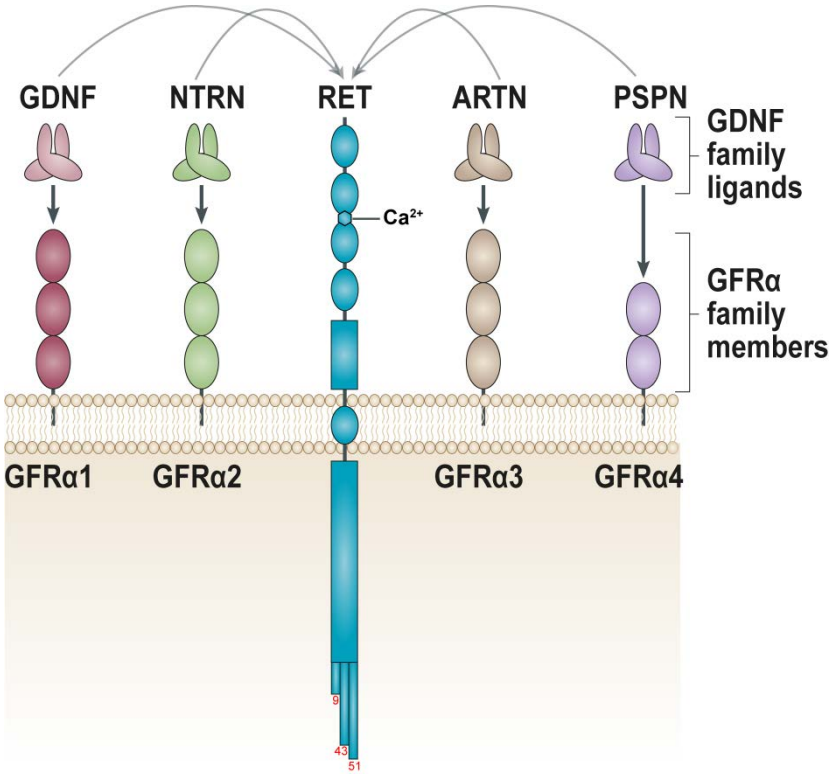
Subsequent studies identified structurally related neurotrophic factors including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4, all f which were clustered into the neurotrophin family of ligands. BDNF and NT-4 bind to TrkB receptors to exert their neurotrophic signaling, whereas NT-3 binds to TrkC. In addition, all of these factors can trigger neuronal death by binding to the p75 neurotrophic receptor ***(Skaper 2012)***. Based on the sequence and structural homology of the neurotrophic factors, they were classified into four main families. Which are neurotrophins; neuropoietic cytokines; recently discovered and are structurally not related to classical neurotrophic factors [mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF)]; and the most important GDNF family of ligands (GFLs) ***(Voutilainen et al. 2015)***. Among the members of GFLs, GDNF and neurturin (NRTN) are two highly potent neurotrophic factors that exert their neuroprotective and neurorestorative effects on DA neurons and have been taken to clinical trials with PD patients ***(Airaksinen & Saarma 2002)***. Biologically active calcitriol, a vitamin D_3 metabolite, has recently been shown to increase the survival of

midbrain DA neurons dissociated from E12 rat mesencephalon, in a dose-responsive manner. Calcitriol was shown to inhibit the apoptotic cell death through upregulation of endogenous GDNF thereby promoting the survival of primary DA cultures ***(Orme et al. 2013)***. Besides calcitriol, quinpirole mediated activation of the dopamine D2 receptor was shown to induce endogenous GDNF expression that was concurrent with temporal increase in the levels of DNA-binding transcription factor, zinc-finger protein 268 ***(Ahmadiantehrani & Ron 2013)*.**

1.4.1 GDNF family of ligands and their signaling

GFLs are members of the TGF-β super family and are critical regulators of DA neuron induction, differentiation, target innervations, and survival both during and after development of DA system. GFLs are secretory proteins like any other neurotrophic factors which include GDNF, NRTN, artemin (ARTN) and persephin (PSPN). GDNF was biochemically purified from a rat glioma cell line based on its ability to promote survival of cultured embryonic DA neurons ***(Lin et al. 1993)***, followed by NRTN ***(Kotzbauer et al. 1996; Horger et al. 1998)***, thereafter ARTN ***(Baloh et al. 1998)*** and PSPN ***(Milbrandt et al. 1998)*** were isolated by database searching. GFL's contain seven cysteine residues with relatively similar spacing and contain three disulfide bonds arranged in a typical head-to-toe conformation and function as homodimers ***(Eigenbrot & Gerber 1997; Airaksinen & Saarma 2002)***. They belong to the cysteine knot growth factor family based on their spatial structure and sequence homology. GFLs are produced in the form of precursors, pre-pro-GFLs. The signal sequence is cleaved upon secretion and the activation of pro-GFLs occurs most likely through additional proteolytic cleavage events. GFLs are concentrated locally through their binding to heparin sulfate side chains of the extracellular matrix proteoglycans preventing their diffusion. GFLs initially bind specifically to soluble or GPI-anchored co-receptors known as GDNF family receptor α's (GFRα's). There are four distinct GFRα's, 1-4 and the expression of each co-receptor is regulated by their corresponding GFLs. Structurally, GFRα's are rich in cysteine residues and consists of three homologous cysteine-rich domain (D) D1, D2 and D3 from the N-terminus. D2 and D3 are packed closely, whereas, D1 is structurally elevated by flexible hinge region ***(Airaksinen et al. 1999; Leppänen et al. 2004; VIRTANEN et al. 2005)***. Although each GFL binds to one preferred GFRα, the co-receptors show a certain degree of promiscuity in their ligand specificities. For example, GDNF binds to GFRα1 with high affinity and it can also bind to GFRα2 and 4 with low affinity and a similar pattern is also observed with NRTN and ARTN (described in Figure 1.9 legend). In contrast, PSPN only binds to GFRα4 with high affinity and appears to have no cross-talk with other co-receptors, at least in mammals ***(Airaksinen et al. 1999; Airaksinen & Saarma 2002)***. However, it is very unlikely that GFL-GFRα complexes could transduce signaling into the cell on their own,

as they lack the intracellular domain. In fact these complexes bind to other transmembrane receptors like Ret (Figure 1.9) ***(Durbec et al. 1996; Parkash et al. 2008)*** in most cases and form a multimeric complex that leads to activation of Ret (Figure 1.10). Alternatively GFL-GFRα complexes also bind to neural cell adhesion molecule (NCAM) ***(Paratcha et al. 2003)*** to exert neurotrophic signaling. Although, GPI-linked proteins through there recently discovered microdomains, were able to mediate intracellular signaling events like activation of Src family kinases and elevation of Ca^{2+} in the immune system, further analysis is necessary to support this phenomenon ***(Airaksinen et al. 1999)***.



1.9 GDNF family ligands and their receptors

Homodimeric GDNF family ligands (GFLs) bind to their respective GPI anchored co-receptors called GDNF family receptor α's (GFR α's) with strong affinity. This complex further binds to the receptor tyrosine kinase Ret to exert downstream neuroprotective signaling. GDNF primarily binds to GFRα1 with high affinity and binds to GFRα2 & 4 with low affinity. NRTN primarily binds to GFRα2 with high affinity and binds to GFRα1 & 4 with low affinity. ARTN primarily binds to GFRα3 and shows low affinity towards GFRα2 & 4. PSPN binds selectively to GFRα4 with high affinity. Image modified from ***(Mulligan 2014)***.

1.5 Life cycle of Ret

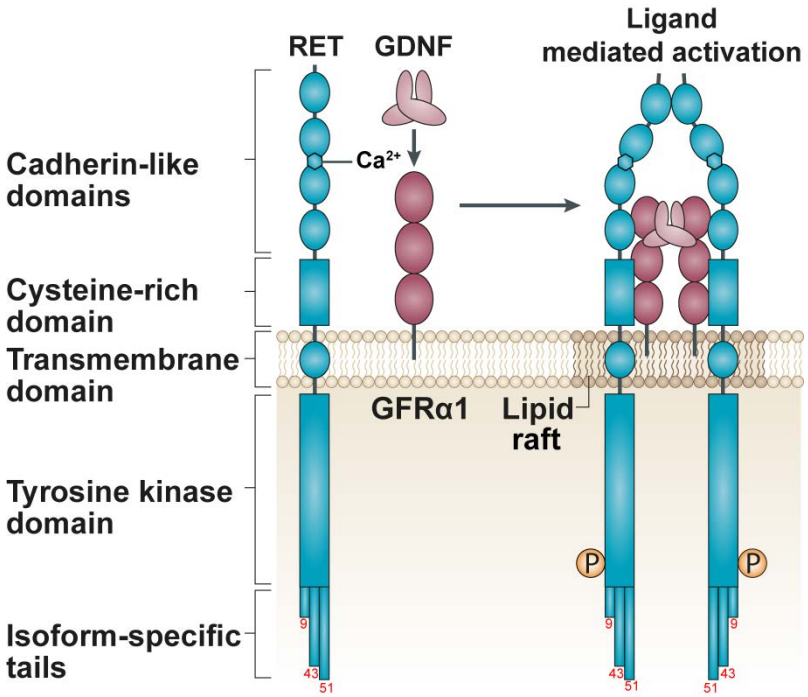
Ret, (**Re**arranged during **t**ransfection) was initially identified in 1985 by Masahide Takahashi and Geoffrey Cooper as an oncogene activated by a gene rearrangement occurred while transfecting DNA from a human lymphoma into mouse NIH3T3 fibroblasts ***(Takahashi et al. 1985)***. Ret is a proto-oncogene encoding a RTK which is a single pass transmembrane

protein. Ret is highly conserved among various species and further, both *Drosophila* Ret and mammalian Ret could activate similar signaling pathways when overexpressed in cultures ***(Abrescia et al. 2005; Airaksinen et al. 2006)***. During early embryogenesis Ret expression is relatively high and it has diverse roles in various tissues. However, Ret expression goes down during adulthood and is restricted to a few organs, derived from the neural crest. Ret regulates the development of sympathetic, parasympathetic, motor, and sensory neurons and is crucial for the postnatal maintenance of nigrostriatal DA neurons. Ret signaling is crucial for enteric nervous system (ENS) morphogenesis, and Ret is necessary for kidney development and plays a role in spermatogenesis outside the nervous system ***(Runeberg-Roos & Saarma 2007)***.

1.5.1 Structure of Ret protein

Ret is characterized by four cadherin-like repeats (residues 29-516) which are important for stabilizing Ret dimers and a cysteine rich region (517-635) which is crucial for protein conformation and ligand binding in the extracellular domain. It contains a hydrophobic transmembrane domain (residues 636-657), an intracellular juxtamembrane domain (residues 658-723), a tyrosine kinase domain (residues 724-1016) and a C-terminal tail (Figure 1.10) ***(Takahashi & Cooper 1987; Takahashi 1988; Runeberg-Roos & Saarma 2007)***. Ret in its extracellular domain contains a binding pocket for Ca^{2+} in between the cadherin repeats 2 and 3. Ca^{2+} binding is necessary for the functional integrity of Ret and its ability to interact with ligand ***(Ibáñez 2013)***. Ret is alternatively spliced at the 3' exons to generate three isoforms, which are Ret9, Ret43 and Ret51 with distinct C-terminal amino acids. Ret9, Ret43, and Ret51 protein isoforms share common amino acid sequences from the N-terminus to residue 1063, they only differ from each other in their C-terminal tails with unique 9, 43 and 51 amino acid residues respectively. Functional roles of Ret9 and Ret51 are well studied *in vitro* and *in vivo* and are evolutionarily highly conserved over a broad range of species. In contrast, Ret43 seems to be expressed only in primates at low levels ***(Carter et al. 2001)***. Both Ret9 and 51 possess functionally distinct features as Ret9 is important for enteric innervation and renal development whereas, Ret51 is required for growth and metabolism of mature sympathetic neurons ***(De Graaff et al. 2001; Tsui-Pierchala et al. 2002)***. The nascent 120kDa Ret protein consists of a signal sequence (residues 1-28) that guides the protein into the ER as it is being synthesized and eventually it is cleaved off co-translationally. The N-terminal cadherin-like domains and cysteine-rich domains of 120kDa Ret are rapidly glycosylated in the ER-lumen to produce an immature 155kDa glycoprotein. Ret protein is further processed within the Golgi apparatus and secretory pathway and the mature 175kDa protein is transported to the plasma membrane for growth factor mediated signaling ***(Hirata et al. 2010; Runeberg-Roos & Saarma 2007)***.

Ret signaling is mediated by a complex network with multiple binding partners such as GFLs and GFRα's. Unlike other RTKs, Ret does not directly bind to its ligands and requires additional co-receptors for activation (Figure 1.10).



1.10 Ret structure and ligand-mediated activation

A schematic structure of Ret depicts four cadherin-like domains, a Ca2+ binding site, and a cysteine rich domain on the extracellular region and a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. After the 1063 amino acid position, short form (Ret9) consists of 9 unique amino acids, long form (Ret51) consists of 51 unique amino acids and the intermediate form (Ret43) consists of 43 unique amino acids tail towards the carboxy terminal. Homodimeric GDNF does not directly interact with Ret, rather it binds to GPI anchored co-receptor, GFR α1, to form a heterodimeric complex, which recruits Ret into lipid rafts and forms a multimeric complex leading to conformational changes that facilitate the binding of Ret monomers via the cadherin domains and lead to receptor dimerization and autophosphorylation of tyrosine residues in the kinase domain. Image modified from ***(Mulligan 2014)***.

1.5.2 Ret activation

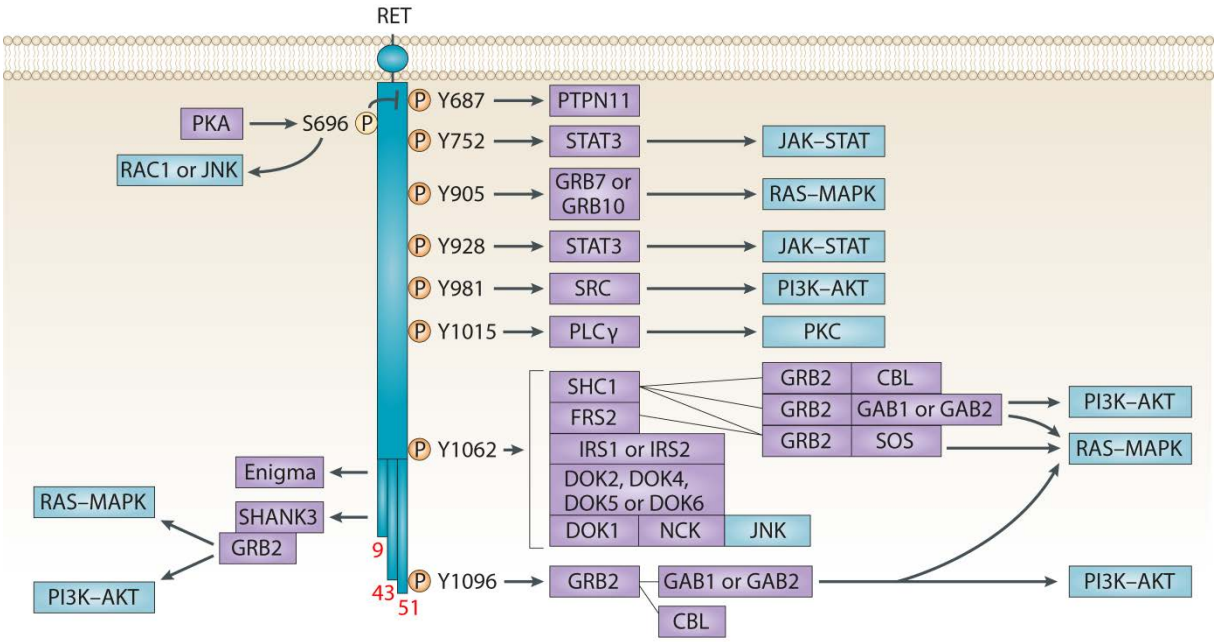
Ret activation occurs with a 2:2:2 stoichiometry within a ternary complex in which initially a soluble homodimeric GFL interacts with its corresponding GPI anchored GFRα co-receptor to form a binary complex. This recruits Ret into lipid rafts, which are membrane microdomains rich in cholesterol and sphingolipids. Lipid rafts concentrate proteins that are modified with saturated lipids and are enriched in signaling proteins that confer high affinity for rafts, including GPI-anchored proteins and doubly acetylated cytoplasmic Src-family kinases crucial for signal transduction. Ret binding to a GFL-GFRα complex leads to

conformational changes in Ret facilitating its dimerization and trans-autophosphorylation of critical tyrosine (Y) residues in its intrinsic kinase domain (Figure 1.10) ***(Airaksinen & Saarma 2002; Mulligan 2014)***. It is not completely clear if the activation of Ret by various GFLs and their cognate co-receptors will stimulate a distinct profile of signaling pathways ***(Airaksinen et al. 1999)***. However, a recent study demonstrated that NRTN-mediated Ret activation is necessary for Retinal function ***(Brantley et al. 2008)***. Mass spectrometry analysis revealed a temporal sequence of Ret autophosphorylation in which residues Y687 and Y1062 flanking the kinase domain are phosphorylated first, followed by autophosphorylation of residues Y900 and Y905 located within the activation loop. This is explained by an inhibitory mechanism involving tethering contacts between the glycine-rich loop, activation loop and αC-helix. Further, this phosphorylation trajectory is altered with oncogenic Ret kinase (M918T) where the late autophosphorylation sites (Y900, Y905) are phosphorylated much earlier than for WT Ret ***(Plaza-Menacho et al. 2014)***.

1.5.3 Ret docking sites for adaptor proteins

Tyrosine residues that are autophosphorylated in Ret protein serve as docking sites for distinct adaptor proteins that trigger the activation of several intracellular signaling cascades (Figure 1.11). Y905 serves as a binding site for Grb7/10 adaptors and Y981 is a docking site for SRC. Both of these tyrosine residues are also involved in the binding of SH2B family proteins, such as SH2B1β. Y1015 binds phospholipase Cγ (PLCγ), which mediates activation of protein kinase C (PKC) signaling crucial for both kidney morphogenesis and the migration of neural progenitors in the developing brain. Y1062 represents a multifunctional docking site for a variety of adaptor proteins including SHC1, FRS2, DOK1/2/4/5/6 and IRS1/2. Binding of SHC and FRS2 promotes the recruitment of other complexes such as GRB2-SOS and GRB2-GAB1/2, which facilitate the activation of RAS/BRAF/MAPK and phosphatidylinositol-3-kinase (PI3K)/Akt pathways respectively. When the adaptor proteins DOK4 and DOK5 are bound to Y1062, MAPK is activated. DOK1 binding is involved in activation of c-Jun N-terminal kinase (JNK). Thus, Y1062-mediated signaling is able to affect a wide range of processes, including promoting cell survival, proliferation, and neurotransmission through PI3K/Akt/NF-κB; growth, differentiation and neurite out growth via JNK, p38MAPK and ERK5 signaling. Y928 phosphorylation recruits signal transducer and activator of transcription 3 (STAT3) that is associated with cell proliferation and transformation. Ret from the lipid rafts promotes binding of SRC kinase which enhances mitogenesis and neuronal survival. Ret phosphorylation on S696 by cAMP-dependent PKA is associated with RAC1 GTPase activation, lamellipodia formation and neural crest cell migration. S696 phosphorylation also regulates the nearby Y687 phosphorylation site in the Ret juxtamembrane region which reduces interaction of protein tyrosine phosphatase non-

receptor type 11 (PTPN11) or SHP2 phosphatase that affects the ability of Ret to induce neural differentiation and survival. Y1096, which is specific for the Ret51 isoform, facilitates the binding of GRB2/GAB complexes and is critical for the activation of PI3K/Akt pathway.



1.11 Ret-mediated signal transduction

A schematic structure of the Ret kinase domain is shown. Indicated autophosphorylated tyrosine residues serve as docking sites for distinct adaptor proteins which mediate the signal transduction intrinsically in both Ret51 and Ret9 isoforms. Image modified from ***(Mulligan 2014)***.

Cytoskeleton-associated proteins, Enigma and SH3 and multiple ankyrin repeat domains protein 3 (SHANK3) interact with Ret9 more strongly than with Ret51 ***(Borrello et al. 2002; Schuetz et al. 2004)***. Ret9 interaction through C-terminal PDZ-binding site with SHANK3 induces constitutive RAS-MAPK and PI3K/Akt signaling. Enigma, a positive regulator of Ret, selectively associates with Y1062 via its LIM domain to localize Ret to specific signaling zones ***(Durick et al. 1998)*** and prevents ubiquitination and degradation of Ret by the CBL family of ubiquitin ligases. Upon ligand-mediated stimulation, Ret phosphorylation persist for up to 15 min and within 120 min Ret internalizes through clathrin coated vesicles and colocalizes with markers of early endosomes (Rab5a) and undergoes predominantly proteasomal degradation ***(Pierchala et al. 2006)***. CBL associates through SHC/GRB2 complexes at the Y1062 docking site and CBL can directly associate with GRB2 adapter proteins at the Y1096 docking site ***(Durbec et al. 1996; Airaksinen et al. 1999; Airaksinen & Saarma 2002; Runeberg-Roos & Saarma 2007; Ibáñez 2013; Mulligan 2014; Kramer & Liss 2015; Melillo & Santoro 2015)*** (Figure 1.11).

1.6 Physiological and pathological GDNF-Ret signaling

Ret expression is primarily seen in neural tissues like peripheral enteric, sympathetic, sensory neurons, and in motor, dopaminergic and noradrenergic neurons. It is also expressed in the developing excretory system (Wolffian duct and branching ureteric bud epithelium), and in differentiating spermatogonia ***(Airaksinen & Saarma 2002; Airaksinen et al. 2006; Ibáñez 2013; Hedayati et al. 2015)***.

1.6.1 GDNF-Ret signaling in renal and spermatogenesis

Consistent with its broad range of expression, Ret is involved in the induction of growth, branching and morphogenesis of the ureteric bud (UB) in the developing metanephric (third stage) kidney. Mutations in Ret are the cause of various forms of renal agenesis and were found in about 35% of patients. Further, absence of Ret or its cognate ligand co-receptors during embryogenesis results in renal agenesis and perinatal lethality. GDNF is secreted in the metanephric mesenchyme that forms a chemotactic gradient through which Ret initiates the branching morphogenesis of UB. Deciphering the underlying mechanisms suggest that signals emanating from Y1015 and Y1062 of Ret seem critical for kidney organogenesis and loss of these GDNF-Ret mediated signaling result in congenital anomalies of the kidneys and the lower urinary tract (CAKUT) pathogenesis ***(Jain, Encinas, et al. 2006; Jain 2009)***. Further, Ret signaling via Y1062 is essential for the maintenance and self-renewal of spermatogonial stem cells and regulation of their differentiation process in testes. Disruption of the Y1062 docking site result in marked atrophy of testes due to reduced production of germ cells, whereas, abnormalities in GDNF-Ret mediated signaling might cause male infertility and testicular germ cell tumors ***(Naughton et al. 2006; Jijiwa et al. 2008)***.

1.6.2 GNDF-Ret signaling in ENS

Neural crest (NC) cells derived from the vagal (colonize entire gut), truncal (populate the foregut) and sacral (colonize post-umbilical bowl) regions contribute to the formation of the ENS. Digestive processes such as motility, exocrine and endocrine secretions are controlled by ENS with two interconnected myenteric and submucous plexuses of neurons that are embedded in the gut wall of the gastrointestinal tract. Ret is expressed in NC cells along with GFRα1 and the migration of vagal neural crest-derived cells is mediated by the spatiotemporal expression of GDNF in the mesenchyme of the gut wall. GDNF-Ret signaling is also essential for the proliferation, differentiation and survival of the target invaded pluripotent ENS progenitors. Mutations causing Ret loss of function give rise to the congenital abnormality Hirschsprung's disease (HSCR). It is characterized by the absence of the parasympathetic ganglionated plexuses of the ENS leading to impaired peristalsis, intestinal obstruction and chronic constipation. Over 100 different mutations scattered all

over the Ret gene have been described in HSCR patients ***(Schuchardt et al. 1994; Manié et al. 2001; Natarajan et al. 2002; Uesaka et al. 2008; Mulligan 2014; Melillo & Santoro 2015)***.

1.6.3 Ret signaling in cancer

Mutations (activating point mutations or genomic rearrangements resulting in fusion proteins) leading to Ret gain of function have been found to be the cause of several different cancers of neuroendocrine origin. Thyroid carcinomas constitute about 3-4% of all human tumors and are categorized into different types. Carcinomas originating from follicular cells are papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC), whereas, medullary thyroid carcinoma (MTC) is derived from parafollicular C-cells ***(Romei et al. 2016)***.

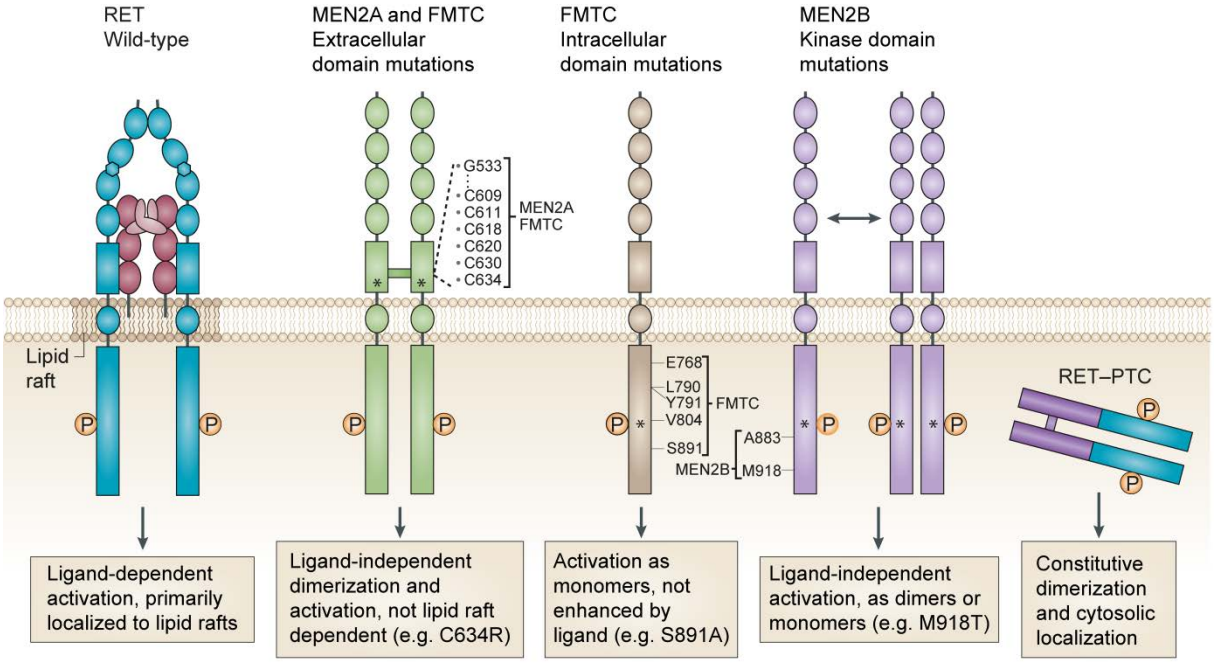
1.6.3.1 Ret/PTC

PTC is the most frequent thyroid cancer that is activated through rearrangements of the *Ret* locus at chromosome 10q11.2 and is named Ret/PTC. The Most common Ret/PTC rearrangements are Ret/PTC1 (fusion between *Ret* and the *CCDC6* gene) and Ret/PTC3 (fusion between *Ret* and the *NCOA4* gene). These oncogenes are composed of the intracellular kinase domain that is encoded by *Ret* 3' sequences fused with 5' sequences of either *CCDC6* or *NCOA4* genes which encode protein dimerization domains. The resulting chimeric Ret oncoproteins reside within the cytosol and promote uncontrolled proliferative signaling (Figure 1.12). Due to their aberrant localization, these Ret/PTC proteins escape normal endosomal trafficking and recycling processes ***(Santoro et al. 2002; Santoro & Carlomagno 2013; Mulligan 2014)***.

1.6.3.2 MEN2 syndromes

MTC originates from calcitonin-producing thyroid parafollicular C-cells and constitutes 5-7% of all thyroid carcinomas. Although, most of the MTC cases are sporadic in nature, around 25% of cases are inherited as an autosomal dominant trait. MTC gives rise to multiple endocrine neoplasia type 2 (MEN2) syndromes, when associated with other organs like parathyroid and adrenal glands. MEN2 is a multi-tumor syndrome that is clinically sub-grouped into MEN2A, MEN2B and Familial MTC (FMTC). Germline or somatic mutations in Ret are the primary cause of MEN2 syndromes and over 90% of MEN2 patients carry one of the more than 70 constitutively active *Ret* mutations associated with this disease ***(Santoro et al. 2004)***. Unlike heterogeneous *Ret* mutations associated with HSCR, cancer associated *Ret* mutations cluster in hot-spots mainly in the cysteine rich domain (exons 10-11) and in the tyrosine rich kinase domain (exons 13-16) (Figure 1.12) ***(de Groot et al. 2006; Wells et al. 2013)***. MEN2A is associated with the point mutations in cysteines, 609, 611, 618 and 620

in exon 10, and 630, 634 in exon 11 of Ret extracellular cysteine rich domain. However, it is most frequently (85%) associated with mutations of cysteine 634 (C634R) in exon 11. Cysteine mutations prevent the formation of intramolecular disulfide bonds, thus free cysteine residues form intermolecular disulfide bonds which mediate ligand-independent constitutive homodimerization leading to activation of Ret (Figure 1.12). MEN2A is characterized by MTC associated with pheochromocytoma and parathyroid hyperplasia or adenomas. FMTC mutations are present in extracellular cysteine rich domain (affecting C609, C611, C618, and C620 in exon 10, and C630 in exon 11) or in the intracellular tyrosine kinase domain (E768, L790, Y791, V804 and S891) of Ret. Mutations affecting non-cysteine amino acids of intracellular Ret, allow phosphorylation and activation of Ret monomers (Figure 1.12). FMTC is characterized by MTC alone with no association with other endocrine or non-endocrine phenotypes and has a late onset of the disease ***(Romei et al. 2012)***.



1.12 Ret activation patterns with respect to point mutations and genomic rearrangements

Ligand-mediated WT Ret activation is tightly regulated, whereas ligand-independent activation of Ret mutant proteins and their functional outcome is uncontrolled. Mutations affecting the Ret extracellular cysteine rich domain (MEN2A/FMTC) lead to constitutive homo-dimerization and activation of Ret mutants. Intracellular Ret mutations associated with FMTC facilitate the activation of Ret monomers and further, ligand binding has no influence over this class of Ret mutants. MEN2B mutations flanking the activation loop of Ret, particularly M918T cause conformational changes in Ret that decrease auto-inhibition and promote auto-activation either as monomer or a dimer. Ligand interaction with this form of Ret mutants will further enhance their activity. Genomic rearrangements in Ret result in chimeric proteins (Ret-PTC) that reside within the cytosol. These proteins are able to dimerize

upstream of Ret kinase domain through the amino terminals of partner protein leading to constitutive activation. Image modified from ***(Mulligan 2014)***.

MEN2B associated mutations are two specific amino acid substitutions M918 (M918T in exon 16) and A833 (A883F in exon 15) residues within the tyrosine kinase domain of Ret (Figure 1.12). In most cases, MEN2B is exclusively associated with M918T and only a small fraction of cases harbor the A883F substitution or very rarely both mutations clustered on the same or two different Ret alleles. Both M918 and A833 residues flank the activation loop of the Ret kinase domain. Mutations in these residues, particularly M918T, greatly enhance the transforming ability by causing increased autophosphorylation either as a monomer or dimer. This leads to complex functional outcomes and the most severe disease phenotypes ***(Jasim et al. 2011)***. The typical characteristics of MEN2B are MTC associated with pheochromocytoma, as well as other developmental anomalies like ganglioneuromas of the intestine and mouth, thickening of corneal nerves, marfanoid habitus and delayed puberty ***(Jhiang 2000; Santoro et al. 2004; Romei et al. 2012; Santoro & Carlomagno 2013; Romei et al. 2016)***. Among the MEN2 subtypes, MEN2B mutations cause the earliest disease onset with poorest survival rate. Furthermore, MEN2B mutations are also seen in 40-65% of sporadic MTC cases ***(Mulligan 2014)***.

1.6.4 GDNF-Ret signaling in DA system development

GDNF/GFRα1/Ret signaling is critical for the development and maintenance of various neuronal sub-populations in both the peripheral and CNS. This is supported by the fact that mice lacking GDNF are lethal at birth and possess deficits in dorsal root ganglion, sympathetic neurons, and these mice completely lack ENS, kidneys and induction of the ureteric bud ***(Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996)***. Further, mice that lack GFRα1 ***(Cacalano et al. 1998; Enomoto et al. 1998)*** and Ret ***(Taraviras et al. 1999; Natarajan et al. 2002; Uesaka et al. 2008)*** are also lethal at birth and display similar phenotypes as described for GDNF-deficient mice. These findings indicate that these proteins function together in transducing signal during development ***(Trupp et al. 1997)***. In contrast, mice lacking other GFLs or its corresponding co-receptors are viable and fertile ***(Airaksinen & Saarma 2002; Paratcha & Ledda 2008)***. In the developing embryo GDNF mRNA is widely expressed in and outside the CNS ***(Golden et al. 1999)***, whereas during adulthood its expression is restricted to few organs ***(Trupp et al. 1997; Golden et al. 1998)***. In the developing brain GDNF expression is observed in various regions with marked increase during P0 and P10 in the striatum ***(Choi-Lundberg & Bohn 1995)***. A similar expression pattern- albeit in lower magnitude- is observed in discrete cell populations of the striatum, thalamic structures, nucleus accumbens, cerebellum and hippocampal regions of the adult brain ***(Schaar et al. 1993; Nosrat et al. 1996; Oo et al. 2005)***. Ret and GFRα1

expression is co-localized in the ventral midbrain during development and adulthood, however, the expression pattern seems to be different from that of GDNF. Interestingly, both Ret and GFRα1 mRNA's are highly expressed in the SNpc and VTA neurons but not detected in the striatal population ***(Marcos & Pachnis 1996; Trupp et al. 1997)***, suggesting that midbrain DA neurons depend on target-derived neurotrophic support for their survival. SNpc DA neurons form connections with GABAergic medium spiny neurons (MSNs), parvalbumin-positive (PV+) interneurons and other cholinergic or somatostatin interneurons in the striatum to modulate their activity. GDNF is secreted at the nerve terminals of SN DA neurons in the striatum, mostly by PV+ interneurons and to a lesser extent by cholinergic interneurons. Subsequently, it is retrogradely transported to the DA cell bodies ***(d'Anglemont de Tassigny et al. 2015)***. Consistent with its expression pattern in the striatum, GDNF was shown to protect SN DA neurons from the biphasic postnatal NCD event by suppressing apoptosis. Numerous pieces of evidence support the idea that GDNF serves as a physiologically limiting neurotrophic factor for midbrain DA neurons (1.3.1.2) during the NCD events ***(Burke et al. 1998; Oo et al. 2003; Burke 2004; Kholodilov et al. 2004)***.

Despite high levels of GDNF expression in various developing brain regions including caudate putamen ***(Strömberg et al. 1993; Nosrat et al. 1996; Golden et al. 1999; Ikeda et al. 1999)***, GDNF seems dispensable during embryonic development of DA neurons. Although mice lacking GDNF die neonatally due to renal agenesis and absence of enteric neurons, GDNF ablation does not seem to affect proliferation and differentiation of DA neurons during embryonic development ***(Moore et al. 1996; Sánchez et al. 1996)***. However, these mice cannot be used to analyze the originally characterized function of GDNF in promoting survival of DA neurons, as they die even before the first phase (P2) of NCD. Similar findings were reported for both Ret and GFRα1 deficient mice, which have a normal number of midbrain DA neurons during embryonic development ***(Marcos & Pachnis 1996; Enomoto et al. 1998)***. However, blocking Ret expression in rat embryos using Ret antisense oligonucleotides resulted in loss of SN DA neurons with decreased DA fibers and dopamine content in the striatum ***(Li et al. 2006)***, whereas studies from DA neuron specific deletion of Ret (Ret-deficient) in mice indicates that Ret is dispensable for normal development of DA neurons ***(Jain, Golden, et al. 2006; Kramer et al. 2007)***. Although, physiological Ret activity is not essential for DA neuron development, the MEN2B mutation (M918T), which leads to constitutive activation of Ret augment nigrostriatal DA neuron numbers. The MEN2B mutation further enhanced the synthesis, storage, release and uptake of dopamine in the striatum of mice. Increased TH and DAT expression in the ventral midbrain and striatum of MEN2B mutant mice are attributed to the increased dopamine synthesis and reuptake respectively in these mice. Further, increased DAT expression in the striatum renders

MEN2B mutant mice more sensitive to the stimulatory effects of cocaine ***(Mijatovic et al. 2007; Mijatovic et al. 2008)***.

1.6.5 GDNF-Ret signaling in DA system maintenance

Studies from GDNF null mice failed to delineate the physiological function of GDNF in midbrain DA neurons due to their neonatal lethality. Although GDNF null mice show normal DA neurons at birth, it could be due to compensatory mechanisms during development or could be that GDNF might serve as target derived neurotrophic factor for mature DA neurons. Spatiotemporal ablation of GNDF in mice circumvents the developmental lethality and other possible compensatory mechanisms. Tamoxifen-induced spatiotemporal deletion of GDNF resulted in selective degeneration of catecholaminergic neurons in SN, VTA and locus coeruleus, which led to impaired locomotor activity ***(Pascual et al. 2008)***. However, a later study using a similar approach to delete GDNF spatiotemporally, reported that GDNF is dispensable for the survival of catecholaminergic neurons ***(Kopra et al. 2015)***. Thus, there is a discrepancy between studies regarding the physiological function of GDNF in DA neurons. True to its initially identified function, exogenous GDNF is known to promote survival of DA neurons after MPTP or 6-hydroxydopamine (6-OHDA) treatment in rodents and primates ***(Aron & Klein 2011)***. GDNF infusion was both neuroprotective and neurorestorative in rodents that were MPTP lesioned or had transected axons of MFB ***(Tomac et al. 1995; Beck et al. 1995)***. In addition, GDNF administration promoted the recovery of DA system without producing evident side effects in MPTP lesioned primates and was also shown to lead to improvements of MPTP induced behavioral symptoms of Parkinsonism ***(Gash et al. 1996; Gash et al. 2005)***. Numerous reports stated the beneficial effects of GDNF on nigrostriatal DA neuron survival in animal models of PD. However, GDNF was so far not able to completely translate these beneficial effects in clinical trials on PD patients ***(d'Anglemont de Tassigny et al. 2015)***.

Although, physiological Ret activity is not essential during the normal development of DA neurons, Ret function is essential for the maintenance of nigrostriatal DA neurons in ageing mice. DA neuron specific ablation of Ret results in age dependent degeneration of the nigrostriatal DA system accompanied with increased glial activation. As a result of decreased DA fibers, these mice showed a decline in striatal dopamine levels resulting in behavioral deficits. Further Ret signaling seems to be important for the size of SNpc DA neurons ***(Kramer et al. 2007; Meka et al. 2015)***. Even though Ret deficiency does not modulate MPTP mediated toxicity, it is important for the subsequent terminal regeneration and sprouting of the DA fibers of the nigrostriatal pathway, likely through endogenous GDNF ***(Kowsky et al. 2007)***. As described earlier, MEN2B mutant mice constitute enlarged nigrostriatal DA system and elevated dopamine levels (1.6.4). In an approach to confirm the

neuroprotective role of Ret signaling, the DA system of these mice was lesioned with neurotoxins. Interestingly, DA cell bodies, but not the axon terminals of constitutively active Ret, MEN2B mutant mice were more resistant to the DA neurotoxins, MPTP and 6-OHDA ***(Mijatovic et al. 2011)***. Although Ret was identified to be the primary canonical receptor for GDNF, it was later shown that GDNF could also signal through alternate receptors like NCAM within the DA system ***(Paratcha et al. 2003)***. However, Ret is essential to mediate the neurotrophic effects of GDNF in the MPTP-lesioned DA system, as overexpression of GDNF failed to mediate neuroprotective and regenerative effect in Ret-deficient mice despite the presence of alternative GDNF receptors ***(Drinkut et al. 2016)***. Mutations in Ret were so far only implicated in cancer and HSCR disease. Although conflicting results do exist regarding the association of GDNF and Ret single-nucleotide polymorphism (SNP) with PD ***(Wirdefeldt et al. 2003; Lücking et al. 2010; Hsieh et al. 2011)***, one study reported a first compound heterozygous case carrying a point mutation in Ret (R144H) and E422 deletion in FKBP52 that could play a potential role in PD ***(Fusco et al. 2010)***.

1.6.6 Ret signaling cooperates with PD associated proteins

Mice deficient for either parkin, PINK1 or DJ-1 show no significant histological alterations making it difficult to delineate the physiological function of these proteins in the DA system. However, these genetic ablations in mice confer susceptibility to other insults, indicating that the physiological function of these proteins can only be uncovered in specific circumstances ***(Raymond H Kim et al. 2005; Frank-Cannon et al. 2008; Moisoi et al. 2014)***. Despite no well characterized mutations in Ret that were directly associated with PD, aged Ret-deficient mice recapitulate PD-like pathology, and constitutive activation of Ret (MEN2B) leads to an augmented nigrostriatal DA system ***(Kramer et al. 2007; Mijatovic et al. 2007)***. These findings suggest that GDNF mediated Ret signaling cooperates with the protein network that is altered in PD. Consistent with this idea, a recent line of reports showed that ARPD associated proteins cooperate with Ret signaling in the maintenance of SNpc DA neurons. In this context, aging mice lacking both DJ-1 and Ret-specifically in the DA system resulted in accelerated loss of SNpc DA cell bodies, but not axons, compared to moderate loss observed in aged Ret-deficient mice. This indicates that DJ-1 is necessary for the survival of SNpc DA cell bodies, but not for their respective striatal fibers under conditions of trophic impairment. Further, using *Drosophila* genetics, DJ-1 was shown to interact with Ret mediated ERK signaling to control eye and wing development ***(Aron et al. 2010)***. DJ-1 is well known as an oxidative stress responsive protein and loss of DJ-1 results in hypoxic conditions which then stabilize a transcription factor, hypoxia-inducible factor-1α (HIF-1α). Post-transcriptional stabilization of HIF-1α was shown to downregulate Ret mRNA. Moreover, DJ-1 protein levels were shown to strongly correlate with Ret expression

indicating that DJ-1 regulates Ret expression in human neuroblastomas ***(Foti et al. 2010)***. Constitutive activation of Ret (MEN2B) was shown to significantly improve the mitochondria related abnormalities and suppress muscle degeneration of *Drosophila* PINK1 mutants. Ret signaling seems to rescue mutant PINK1 deficits independently of parkin as MEN2B was not able to rescue the abnormalities of *Drosophila* parkin mutants ***(Klein et al. 2014)***. Recent evidence suggests that ageing mice lacking parkin, and Ret specifically in the DA system exhibit enhanced nigrostriatal DA degeneration compared to single aged parkin and Ret-deficient mice. Parkin/Ret double deficient mice evinced striatal dopamine depletion and biochemical alterations like reduced mitochondrial complex I activity and cellular ATP levels as early as 3-6 months of age compared to mice singly-deficient for parkin or Ret. Further, overexpression of WT human parkin (h-parkin) in Ret-deficient mice, compensated for Ret loss by rescuing the DA system of aged Ret-deficient mice. Either parkin or Ret knock down (KD) in SH-SY5Y cultures were shown to impair mitochondrial morphology and bioenergetics, whereas overexpression of parkin rescued Ret-KD mediated mitochondrial deficits or GDNF stimulation restored parkin-KD mediated mitochondrial deficits. This rescue mechanism was suggested to be mediated through activation of PI3K/NF-κB signaling ***(Meka et al. 2015)***. These findings suggest that Ret functions together with other PD-associated proteins in the survival of nigrostriatal DA neurons.

2. Purpose of thesis project

Mutations that impair parkin function are one of the causes for familial forms of PD, characterized by the major loss of nigrostriatal DA neurons. Further, in aged mice lacking the GDNF receptor, Ret signaling evinces loss of nigrostriatal DA neurons, whereas, constitutive Ret signaling (MEN2B) results in an augmented nigrostriatal DA system in mice. Recent evidence supports the survival function of parkin in DA neurons, where parkin loss exacerbates the phenotype of aged Ret-deficient mice. Conversely, transgenic overexpression of parkin fully compensates for the Ret loss. These findings argue for a genetic cross-talk between parkin and Ret in the DA system. The main purpose of the study was to further elucidate the cross-talk of parkin and Ret in the context of DA system, with a view to comprehend how parkin could alter trophically impaired (loss of Ret activity) or trophically reinforced (constitutive Ret activity) DA neurons. Thus, the specific aims of this project were:

1. To characterize additional phenotypes of parkin and Ret doubly deficient mice and singly Ret-deficient mice.
2. To characterize parkin-deficient MEN2B mice in order to elucidate if parkin loss could normalize the MEN2B phenotype with respect to the augmented DA system and related anomalies.
3. To study the developmental role of parkin in the establishment of an augmented DA system in MEN2B mutant mice.
4. To unravel the molecular mechanisms underlying the parkin and Ret cross-talk in connection with the possible converging signaling cascades of parkin and Ret in mediating DA neuron survival.

3. Materials and Methods

A. Materials

3.1 Reagents

Table 3.1 General reagents and pharmaceutical substances

Substance	Company	Order number
1,4-dithiothreitol (DTT)	Carl Roth	6908.2
2-Mercaptoethanol	Sigma-Aldrich	M3148-250ML
3,4-Dihydroxybenzylamine	Aldrich	358781
Acrylamide	Carl Roth	3029.1
Agarose	BIOzym	840040
Ammonium chloride	Carl Roth	K298.1
Ammonium Persulfate	Appli Chem	2941
Ampicilin	Carl Roth	K 029.3
Bovine serum albumin	Appli Chem	A1391.0500
Bromophenol blue	Carl Roth	A512.1
Calcium chloride	Carl Roth	HN04.1
Celvol® 205	Celanese chem	4693132001
Complete, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	9908.1
Coumaric acid	Carl Roth	239764-1GM
Cycloheximide	Merck Chemicals	D-21490
DAPI	Mol Probes	CN75.2
Diaminobenzidine (DAB)	Carl Roth	4984.1
Dipotassium hydrogen phosphate	Merck	105104
Disodiumhydrogenphosphate	Carl Roth	D2650
DMSO	Sigma-Aldrich	R0186
dNTP Set, 100mM Solutions	Thermo Fisher Scientific	8043.2
EDTA	Carl Roth	3054.2
EGTA	Carl Roth	5054.1
Ethanol	Carl Roth	7870.1
Ethidiumbromide	Carl Roth	E9129
Ethylene glycol	Sigma-Aldrich	F2637
Ficoll Type 400	Sigma-Aldrich	0100-01
Flouromount G	Southern biotech	49629
Glutaraldehyde	Sigma-Aldrich	3783.2
Glycerol	Carl Roth	
Hematoxylin	Merck	1.05174
HEPES	Appli Chem	A3268.0250

Substance	Company	Order number
Hydrochloric acid	Carl Roth	X942.2
Hydrogen peroxide	Merck	1.07209
Ketamine	Albrecht GmbH	
Luminol	Carl Roth	4203.1
Magnesium chloride	Carl Roth	2189.1
MG132	Sigma-Aldrich	C2211-5MG
Paraformaldehyde	Appli Chem	A3813.1000
Pepsin	Appli Chem	A4289
Perchloric acid	Appli Chem	A0539
PHOSSTOP (Phosphatase inhibitors)	Sigma-Aldrich	4906837001
Ponceau S	Sigma-Aldrich	P-7170-1l
Potassium chloride	Carl Roth	6781.1
Potassium dihydrogen phosphate	Merck	1.12034
Rompun	Bayer Vital GmbH	
Sodium chloride	Carl Roth	3957.2
Sodium dodecyl sulfate	Carl Roth	5136.1
Sodium hydroxide	Carl Roth	P031.2
Sucrose	Carl Roth	9097.1
TEMED	Carl Roth	9351.1
Tissue-Tek® O.C.T.™	SAKURA Inc.	4583
Tris(hydroxymethyl)aminomethane	Merck	108382
Triton X-100	Carl Roth	3051.2
Tween20	Carl Roth	9127.1
Xylene cyanol dye solution	Sigma-Aldrich	B3269
Xylol	Carl Roth	4436.1

Table 3.2 General buffers and solutions

Buffer/solution	Formulation
1X (0.1 M) PBS	10 mM Na_2HPO_4; 2 mM KH_2PO_4; 0.137 M NaCl; 2.7 mM KCl; pH 7.4
1X (0.1 M) TBS (TBS-T)	50 mM Tris pH 7.4; 150 mM NaCl (+ 0.1 % (v/v) Tween20)
Tail lysis buffer	A: 5 M NaOH; 0.5 M EDTA; B: 1 M Tris-HCl pH 5.0
10X PCR buffer	1M KCl; 1M Tris HCl pH 9.0; 1M $MgCl_2$
DNA loading buffer	3% (w/v) Ficoll Type 400; 0.05 % (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol FF
50X TAE buffer	2 M Tris pH 7.8; 0.5 M NaOAc; 50 mM EDTA
Protein extraction buffer	50 mM Tris-Hcl pH 7.5; 150 mM NaCl; 0.5 % Triton X-100; 20 mM NaF; 1 mM Orthovanadate; Complete™ EDTA free protease inhibitor cocktail (Sigma-Aldrich); PHOSSTOP (Sigma-Aldrich)

Buffer/solution	Formulation
Protein loading buffer (6X)	125 mM Tris-HCl pH 6.8; 20 % (v/v) glycerol; 4 % (w/v) SDS; 0.02 % (w/v) bromophenol blue; 250 mM DTT
Nuclear extraction buffer	0.25M HEPES pH 7.9; 0.84 M NaCl; 1.5 mM $MgCl_2$; 0.4 mM EDTA; 0.5 mM DTT was added freshly
APS (10%)	1 gm APS; 10 mL MPW H_2O
Blocking solution	5% BSA; in TBST (for western)
ECL solution	1 M Tris pH 8.0; 3 µl of 30 % H_2O_2; 50 µl of 250 mM luminol (DMSO); 25 µl 90 mM coumaric acid (DMSO) for 10ml ECL solution
Potassium phosphate buffer	80.2 ml 1M K_2HPO_4; 19.8 ml 1M KH_2PO_4; pH 7.4; make to 1000 ml with distilled water
Citrate phosphate buffer	Citric acid 4.7 gm; 9.2 gm Na_2HPO_4; make to 1000 ml with distilled water
PBS (Ca^{2+}/Mg^{2+})	1X PBS; 0.5 mM $CaCl_2$; 2mM $MgCl_2$
Glycine buffer	20 mM glycine; 1X PBS; 0.5 mM CaCl2; 2mM MgCl2
PFA (4%)	PBS (warm up to 70°C); 40 g PFA; 350 µL 5M NaOH; 150 µL Conc HCl- pH 7.5
Sucrose solution (IHC)	30 gm Sucrose; 100ml PBS; filter sterilize
Brain embedding solution	10:1-egg yellow : sucrose mix; 0.5ml 25% glyceraldehyde to 10ml mix for polymerization
Celvol® mounting medium	Celvol®205 125 g/L; glycerol 25 % (v/v); in 0.1 M PBS pH 7.4
Cryoprotection solution	30% glycerol (v/v); 30 % ethylene glycol (v/v); in 0.1 M PBS pH 7.4
Blocking solution (IHC)	5% BSA; 0.3% Triton X-100 in TBS
Carrier solution (IHC)	2% BSA; 0.1% Triton X-100 in TBS (primary and secondary antibodies)
DAB solution	1 mg/ml DAB; 10ul H_2O_2 in 10 ml PBS or TBS
HPLC lysis buffer	0.1M perchloric acid; 0.5 mM EDTA; 100 ng/ml 3,4-dihydroxybenzylamine (internal standard)
Mitochondria extraction buffer	320 mM Sucrose; 5 mM Tris pH 7.4; 2 mM EGTA with protease inhibitor cocktail

Table 3.3 Molecular biological kits

Kits	Company	Order number
ATP Assay Kit (Colorimetric/Fluorometric)	Abcam	ab83355
BCA Protein Assay kit	Thermo Fisher Scientific	23225
Complex I Enzyme Activity Microplate Assay Kit	Abcam	ab109721
Dynabeads® Protein G for Immunoprecipitation	Thermo Fisher Scientific	10004D
Dynabeads® M-280 Streptavidin	Thermo Fisher Scientific	11205D
iBlot® Transfer Stack, PVDF, regular size	Thermo Fisher Scientific	IB4010-31
PureLink® HiPure Plasmid Filter Maxiprep Kit	Thermo Fisher Scientific	K210017

Kits	Company	Order number
Subcellular Protein Fractionation Kit	Thermo Fisher Scientific	78840

Table 3.4 Primary antibodies

Antibodies	Company	Dilution	Secondary	Order number
Akt (pan)	Cell Signaling Technology	1:1000	Anti-Rabbit	4691S
Anti- PGC-1	Merck Chemicals	1:1000	Anti-Rabbit	AB3242
Anti-c-Ret (long isoform)	ZYTOMED	1:1000	Anti-Rabbit	A01-8128
Anti-c-Ret (short isoform)	ZYTOMED	1:1000	Anti-Rabbit	A01-8121
GM130 (Dr. Sandra Markmann)	BD Biosciences	1:1000	Anti-Mouse	610822
Histone	Cell Signaling Technology	1:1000	Anti-Rabbit	9649S
LC3	Novus Biologicals	1:2000	Anti-Rabbit	NB100-2220
Mitofilin	Novus Biologicals	1:1000	Anti-Rabbit	NB100-1919
NCAM	Hybridomabank USA	1:500	Anti-Mouse	5B8
Nurr1	Santa Cruz Biotechnology	1:500	Anti-Rabbit	sc-991
p44/42 MAP Kinase (ERK)	Cell Signaling Technology	1:1000	Anti-Rabbit	4695S
p62	Enzo Life Sciences	1:1000	Anti-Rabbit	BML-PW9860
Parkin	Santa Cruz Biotechnology	1:1000	Anti-Mouse	sc-32282
PARP	Cell Signaling Technology	1:1000	Anti-Rabbit	9532S
PDI (Dr. Sandra Markmann)	Assay Designs	1:1000	Anti-Mouse	SPA-891
Phospho-Akt (Ser473)	Cell Signaling Technology	1:1000	Anti-Rabbit	4060S
Phospho-p44/42 (Erk1/2) (T202/Y204)	Cell Signaling Technology	1:1000	Anti-Rabbit	9101S
Phospho-S6 Ribosomal Protein (Ser235/236)	Cell Signaling Technology	1:1000	Anti-Rabbit	2211S
Ret	Lab-made	1:1000	Anti-Rabbit	Lab-made
S6 Ribosomal Protein	Cell Signaling Technology	1:1000	Anti-Mouse	2317S
Tubulin, alpha	Sigma-Aldrich	1:20000	Anti-Mouse	T9026
Tyrosine Hydroxylase	Acris	1:1000	Anti-Mouse	22941
β-Actin	Santa Cruz Biotechnology	1:1000	Anti-Rabbit	sc-130657
β-catenin	Santa Cruz Biotechnology	1:1000	Anti-Rabbit	sc-7199

Table 3.5: Secondary antibodies/IgG controls/signal enhancer

Secondary antibodies/IgG control/signal enhancer	Company	Dilution	Order number
anti-Mouse IgG (H+L)	dianova	1:10000	115-035-146
anti-Rabbit IgG (H+L)	dianova	1:10000	111-035-144
Horse anti-Mouse IgG biotinylated	LINARIS	1:200	BA-2001
Horse anti-Rabbit IgG biotinylated	LINARIS	1:200	BA-1100

Secondary antibodies/IgG control/signal enhancer	Company	Dilution	Order number
normal mouse IgG	Santa Cruz Biotechnology	Control	sc-2025
normal rabbit IgG	Santa Cruz Biotechnology	Control	sc-2027
Vectastain® ABC-HRP Standard	LINARIS	1:200	PK-4000

Table 3.6: Cell culture reagents

Substance	Company	Order number
DMEM	Thermo Fisher Scientific	61965026
DPBS	Thermo Fisher Scientific	14190094
FBS	Lonza	DE14-802F
Glutamax	Thermo Fisher Scientific	35050038
HEPES (1M)	Thermo Fisher Scientific	15630056
MEM Non-Essential Amino Acids Solution	Thermo Fisher Scientific	11140035
Opti-MEM® I Reduced Serum Medium	Thermo Fisher Scientific	11058021
Penicilin-Streptomycin	Thermo Fisher Scientific	15140122
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific	11360039
Trypsin-EDTA	Thermo Fisher Scientific	25300054
Lipofectamine® 2000 Transfection Reagent	Thermo Fisher Scientific	11668019

B. Methodology

3.2 Transgenic animals

Animal experiments were performed according to the German Animal Welfare Act and approved by the local authorities of the state of Hamburg and the animal care committee of the University Medical Center Hamburg-Eppendorf. Mice were housed either in the mouse facility of the Center for Molecular Neurobiology Hamburg (ZMNH) or in UKE mouse facility under standard conditions at 22°C and 40-50% humidity in a 12 hr day/night cycle with access to pelleted diet and water *ad libitum.* Changing of cages, water and food supply, breedings, weanings, collection of biopsies and other routines were done by the animal care takers of the UKE mouse facility.

Table 3.7 Transgenic mouse lines

Target Allele	Mouse line generation
DAT Cre Bac	Knock-in of Cre recombinase into the 5' UTR of the exogenous DAT locus within a bacmid, facilitating Cre expression specifically in DA neurons ***(Parlato et al. 2006)***.
$Ret^{lx/lx}$	Ret was targeted with conditional loxP sites flanking exon 12 ***(Kramer et al. 2006)***.

Target Allele	Mouse line generation
Parkin-deficient	Targeted deletion of exon and parts of intron 3 ***(Itier et al. 2003)***.
MEN2B	Corresponding MEN2B mutation (M919T) was introduced within the endogenous mouse Ret allele using Cre/loxP site-specific recombination ***(Smith-Hicks et al. 2000)***.
WT human parkin	Overexpression of WT human parkin was driven by mouse prion promoter ***(Meka et al. 2015)***

All mice were maintained on a C57BL/6J background

3.2.1 Genotyping

Tail biopsies were obtained from new born pups and incubated at 95°C with 100µl of tail lysis buffer A (Table 3.2) for 20 min till it is completely digested. Samples were vortexed thoroughly and centrifuged for the debris to settle, and were neutralized with 100µl of tail lysis buffer B (Table 3.2) and vortexed thoroughly. DNA was stored at 4°C until the PCR analysis. PCR samples were analyzed by agarose gel electrophoresis resolved in 1 or 2 % agarose gels based on the amplicon size.

Table 3.8 PCR reaction

Constituents	50 µl PCR Reaction (1X)
200 nM Forward primer	1 µl
200 nM Reverse primer	1 µl
10X PCR buffer	5 µl
10 µM dNTPs mix	5 µl
Taq (homemade)	1 µl
Distilled water	36 µl
DNA	2 µl

Table 3.9 Genotyping PCR primers

Primer name	Sequence (5' to 3')	Amplicon size	Tm
Ret^{lx} forward	CCA ACA GTA GCC TCT GTG TAA CCC C	300 bp (WT); 350 bp (Ret^{lx})	62°C
Ret^{lx} reverse	GCA GTC TCT CCA TGG ACA TGG TAG		
Ret^{rec} forward	CGA GTA GAG AAT GGA CTG CCA TCT CCC	600 bp	62°C
Ret^{rec} reverse	ATG AGC CTA TGG GGG GGT GGG CAC		
Parkin WT forward	CTC CTG GAC TCC CAT ATG GAG CCC	300 bp	65°C
Parkin WT reverse	CCT TCT GTT GCT CCA CTG GCA GAG		
Parkin KO forward	GAA CGA GAT CAG CAG CCT CTG TTC C	350 bp	65°C
Parkin KO reverse	CCT TCT GTT GCT CCA CTG GCA GAG		
MEN2B forward	CCT CTC ACA CAC CAC AAC C	300 bp (WT Ret); 350 bp (MEN2B)	60°C
MEN2B reverse	GCT CAG TCT GAG ATG CTG GG		

Primer name	Sequence (5' to 3')	Amplicon size	Tm
WT human parkin forward	ACC TGC AGG CAG GCA ACG CTC AC	361 bp	64°C
WT human parkin reverse	GCA GGG AGT AGC CAA GTT GAG GG		

3.2.2 Tissue preparation

For immuno-histological stainings, mice were deeply anesthetized with ketamine and xylazine mix, 240/30mg/kg; 450 µl/30g body weight (intraperitoneal). After ensuring the absence of any nociceptive responses to tail and toe-pinching, a lateral incision was made beneath the rib cage and above the abdomen. The diaphragm and both sides of the rib cage were cut opened towards the collarbone, to get free access to the heart. Mice were then transcardially perfused with ice cold PBS and then with 4% ice cold PFA (fixative) for 20 min respectively, by inserting the needle (30 gauge) into the posterior tip of the left ventricle. Subsequently, an incision was made at the right atrium to allow outflow of the blood. The constant flow of ice cold PBS or fixative are regulated at a flow rate of 2.5 ml/min through a peristaltic pump for adult mice and 1ml/min for postnatal day (P) 4 or P16 pups. Tail biopsies were collected for re-genotyping of mice before perfusing with the fixative.

For biochemical analysis, mice were killed by cervical dislocation and were then instantly decapitated for collecting fresh brains. In order to collect fresh and fixed brains, the skull was cut through the midline suture of both the brain hemispheres in the posterior-anterior direction without damaging the brain. Skull plates were carefully removed and brain was gently taken out by cutting the optic nerves and other connections along the ventral surface of the brain. Fresh brains (biochemical analysis) were collected in a 12 well plate flash frozen in liquid nitrogen, eventually transferred to -80°C freezer and fixed brains (immune-histological stainings) were incubated overnight in 15ml tubes containing 4% PFA. Post fixation brains were washed in cold PBS and impregnated with 30% sucrose. Left and right brain halves were embedded separately in egg yolk with 10% sucrose and 5% glutaraldehyde. Post polymerization, blocks were allowed to rapidly freeze on dry ice and eventually transferred to -80°C for later analysis. Using cryostat, 30 µm thick coronal sections were cut and preserved in cryoprotection solution and stored in -20°C freezer for immune-histological stainings.

Adrenal glands form P16 mice were dissected and fixed in 4% PFA for overnight and washed in cold PBS and impregnated with 30% sucrose. Adrenal glands were embedded in Tissue Tek® O.C.T™ and flash frozen on dry ice and stored in -80°C. Using cryostat, 20 µm thick transverse sections were cut and directly mounted on the SuperFrost™ Plus slides.

3.2.3 Hematoxylin staining

Pre-mounted sections of adrenal glands were left in 4% PFA for 20 min and incubated at 50°C for 1 hr. Post rehydration, sections were immersed for 3 min in hematoxylin solution and rinsed with 0.1% HCl solution. Sections were washed for 2 min in running tap water and then in an ascending series of alcohol (50%, 70%, 80%, 100% ethanol) and finally in xylene. Sections were dried briefly and cover-slipped using Eukitt mounting medium.

3.2.4 Immunostaining

Free floating ventral midbrain sections were washed in TBS and then incubated for 1 or 2 hr in IHC blocking solution at RT and transferred to respective primary antibody prepared in IHC carrier solution for overnight incubation at 4°C. Sections are washed for three times with TBS (5 min each) post primary antibody incubation and the sections were transferred to biotin-coupled secondary antibodies prepared in IHC carrier solution for 1 hr RT incubation. After three washes in TBS, sections were incubated for 1 hr at RT with horseradish peroxidase coupled avidin-biotin complexes prepared at least 20 min prior. Sections were washed for three times with TBS, post incubation and developed with HRP substrate Diaminobenzidine (DAB) solution with hydrogen peroxide prepared in TBS. Sections were developed until a brown precipitate (signal) was seen (Buffer compositions and respective antibody details, refer to Table 3.2; 3.4 & 3.5).

3.2.5 Stereological quantification

Stereological countings were done for TH positive neuronal population in the serial sections of SN and VTA separately with a section interval of six, using an optical fractionator probe of the StereoInvestigator software (MicroBrightField, Williston, Vermont, United States). Counting was done unbiased for genotype, using an oil immersion 63X objective, a counting frame of 50X50 µm, and a grid size of 100X100 µm as previously described ***(Kramer et al. 2007)***. Data in figure 4.2 and 4.6 was generated along with Praveen Meka. Figure 4.7.1 was generated by Praveen Meka.

3.2.6 Soma size quantification

For soma area quantification, midbrain coronal sections stained for TH were analyzed with a 63X objective using a bright field microscope. Cells were randomly selected with the optical fractionator probe and the soma area of SN and VTA neurons was determined using the nucleator probe in the StereoInvestigator software. All prominent TH positive neurons in the SN and VTA were analyzed by two independent investigators blinded for genotype ***(Aron et al. 2010)*.**

3.3 Tissue preparation for biochemical analysis

For punching striatum tissue sample from fresh frozen brains, 2 mm thick coronal slice was cut with coordinates approximately from bregma -0.5 to +1.5 mm, subsequently, using a sample corer (FST #18035-02) with an inner diameter of 2 mm, striatum was punched. For punching SN tissue sample from ventral midbrain tissue, 1.5 mm thick coronal slice was cut with coordinates approximately from bregma -3.8 to -2.3, subsequently, using a sample corer (FST #18035-01) with an inner diameter of 1 mm, SN was punched.

3.3.1 Immunoprecipitation

Immunoprecipitation experiments in both cultured cells and mouse tissue homogenate were done using magnetic beads (Dynabeads® Protein G for Immunoprecipitation) in accordance with the instructions of the manufacturer. Briefly, antibodies against specific proteins were allowed to couple with protein-G immobilized on magnetic beads in citrate phosphate buffer with pH 5.0. Antibody-bead complexes were washed post coupling and incubated overnight at 4°C with cell lysates or tissue homogenates. Post incubation, the complexes were washed again and subjected to immunoblotting and probed with antibodies specific to co-immunoprecipitated proteins.

3.3.2 Sample preparation for immunoblotting

Punched tissue (striatum, or SN/VTA) was homogenized on ice, using dounce homogenizers in 250 µl ice cold protein extraction buffer (Table 3.2) with protease and phosphatase inhibitors. Resulting brain lysates were centrifuged at 14000 g for 10 min at 4°C to obtain Triton X-100 soluble (supernatant) and insoluble (pellet) fractions. Supernatants were aliquoted for protein estimation and the rest was either frozen at -80°C for future use or added 6X protein loading buffer (Table 3.2) and denatured by boiling for 10 min at 95°C before loading equal amount of protein on to the gels. Protein concentrations were determined according to the manufacturer guidelines using BCA Protein assay kit (Pierce) before adding the 6X protein loading buffer.

3.3.3 Immunoblotting

Tissue lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out as described by Lammeli and Farre (1973)**.** Proteins are denatured in the presence of SDS and a thiol reducing agent, DTT (Table 3.1). Polypeptides migrate on the polyacrylamide gel according to their size, since most proteins in this state bind to SDS in a constant weight ratio such that the charge of all polypeptides is identical. Using Hoefer SE 600/400 16-cm system with 1.5 mm spacers and 10 or 15 well Teflon combs, 10% (w/v) resolving and 5% stacking acrylamide mini gels were prepared accordingly.

Table 3.10 Gel formulation

Constituents	10% resolving gel (10 ml)	4% stacking gel (5 ml)
30% acrylamide stock	3.3 ml	0.65 ml
Tris 1.5M pH 8.8; 0.4% SDS	2.6 ml	
Tris 0.5M pH 6.8; 0.4% SDS		1.3 ml
Distilled water	4 ml	3.05 ml
APS 10%	50 µl	50 µl
TEMED	5 µl	5 µl

Denatured protein samples as described earlier (3.3.2) were loaded on to the gel along with a prestained molecular weight protein marker. Buffer reservoirs were filled with electrode buffer and electrophoresis was carried out using constant voltage at 120V, till the bromophenol blue dye front diffuses from the bottom of the gel. The proteins on the gel were then transferred onto PVDF membrane according to the manufacturer instructions using iBlot® Transfer Stack, PVDF, regular size. Transfer efficiency of the proteins was assessed by Ponceau S staining and the membranes are then washed.

Membranes were blocked with blocking solution (Table 3.2) at RT for 1 hr with gentle rocking and the membranes were briefly washed and transferred to respective primary antibody (Table 3.4) diluted in blocking solution and incubated either at RT for 2 hr or overnight at 4°C with constant shaking. Membranes were washed with TBST for five times for 5 min and incubated with corresponding HRP-conjugated secondary antibody (Table 3.5) diluted in blocking solution at RT for 1 hr. After incubation, membranes were washed with TBST for five times for 5 min and one TBS wash for 5 min. Immunodetection was obtained by incubating the membrane for at least 3 min in ECL solution (Table 3.2) and the signal was detected with an Image Reader LAS-4000 mini system. The chemiluminescent signals were quantified using the LI-COR Image Studio Lite software. After detecting the phosphorylated proteins, membranes were stripped using stripping solution (Table 3.2) for 30 min at 50°C and the membranes were washed for 5 min five times in order to reprobe for corresponding non-phosphorylated proteins.

3.3.4 Measurement of total ATP levels in mouse brain

As described earlier (3.3), SN was punched from the ventral midbrain of mice and were homogenized in 200 µl of ice cold ATP assay buffer provided in the kit (Table 3.3) using dounce homogenizers. Small aliquot was kept for determining the protein concentration using BCA Protein assay kit (Pierce) and the remaining was centrifuged at 14000g for 15 min. Supernatant was aliquoted and 50 µl was used for the fluorometric ATP assay

according to the manufacturer instructions. The values obtained from the fluorescence reader were normalized to total protein levels from BCA.

3.3.5 Mitochondrial enrichment and complex I activity measurement

SN was punched from the ventral midbrain of mice as described earlier (3.3), and was homogenized in 1 ml of ice cold homogenization buffer (320 mM Sucrose; 5 mM Tris pH-7.4; 2 mM EGTA including protease inhibitor cocktail) in dounce homogenizer. The homogenates were centrifuged at 2000g for 3 min at 4°C to remove cell debris and nuclei. Supernatants were centrifuged at 12000g for 10 min at 4°C to pellet mitochondria and synaptosomes. The pellet was suspended in 1 ml of homogenization buffer containing 0.02% w/v of digitonin to disrupt synaptosomal membranes and release trapped mitochondria ***(Palacino et al. 2004)***. The resuspended samples were centrifuged for 10 min at 12000g to pellet mitochondria, which were again resuspended in 100 µl of homogenization buffer and protein content was determined by BCA Protein assay kit (Pierce). Complex I enzyme activity assay was performed using the complex I enzyme activity microplate assay kit (Table 3.3) according to the manufacturer's instructions with 30 µg protein from the enriched mitochondrial preparations. This experiment was done in mutual aid with Helia Aboutalebi.

3.3.6 Total striatal dopamine measurements

Striatal tissue was punched as described in (3.3), and homogenized with dounce homogenizers in 0.1 M perchloric acid containing 0.5 mM disodium EDTA and an internal standard, 100ng/ml 3,4-dihydroxybenzylamine. The homogenates were then centrifuged at 50,000g for 30 min and the supernatants were used for HPLC measurements, whereas, pellets were resuspended in protein extraction buffer for determining the protein concentrations (BCA Protein assay kit Pierce). To determine the concentrations of monoamines and its metabolites, supernatants were subjected to reversed phase HPLC with electrochemical detection as previously described ***(Yang & Beal 2011)*** with few modifications ***(Meka et al. 2015)***. Supernatants were filtered through 0.22 µM PVDF filters and 20 µl samples were injected in to a C18 reverse-phase HR-80 column (ESA, Bedford, MA). The applied electrochemical potentials for determining the chromatograms of monoamines and its metabolites were: Conditioning cell +10 mV; Analytical cell channel1 +50 mV/channel2 +360 mV. The retention times of the metabolites measured were, DOPAC (3.80 min), 3,4-dihydroxybenzylamine (internal standard) (4.25 min), dopamine (6.88 min) and HVA (10.30 min). Concentrations of monoamines and its metabolites were calculated from peak areas that were normalized to the peak areas of the internal standard to obtain total amount of dopamine and its metabolites present in the 20 µl sample volume. These final values were represented as ng/mg of striatal protein. This experiment was done in collaboration with Dr. Barbara Finckh who did the HPLC measurements.

3.4 Behavioral studies

Only adult male mice aged between 3-6 months were used for behavioral analysis in this study. All animals were housed separately in individual cages under standard conditions in a reversed 12 hr day/night cycle with access to pelleted diet and water *ad libitum.* Behavioral analysis was done in a separate room during the night cycle of animals with a light intensity of 12 lux as described earlier ***(Meka et al. 2015)***. All equipment were cleaned after each mouse was tested, by wiping with soap, then water solution, dried and then with 70% ethanol and finally dried. Behavioral analysis was done in mutual aid with Sai Sneha Priya Nemani.

3.4.1 Open field test

To test locomotor activity and exploratory behavior, mice with no prior training were subjected to open field with 50X50 cm arena enclosed by 40 cm high walls. Mice were placed in to the center of the arena and allowed to roam freely for 10 min and their activity was monitored by an observer and using the Etho Vision software (Noldus, Sterling, USA).

3.4.2 Elevated plus maze

The arena of plus maze is elevated 75 cm from the floor level with 30 cm long and 5 cm wide arms, connected by a 5X5 cm center. Two opposing arms are bordered by 15 cm high walls (closed arms), and the other two arms (open arms) are bordered by a 2 mm rim. Each mouse was placed in the center facing an open arm and the activity was recorded for 5 min. Following parameters were analyzed from the pre-recorded videos. Number of entries and duration into the open and closed arms (calculated when all four paws were on an arm), Self-grooming, rearing on walls and stretch attend posture (calculated when mice extend into other areas only moving forepaws and not rear paws) ***(Trullas & Skolnick 1993)***.

3.4.3 Pole test

To test motor coordination, mice were placed on top of a vertical 60 cm long rod made of rough wood with a diameter of 7 mm. To motivate the mice to climb down the pole, nesting material of the animal's home cage was placed at the bottom of the pole. Mice were placed grasping the rod with all the paws with the head pointing upwards. Each mouse was tested in three consecutive trials with time interval of 30 sec during which mouse was placed into its home cage. The time required for each mouse to turn 180° and climb down the pole with head pointing downwards was recorded and eventually evaluated as previously described ***(Freitag et al. 2003)***.

3.5 *In vitro* studies

Table 3.11 Cell culture medium

Cell line	Medium composition
SH-SY5Y	DMEM (Table 3.6); 10%FBS; 1X Non-Essential Amino Acids; 1X Sodium pyruvate; 1X Penicilin-Streptomycin
MZCRC1	DMEM; 10%FBS; 1X Penicilin-Streptomycin
HeLa	DMEM; 10%FBS; 1X Penicilin-Streptomycin
SK-N-BE(2)	DMEM; 10%FBS; 1X Penicilin-Streptomycin

Table 3.12 Plasmids and siRNA oligos

Plasmid/siRNA oligos	Source
mCherry	Dr. Kramer
MEN2B	Prof Dr. Saarma
WT human parkin	Prof. Dr. Winklhofer
Parkin Q311Stop	Prof. Dr. Winklhofer
Parkin G430D	Prof. Dr. Winklhofer
Parkin R42P	Prof. Dr. Winklhofer
Parkin W453Stop	Prof. Dr. Winklhofer
Parkin Δ-1-79	Prof. Dr. Winklhofer
Human parkin stealth siRNA	HSS107594, Invitrogen
Stealth siRNA negative control, Medium GC	12935300, Invitrogen

3.5.1 Transformation

For each transformation reaction, one vial of frozen DH5α competent cells were thawed on ice to which plasmid DNA construct was added and mixed gently and incubated on ice for 30 min. Subsequently, the mixer was incubated in water bath at 42°C for 60 seconds in order to facilitate heat shock and allow the DNA into the cells. 500 µl of SOC medium was added and the transformation mix was incubated in a shaking incubator at 37°C, 220 rpm, for 45 min. 50-75 µl of transformation mixture was plated on the LB agar plates containing the appropriate selection marker (ampicillin) and plates were incubated at 37°C for 12-14 hr. Transformed colonies were picked for plasmid isolation.

3.5.2 Isolation of plasmid DNA

Colonies were picked from LB plates containing ampicillin and grown overnight in 10 ml of LB broth containing ampicillin. Following day, 2 ml of the culture was aliquoted for maxi

preparation and the rest was used for isolating the plasmid DNA using the Qiagen mini prep plasmid DNA isolation kit in accordance with the manufacturer's instructions. Plasmid DNA obtained was sequenced with respective primers to confirm the identity of the plasmid DNA. After confirming the plasmid identity, aliquoted 2 ml culture was inoculated into 250 ml of LB broth containing ampicillin and grown overnight at 37°C with constant shaking. Plasmid DNA was isolated from the culture according to the manufacturer's instructions (PureLink® HiPure Maxiprep Kit).

3.5.3 Culturing and transfection of cell lines

SH-SY5Y cultures were kindly provided by Prof. Dr. Winklhofer (DSMZ No. ACC 209); MZCRC1 cultures were kindly provided by Dr. Alex Knuth; HeLa cultures were kindly provided by Dr. Uwe Borgmeyer; All cultures were supplemented with the respective DMEM medium mentioned in Table 3.11 and were maintained at 37°C with 5% CO_2. Trypsin EDTA was used for trypsinization while passaging the cells. SH-SY5Y cultures express Ret and parkin endogenously and MZCRC1 cultures harbor an endogenous point mutation in exon 16 of Ret at position 918, methionine to threonine (M918T) giving raise to MEN2B like phenotype. For RNA interference experiments these cultures were reverse transfected with either stealth siRNA against human parkin or negative siRNA control using Lipofectamine® 2000 in accordance with the manufacturer's instructions. Cultures were incubated for 48 hr for efficient knock down (KD) of parkin before harvesting the cultures.

HeLa cultures do not express either Ret or parkin endogenously, thus the cells were co-transfected with plasmid DNA constructs listed in Table 3.12, using Lipofectamine® 2000 following the manufacturer's instructions. Cultures were incubated for 48 hr post transfection for the transgene to express before harvesting. Both SH-SY5Y and HeLa cultures harvested were processed for biochemical analysis as described in (3.3.2-3.3.3).

3.5.4 Treatments in SH-SY5Y and HeLa cultures

For some turn-over experiments, SH-SY5Y cultures were reverse transfected with stealth siRNA against human parkin or negative siRNA control and harvested 48 hr post transfection. To decipher the role of parkin in regulating Ret protein levels, transfected cultures were treated with cycloheximide (CHX) at 100 mg/ml concentration. CHX was added to cells at 0, 3 or 6 hr and left for incubation prior to harvesting. HeLa cultures were co-transfected with mCherry or parkin along with MEN2B and harvested 48 hr post transfection. To decipher the role of parkin in regulating MEN2B protein levels, transfected cultures were treated with either MG132 (10 µM) or ammonium chloride (NH_4Cl) (10 mM) 8 hr prior to harvesting. Both SH-SY5Y and HeLa cultures harvested were processed for biochemical analysis as described in (3.3.2-3.3.3).

3.5.5 Subcellular fractionation

SK-N-BE(2) cultures endogenously express high Ret protein and therefore used for Ret nuclear localization studies. Subcellular fractionation of SK-N-BE(2) cultures was done using a kit (Subcellular Protein Fractionation Kit-Table 3.3). Following the manufacturer instructions for the fractionation yields segregation and enrichment of proteins from five different cellular compartments namely, cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal. Mouse tissue was homogenized in 0.1M potassium phosphate buffer with protease inhibitor cocktail and centrifuged at 1000g for 10 min to obtain post nuclear pellet (PNS) and nuclear pellet. Nuclear pellet was thus homogenized in nuclear extraction buffer and centrifuged for 10 min at 4°C to obtain nuclear proteins in the supernatant. Protein concentration was determined from the fractions obtained and were subjected to immunoblotting as described earlier (3.3.2-3.3.3). Fraction purity was assessed by probing for marker proteins highly abundant in respective sub cellular compartments.

3.5.6 Cell surface biotinylation

Biotinylation experiments were performed on intact SK-N-BE(2) cultures. Briefly, cultures were washed with cold PBS containing Ca^{2+}/Mg^{2+} and further incubated with sulfo-NHS-LC-Biotin solution at RT for at least 10 min. Biotin solution was discarded and the cultures were incubated with glycine buffer for at least 10 min at RT and this step was repeated for a couple of times. Glycine buffer was replaced with warm culturing medium without 10% FBS and stimulated with GDNF and incubated at 37°C with 5% CO_2 for respective time points and then harvested. Cell pellets were washed with PBS and subjected to subcellular fractionation as described earlier (3.5.3). Protein concentration was determined and biotin conjugated proteins were immunoprecipitated using streptavidin magnetic beads (Dynabeads® M-280 Streptavidin) from various subcellular fractions. Manufacturer instructions were followed to pull down biotinylated proteins using streptavidin magnetic beads and later subjected to immunoblotting (3.3.2-3.3.3).

3.6 Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed with GraphPad Prism 5.0. Comparison of two independent groups was performed using two-tailed, unpaired Student's t-test and to compare more than two experimental groups, ANOVA, followed by Tukey's or Newman-Keuls post hoc test was used. The mean difference that is smaller than p-value 0.05 between the groups was considered statistically significant.

4. Results

A. Parkin deletion leads to age dependent progressive nigrostriatal degeneration in Ret-deficient mice

4.1 Generation and further characterization of parkin and Ret doubly deficient mice

Ret-null mice are predisposed to perinatal lethality since Ret signaling is essential for renal organogenesis and enteric neurogenesis ***(Schuchardt et al. 1994; Uesaka et al. 2008; Golden et al. 2010)***. Therefore, conditional Ret-deficient mice were generated using the Cre/loxP system, through which Ret can be exclusively deleted in a cell-type specific manner. DA neuron specific deletion of Ret is achieved by crossing mice carrying a transgene which expresses Cre recombinase under the control of DA system specific promoter with mice carrying the floxed allele for Ret ($Ret^{lx/lx}$ mice) ***(Kramer et al. 2006; Kramer et al. 2007)***. Since the aim is to generate a DA neuron specific ablation of Ret, here the expression of Cre recombinase is driven by the promoter of DAT and the expression of DAT is restricted to the DA neuronal population. Deletion of Ret gene in the DA neurons can be achieved early during embryonic day 13.5 (E13.5) with Cre recombinase expression (**D**AT-**C**re recombinase in **B**ACmid; DCB) ***(Parlato et al. 2006; Turiault et al. 2007)***, thereby attaining DCB-$Ret^{lx/lx}$ (hereinafter referred to as Ret-/-) mice ***(Meka et al. 2015)*** (Figure 4.1A). However, Ret-deficient mice used here are different from the mice in Kramer et al. 2007 and the reasoning behind this was that the insertion of the Cre transgene directly into the endogenous DAT locus, perturbed the functional DAT allele in these mice. This resulted in decrease of DAT protein levels in an age-dependent manner leading to hyperactive behavior in these mice ***(Turiault et al. 2007; Kramer et al. 2007)***. Using DCB circumvents this behavior, since the Cre expression is regulated by an exogenous DAT promoter within the bacmid thereby leaving the endogenous DAT alleles intact.

To explore the synergistic function of both Ret and parkin, Ret-deficient mice were cross-bred with mice deficient for parkin (parkin-/-) ***(Itier et al. 2003)*** to get parkin and Ret double deficient mice (hereinafter referred to as parkin-/-; Ret-/-) (Figure 4.1B).

Both single (Ret-/-) and double (parkin-/-; Ret-/-) deficient mice were viable and fertile. Ret-deficient and parkin; Ret double deficient mice along with the control mice (DCB or $Ret^{lx/lx}$) were aged 3–6 months, 12 months and 24 months, thereafter, used for analyzing the alterations in the DA system of these mice either due to deletion of Ret alone or parkin and Ret together. Basic and phenotype characterization of these mice were shown in Praveen

Meka's doctoral thesis. However, they were further characterized to explore additional phenotypes which were not addressed by Dr. Meka in his thesis.

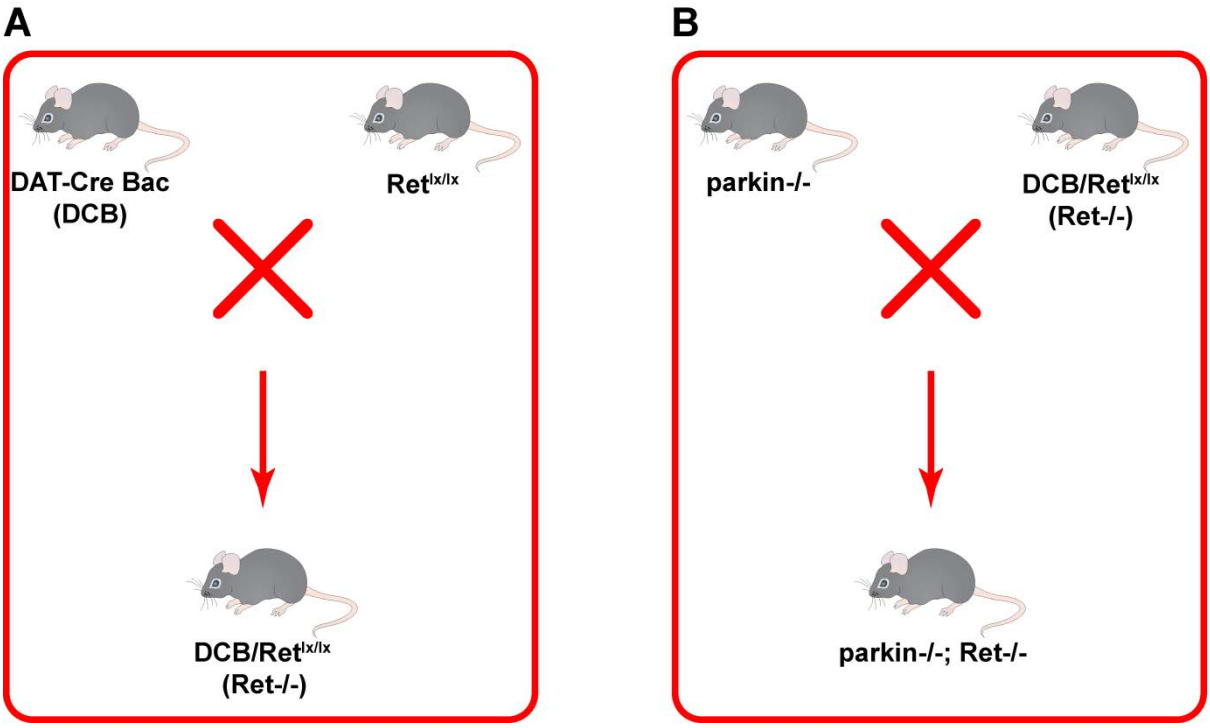


Figure 4.1 Schemes representing the generation of Ret-/- and parkin-/-; Ret-/- mice

Cell-type specific ablation of Ret is achieved by crossing DAT-Cre BAC (DCB) mice which were obtained from Günther Schütz and $Ret^{lx/lx}$ ***(Kramer et al. 2006)*** mice to generate DAT-Cre BAC- $Ret^{lx/lx}$ (Ret-/-) offspring (**A**) and these Ret-/- mice were further crossed with parkin null mice which were obtained from Alexis Brice to generate mice deficient for both Ret and parkin (parkin-/-; Ret-/-) (**B**) to understand the synergistic role of Ret and parkin in the survival of nigrostriatal DA neurons.

4.2 Synergistic function of Ret and parkin is required for the survival of nigrostriatal DA neurons

Mice lacking Ret signaling specifically in DA neurons exhibit late on-set and age dependent nigrostriatal degeneration of DA system ***(Kramer et al. 2007)***. However, mice lacking parkin function do not show DA neurodegeneration ***(Goldberg et al. 2003; Itier et al. 2003)***, and the absolute requirement of parkin in the survival of DA neurons *in vivo* remains unclear. To establish the survival function of parkin in DA neurons of tropically impaired (Ret-deficient) mice, parkin and Ret double deficient mice were generated. To detect the morphological alterations in the DA system, coronal midbrain sections of 24 month-old Ret-deficient, parkin and Ret doubly deficient mice along with their respective age-matched controls (Figure 4.2 A) were stained for TH, a marker for DA neurons. Stereological quantification using the optical fractionator probe revealed the total number of DA neurons both in the SNpc and the VTA of mice with various genotypes. As mentioned earlier, parkin-deficient mice showed no

alterations in the total number of DA neurons compared to DCB control mice. Whereas Ret-deficient mice showed a significant 21% loss of DA neurons specifically in the SNpc region (Figure 4.2 B), the number of neurons in the adjacent VTA remained unchanged (Figure 4.2 C), as reported earlier ***(Kramer et al. 2007)***.

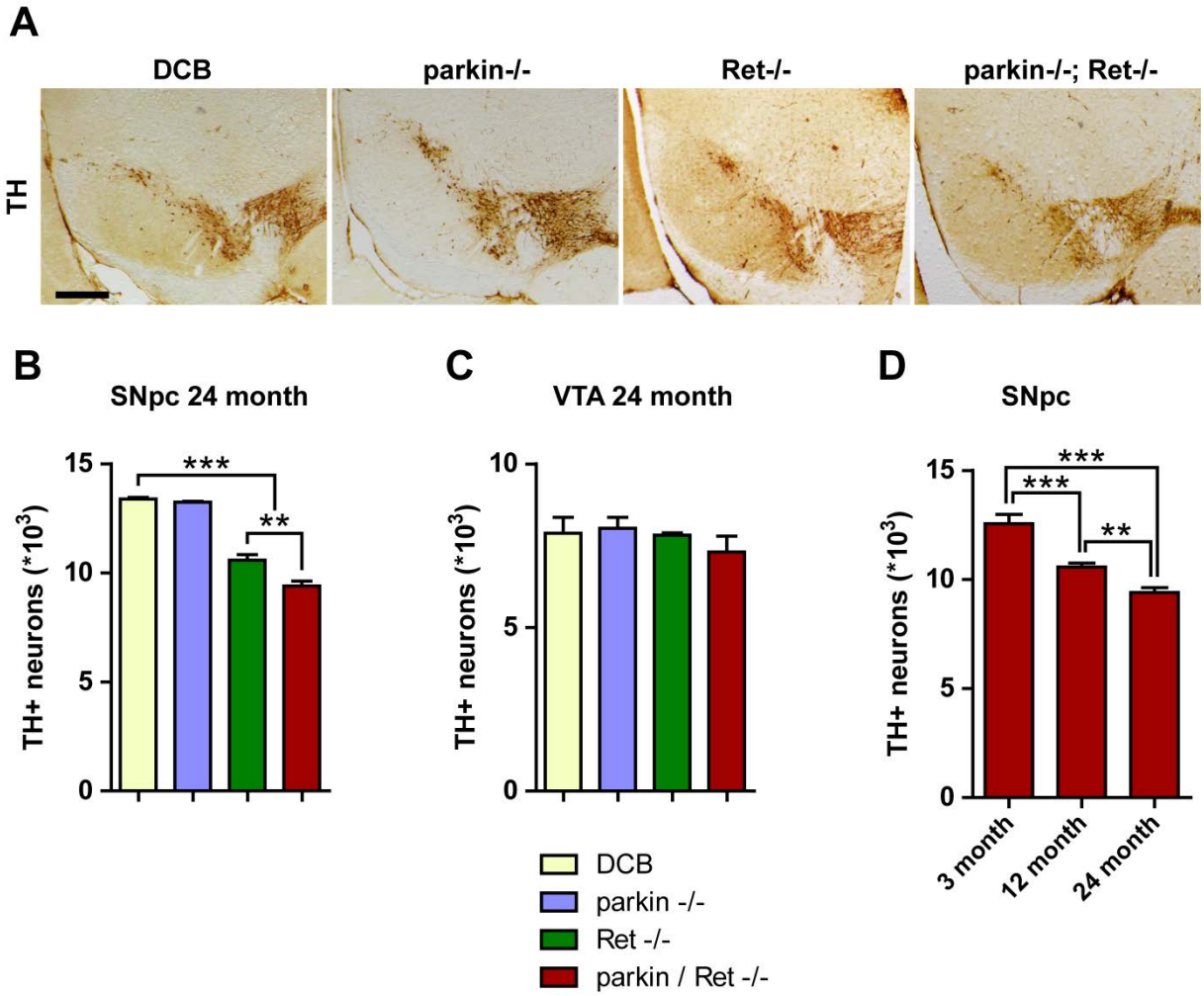


Figure 4.2 Parkin and Ret doubly deficient mice show more pronounced loss of DA neurons compared to Ret singly deficient mice

Photomicrographs of coronal brain sections from 24 month-old DCB (control) parkin-/-, Ret-/- and parkin-/-; Ret-/- mice showing DA neurons in the SNpc and VTA for the DA marker TH (scale bar=250µm) (**A**). Stereological quantifications of TH+ neurons of SNpc at age of 24 month revealed a 21% cell loss in Ret-/- mice and 30% loss in parkin-/-; Ret-/- compared to their age-matched controls (DCB or parkin-/-), n=3–6 mice per genotype (**B**); TH+ neurons of VTA at age of 24 month revealed no alterations among the genotypes, n=3–6 mice per genotype (**C**); TH+ neurons of SNpc showing an age dependent progressive degeneration of DA neurons in parkin-/-; Ret-/- mice showing 16% loss at 12 month followed by 25% loss at 24 month age compared to 3–6 month-old parkin-/-; Ret-/- mice (**D**), n=5-6 mice per genotype. Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

Deletion of parkin in Ret-deficient mice showed an enhanced degeneration with a 30% loss in the total number of SNpc DA neurons (Figure 4.2 B) and, as observed in the single Ret-deficient mice, the number of VTA DA neurons in parkin and Ret double deficient mice was also unaltered (Figure 4.2 C). Thus, deletion of parkin in Ret-deficient mice aggravates the Ret deficiency phenotype, pointing to a critical cell survival function of parkin under conditions of trophic impairment in DA neurons. Besides augmented degeneration of DA neurons, parkin ablation in Ret-deficient mice lead to an age-dependent progressive neurodegeneration specifically in the SNpc region (Figure 4.2 D) that is reminiscent of PD.

4.3 Ret loss perturbs mitochondrial complex I activity in the SN of aged mice

Impaired mitochondrial function is implicated in most neurodegenerative disorders including PD ***(Winklhofer & Haass 2010)***. Parkin is one of the key regulators in maintaining mitochondrial homeostasis ***(Pilsl & Winklhofer 2012; Corti & Brice 2013)***. Recent evidence suggests that either parkin knock down (KD) or Ret-KD alone can lead to a mitochondrial fragmentation phenotype in SH-SY5Y cultures. However, no alterations in either complex I activity or in ATP levels are observed in 3–6 month-old Ret-deficient or in the parkin-deficient mice compared to their age-matched controls (DCB) ***(Meka et al. 2015)***. This lead to the question if aged Ret-deficient mice might show mitochondria-related alterations that are not evident in 3–6 month-old mice. To further understand the role of Ret activity in maintaining the mitochondrial integrity in aged mice, mitochondria enriched fractions were obtained from the SN of 12 month-old Ret-deficient mice and mitochondrial complex I activity was measured.

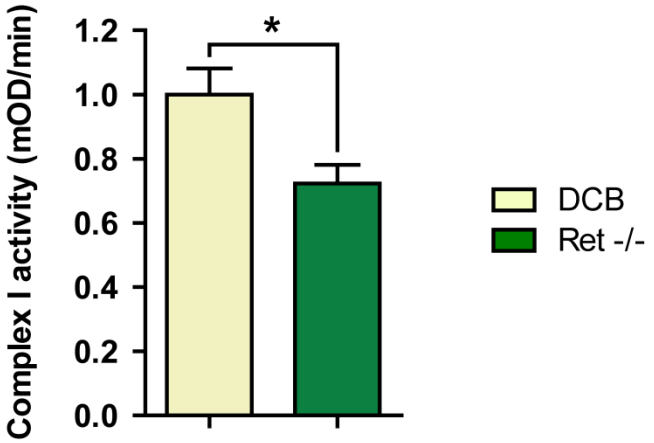


Figure 4.3 Ablation of Ret in the DA neurons impairs mitochondrial complex I activity in an age-dependent manner

Measurements of mitochondrial complex I activity in the mitochondria enriched fractions obtained from the SN of 12 month-old Ret-/- mice compared to their age-matched DCB controls, n=4-5 per genotype; Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student's t-test.

A consistent and significant 28% reduction in complex I activity in 12 month-old Ret-deficient mice was observed compared to their age-matched DCB controls (Figure 4.3). These observations suggested for the first time an important physiological function of Ret in maintaining mitochondrial activity *in vivo*.

4.4 Autophagy is unaffected in parkin–Ret doubly deficient mice

Autophagy is observed to be dysregulated in the brains of PD patients and in a few animal models of PD ***(Lynch-Day et al. 2012)***. Since ablation of parkin in Ret-deficient mice could recapitulate most of the characteristics of PD in humans ***(Meka et al. 2015)***, parkin and Ret doubly deficient mice might be a better model to understand the pathogenesis of PD.

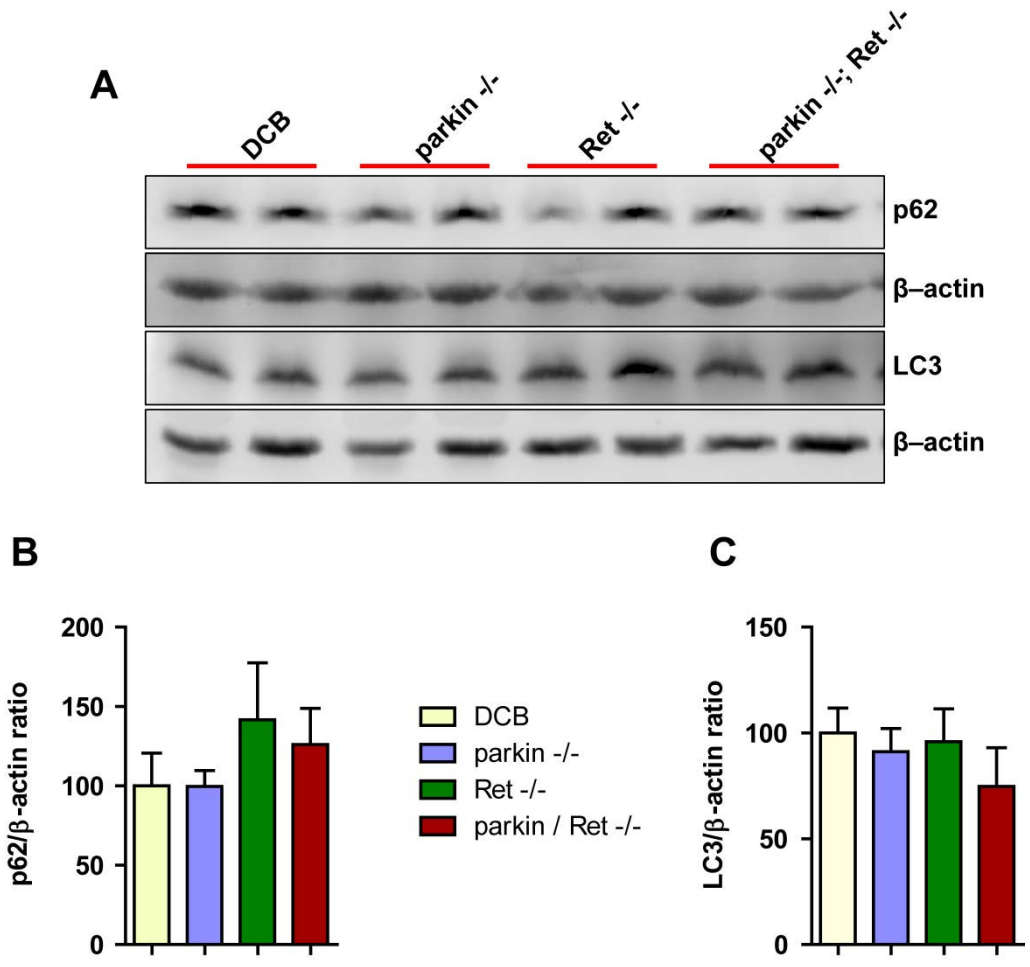


Figure 4.4 Autophagy is unaffected in the DA system of parkin-Ret doubly deficient and parkin or Ret singly deficient mice

Western blots of (**A**) ventral midbrain lysates depicting expression of the indicated proteins from 24 month-old DCB control, parkin or Ret single mutant and parkin–Ret double mutant mice, n=4 mice per genotype; Quantification of the relative expression of p62 to β-actin (**B**); LC3 to β-actin (**C**) of indicated genotypes. Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

Parkin along with other PD-linked proteins PINK1 and α-synuclein ***(Lynch-Day et al. 2012)*** are linked to autophagy, this provoked to check if autophagy, one of the key aspects that are impaired during the course of the disease is also affected in parkin and Ret double deficient mice. Both p62 and LC3 are reliable and widely examined markers for impaired autophagy ***(Pankiv et al. 2007)*** and accumulation of p62 and LC3 is strikingly elevated when autophagy is blocked. To address this question, ventral midbrain was punched from DCB controls, parkin-deficient mice, Ret-deficient mice and parkin and Ret double deficient mice, and the lysates were immunoblotted to assess p62 and LC3 levels in 24 month-old mice. However, no marked increase in either p62 or in LC3 levels were observed among the genotypes (Figure 4.4 A–C) indicating that autophagy is not significantly affected in these mice.

B. Parkin ablation normalizes the enlarged DA system in MEN2B mutant mice

4.5 Generation and characterization of parkin-deficient MEN2B mice

To confirm the functional cross-talk of both parkin and Ret, and to further investigate the function of parkin in the developmental context of DA system, mice carrying a constitutively active form of Ret–multiple endocrine neoplasia type 2 B (MEN2B) were used. MEN2B is a constitutively active form of Ret due to a point mutation at position (Met 918 Thr). Mice expressing MEN2B show an enlarged DA system and elevated dopamine levels in the striatum ***(Mijatovic et al. 2007)*** during adulthood. In order to assay the role of parkin during development of DA system and to analyze if parkin deletion in MEN2B mutant mice could normalize the Ret gain-of-function phenotype, parkin-deficient mice ***(Itier et al. 2003)*** were crossed with heterozygous MEN2B mutant mice as homozygotes of male MEN2B mutant mice displayed infertility ***(Smith-Hicks et al. 2000)*** to generate parkin-deficient MEN2B mice (Figure 4.5 A). Parkin-deficient MEN2B mice were viable and either 3–6 month-old mice or the early postnatal 4–16 days old parkin-deficient MEN2B pups along with their respective controls were used to analyze the DA system or the adrenal gland morphology.

4.6 Deletion of parkin normalizes the enlarged DA system in the adult MEN2B mutant mice

To confirm the functional cross-talk of both parkin and Ret during the development of the DA system, parkin-deficient MEN2B mice along with their respective controls were stereologically analyzed for the total number of DA neurons in adult midbrain coronal sections stained for TH.

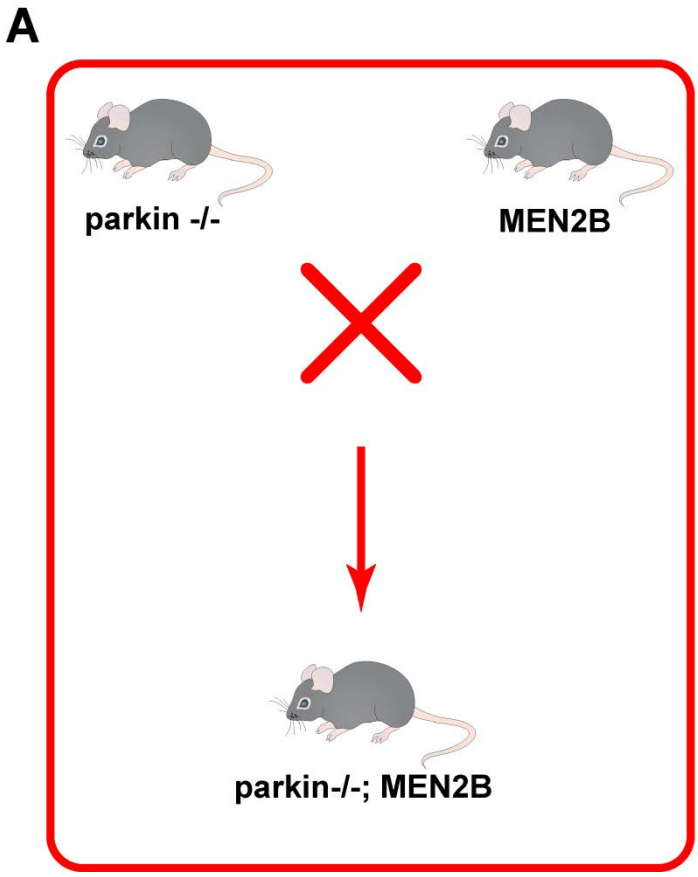


Figure 4.5 Scheme representing the generation of parkin-deficient MEN2B mice
Parkin-deficient (parkin-/-) mice were obtained from ***(Itier et al. 2003)*** and crossed with constitutively active form of Ret–MEN2B mutant mice (MEN2B) which were obtained from Frank Costantini ***(Smith-Hicks et al. 2000)*** to generate parkin-deficient MEN2B mice (parkin-/-; MEN2B).

In agreement with previous findings ***(Mijatovic et al. 2007)***, stereological quantification of MEN2B mutant mice revealed a significant 14% increase in total number of DA neurons specifically in the SNpc. In contrast, the total number of DA neurons in the adjacent VTA neurons was unaltered when compared to age-matched WT control mice. However, as shown in the earlier results (Figure 4.2) and from the previous studies ***(Itier et al. 2003; Goldberg et al. 2003)***, no significant differences were observed in the total number of DA neurons in parkin-deficient mice when compared to WT control mice. Parkin-deficient MEN2B mice showed a significant normalization of around 12% in the number of DA neurons in the SNpc when compared to MEN2B mutant mice and no significant alterations were observed in the VTA region of parkin-deficient MEN2B mice stating that Ret gain-of-function is confined to SNpc neurons (Figure 4.6 A–C). These *in vivo* findings suggest that loss of parkin exacerbates the Ret loss-of-function phenotype and normalizes the Ret gain-of-function phenotype in the DA system, further supporting the functional cross-talk of parkin and Ret.

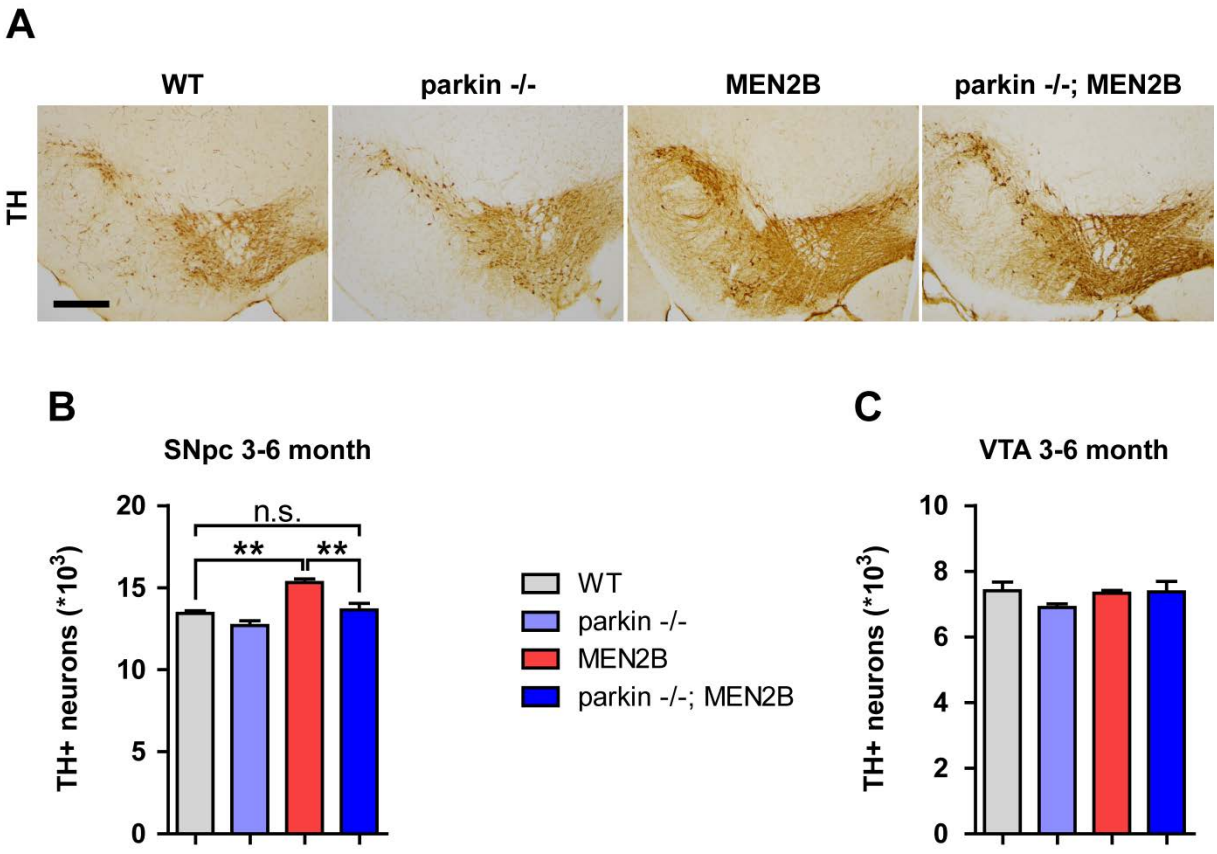


Figure 4.6 Parkin deletion normalizes the increased nigral DA neurons in the MEN2B mutant mice

Photomicrographs of coronal brain sections from 3–6 month-old WT (control), parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice showing DA neurons in the SNpc and VTA stained for the DA marker TH (scale bar=200 µm) (**A**). Stereological quantifications of TH+ neurons of SNpc at age of 3–6 month revealed a 14% increase in MEN2B mutant mice compared to their age-matched WT controls and 12% normalization in parkin-/-; MEN2B mice compared to MEN2B mutant mice, n=3 – 4 mice per genotype (**B**); TH+ neurons of VTA at age of 3–6 month revealed no alterations among the genotypes, n=4 mice per genotype (**C**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

4.7 Parkin deletion normalizes the enlarged DA system during postnatal development in MEN2B mutant mice

Parkin deficiency normalizes the effect of MEN2B mutation in the SNpc DA neurons (Figure 4.6). To understand whether this normalization is an early or a late postnatal effect, the DA system of mice at postnatal day (P) 4 and 16 were analyzed (Figure 4.7.1; 4.7.2). As it was unclear when exactly MEN2B phenotype appears during the development of DA system, coronal midbrain sections were stained for TH from control (WT) and MEN2B mutant mice at the age of P4 (Figure 4.7.1 A). We then stereologically quantified the total number of DA neurons. No significant alterations in the number of DA neurons in SNpc and VTA are

observed at the age of P4 in the MEN2B mutant mice when compared to their age-matched control (WT) mice (Figure 4.7.1 B–C), suggesting the lack of MEN2B phenotype in the DA system during the early developmental stage, P4.

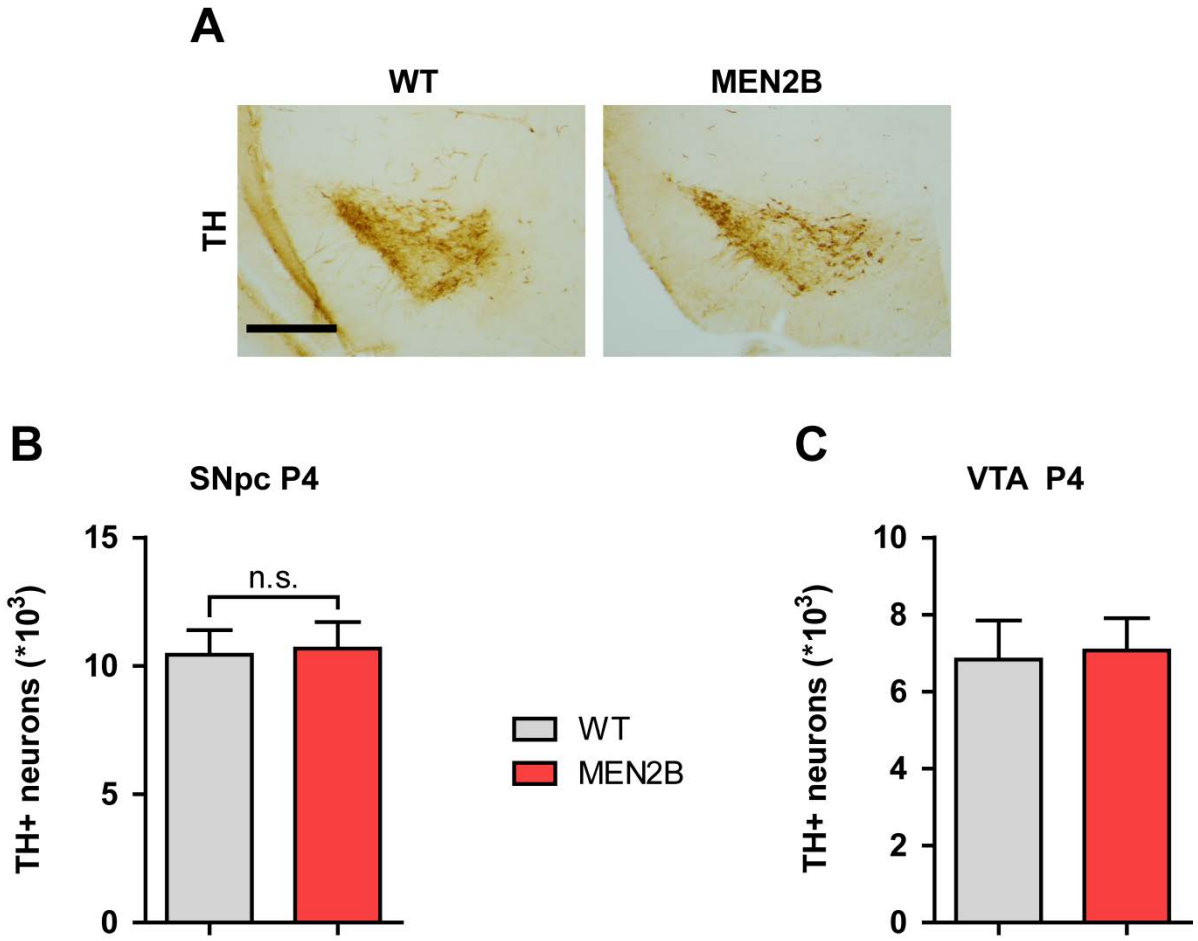


Figure 4.7.1 No evidence of MEN2B phenotype at the age of P4

Photomicrographs of coronal brain sections from postnatal day 4 (p4) old control (WT) and MEN2B mutant mice showing DA neurons in the SNpc and VTA for the DA marker TH (scale bar=200 µm) (**A**). Stereological quantifications of TH+ neurons of SNpc at age of p4 revealed no significant alterations in MEN2B mutant mice compared to their age-matched WT controls, n=3–4 mice per genotype (**B**); TH+ neurons of VTA at age of p4 revealed no alterations among the genotypes, n=3–4 mice per genotype (**C**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student's t-test.

As the MEN2B phenotype was not observed at P4, the DA system of P16 mice was analyzed for a MEN2B phenotype in MEN2B mutant mice. In parallel, we also asked whether a MEN2B phenotype at P16- if observed- could be normalized in parkin-deficient MEN2B mice. TH immunolabeled coronal midbrain sections from control (WT), parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice at the age of P16 (Figure 4.7.2 A) were stereologically analyzed for the total number of DA neurons in the SNpc and VTA. A

significant increase of 55% DA neurons in the SNpc of MEN2B mutant mice was observed compared to their age-matched controls (WT) and further deletion of parkin showed a significant 44% normalization of the number of SNpc DA neurons in MEN2B mutant mice (Figure 4.7.2 B). However as observed earlier for the 3–6 month adult mice (Figure 4.6 C), no significant alterations were observed in the total number of VTA DA neurons (Figure 4.7.2 C). Together with our earlier findings from the 3–6 month-old mice, these findings from P16 mice support the model for genetic cross-talk between parkin and Ret. Further this finding indicates the role of parkin in normalizing the oncogenic MEN2B phenotype concerning the DA system in mice.

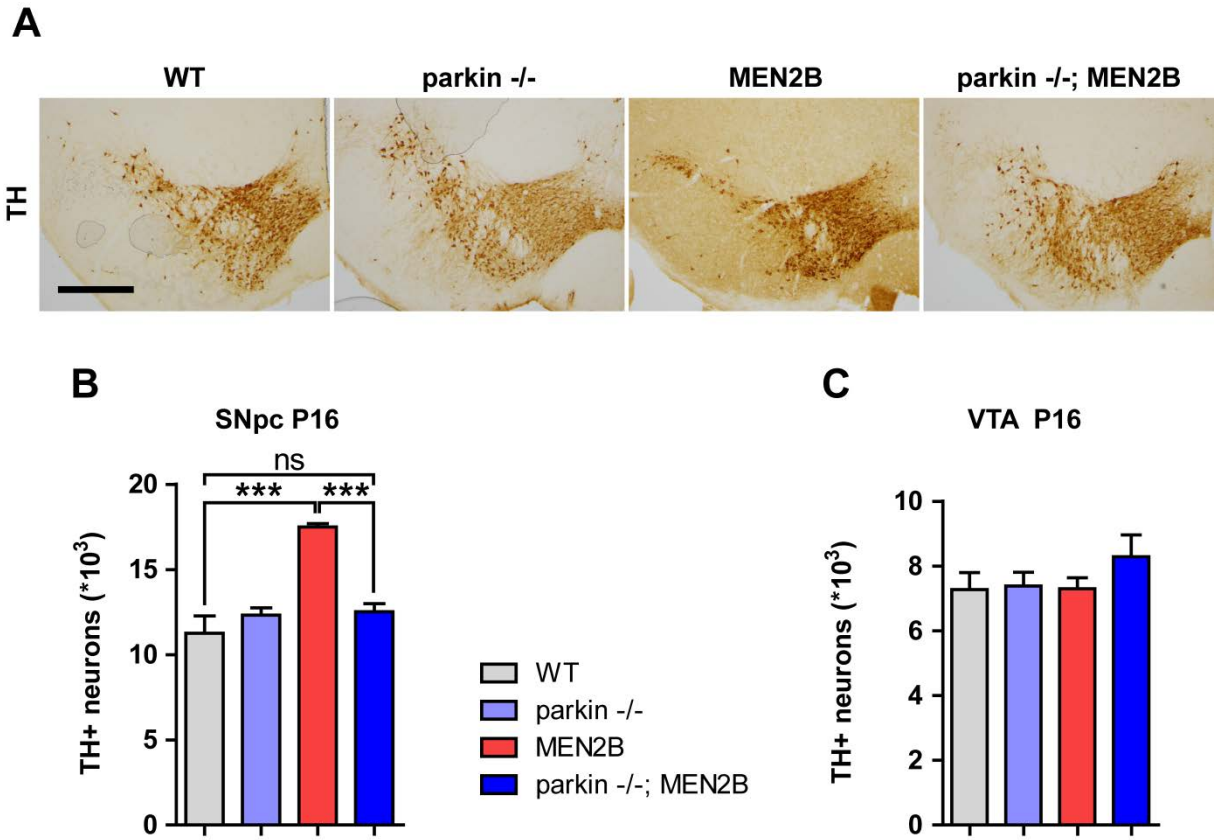


Figure 4.7.2 Augmented DA system at the age of P16 in MEN2B mutant mice and lack of parkin normalizes MEN2B phenotype during development

Photomicrographs of coronal brain sections from postnatal day 16 (P16) old WT (control), parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice showing DA neurons in the SNpc and VTA stained for the DA marker TH (scale bar=200 µm) (**A**). Stereological quantifications of TH+ neurons of SNpc at age of postnatal day 16 (p16) revealed a 55% increase in MEN2B mutant mice compared to their age-matched WT controls and a significant 44% normalization in parkin-/-; MEN2B mice compared to MEN2B mutant mice, n=4 mice per genotype (**B**); TH+ neurons of VTA at age of postnatal day 16 (p16) revealed no significant alterations among the genotypes, n=4 mice per genotype (**C**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

4.8 Constitutive activation of Ret leads to increased soma size of SNpc DA neurons

Deletion of Ret specifically in the DA system leads to reduced soma size of the SNpc DA neurons in addition to age-dependent neurodegeneration ***(Aron et al. 2010; Meka et al. 2015)***. Indeed, it has been reported that reduced soma size of the SNpc DA neurons is one of the primary alterations that were observed in 3–6 month-old, Ret-deficient mice preceding neurodegeneration. This reduced cell size could be one of the detrimental effects that lead to reduced cell membrane capacitance and therefore cell death with age in Ret-deficient mice. Thus, Ret activity appears also to be essential to maintain proper cell size and membrane capacitance in DA neurons. Further investigation was carried out to prove the fact that Ret activity directly correlates with the DA cell soma size. Using the nucleator probe of the Stereo Investigator program, soma size of the TH immunoprobed DA neurons in the SNpc and the VTA were measured for 3–6 month-old parkin-deficient mice, parkin-deficient MEN2B and MEN2B mutant mice. In line with the previous findings that Ret activity corresponds to DA cell soma size, MEN2B mutant mice showed a significant 21% increase in the cell soma size for DA neurons of the SNpc when compared to parkin-deficient mice (Figure 4.8 A).

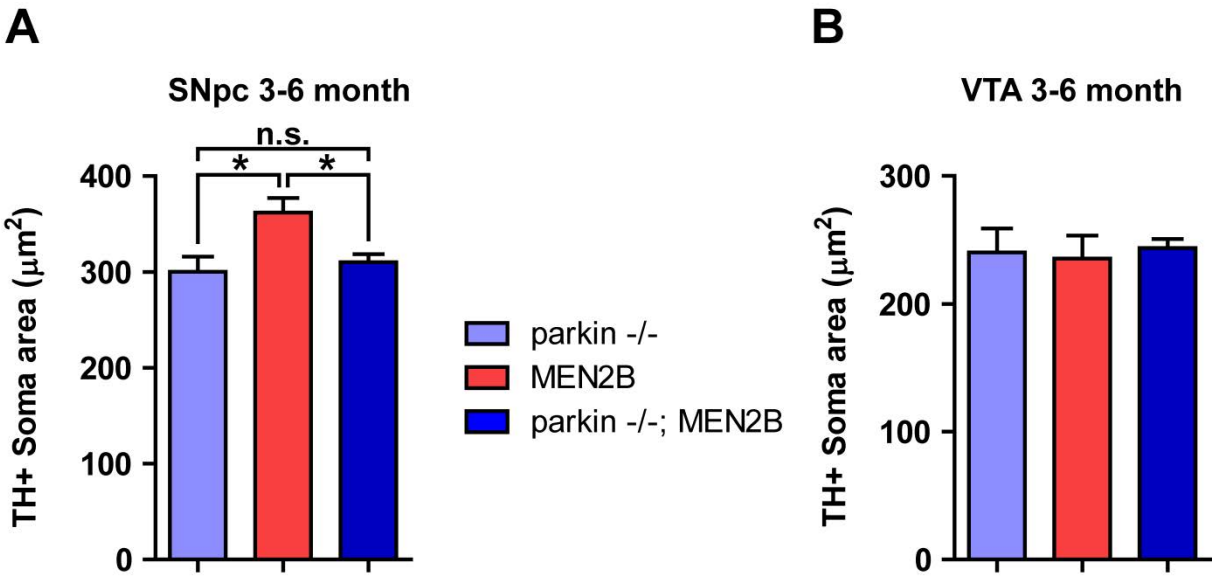


Figure 4.8 MEN2B-mediated morphological alterations are normalized by deletion of parkin in MEN2B mutant mice

Quantifications of cell soma area of TH positive neurons in the SN and VTA of 3–6 month-old parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice (A–B); Soma size measurements of TH+ neurons of SN revealed a 21% increase in MEN2B mutant mice compared to parkin -/- mice and a significant 17% normalization in parkin-/-; MEN2B mice observed compared to MEN2B mutant mice, n=3–4 mice per genotype (**A**); Soma size measurements of TH+ neurons of VTA revealed no significant alterations among the genotypes, n=3–4 mice per genotype (**B**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

Further, in concurrence with the previous findings (Figure 4.6; 4.7.2), deletion of parkin in MEN2B mutant mice significantly normalized the Ret activity-dependent increase of cell soma size by 17% compared to MEN2B mutant mice in the SNpc (Figure 4.8 A). However, no significant alterations were observed in the soma size of the VTA DA neurons (Figure 4.8 B). Since no histological and morphological alterations were observed in parkin-deficient mice as reported earlier ***(Itier et al. 2003; Goldberg et al. 2003; Meka et al. 2015)*** and from the current study (Figure 4.2, 4.6 and 4.7.2) parkin-deficient mice were considered as controls in this experiment.

4.9 Altered mitochondrial function in MEN2B mutant mice

One possibility to explain the phenotype of MEN2B mutant mice in the DA system is through elevated mitochondrial bioenergetics. The likely hypothesis is that the constitutively active form of Ret, MEN2B, along with the complementary function of parkin leads to elevated mitochondrial activity and there by an increase in total ATP levels rendering an augmented nigrostriatal DA system. Further ablation of parkin could be normalizing this phenomenon ***(Palacino et al. 2004; Stichel et al. 2007)***. The fact that absence of Ret perturbs mitochondrial activity both *in vitro* ***(Klein et al. 2014)*** and *in vivo* (Figure 4.3), and that parkin is well known to regulate mitochondrial homeostasis ***(Corti & Brice 2013; Pilsl & Winklhofer 2012)*** supports this.

To evaluate this hypothesis, mitochondria enriched fractions were prepared from the SN of 3–6 month-old controls (WT), parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice, and mitochondrial complex I activity was measured. MEN2B mutant mice showed a significant 26% decrease in mitochondrial complex I activity compared to WT controls (Figure 4.9 A). This loss in complex I activity was rescued if parkin is deleted in MEN2B mutant mice (Figure 4.9 A). Parkin-deficient mice showed a tendency toward a decrease in complex I activity. However, it was not significant (Figure 4.9 A) ***(Meka et al. 2015)***. To further understand these unexpected results, total ATP levels were measured from the SN tissue lysate of all the mutant mice including the WT controls at 3–6 month age. No significant alterations were observed in the total ATP levels of MEN2B mutant mice when compared to WT controls (Figure 4.9 B). A tendency to have a decrease in ATP levels was observed in parkin-deficient mice compared to WT controls and this was significantly rescued in parkin-deficient MEN2B mice (Figure 4.9 B).

Altogether, these findings suggest that mitochondrial bioenergetics were altered in MEN2B mutant mice. However, these findings did not correlate with the enlarged DA system observed in MEN2B mutant mice and how parkin ablation is normalizing this phenotype.

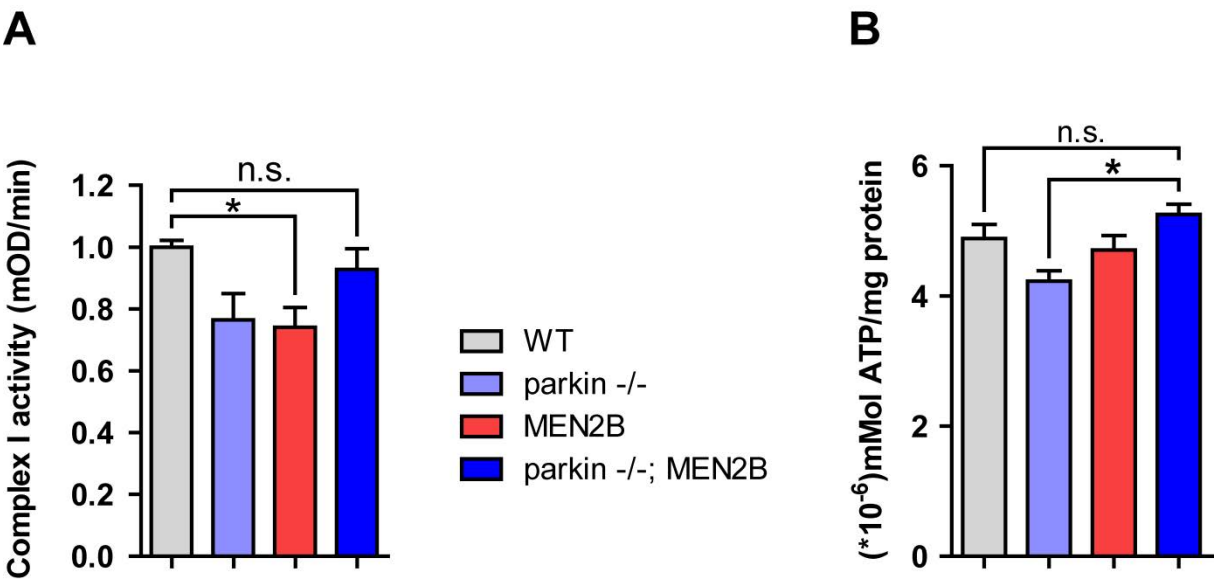


Figure 4.9 Altered mitochondrial functions are rescued by deleting parkin in MEN2B mutant mice

Quantifications of mitochondrial activity and ATP levels from the SN of 3–6 month-old WT controls, parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice (A–B); Mitochondrial complex I enzyme activity measurements from the mitochondria enriched fractions obtained from the SN revealed a significant 26% decrease in MEN2B mutant mice compared to WT controls and parkin-/-; MEN2B mice showed a 19% rescue in complex I activity compared to MEN2B mutant mice. 24% decrease in complex I activity is observed in parkin-/- mice compared to controls, n=5 mice per genotype (**A**); Measurements of total cellular ATP levels in the SN revealed no alterations in MEN2B mutant mice, a tendency of decrease in ATP levels observed in parkin-/- mice is significantly rescued in parkin-/-; MEN2B mice, n=4–5 mice per genotype (**B**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Tukey's post hoc test.

4.10 Parkin deletion has no effect on the elevated dopamine levels in MEN2B mutant mice

Constitutive Ret activity results in elevated DA levels in the brains of MEN2B mutant mice. These findings were further supported by increased TH (rate limiting enzyme for dopamine synthesis) protein expression in the DA system of MEN2B mutant mice ***(Mijatovic et al. 2007)***. To investigate if deletion of parkin has any effect on the elevated dopamine levels in MEN2B mutant mice, total levels of dopamine and its metabolites were measured using HPLC–ECD system from striatal lysates of 3–6 month-old WT controls, parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice. In agreement with earlier findings reported for MEN2B mutant mice ***(Mijatovic et al. 2007)***, a profound increase in the total dopamine by 75% (Figure 4.10 A) and its metabolites DOPAC by 158% (Figure 4.10 B) and homovanillic acid (HVA) by 97% (Figure 4.10 C) were observed in the striatal tissue compared to WT controls (Figure 4.10 A–C).

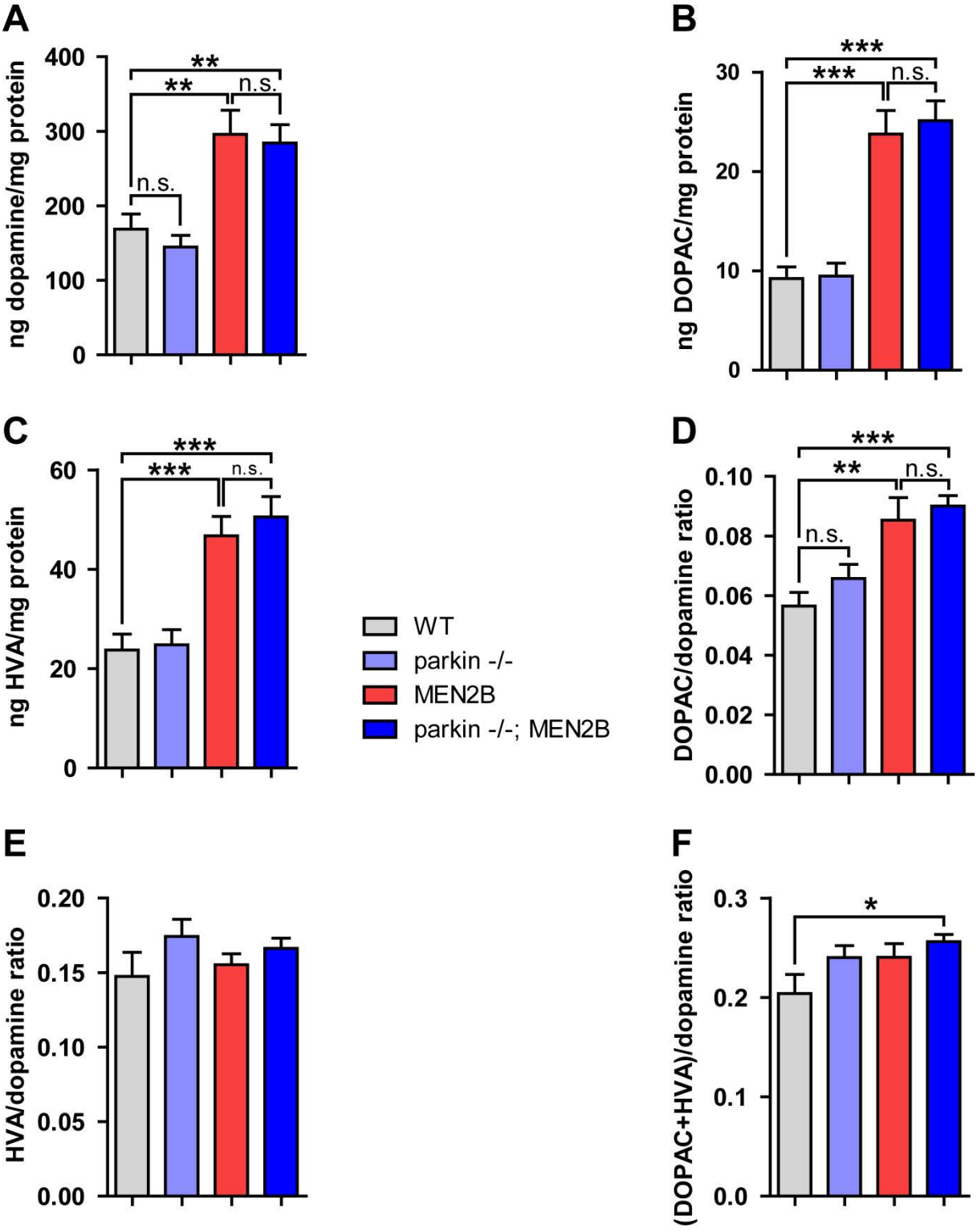


Figure 4.10 Deletion of parkin increases turnover of dopamine without affecting the elevated dopamine levels in MEN2B mutant mice

Quantifications of total dopamine and its metabolites from the striatum of 3–6 month-old WT controls, parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice using HPLC, n=10–14 mice per genotype (**A–C**); Measurements of total dopamine levels from the striatal lysates reveal a significant 75% increase in MEN2B mutant mice compared to WT controls (**A**); Total DOPAC levels reveal a significant 158% increase in MEN2B mutant mice compared to WT controls (**B**); Total HVA levels reveal a significant

97% increase in MEN2B mutant mice compared to WT controls (**C**); parkin-/-; MEN2B mice showed no alterations in either dopamine, DOPAC or HVA levels compared to MEN2B mutant mice (**A–C**); Increase in DOPAC/dopamine ratio with no alterations in HVA/dopamine ratio reveals DOPAC increase is higher than that of the HVA levels in both MEN2B mutant and parkin-/-; MEN2B mice (**D–E**); (DOPAC+HVA)/dopamine ratio implies a significant increase in the dopamine turnover in parkin-/-; MEN2B mice compared to WT control mice (**F**). Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

DOPAC is a metabolite of dopamine, which is mainly produced intracellularly ***(Meiser et al. 2013)*** and HVA is formed outside the DA neurons and is a secondary metabolite of dopamine derived either from DOPAC or 3-MT (3–methoxytyramine) ***(Meiser et al. 2013)***. However deletion of parkin in MEN2B mutant mice seemed to have no effect on the elevated level of dopamine (Figure 4.10 A) or its metabolites DOPAC (Figure 4.10 B) and HVA (Figure 4.10 C) in the striatal tissue compared to MEN2B mutant mice (Figure 4.10 A–C). The increase in the DOPAC/dopamine ratio by 51%, with no changes in HVA/dopamine ratio, indicates a marked increase in DOPAC levels relative to HVA levels in MEN2B mutant mice (Figure 4.10 D–E) and this does not appear to be altered when parkin is deleted in MEN2B mutant mice (Figure 4.10 D–E). However, deletion of parkin significantly increased the turnover of total dopamine by 26% in the striatal tissue of MEN2B mutant mice when compared to WT controls (Figure 4.10 F). Taken together, parkin deletion does not fully normalize the elevated dopamine levels but, significantly increases the turnover of total dopamine in the ST of MEN2B mutant mice.

4.11 MEN2B induced behavioral anomalies were partially rescued by deleting parkin in MEN2B mutant mice

After analyzing the cellular and physiological alterations that are normalized with parkin deletion in MEN2B mutant mice, further investigations were carried out to determine if deletion of parkin could also normalize MEN2B-induced behavioral alterations. Open-field (OF), elevated plus maze (EPM) and pole tests were performed to measure the alterations in general locomotor activity, anxiety and motor coordination, respectively in 3–6 month-old mice. Spontaneous locomotor activity of four mice (with controls and the mutant groups together) at a time was recorded using a video-tracking system on the open-field arenas, with each arena 50X50X40 cm in size for duration of 10 min. Recordings were analyzed using the Ethovision (Noldus) software to reveal different parameters in movement related behavioral alterations between the WT controls and mutant mice. No significant alterations were observed in the total distance traveled among the indicated genotypes from the 10 min long recordings (Figure 4.11.1 A).

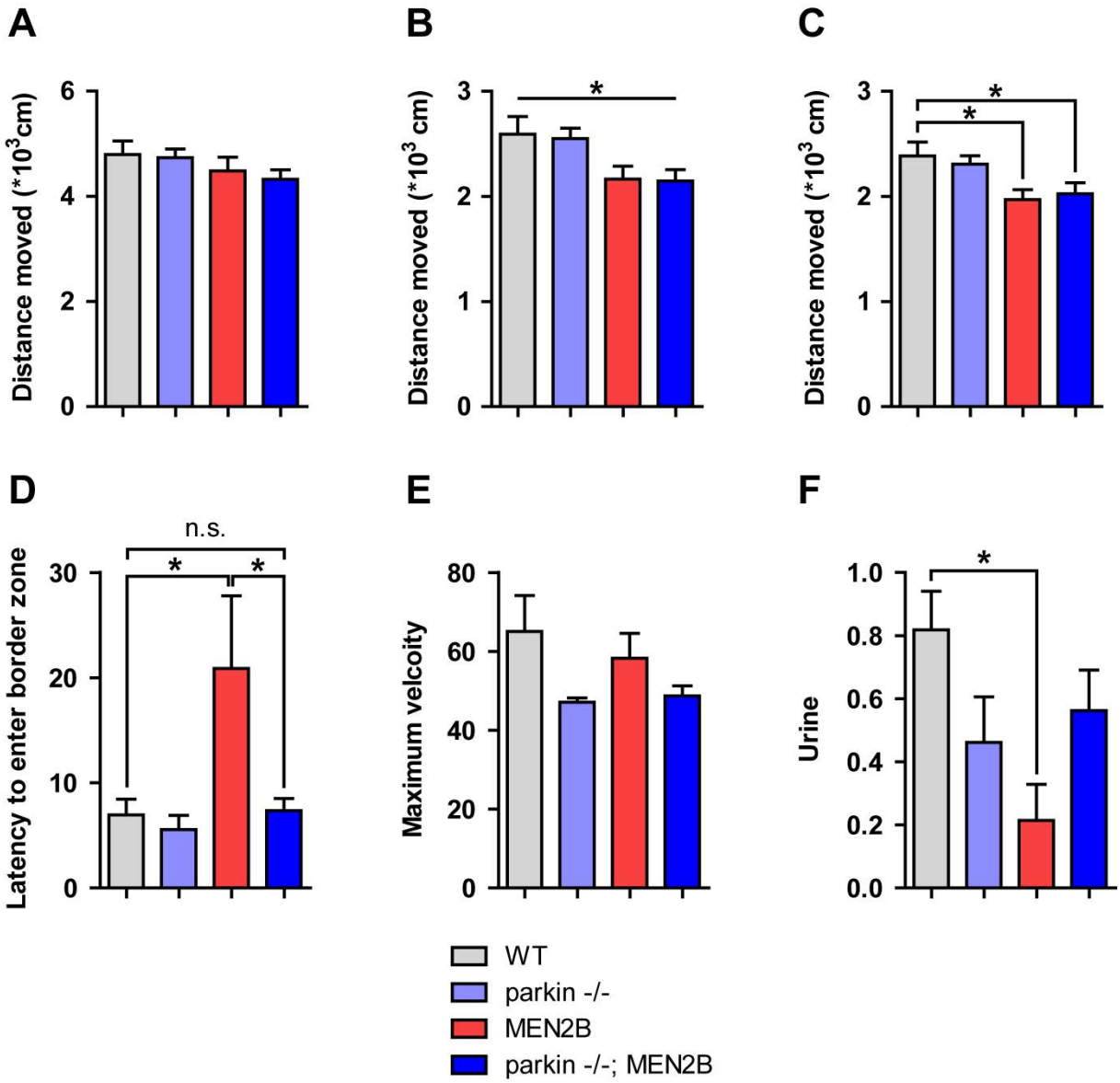


Figure 4.11.1 Parkin ablation rescues behavioral abnormalities in MEN2B mutant mice

Performance of 3–6 month-old WT controls, parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice in the open field arena, n=13–19 mice per genotype (**A–F**); Quantifications showing the horizontal activity of mice with indicated genotypes for a 10 min duration revealing no significant alterations (**A**); Horizontal activity measured for 0–5 min revealing a significant alterations in mice with indicated genotypes (**B**); Horizontal activity measured for 5–10 min revealing a significant decrease in MEN2B mutant and parkin-/-; MEN2B mice compared to WT controls (**C**); Maximum velocity at which the mice moved is not altered significantly among the genotypes (**D**); Latency to enter the border zone is significantly high in MEN2B mutant mice compared to WT controls and deletion parkin normalized this behavior in MEN2B mutant mice (**E**); Urine secretion during the trial in MEN2B mutant mice is significantly low compared to WT controls that is normalized by deleting parkin in MEN2B mutant mice (**F**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

In line with previous reports MEN2B mutant mice, despite elevated dopamine levels in the brain, showed no difference in the total distance moved ***(Mijatovic et al. 2007)***. However, when the total distance was analyzed using a binning of 0–5 min and 5–10 min separately, a significant reduction in the spontaneous locomotor activity was observed in MEN2B mutant mice when compared to WT controls at both time intervals (Figure 4.11.1 B–C). Deletion of parkin has no influence on the decreased locomotor activity in MEN2B mutant mice (Figure 4.11.1 A–C). Though the mean velocity at which the mice were moving did not alter for 10 min duration, however, maximum velocity at which parkin-deficient mice and parkin-deficient MEN2B mice were moving showed a tendency to decrease as compared to WT controls and MEN2B mutant mice, but this was not significant (Figure 4.11.1 E). Besides decreased locomotor activity, MEN2B mutant mice took significantly more time to enter the border zone when compared to WT controls and parkin-deficient mice. This behavior is effectively rescued by deleting parkin in MEN2B mutant mice (Figure 4.11.1 D). Differences in defecation and urination in mice during behavioral analysis is usually measured as a sign of individual differences in emotionality. The amount of urine excretion during the trial was significantly lower in MEN2B mutant mice compared to WT controls and this phenotype is rescued by deleting parkin in MEN2B mutant mice (Figure 4.11.1 F).

EPM is a simple method for assessing anxiety responses in mice. The elevated maze consists of four arms, two open and two closed that are arranged to form a plus. Maze was elevated at height of 75 cm from the floor. Each mouse was placed at the center facing an open arm and the activity was recorded for 5 min using a video-tracking system. Among all the parameters analyzed from the recordings, no significant alterations between genotypes were observed in total open arm entries (when all the four paws of the mouse were on an open arm), self-grooming, or rearing on walls (Figure 4.11.2 A–C). MEN2B mutant mice showed a significant increase in stretch attend posture compared to WT controls and parkin-deficient mice, and this behavior is completely rescued when parkin is deleted in MEN2B mutant mice (Figure 4.11.2 D).

Pole test was done to assess the motor co-ordination behavior while mice are climbing down a vertical pole ***(Freitag et al. 2003)***. Each mouse was placed on the top of the pole (60 cm long; 7mm in diameter) with head facing upward and then allowed to take a 180° turn and climb down the pole. From three consecutive trials done for each mouse, a score is given from the recordings based on the manner they descended from the top of the pole (falling vs. climbing down after 180° turn). Another parameter analyzed was the time taken for each mouse to reach the platform with all four paws. No significant alterations were observed among WT controls, parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice in the manner they descended from the top of the pole (Figure 4.11.2 E). However, the latency to

climb down the vertical pole in MEN2B mutant mice was significantly higher as compared to WT controls and deleting parkin rescued this abnormal behavior in MEN2B mutant mice (Figure 4.11.2 F).

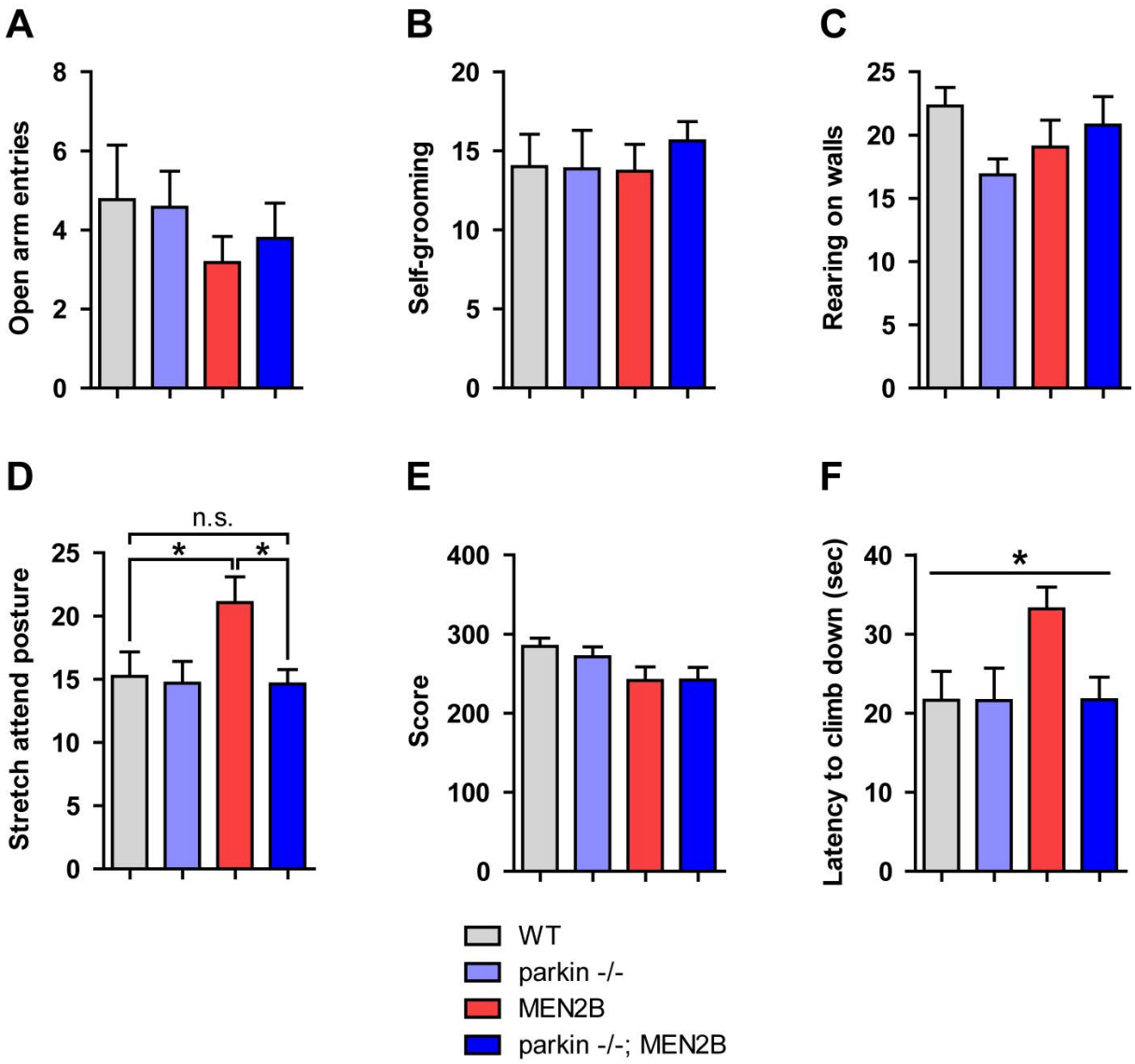


Figure 4.11.2 Parkin ablation rescues movement initiation abnormality in MEN2B mutant mice
Performance of 3–6 month-old WT controls, parkin-/-, MEN2B mutant and parkin-/-; MEN2B mice in the EPM and pole test, n=13–19 mice per genotype (**A–F**); Quantifications showing the total number of open arm entries in mice with indicated genotypes for a 5 min duration reveal no significant alterations (**A**); Self grooming and rearing on walls is unaffected in mice with indicated genotypes (**B–C**); Stretch attend posture is significantly increased in MEN2B mutant mice compared to WT controls and significantly normalized when parkin is deleted in MEN2B mutant mice (**D**); Score given to rotate and climb down the vertical pole is not changed in mice with indicated genotypes (**E**); Latency to climb down the vertical pole is significantly high in MEN2B mutant mice compared to WT controls. However deletion of parkin rescued this phenotype in MEN2B mutant mice (**F**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

Taken together, behavioral assessment data from open field, EPM, and pole test suggest that MEN2B mutant mice exhibit difficulties in initiating movement and deletion of parkin significantly rescues these behavioral abnormalities in MEN2B mutant mice.

4.12 Adrenal gland morphology in MEN2B mutant mice is partially rescued by deleting parkin

MEN2B mutant mice evince bilateral adrenal gland malformation postnatally ***(Smith-Hicks et al. 2000)***. Having established the synergistic function of parkin and Ret in the DA system using different mouse models, we wanted to test whether parkin and Ret cross-talk could be extended to the periphery beyond the CNS. Thus, the adrenal gland morphology of parkin-deficient MEN2B mice was analyzed.

Hematoxylin staining of transverse sections of adrenal glands from parkin-deficient MEN2B, MEN2B mutant and WT control mice was performed at the age of P14. Adrenal gland morphology in MEN2B mutant mice was bilaterally distorted, confirming the earlier findings ***(Smith-Hicks et al. 2000)***. Moreover, the cortex region is not completely enclosed over the medulla region in MEN2B mutant mice (middle panel), unlike WT control mice (left panel), where the cortex is completely enclosed over the medulla region (Figure 4.12). Although, deletion of parkin only partially rescued the distorted morphology of adrenal glands in MEN2B mutant mice, two out of three parkin-deficient MEN2B mice showed almost complete enclosure of medulla region with the adrenal cortex (right panel) (Figure 4.12) when compared to age-matched MEN2B mutant mice. Together, these findings suggest the possibility that parkin and Ret synergistically function outside the DA system. Nevertheless, further experiments analyzing whether parkin deletion can normalize other MEN2B phenotypes outside DA system are needed before concluding that parkin and Ret cross-talk is a general phenomenon outside the CNS ***(Smith-Hicks et al. 2000)***.

C. Molecular mechanisms underlying parkin and Ret cross-talk

4.13 Parkin governs Ret/MEN2B protein levels in the DA system of mice

Unraveling the molecular mechanisms underlying the synergistic function of parkin and Ret in the DA system could provide clues of how parkin mutations and lack of neurotrophic support can lead to PD. In this context, the first question addressed was, whether parkin can regulate Ret or MEN2B protein levels in the mouse DA system. Ret expression was assessed by immunoblotting using striatal lysates obtained from a 3–6 month-old WT controls, parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice (Figure 4.13 A).

Despite no histological alterations in the DA system ***(Itier et al. 2003; Goldberg et al. 2003)***, a significant 22% loss in the relative levels of Ret was observed in the striatal lysates of parkin-deficient mice compared to age-matched WT controls (Figure 4.13 A & F).

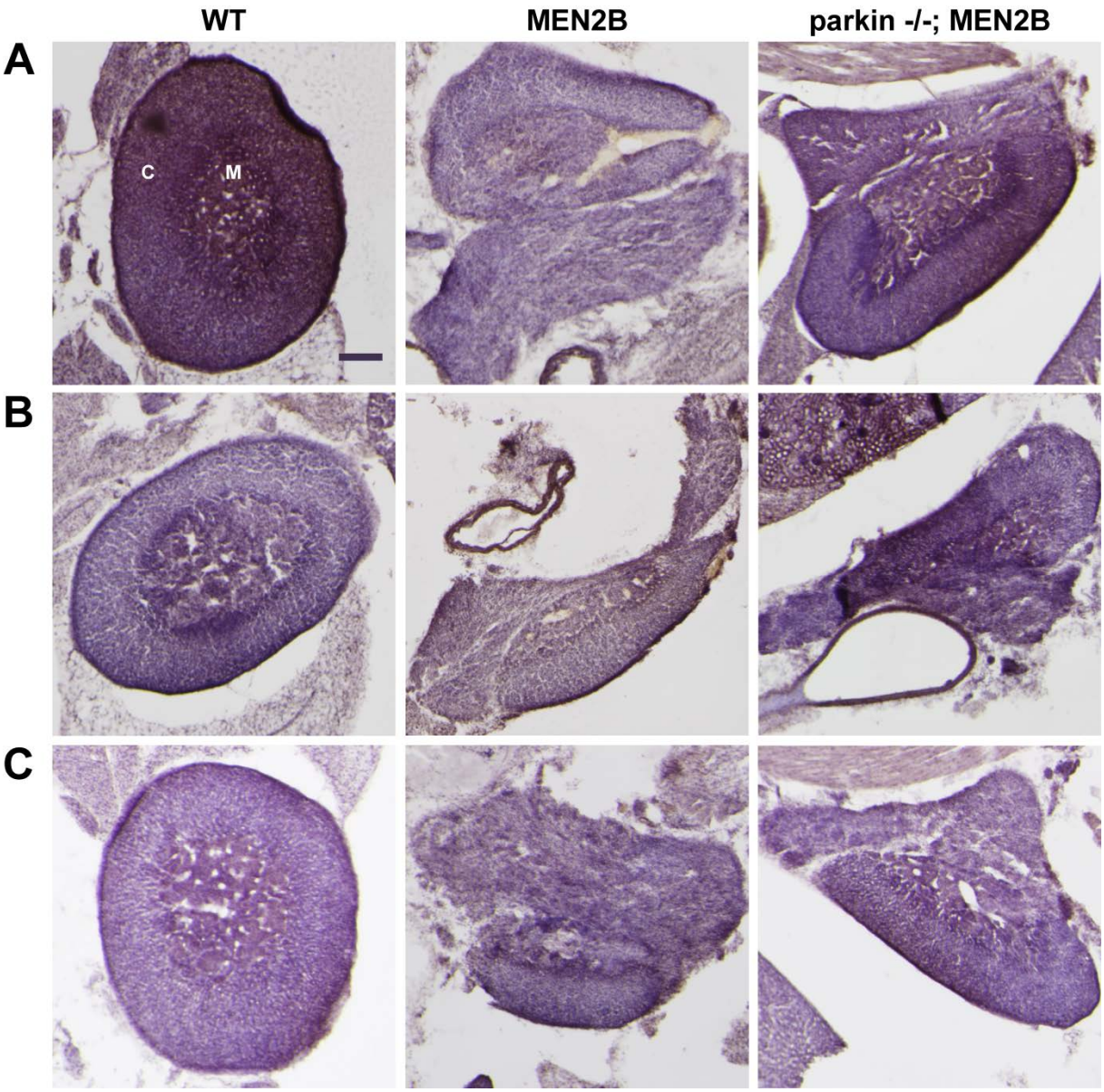


Figure 4.12 Parkin ablation partially rescued MEN2B-mediated anomalies of the adrenal glands in MEN2B mutant mice

Photomicrographs of transversal adrenal gland sections at the age of P14 from WT (control), MEN2B mutant and parkin-/-; MEN2B mice stained with hematoxylin to visualize, morphological changes of the adrenal gland cortex (C) and medulla (M), n=3 mice per genotype (scale bar=200 µm). Panels A, B, C represent sections from different mice of indicated genotypes. Adrenal glands morphology from WT mice shows a clear distinction between the medulla and the cortex region. MEN2B mutant mice display distorted morphology of the adrenal glands and this phenotype is partially rescued when parkin is deleted in MEN2B mutant mice.

MEN2B mutant mice showed a significant 74% increase in total Ret protein levels in the striatal lysates compared to age-matched WT controls. Although parkin-deficient MEN2B mice showed a significant 44% normalization of Ret protein levels compared to MEN2B mutant mice, there was still 30% significant increase in parkin-deficient MEN2B mice compared to WT controls (Figure 4.13 A & B). Alternative splicing of Ret results in three distinct isoforms, of which only Ret9 (short isoform) and Ret51 (long isoform) are well studied ***(De Graaff et al. 2001; Tsui-Pierchala et al. 2002; Richardson et al. 2012)***, both Ret9 and Ret51 isoform levels were assessed in the striatal lysates of the indicated genotypes to gain insight into which of these Ret isoforms are regulated by parkin. A significant 36% increase in Ret51 levels were observed in MEN2B mutant mice compared to WT controls and deletion of parkin showed only a 13% decrease in Ret51 levels in MEN2B mutant mice (Figure 4.13 A & C). A significant 94% increase in Ret9 levels were observed in MEN2B mutant mice compared to WT controls and deletion of parkin showed only a 21% decrease in Ret9 levels in MEN2B mutant mice (Figure 4.13 A & D). However, no significant differences were observed in either Ret51 or Ret9 levels in parkin-deficient mice compared to WT controls (Figure 4.13 A, C & D). As the results from the immunoblot analysis shows, the basal expression of Ret51 is two-fold higher than the Ret9 expression in WT control mice (Figure 4.13 A & G). Conversely, MEN2B mutation lead to nearly one-fold increase Ret9 expression, whereas only 36% increase in Ret51 expression was observed in MEN2B mutant mice compared to the basal levels in WT controls (Figure 4.13 A, C & D). TH expression was shown to be increased in MEN2B mutant mice ***(Mijatovic et al. 2007)***, In agreement with these findings 45% increase in TH expression was observed in MEN2B mutant mice compared to WT controls. However deleting parkin significantly normalized TH expression by 29% in MEN2B mutant mice compared to single MEN2B mutant mice (Figure 4.13 A & E). Parkin protein levels were significantly lower by 44% in MEN2B mutant mice compared to WT controls (Figure 4.13 A & H). Together, all of these results indicate that parkin can regulate Ret protein levels under physiological conditions and deletion of parkin can significantly normalize the increased Ret expression in MEN2B mutant mice.

4.14 No alterations in Ret transcriptional regulators, mitochondrial biogenesis, or autophagic markers in the DA system of MEN2B mutant mice

Nurr1 is a transcriptional regulator of Ret and has been shown to induce Ret expression in the DA system ***(Wallén A et al. 2001; Galleguillos et al. 2010; Volakakis et al. 2015)***. However, no significant alterations in Nurr1 protein levels were observed in the striatal lysates of indicated genotypes (Figure 4.14 A & B) indicating that MEN2B-induced Ret expression could be independent of Nurr1.

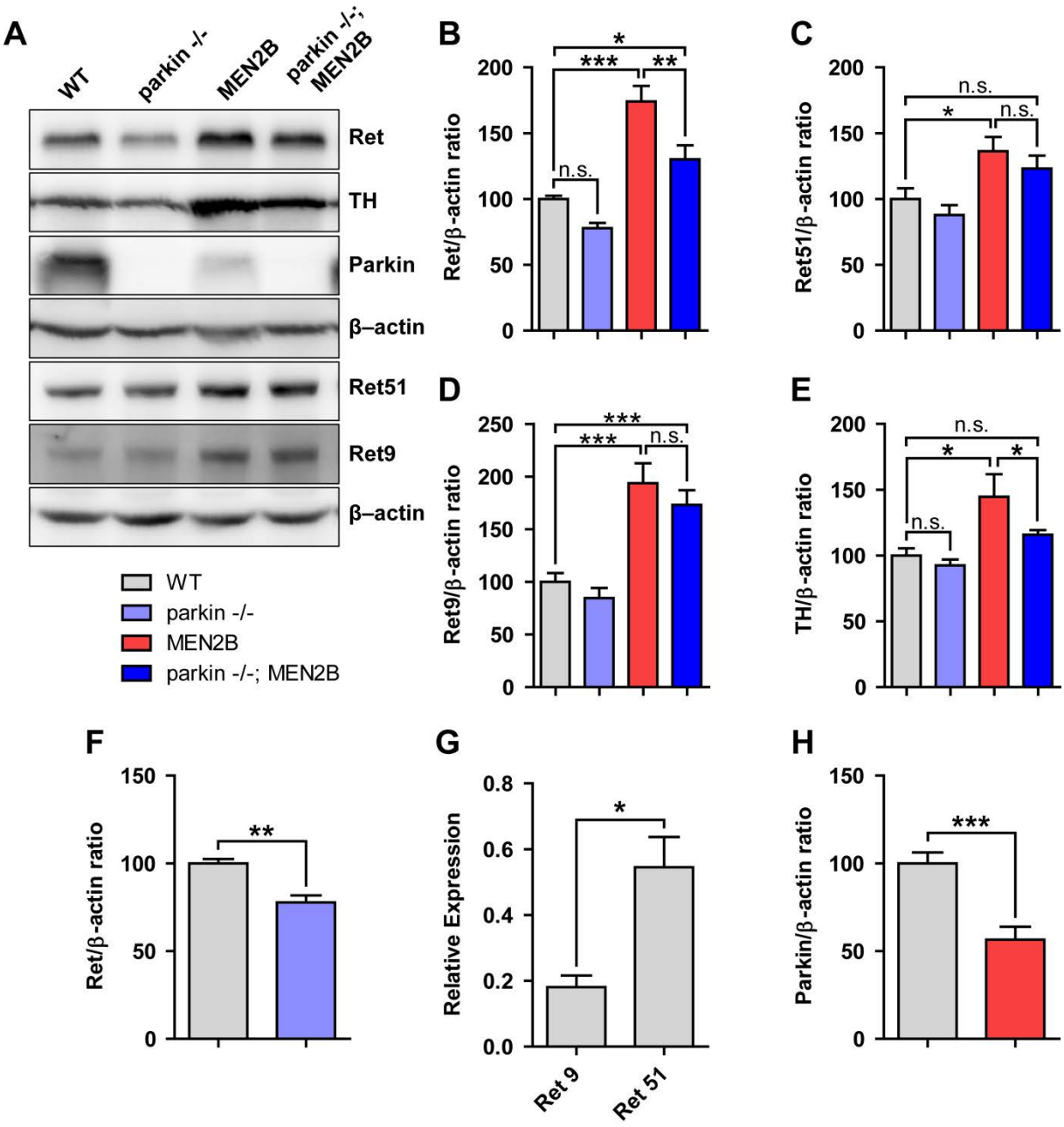


Figure 4.13 Parkin regulates Ret/MEN2B protein levels in the mouse DA system

Immunoblots of (**A**) striatal lysates depicting expression of the indicated proteins from 3–6 month-old WT controls, parkin -/-, MEN2B mutant and parkin -/-; MEN2B mice; Quantification of the relative expression of Ret to β-actin ratio showed a significant 74% increase in Ret protein levels in MEN2B mutant mice and deletion of parkin significantly normalized Ret protein levels by 44% in MEN2B mutant mice, n=5 mice per genotype (**B**); Ret51 to β-actin ratio reveal a significant 36% increase in Ret51 protein levels in MEN2B mutant mice, n=9 mice per genotype (**C**); Ret9 to β-actin ratio reveal a significant 94% increase in Ret9 protein levels in MEN2B mutant mice, n=9 mice per genotype (**D**); TH to β-actin ratio showed a significant 45% increase in TH protein levels in MEN2B mutant mice and deletion of parkin significantly normalized TH protein levels by 29% in MEN2B mutant mice (**E**); Ret to β-actin ratio shows a significant 22% decrease in Ret protein levels in parkin -/- mice compared to WT controls when analyzed separately from graph **B**, n=5 mice per genotype (**F**); Basal expression of

Ret51 to β-actin ratio is 200% higher than Ret9 to β-actin ratio in WT control mice, n=4 mice per genotype (**G**); Parkin to β-actin ratio reveal a significant 44% decrease in MEN2B mutant mice compared to WT controls, n=8–9 mice per genotype (**H**). Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test or 1 way ANOVA with Newman–Keuls post hoc test.

However, further analysis of Nurr1 transcriptional activity might provide more insights in this context. Mitofilin, a subunit of the mitochondrial inner membrane protein localized to cristae junctions ***(Zerbes et al. 2012; Jans et al. 2013; Pfanner et al. 2014)*** was also unaltered in the striatal lysates of indicated genotypes (Figure 4.14 A & C). Levels of Peroxisome proliferator-activated receptor γ coactivator protein–1α (PGC–1α), a well-known master regulator of mitochondrial biogenesis and is implicated in PD ***(Fernandez-Marcos & Auwerx 2011; Pacelli et al. 2015; Jiang et al. 2016)***, were not significantly altered in the striatal lysates of the indicated genotypes (Figure 4.14 A & D). Further, in concurrence with the previous findings (Figure 4.4), no significant differences in LC3I, LC3II and p62 levels were observed in the striatal lysates of parkin-deficient, MEN2B mutant and parkin-deficient MEN2B mice compared to WT controls indicating that autophagy is unaffected in these mice (Figure 4.14 A & E–G).

4.15 Physical interaction of parkin and Ret in the DA system of mice

Parkin's ability to positively regulate Ret/MEN2B protein levels emphasizes how deletion of parkin normalizes MEN2B phenotype in the DA system of mice. Recent studies in this direction report that parkin could regulate the trafficking and signaling of a receptor tyrosine kinase (RTK), EGFR through interaction and ubiquitination of its adaptor protein EPS15 that trigger endocytosis of EGFR ***(Fallon et al. 2006)***. Having seen the tight genetic cross-talk between parkin and Ret in different mouse models, further investigations were done to analyze whether parkin could also interact and regulate the trafficking and signaling of Ret.

As a preliminary experiment, immunoprecipitation (IP) of Ret was carried out using commercially available antibodies along with a lab-made polyclonal and monoclonal antibodies against Ret (Figure 4.15.1). For this experiment SK-N-BE(2) cultures were used as expression of Ret is highly abundant in this cell line. Efficient Ret IP was observed only with the lab-made monoclonal antibody and partial Ret IP was observed with lab-made polyclonal antibody. Parkin was co-immunoprecipitated (Co-IP) with lab-made Ret monoclonal antibody and partially with lab-made Ret polyclonal antibody (Figure 4.15.1). After finding the evidence for parkin and Ret interaction in the SK-N-BE(2) cultures, these findings were confirmed *in vivo*. Striatal punches were obtained from the 3–6 month-old WT control mice. IP of parkin from the striatal lysates of WT control mice confirmed the

interaction of parkin and Ret (Figure 4.15.2 A), indicating that parkin could regulate Ret endocytosis in the DA system of mice.

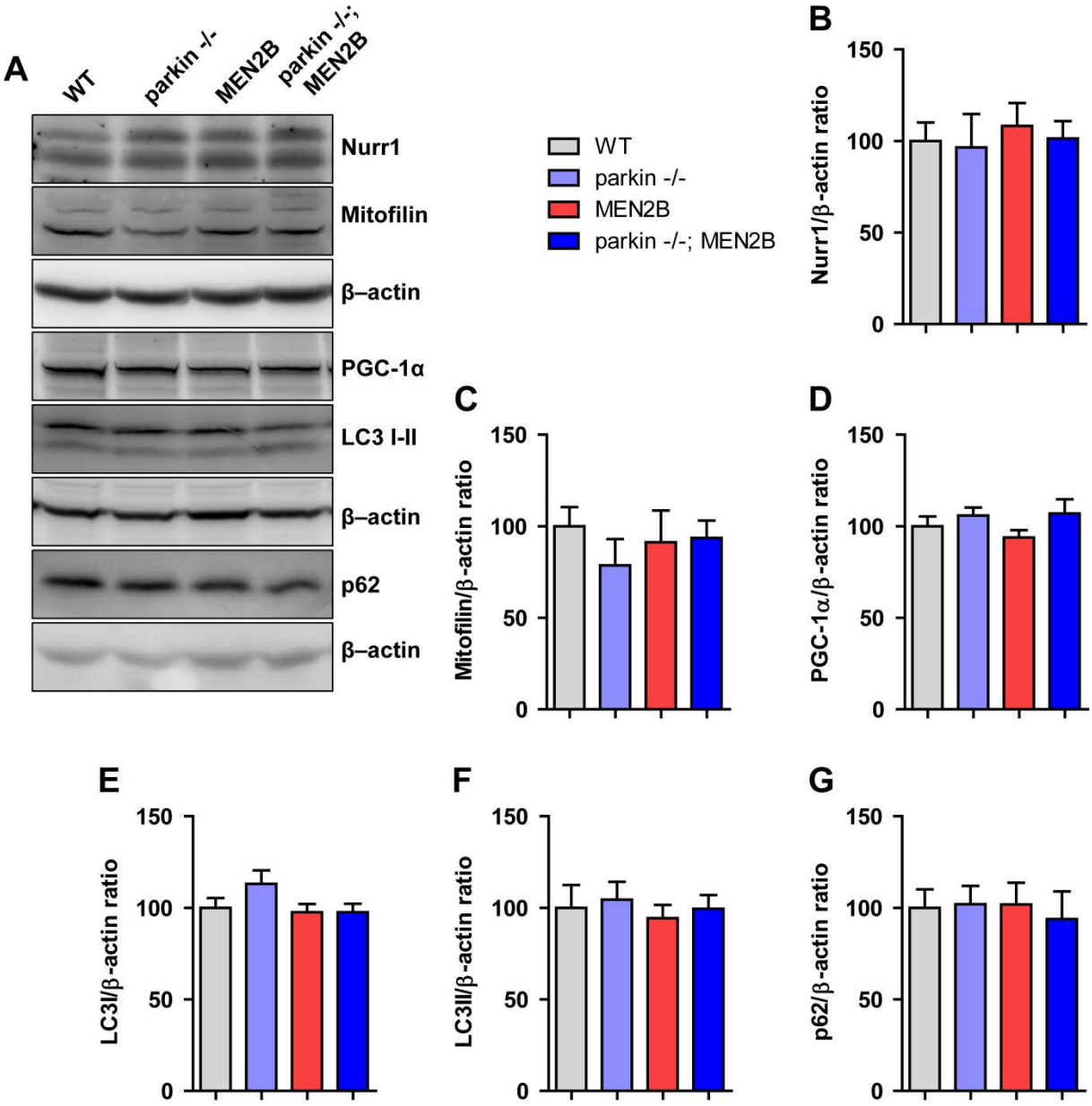


Figure 4.14 Markers for mitochondrial biogenesis and autophagy are not altered in MEN2B mutant mice

Immunoblots of (**A**) striatal lysates depicting expression of the indicated proteins from 3–6 month-old WT controls, parkin -/-, MEN2B mutant and parkin -/-; MEN2B mice, n=5 mice per genotype; Quantification of the relative expression of Nurr1 to β-actin ratio (**B**); Mitofilin to β-actin ratio (**C**); PGC–1α to β-actin ratio (**D**); LC3I to β-actin ratio (**E**); LC3II to β-actin ratio (**F**); p62 to β-actin ratio (**G**) revealed no significant alterations among the genotypes. Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test or 1 way ANOVA with Newman–Keuls post hoc test.

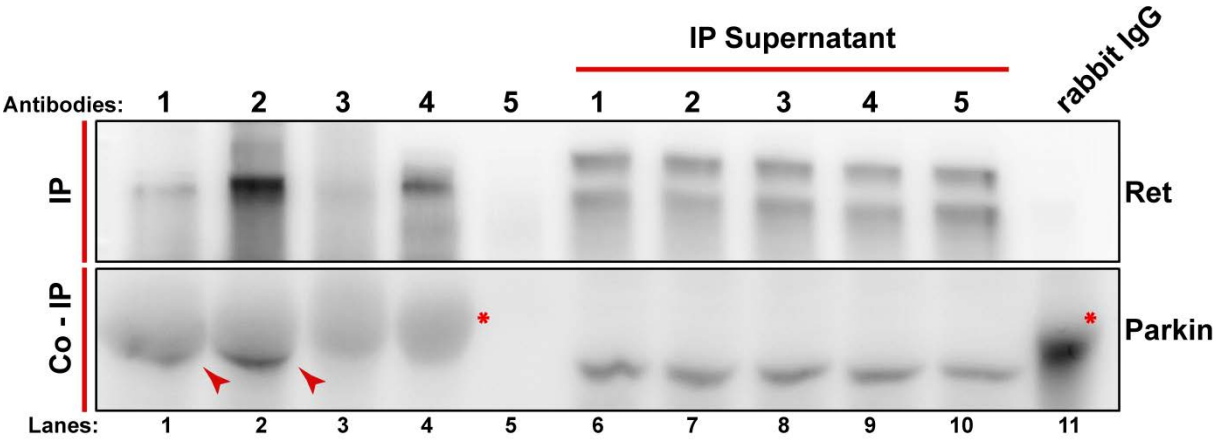


Figure 4.15.1 Ret and parkin interaction in SK-N-BE(2) cells

Immunoblots depicting Ret and parkin interaction; Immunoprecipitation of Ret (IP blot) was done using lab-made Ret polyclonal (1); lab-made Ret monoclonal (2); Ret (H300) polyclonal (3); Ret (C20) polyclonal (4); Ret (C31B4) monoclonal (5) antibodies from SK-N-BE(2) cultures and were shown on the left side of the blot (lanes 1–5); Co-immunoprecipitation of parkin (Co-IP blot) (arrow heads) was only observed in lane 2 and partially in lane 1 where Ret is efficiently immunoprecipitated. Supernatants from the immunoprecipitated samples were also loaded to ensure equal amount of protein in all lanes and shown on the right side of the blot (lanes 6–10) along with a rabbit IgG negative control as depicted in lane 11. IgG long chain on the blots is indicated by red-asterisk (*).

To analyze if this interaction might be a Ret-isoform specific interaction, blots were probed with isoform specific antibodies for Ret51 and Ret9. Since basal levels of the long isoform of Ret are higher in the DA system (Figure 4.13 A & G), Ret51 interaction was confirmed. Due to the low abundancy of the short isoform Ret9 interaction with parkin was very weak and not conclusive, (Figure 4.15.2 A). These findings were further confirmed by Co-IP of parkin with Ret from the striatal lysates of WT control mice (Figure 4.15.2 B). Together, these findings indicate that parkin could regulate the internalization and signaling of Ret in the DA system of mice.

4.16 Nuclear translocation of Ret

RTKs are shown to be trafficking from the cell surface to the nucleus mostly in response to ligand binding. Either an intact receptor is translocated to the nucleus or an intracellular domain fragment of the receptor is generated at the cell surface ***(Lemmon & Schlessinger 2010; Carpenter & Liao 2013)***. To get an insight into trafficking of Ret from the cell surface to the nucleus, sub-cellular fractionation was carried out using SK-N-BE(2) cultures with a commercially available subcellular protein fractionation kit. Four subcellular compartments namely cytosolic, membrane, nuclear soluble and nuclear insoluble or chromatin-bound enriched fractions were obtained and the fractions along with a whole cell lysate were

immunoblotted and probed for the Ret protein using a lab-made monoclonal antibody generated against the extracellular domain (amino terminal) of Ret (Figure 4.16.1 A).

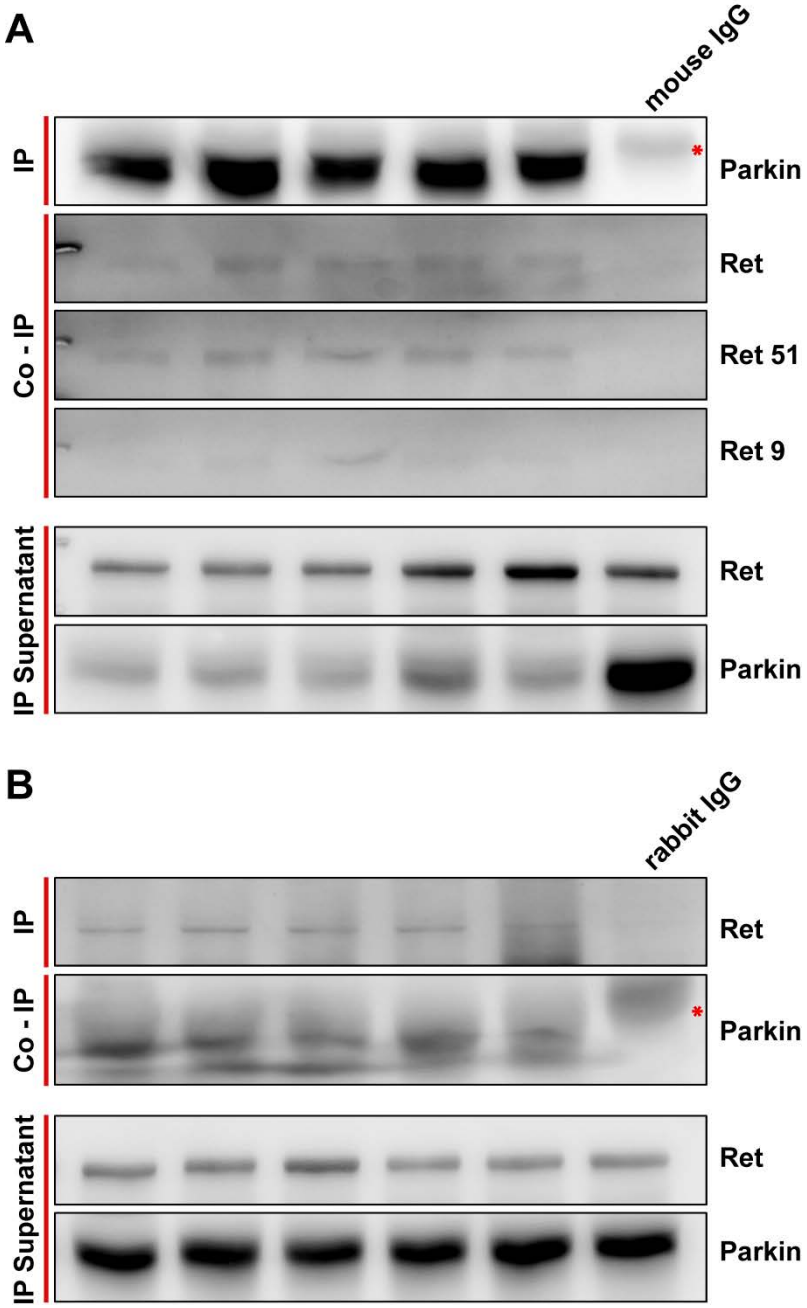


Figure 4.15.2 Parkin and Ret interaction in the mouse DA system

Immunoblots depicting Ret and parkin interaction from the striatal lysates of 3–6 month-old WT controls, n=6 mice (**A–B**); Immunoprecipitation of parkin (IP blot) along with mouse IgG control confirming the co-immunoprecipitation of Ret along with its long isoform Ret51 and a very weak interaction with its short isoform Ret9 (Co-IP blots). Supernatants from the immunoprecipitated samples were also loaded to ensure equal amount of protein in all lanes **(A)**; Immunoprecipitation of Ret (IP blot) along with rabbit IgG control confirming the co-immunoprecipitation of parkin (Co-IP blot). Supernatants from the immunoprecipitated samples were also loaded to ensure equal amount of protein in all lanes (**B**); IgG long chain on the blots is indicated by red-asterisk (*).

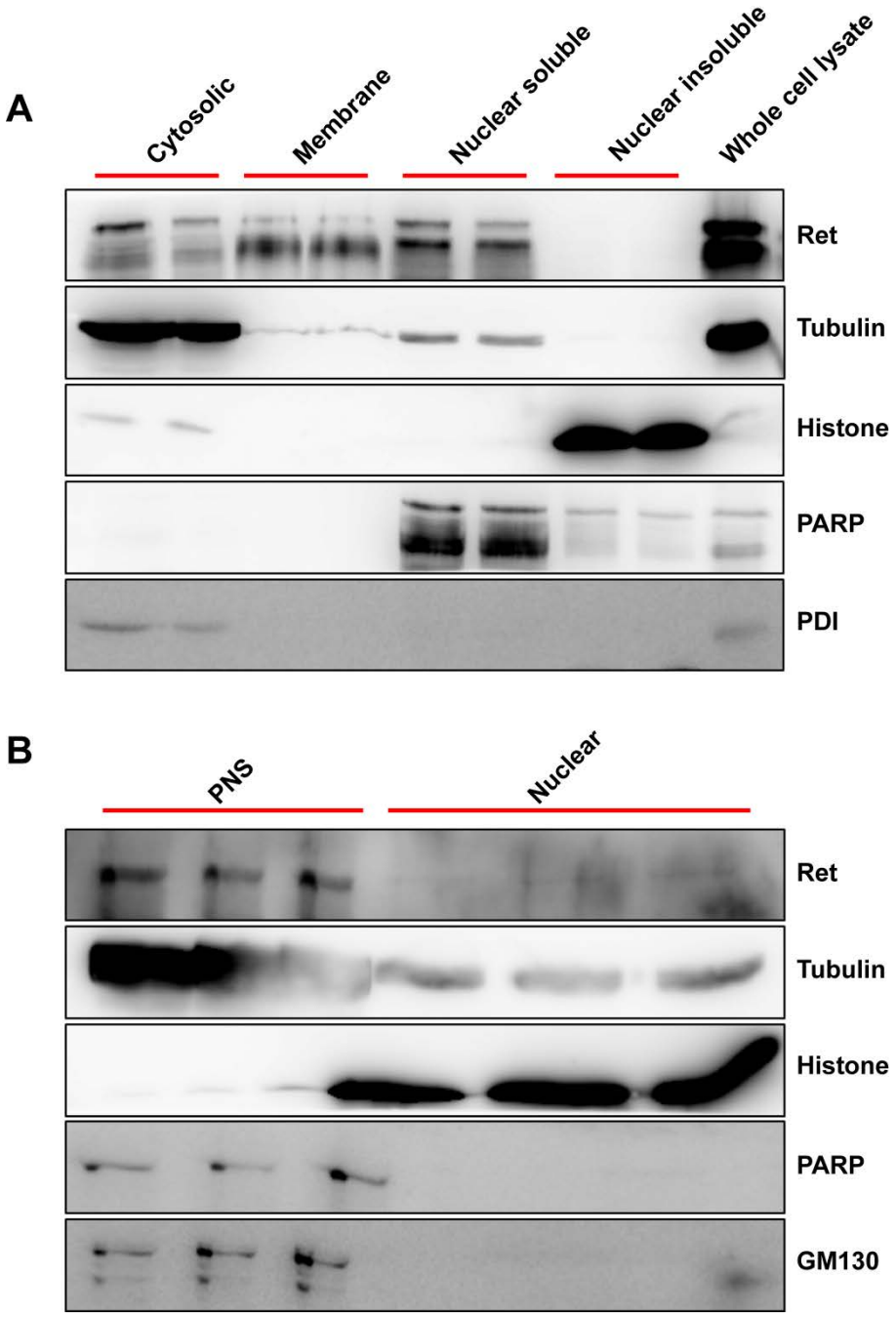


Figure 4.16.1 Ret nuclear localization in the DA system of mice

Immunoblots depicting (**A–B**) Ret nuclear localization in SK-N-BE(2) cultures (**A**); and Ret nuclear presence in 3–6 month-old mouse ventral midbrain tissue, n=3 (**B**); Various sub-cellular compartmental markers ensuring the fractionation purity-Tubulin for cytosol; Histone for nuclear insoluble or chromatin bound; PARP for nuclear fraction; PDI for endoplasmic reticulum; GM130 for Golgi apparatus. PNS= post-nuclear supernatant in this figure.

Immunoreactivity for Ret was observed in all the fractions (cytosolic, membrane and nuclear soluble) except in the nuclear insoluble or chromatin-bound fractions (Figure 4.16.1 A). To confirm the nuclear localization of Ret *in vivo* in mice, post-nuclear supernatant and nuclear fractions were extracted separately from the ventral midbrain of 3–6 month-old WT control

mice and the fractions were subjected to immunoblotting and further probed for Ret (Figure 4.16.1 B). Ret immunoreactivity was observed in both post-nuclear supernatant and in the nuclear fractions (Figure 4.16.1 B). Though these findings suggested that Ret is translocated into the nucleus like other RTKs, further investigations were done to know if this nuclear localization is ligand mediated. Specifically, the classical cell-surface biotinylation approach was adapted to elucidate if Ret nuclear localization is ligand-mediated or ligand-independent. SK-N-BE(2) cultures were surface biotinylated using a water soluble EZ-link Sulfo NHS-LC-Biotin and glial cell-derived neurotrophic factor (GDNF) stimulation was carried out at 0, 10, 30, 60 min and then the cultures were harvested. Following subcellular fractionation, biotinylated proteins were pulled down using streptavidin M-280 Dynabeads® and subjected to immunoblotting.

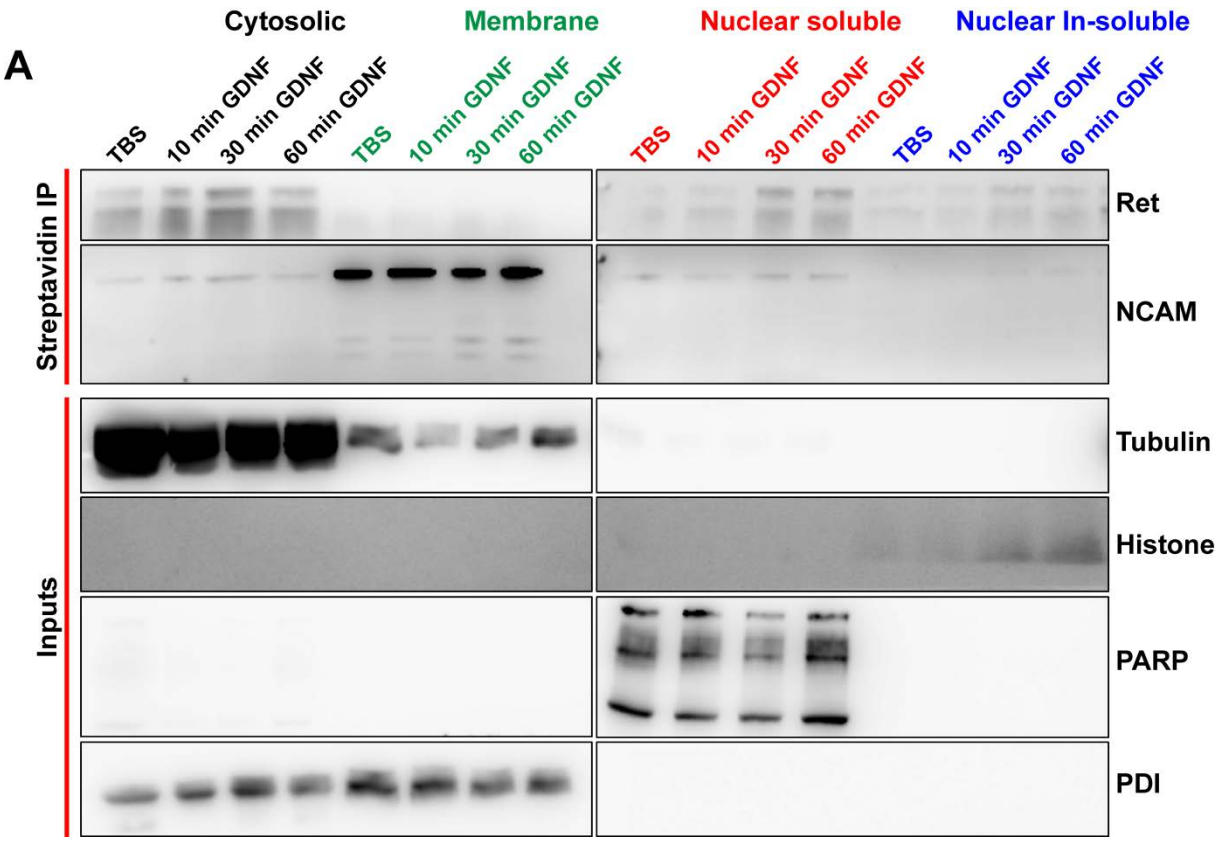


Figure 4.16.2 Ligand-mediated Ret nuclear localization

Immunoblots depicting Ret and NCAM nuclear localization in SK-N-BE(2) cultures in a ligand-dependent manner; Various sub-cellular compartmental markers ensure the fractionation purity: Tubulin for cytosol; Histone for nuclear in-soluble or chromatin bound; PARP for nuclear fraction; PDI for endoplasmic reticulum.

Fraction purity was ensured by subjecting the Inputs from the subcellular fractionation to immunoblotting, probing for various subcellular compartmental markers. Tubulin was enriched in cytosolic fraction, histone was enriched in the chromatin-bound or nuclear-

insoluble fraction and poly (ADP-ribose) polymerase (PARP) was enriched in nuclear soluble compartment for cultures (SK-N-BE(2)) and the post-nuclear supernatant in the mouse tissue. Protein disulfide isomerase (PDI) is enriched in the cytosolic and membrane fractions and GM130 is enriched in the post-nuclear supernatant in the mouse tissue (Figures 4.16.1 & 4.16.2). Nuclear localization of Ret was observed at all time-points in the nuclear soluble fraction as well as in the nuclear insoluble fraction, increasing over time (Figure 4.16.2). As a control, NCAM, which is known to translocate into the nucleus ***(Kleene et al. 2010)*** was monitored in the nuclear soluble and nuclear in-soluble fraction of SK-N-BE(2) cultures and an increase in nuclear translocation of NCAM was noticed over time (Figure 4.16.2). However, further validation is needed to confirm the quantitative results as the observations were from one experiment.

4.17 Parkin regulates Ret/MEN2B protein levels in cultured cells

Parkin-deficiency leads to a decrease of Ret protein levels in the DA system of mice (Figure 4.13 A, B & F). To gain mechanistic insight into the cross-talk of parkin and Ret, an *in vitro* model system was adapted. SH-SY5Y cultures were used due to the advantage that both parkin and Ret are endogenously expressed. In order to elucidate that parkin regulates Ret protein levels in these cultures and also to recapitulate the findings from the mouse models (Figure 4.13 A, B & F), SH-SY5Y cultures were transiently transfected with either a control siRNA or siRNA specific to parkin. After incubation for 48 hr the efficiency of parkin-KD was assessed using immunoblotting. Parkin protein levels were significantly reduced by 60% in the parkin siRNA-transfected lysates compared to control siRNA-transfected lysates (Figure 4.17 A & D). Ret protein levels were significantly decreased by 52% in the parkin-KD lysates (Figure 4.17 A & B), thus confirming the data from the mouse models (Figure 4.13 A, B & F). In contrast to the in vivo data (Figure 4.13 A & C), Ret51 levels were significantly lower by 41% in the parkin-KD SH-SY5Y lysates (Figure 4.17 A & C) and reminiscent to mouse models, Ret9 short isoform basal levels were much lower than Ret51 and were actually below the detection limit in the SH-SY5Y lysates. A strong correlation was observed between the amount of parkin in the parkin-KD lysates and the loss of Ret protein (Figure 4.17 A, B, D & E). MZCRC1, a cell line endogenously expressing MEN2B ***(Cooley et al. 1995; Zhu et al. 2010; Bagheri-Yarmand et al. 2015)*** were transiently transfected with either control siRNA or parkin siRNA and MEN2B protein levels were assessed 48 hr post transfection. As observed in the mice, where parkin deletion normalizes the MEN2B/Ret protein levels in MEN2B mutants (Figure 4.13 A–B), parkin-KD in MZCRC1 cells resulted in a significant 18% decrease in MEN2B protein levels compared to control siRNA-transfected cultures (Figure 4.17 F–G). Together, these findings indicate that the presence of parkin stabilizes Ret/MEN2B protein levels both *in vivo* and *in vitro*.

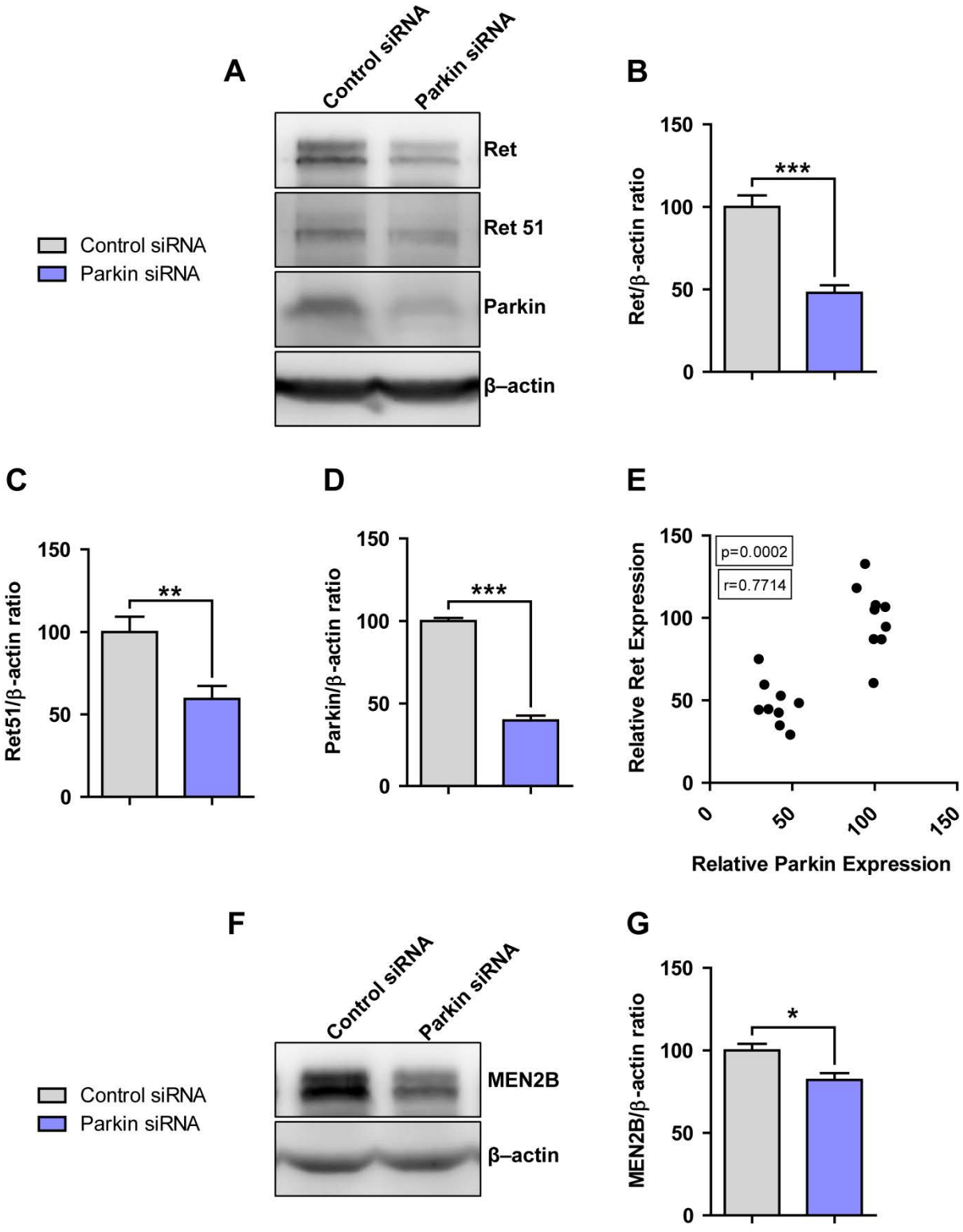


Figure 4.17 Parkin-KD induced Ret/MEN2B protein loss in cultured cells

SH-SY5Y or MZCRC1 cultures were transiently transfected with either control-siRNA or siRNA-specific to parkin and the lysates were subjected to immunoblotting to assess the depicted proteins (**A–G**); Representative immunoblots illustrating various proteins (**A**); Quantification of the relative expression of Ret to β-actin ratio showed a significant 52% decrease due to parkin-KD, n=9 (**B**); Ret51 to β-actin ratio reveal a significant 41% decrease due to parkin-KD, n=6 (**C**); Parkin to β-actin ratio show a significant 60% parkin siRNA induced knock down compared to control siRNA, n=9 (**D**); Strong

positive correlation between the amount of parkin-KD and the amount Ret protein loss with a p value 0.0002 and a Pearson r=0.7714 (**E**); western blots from MZCRC1 lysates (**F**); MEN2B to β-actin ratio showing a significant 18% decrease of MEN2B protein levels mediated by parkin-KD, n=6 (**G**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test.

4.18 Pathogenic parkin mutants fail to regulate Ret protein levels in cultured cells

Mutations in parkin leading to loss of function are known to be responsible for autosomal recessive PD ***(Lubbe & Morris 2014)***. Considering the ability of parkin to regulate Ret protein levels, it is important to analyze the ability of pathogenic parkin mutants to regulate Ret/MEN2B protein levels. To this end, HeLa cultures were used since both parkin and Ret/MEN2B are not expressed endogenously. HeLa cultures were transiently transfected with either MEN2B in combination with mCherry as control or MEN2B in combination with WT human parkin or parkin mutant variants (Parkin–Q311Stop, G430D, R42P, W453Stop, Δ1–79). All parkin mutant variants used here were well characterized previously by Konstanze F. Winklhofer's group ***(Henn et al. 2005)***. Co-transfected cultures were incubated for 48 hr and the lysates were then subjected to immunoblotting and Ret/MEN2B expression levels were assessed. An increase of 26% in Ret/MEN2B protein levels was observed in the presence of WT human parkin compared to the control condition, whereas no consistent increase was observed in Ret/MEN2B protein levels in the presence of various parkin mutants (Q311Stop, G430D, R42P, W453Stop, Δ1–79) respectively (Figure 4.18 A–B). Due to the marginal increase in Ret/MEN2B protein levels in the presence of WT human parkin, no significant alterations were observed, when all the conditions were analyzed together using 1 way ANOVA (Figure 4.18 A–B). However, a significant increase was only observed in Ret/MEN2B protein levels, when control condition and MEN2B co-expressed with WT human parkin condition was analyzed separately using Student-t test (Figure 4.18 A & C). No such significance was observed in Ret/MEN2B protein levels, when MEN2B was co-expressed with various parkin mutants and are analyzed independently with control condition (Figure 4.18 A & D–H).

These findings further support the ability of parkin to positively regulate Ret/MEN2B protein levels and emphasize the importance of parkin's physiological function as the pathogenic parkin mutants were not able to do so (Figure 4.18).

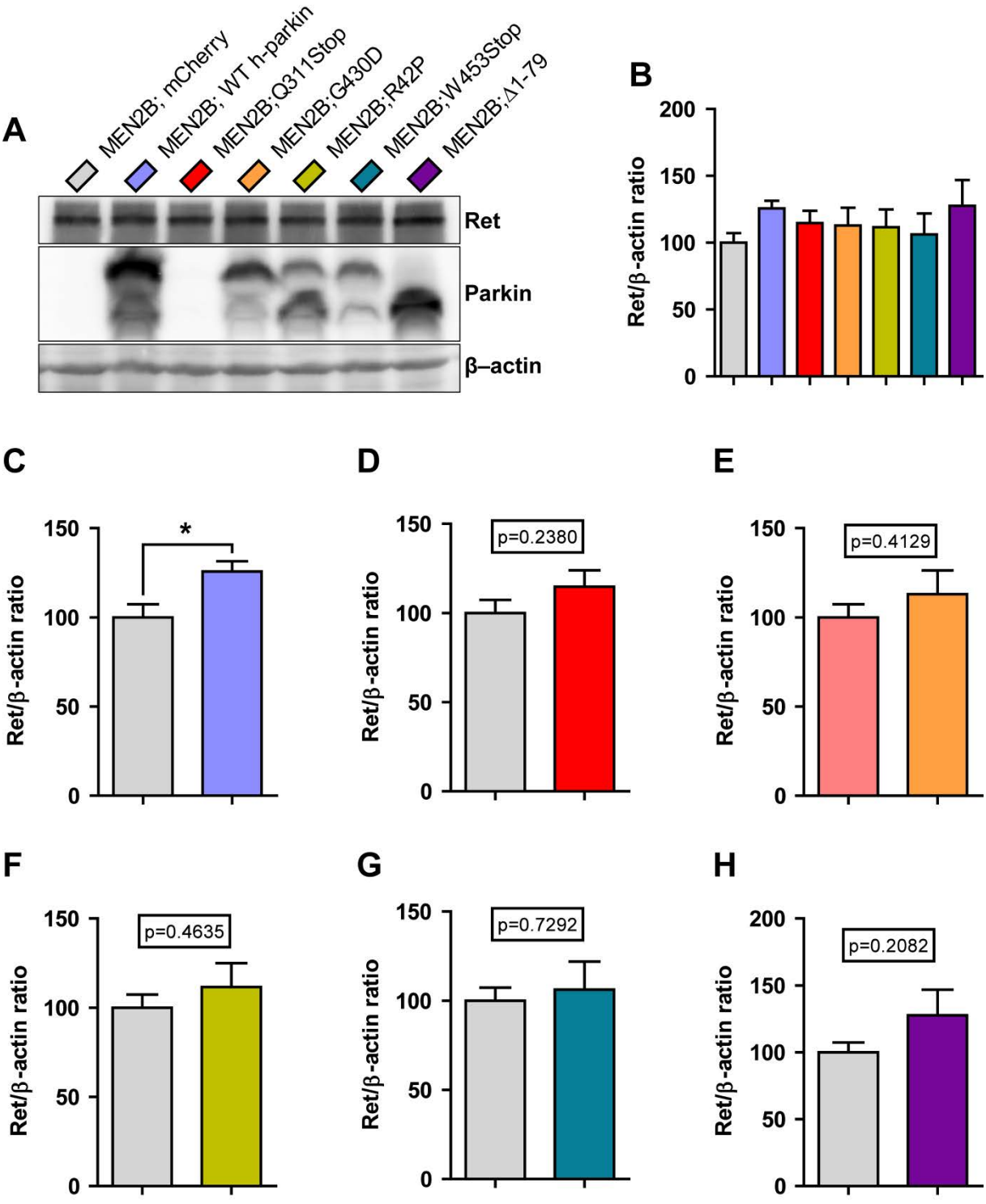


Figure 4.18 Physiological function of parkin is important to regulate Ret/MEN2B protein levels

HeLa cultures were transiently co-transfected with either MEN2B with mCherry as control or MEN2B with WT human parkin or MEN2B with parkin mutant variants and the lysates were immunoblotted to assess Ret/MEN2B protein levels, n=6 (**A–H**); Representative Immunoblots depicting various proteins (**A**); Quantification of the relative expression of Ret to β-actin ratio reveal a consistent but not significant increase in Ret/MEN2B protein levels in the presence of WT human parkin, whereas no such increase was observed in the presence of various parkin mutants (**B**); Ret to β-actin ratio reveal a significant 26% increase in the presence of WT human parkin (**C**); Ret to β-actin ratio was not

altered in the presence of parkin mutant (Q311Stop) (**D**); Ret to β-actin ratio shows no significant differences in the presence of parkin mutant (G430D) (**E**); Ret to β-actin ratio reveals no significant differences in the presence of parkin mutant (R42P) (**F**); Ret to β-actin ratio shows no significant alterations in the presence of parkin mutant (W453Stop) (**G**); Ret to β-actin ratio reveals no significant alterations in the presence of parkin mutant (Δ1–79) (**H**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test or 1 way ANOVA with Newman–Keuls post hoc test.

4.19 Ret degradation is not regulated by parkin

Findings from both *in vivo* (Figure 4.13 A, B & F) and *in vitro* (Figure 17; 18 A, C) analyses corroborate the fact that parkin could regulate Ret protein levels. However there are at least three possible mechanisms how parkin could be regulating Ret protein levels, transcriptionally, translationally or during the degradation of Ret. It has already been reported for a similar RTK, EGFR, that parkin delays its internalization, and thereby prolonging the EGFR mediated downstream signaling and ultimately reducing its turnover rate ***(Fallon et al. 2006)***. To see whether parkin might use a similar mechanism to affect Ret protein levels, the impact of parkin on Ret protein turnover was analyzed.

Parkin-mediated Ret degradation was assessed by treating SH-SY5Y cultures with cycloheximide (CHX) at various time-points to block translation of new Ret protein and Ret decay levels was measured in the presence or absence of parkin. This was achieved by transiently transfecting SH-SY5Y cultures with control or parkin-specific siRNA and the cultures were incubated for 48 hr before harvesting. Transfected cultures were then treated with CHX at various time intervals 0, 3 and 6 hr before harvesting at 48 hr after transfection (Figure 4.19.1 A) and the lysates were subjected to immunoblotting. CHX induced blockage of translation was evident from the decline in Ret protein levels over 0, 3 and 6 hr time-points in both control and parkin siRNA-transfected lysates (Figure 4.19.1 B–C). Ret protein levels, assessed from the lysates revealed a significant 38% parkin induced loss of Ret protein levels at 0 hr (CHX) time-point in parkin siRNA-transfected lysates compared to control siRNA-transfected lysates (Figure 4.19.1 B–C). At 3 hr (CHX) time-point Ret protein levels decreased drastically by 68% due to CHX treatment in the control siRNA-transfected lysates compared to control at 0 hr (CHX) time-point. However there was only an additional 10% Ret protein loss in parkin siRNA-transfected lysates at 3 hr (CHX) time-point compared to same 3 hr (CHX) control time-point (Figure 4.19.1 B–C).At 6 hr (CHX) time-point Ret protein levels further decreased drastically by 85% due to CHX treatment in the control siRNA-transfected lysates compared to control at 0 hr (CHX) time-point. However, there was only an additional 6% Ret protein loss in parkin siRNA-transfected lysates at 6 hr (CHX) time-point compared to same 6 hr (CHX) control time-point (Figure 4.19.1 B–C).

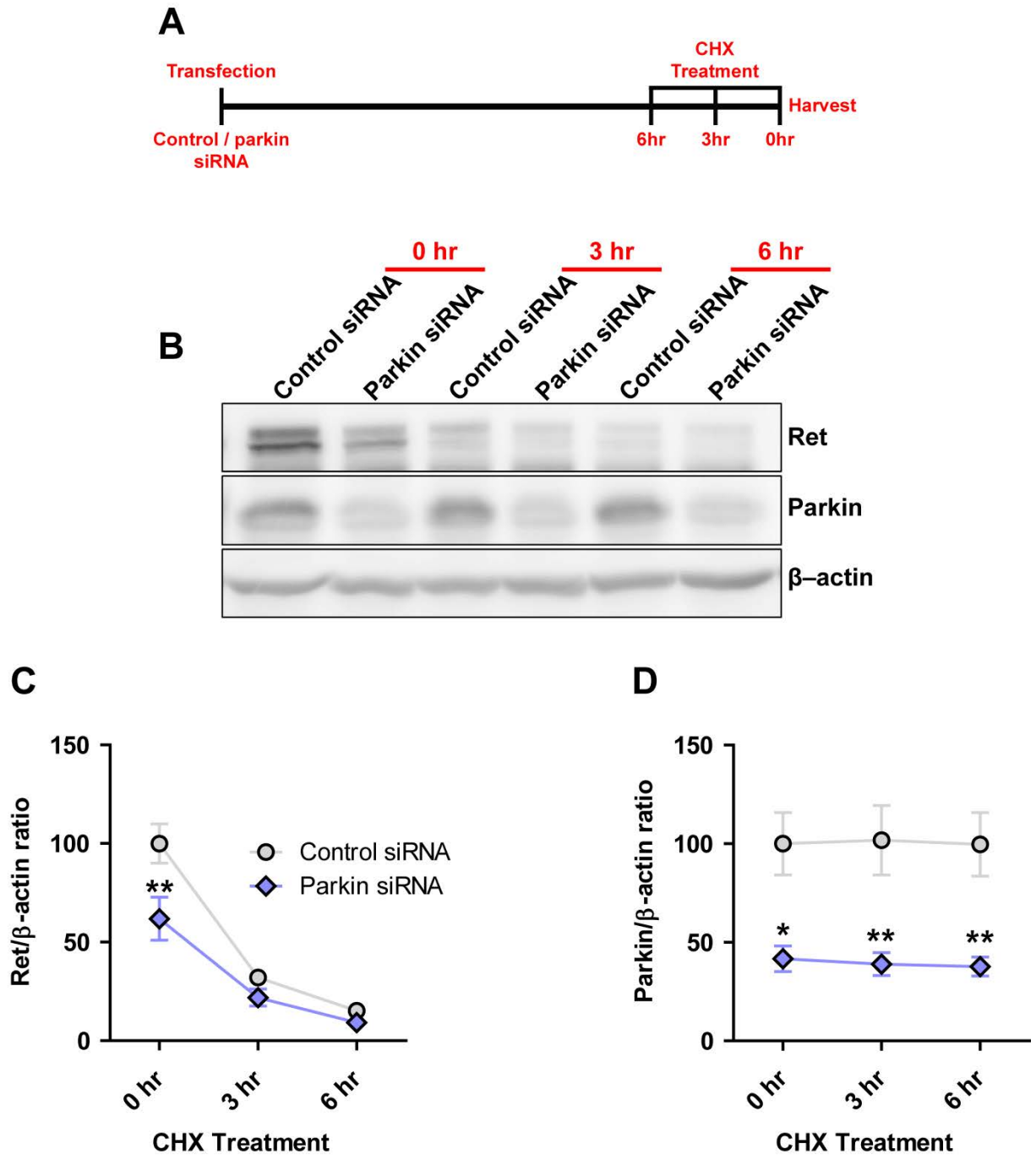


Figure 4.19.1 Ret-degradation is not affected by parkin

SH-SY5Y cultures were transiently transfected with either control or siRNA specific to parkin and treated with CHX (100 mg/ml) for the described time-period to assess Ret decay in presence or absence of parkin, n=4 (**A–D**); Scheme representing experimental paradigm (**A**); Representative Immunoblots illustrating various proteins (**B**); Quantification of the relative expression of Ret to β-actin ratio revealed a profound decline in Ret protein levels over the depicted time-points due to CHX treatment in both the groups. However no significant alterations were observed in the Ret decay due to parkin loss other than 0 hr CHX time-point (**C**); Parkin to β-actin ratio shows a significant 58% KD efficiency, however CHX has no effect on parkin protein levels (**D**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 2 way ANOVA with Bonferroni post hoc test.

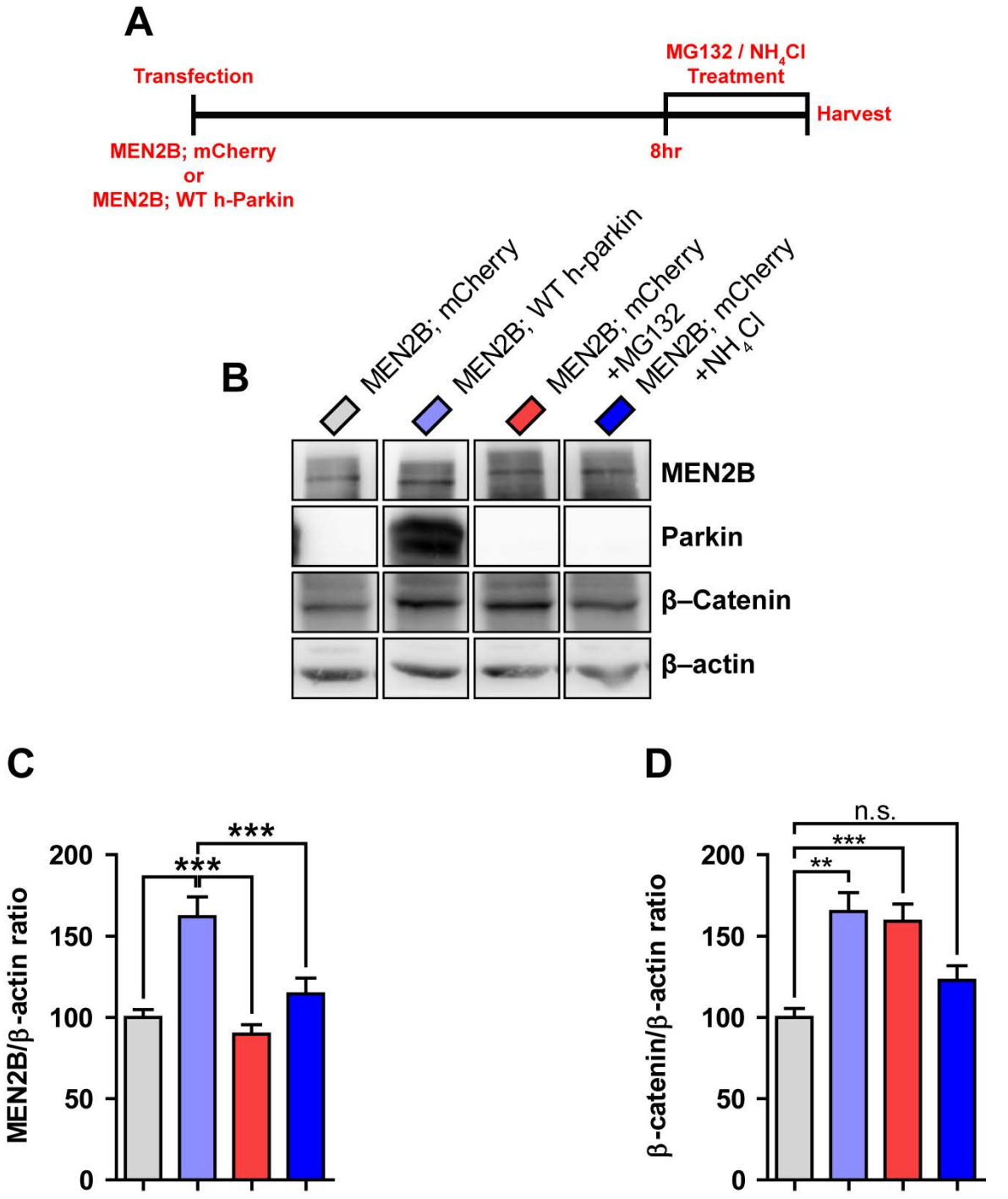


Figure 4.19.2 MEN2B-degradation is not affected by parkin

HeLa cultures were transiently co-expressed with MEN2B in combination with parkin or mCherry and treated with either MG132 (10 µM) or NH_4Cl (10 mM) for 8 hr prior to harvesting, n=9 (**A–D**); Scheme representing experimental paradigm (**A**); Representative Immunoblots illustrating various proteins (**B**); Quantification of the relative expression of MEN2B to β-actin ratio revealed a significant increase in the presence of parkin and no alterations observed when treated with MG132 or NH_4Cl (**C**); β-Catenin to β-actin ratio showed a significant increase in response to MG132 and parkin (**D**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

Parkin siRNA led to a significant decrease of parkin protein levels by 58%, but CHX treatment revealed no significant effect on parkin protein levels suggesting that parkin is a more stable protein as compared to Ret (Figure 4.19.1 B & D). These findings suggest that despite CHX induced decline of Ret protein levels no significant alterations were observed in the decay of Ret protein levels in the parkin siRNA conditions compared to control siRNA conditions suggesting that Ret degradation is not regulated by parkin (Figure 4.19.1).

To further corroborate these findings with earlier findings (Figure 4.18 A & C) that parkin could positively regulate MEN2B protein levels, MEN2B was transiently co-expressed with either mCherry or parkin in HeLa cultures as these proteins were not expressed endogenously in these cultures. The transfected cultures were further incubated for 48 hr post-transfection for the transgene expression and 8 hr prior to harvesting, transfected cultures were treated with either a proteasome blocker (MG132) or lysosome blocker (ammonium chloride) (Figure 4.19.2 A) and the lysates were subjected to immunoblotting. MEN2B protein levels were assessed from the lysates and there was a significant increase in MEN2B protein levels in the presence of parkin compared to MEN2B with mCherry as control. However, no significant alterations were observed in the MEN2B with mCherry groups when treated with either MG132 or ammonium chloride to block the proteasome or lysosome, respectively (Figure 4.19.2 B–C). Efficiency of proteasome blockage was assessed based on the β-catenin accumulation ***(Ahmad et al. 2014)*** (Figure 4.19.2 B & D). Taken together, these findings indicate that Ret/MEN2B protein levels were not regulated by parkin at the degradation level.

4.20 Ret is not transcriptionally regulated by parkin

Since parkin did not seem to regulate Ret degradation, transcriptional control of Ret by parkin was then assessed. Parkin is known for transcriptionally regulating genes that are involved in mitochondrial biogenesis through E3 ubiquitin ligase activity ***(Castillo-Quan 2011; Shin et al. 2011)***. However, recently it was shown that Ret is not transcriptionally regulated by parkin and the data shown here is adapted from Meka et al 2015, in order to support the findings in the current study (Figure 4.20 A).

This particular experiment was done in Prof. Konstanze Winklhofer's lab to analyze the siRNA-mediated KD effect of Ret, parkin or Ret and parkin together on total ATP levels in SH-SY5Y cultures ***(Meka et al. 2015)***. Altogether these findings indicate that parkin does not control the transcription or degradation of Ret and the possible alternative could be that Ret is translationally regulated by parkin.

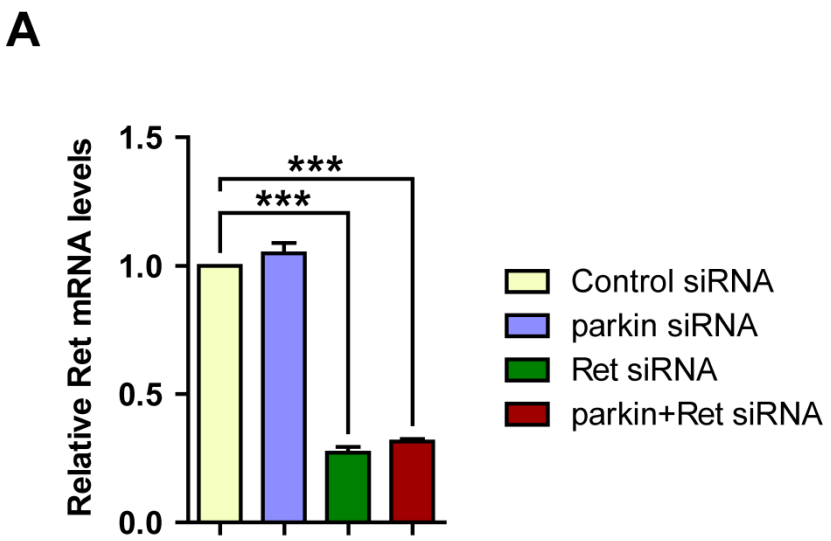


Figure 4.20 Ret transcription is not controlled by parkin

Quantification of the relative mRNA levels in SH-SY5Y cells transiently transfected with Ret-siRNA or parkin-siRNA or both and Ret mRNA levels were assessed (**A**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

4.21 GDNF–Ret signaling is compromised without parkin

Ret has roles in cell growth, differentiation and survival of several tissues and cell types ***(Kodama et al. 2005; Mulligan 2014)***. Ligand-induced activation of Ret is well known to mediate downstream activation of PI3K/Akt and MAP Kinase/ERK signaling pathways ***(Richardson et al. 2006)***. To further facilitate the understanding of whether parkin-KD mediated Ret protein loss (Figure 4.17) perturbs Ret downstream signaling, parkin-KD SH-SY5Y cultures were analyzed for Ret immediate downstream targets using immunoblotting. Since parkin is presumed to regulate Ret protein levels translationally, ribosomal protein S6 (rpS6) phosphorylation (S235/236) levels were assessed first (Figure 4.21 A). rpS6 is a subunit of 40S ribosome and is directly phosphorylated by p70S6K at several sites including Serine residues 235 and 236 that is associated with translation at the level of mRNA binding ***(Ferrari et al. 1991; Dufner & Thomas 1999)***. Early chemical protection and UV cross linking studies revealed that rpS6 is localized at the interface between the 40S and 60S ribosomal subunits and interacts with tRNA, initiation factors and mRNA. These studies acknowledge the notion that phosphorylated-rpS6 may influence the binding of mRNA to the 40S subunit ***(Nygård & Nilsson 1990)***.

Parkin-KD in SH-SY5Y cultures led to a significant 57% loss in phosphorylated-rpS6 levels compared to control conditions with no significant alterations in the total rpS6 protein levels (Figure 4.21 A & B–D). Growth factor dependent activation of both Akt and ERK are known to negatively regulate tuberous sclerosis complex 2 (TSC2) leading to Ras homologue enriched in brain (Rheb) activation, that triggers mTOR activation contributing to the

activation of protein synthesis ***(Zoncu et al. 2011; Ebert & Greenberg 2013)***. One of the downstream targets of Ret is ERK1/2 and parkin-KD induced 62% loss of phosphorylated-ERK (pERK) (Thr202/Tyr204) levels in SH-SY5Y cultures compared to control conditions. No significant alterations in total ERK protein levels were observed (Figure 4.21 A & E–G).

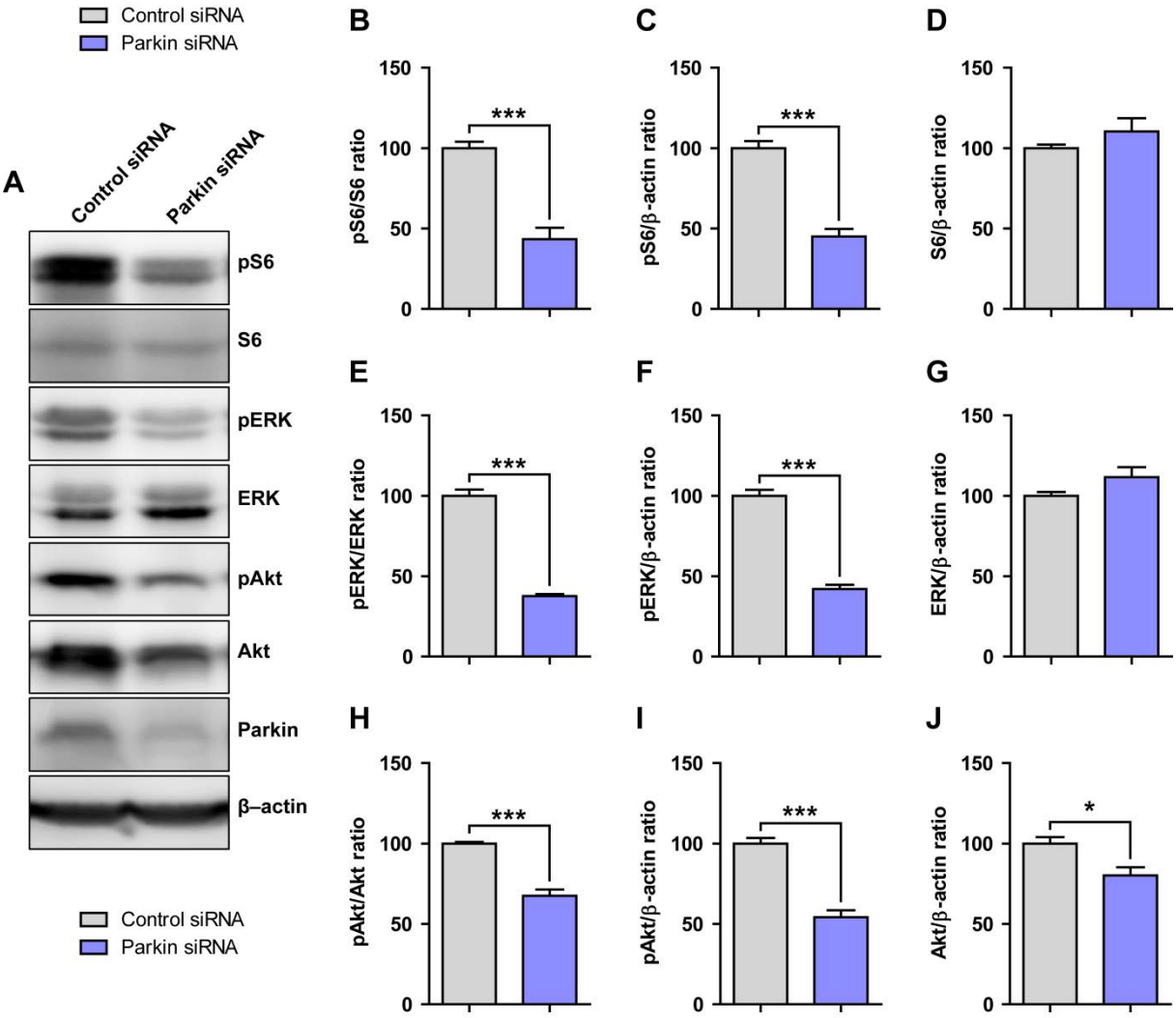


Figure 4.21 Knock down of parkin diminishes GDNF-Ret signaling

SH-SY5Y cultures were transiently transfected with either control siRNA or siRNA specific to parkin and the lysates were subjected to immunoblotting to assess the depicted proteins (**A–J**); Representative immunoblots illustrating various proteins (**A**); Quantification of the relative expression of phosphorylated-rpS6 to rpS6 ratio (pS6/S6), phosphorylated-rpS6 to β-actin ratio (pS6/β-actin) and rpS6 to β-actin ratio (S6/β-actin) reveal a significant loss of 57%, 55% and no changes respectively, due to parkin-KD (**B–D**); pERK to ERK ratio, pERK to β-actin ratio and ERK to β-actin ratio show a significant loss of 62%, 58% and no changes respectively, due to parkin-KD (**E–G**); pAkt to Akt ratio, pAkt to β-actin ratio and Akt to β-actin ratio reveal a significant loss of 33%, 46% and 20% loss respectively, due to parkin-KD (**H–J**); Representative parkin-KD efficiency blots are shown (**A**) and quantifications are depicted in Figure 17 D; Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test.

Another important downstream target of Ret and a central kinase that is implicated in both PD and cancer is Akt ***(Durgadoss et al. 2012; Ahmad et al. 2014; Mulligan 2014)***. A significant loss of 33% was observed in phosphorylated-Akt (pAkt) S473 levels in the parkin-KD conditions compared to control conditions. Parkin-KD also led to a significant decrease of 20% in total Akt levels (Figure 4.21 A & H–J). Together, these results imply that signaling pathways implicated in translational control are affected in parkin-KD cells. Accordingly, they provide some support for the conjecture that Ret protein levels are translationally regulated directly or indirectly by parkin.

4.22 Constitutively active–Ret signaling is normalized by deletion of parkin in the DA system of mice

Normal Ret signaling is important for the maintenance and regeneration of nigrostriatal DA neurons ***(Kramer et al. 2007; Meka et al. 2015; Drinkut et al. 2016)***. Constitutive activation of Ret–MEN2B leads to an increase in DA neurons specifically in SNpc leading to a substantial increase in DA innervations in the ST ***(Mijatovic et al. 2007)***. Considering augmented DA system in MEN2B mutant mice is a consequence of constitutive downstream activation of oncogenic MEN2B, well acknowledged Ret/MEN2B downstream signaling molecules were analyzed (Figure 4.22).

However, the present study demonstrates that parkin reinforces MEN2B to promote an enlarged nigrostriatal DA system as deletion of parkin normalized this augmented nigrostriatal DA system in MEN2B mutant mice (Figure 4.6; 4.7.2). Further analysis was carried out to gain insight into if parkin ablation could normalize the constitutive Ret signaling in MEN2B mutant mice. Phosphorylation levels of rpS6, ERK and Akt were assessed in the striatal lysates of 3–6 month-old WT controls, parkin-deficient mice, MEN2B mutants and parkin-deficient MEN2B mice. A significant 53% increase in the phosphorylation of rpS6 levels was observed in MEN2B mutant mice as anticipated and parkin ablation significantly normalized phosphorylated-rpS6 levels by 43% in MEN2B mutant mice along with a significant decrease of total rpS6 levels in parkin-deficient MEN2B mice compared to WT controls (Figure 4.22.1 A–D). MEN2B mutant mice exhibit a 111% increase in pERK levels in the striatal lysates and parkin deletion partially normalized by 31% in MEN2B mutant mice and no significant alterations were observed in total ERK levels (Figure 4.22.2 A–D). A significant 60% increase in the pAkt levels was observed in MEN2B mutant mice and parkin ablation significantly normalized pAkt levels by 37% in the striatal lysates of MEN2B mutant mice. Total Akt levels were not altered among the genotypes (Figure 4.22.2 A & E–G). Thus, parkin ablation normalizes constitutively active Ret signaling (Figure 4.22), histological alterations (Figure 4.6), and the behavioral abnormalities (Figure 4.11) in MEN2B mutant mice.

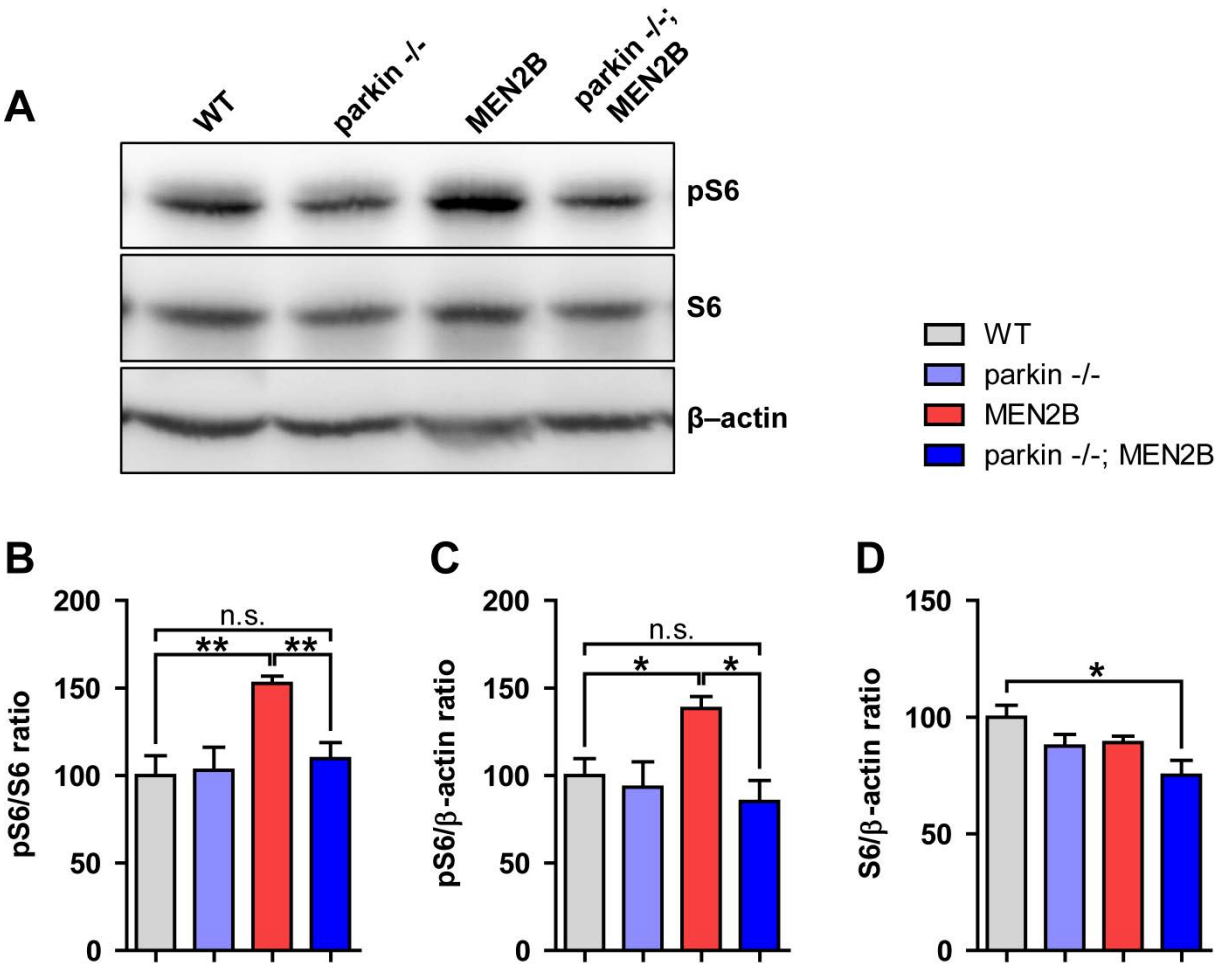


Figure 4.22.1 Parkin normalizes elevated phosphor-rpS6 levels in the DA system of mice

Immunoblots of (**A**) striatal lysates depicting expression of the indicated proteins from 3–6 month-old WT controls, parkin -/-, MEN2B mutant and parkin -/-; MEN2B mice; Quantification of the relative expression of phosphorylated-rpS6 to rpS6 ratio (pS6/S6) reveal a significant 53% increase in MEN2B mutant mice and parkin deletion normalized by 43% (**B**); phosphorylated-rpS6 to β-actin ratio (pS6/β-actin) increased by 38% and deletion of parkin normalized by 47% (**C**); rpS6 to β-actin ratio (S6/β-actin) is not altered in MEN2B mutant mice however, deletion of parkin lead to 25% decrease in total rpS6 levels in MEN2B mutant mice, n=5 mice per genotype (**D**) Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, 1 way ANOVA with Newman–Keuls post hoc test.

4.23 Ret and parkin target common signaling pathways

Parkin ablation results in exacerbating the progressive neurodegeneration of nigrostriatal DA neurons in Ret-deficient mice (Figure 4.2) whereas, parkin–overexpression is shown to be neuroprotective in Ret-deficient mice as it rescues all the known phenotypes of aged Ret-deficient mice ***(Meka et al. 2015)***. Normalization of MEN2B induced oncogenic signaling by deletion of parkin (Figure 4.22) and perturbed Ret downstream signaling in parkin-KD SH-SY5Y cultures (Figure 4.21) prompted to elucidate the effect of parkin overexpression on Ret downstream signaling in mice.

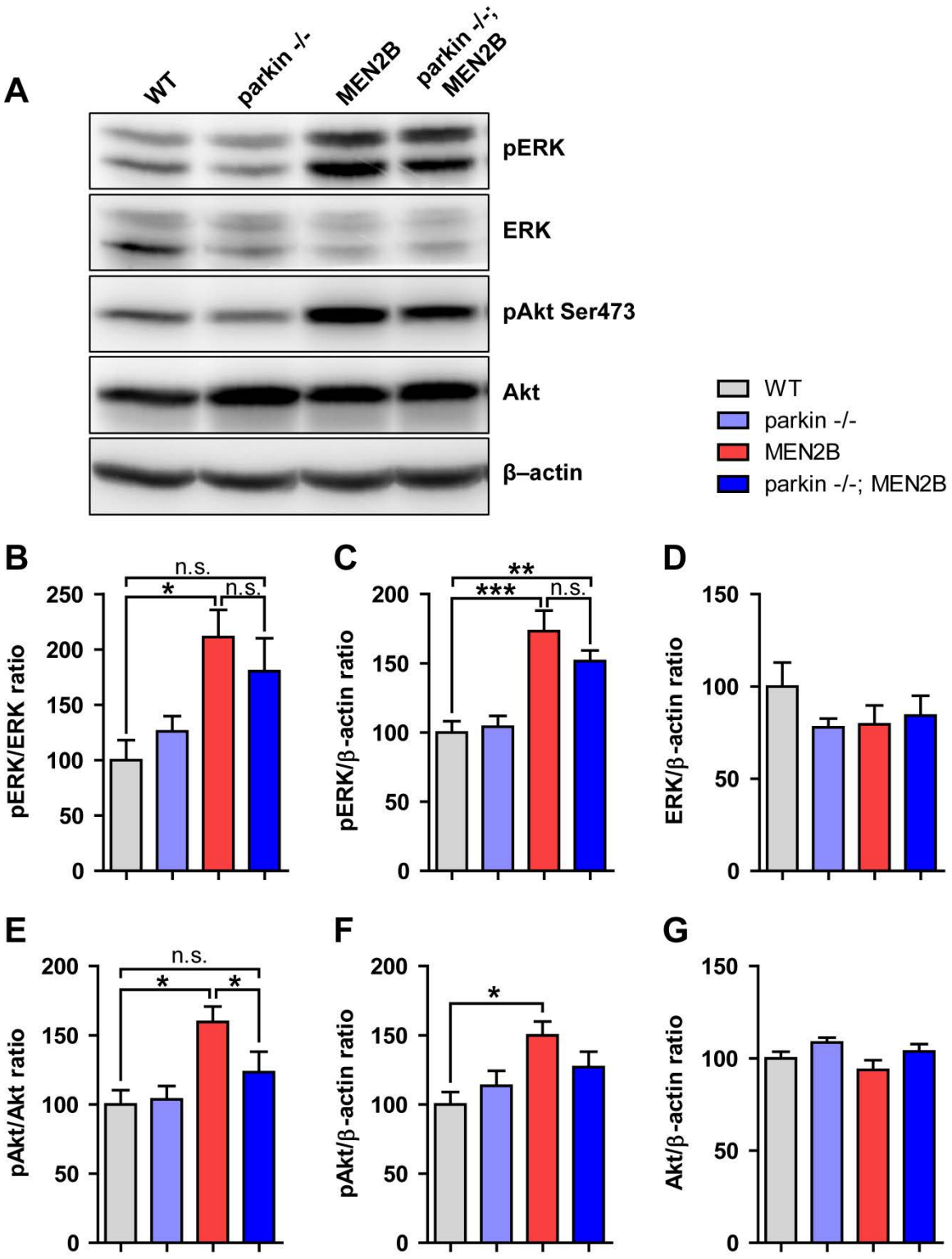


Figure 4.22.2 Parkin normalizes oncogenic MEN2B signaling in the DA system of mice

Immunoblots of (**A**) striatal lysates depicting expression of the indicated proteins from 3–6 month-old WT controls, parkin -/-, MEN2B mutant and parkin -/-; MEN2B mice, n=5 mice per genotype; Quantification of the relative expression of pERK to ERK ratio reveal a significant 111% increase in MEN2B mutant mice and parkin deletion normalized by 31% (**B**); pERK to β-actin ratio increased by 73% and deletion of parkin normalized by 22% (**C**); ERK to β-actin ratio is not altered among the genotypes (**D**); pAkt to Akt ratio reveals a significant 60% increase in MEN2B mutant mice and parkin

deletion normalized by 37% (**E**); pAkt to β-actin ratio increased by 50% and deletion of parkin normalized by 23% (**F**); Akt to β-actin ratio is not altered in the different genotypes (**G**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test or 1 way ANOVA with Newman–Keuls post hoc test.

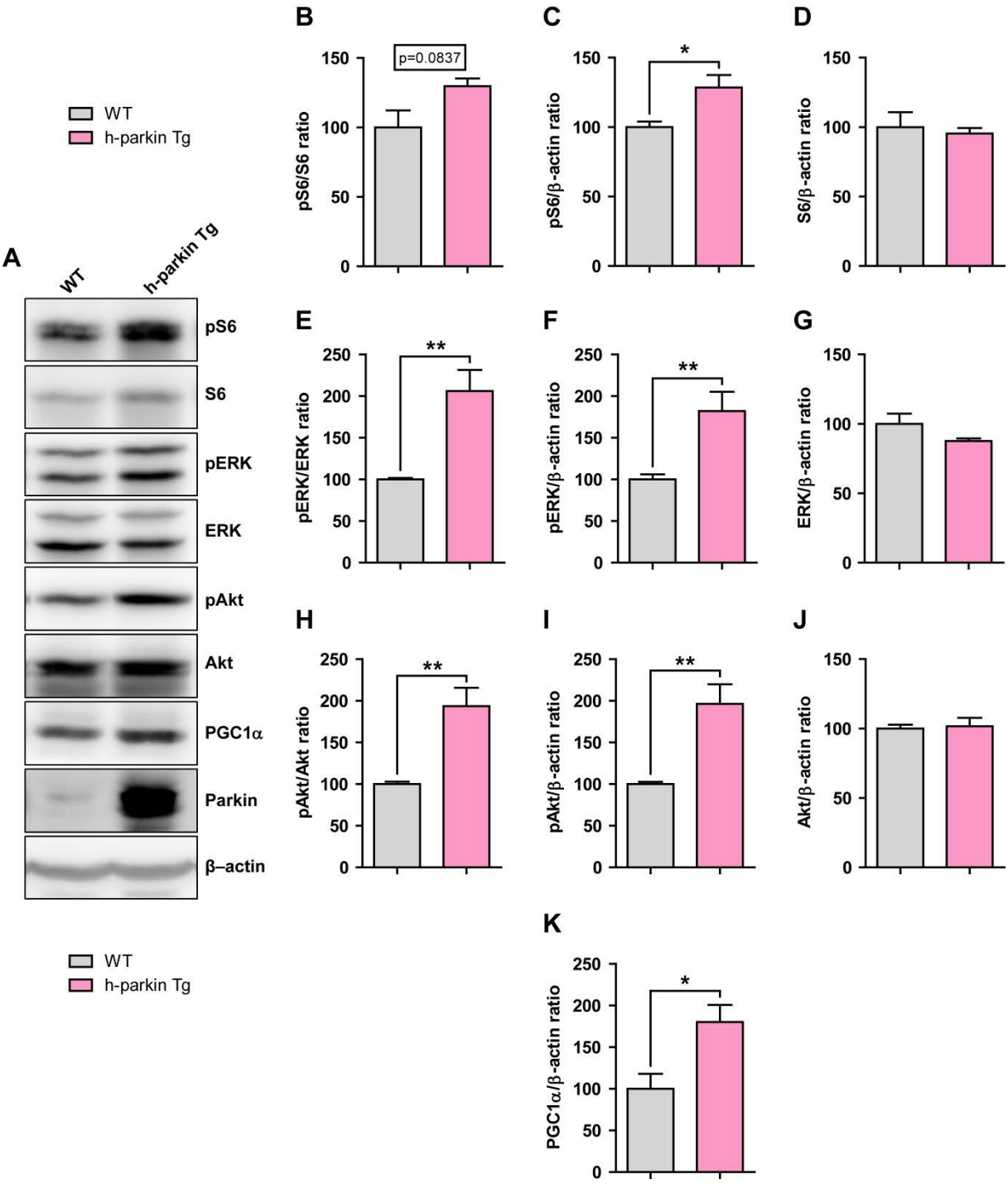


Figure 4.23 Overexpression of parkin complements GDNF-Ret signaling in mice

Immunoblots of (**A**) striatal lysates depicting expression of the indicated proteins from 3–6 month-old WT controls and h-parkin Tg mice, n=4–5 mice per genotype (**A–K**); Quantification of the relative expression of phosphorylated-rpS6 to rpS6 ratio (pS6/S6), phosphorylated-rpS6 to β-actin ratio (pS6/ β-actin) and rpS6 to β-actin ratio (S6/ β-actin) reveal an increase of 30% (p=0.0837), 28.4% significant

increase and no changes respectively, in h-parkin Tg mice (**B–D**); pERK to ERK ratio, pERK to β-actin ratio and ERK to β-actin ratio show a significant increase of 106%, 82% and no changes respectively in h-parkin Tg mice (**E–G**); pAkt to Akt ratio, pAkt to β-actin ratio and Akt to β-actin ratio reveal a significant increase of 93%, 96% and no changes respectively, in h-parkin Tg mice (**H–J**); PGC–1α to β-actin ratio reveal a significant increase of 80% in h-parkin Tg mice (**K**); Data are represented as mean +/- SEM; n.s. (not significant), *p≤0.05, ***p ≤0.0005, Student-t test.

Striatal punches from 3–6 month-old WT controls and human-parkin overexpressing transgenic (h-parkin Tg) mice that are previously characterized ***(Meka et al. 2015)*** were obtained and the lysates were subjected to immunoblotting. In line with earlier observations (Figure 4.22.1), elevated phosphorylated-rpS6 levels were observed in h-parkin Tg mice compared to age-matched WT controls and the total rpS6 levels did not alter (Figure 4.23 A & B–D). The phosphorylation levels of ERK, one of Ret's downstream targets, were markedly increased by 106% in the striatal lysates of h-parkin Tg mice compared to WT controls with no significant differences in the total ERK levels (Figure 4.23 A & E–G). Another immediate downstream target of Ret is Akt, striatal lysates of h-parkin Tg mice displayed a significant 93% increase in pAkt levels whereas no changes were observed in total Akt levels (Figure 4.23 A & H–J) supporting the previous findings from parkin-KD SH-SY5Y cultures and parkin-deficient MEN2B mice.

It is shown that parkin leads to proteasomal degradation of the transcriptional repressor, PARIS (ZNF746) which de-represses the expression of PGC–1α and nuclear respiratory factor 1 (NRF1), both of which lead to mitochondrial biogenesis ***(Shin et al. 2011)***. In agreement with this, PGC–1α is significantly upregulated by 80% in the striatal lysates of h-parkin Tg overexpressing mice compared to WT controls (Figure 4.23 A & K).

Overall, these findings suggest that both parkin and Ret could target common signaling pathways and also helps to understand the mechanism of how parkin overexpression complements Ret loss of function and rescues the phenotype of aged Ret-deficient mice.

5. Discussion

Neurotrophic factors play an important regulatory role in the development and maintenance of specific neuronal populations. These factors have emerged as the most potent mediators of neuronal survival and are being considered as promising therapeutic agents for neurodegenerative disorders like PD. In the case of DA neurons, GDNF and its receptor Ret has been shown to be important for their survival and maintenance ***(Lin et al. 1993; Kordower et al. 2000; Kramer et al. 2007)***. Although the critical function of Ret is evident for the maintenance of DA neurons, the nature of this function still remains elusive. The current study emphasizes the essential role of Ret in the maintenance of mitochondria and how Ret signaling cooperates with parkin, a protein that is implicated in PD during the development and maintenance of DA neurons in mice. We used mouse genetics to analyze both loss of function of parkin in trophically impaired (Ret-deficient) DA neurons during ageing and loss of function of parkin in trophically reinforced (constitutively active Ret, MEN2B) DA neurons during development and adulthood. We found that synergistic function of parkin and Ret is required for the survival of nigrostriatal DA neurons during ageing, indicating a genetic interaction between the two genes. Further we found that parkin cooperates with MEN2B in establishing the augmented DA system during development indicating that parkin is essential for the MEN2B phenotype during the development of DA neurons. Further, we investigated the molecular mechanisms how parkin and Ret together, trigger the survival of DA neurons both in mice and in the cultured cells. In biochemical analysis using mouse striatal punches and cultured cells, we found that parkin may translationally regulate Ret protein levels and both parkin and Ret target common signaling pathways in order to facilitate cell survival in cultures as well as in mice.

5.1 Ret assures proper mitochondrial function in aged mice

Importance of the physiological function of Ret in the nigrostriatal DA system and its interaction with PD-linked proteins was well established previously ***(Kramer et al. 2007; Aron et al. 2010; Klein et al. 2014)***. However, not all the functions of Ret in the DA system were understood in the previous studies due to the insertion of Cre recombinase transgene into the 5'-UTR of the endogenous DAT locus, resulting in the disruption of one of the functional DAT-alleles in those mice, which led to a decrease in DAT protein levels and behavioral alterations over age ***(Kramer et al. 2007)***. These issues were addressed in the current study by using a bacmid carrying a Cre recombinase under control of the exogenous DAT promoter, leaving the endogenous DAT alleles undisturbed ***(Parlato et al. 2006; Turiault et al. 2007)***. Characterizing the Ret-deficient mice generated using the DCB mice revealed additional phenotypes of Ret-deficient mice, like decreased dopamine levels and behavioral alterations (Meka thesis 2014).

Ret is expressed in all the DA neurons of the ventral midbrain (SN and VTA), whereas deletion of Ret in the DA neurons leads to specific degeneration of SNpc DA neurons, but not VTA DA neurons during ageing. Thus, certain specific requirements important for the survival of SNpc DA neurons are coordinated by Ret. For example, SNpc DA neurons are autonomous pacemakers and highly dependent on L-type voltage-dependent Ca^{2+} channels (Cav1.3 type). The perpetual activity of these channels in the SNpc DA neurons results in elevated intracellular Ca^{2+} influx and thereby leads to elevated bioenergetic requirements. In particular, there is increased mitochondrial oxidative phosphorylation and as a consequence, elevated ROS production in these neurons ***(Surmeier 2007; Surmeier et al. 2011; Pacelli et al. 2015)***. A compromised anti-oxidant system during ageing could further lead to increased oxidative stress, hastening death of SNpc DA neurons. Taken together, elevated bioenergetic requirements could be one of the causes for selective degeneration of SNpc DA neurons and additionally mitochondria are directly involved in Ca^{2+} buffering ***(Mattson et al. 2008; Saxton & Hollenbeck 2012)***. A constitutively active form of Ret, MEN2B, was shown in *Drosophila* to rescue the muscle degeneration and more prominently disintegration of mitochondria along with loss of ATP levels caused by the pathogenic mutations in PINK1, supporting the significance of Ret function in the maintenance of mitochondrial activity ***(Klein et al. 2014)***, possibly through its downstream target Akt. Growth factor-dependent activation of Akt was shown to translocate active Akt into mitochondria via the proton-gradient across the membranes and was found to reside in the matrix, inner and outer membranes to phosphorylate its substrates, like β-subunit of ATP synthase ***(Jope & Bijur 2003)***. In line with these findings, aged Ret-deficient mice showed a significant decrease in the mitochondrial complex I activity measured from enriched mitochondrial fractions obtained from the SN in the current study (Figure 4.3), further supporting the notion that Ret function is necessary for mitochondrial complex I activity and ascribing elevated bioenergetic requirements being the cause of selective degeneration. Interestingly, other studies attributed the possible functions of GDNF and its receptor Ret in transiently regulating A-type K+ channels in the midbrain DA neurons and different types of voltage-gated Ca^{2+} channels in the dorsal root ganglionic neurons, respectively, via MAP kinase/ERK-dependent pathways ***(Yang et al. 2001; Woodall et al. 2008)***.

5.2 Parkin is required for the survival of nigrostriatal DA neurons

One of the major advances in PD research was the identification of familial PD-associated genes and the characterization of their biochemical mechanisms ***(Cookson 2005; Gupta et al. 2008; Lubbe & Morris 2014)***. In contrast to the well-established roles of parkin, PINK1 and DJ-1 *in vitro,* transgenic mouse models lacking these proteins failed to fully recapitulate the disease pathogenesis ***(Itier et al. 2003; Goldberg et al. 2003; Yamaguchi & Shen***

2007; Gispert et al. 2009; Dawson et al. 2010). More importantly, these mice exhibit no histological alterations in the nigrostriatal DA neurons making it difficult to study their function for the survival of the SNpc DA which degenerate during the course of PD. Despite no DA neuronal loss per se, mice lacking parkin, PINK1 and DJ-1 seem to be especially susceptible to neuronal stressors ***(Raymond H Kim et al. 2005; Frank-Cannon et al. 2008; Moisoi et al. 2014)*** suggesting that loss of parkin, PINK1 and DJ-1 proteins sensitize neurons to the effect of other insults such as trophic impairment, indicating that PD is a multifactorial disorder with evolving layers of complexity.

To understand the physiological importance of parkin in the maintenance of DA system in the trophically impaired (Ret-deficient) DA neurons, parkin and Ret double deficient mice were generated by crossing parkin-deficient mice with conditional Ret-deficient mice (Figure 4.1). As PD being an age related disorder, the DA system of parkin and Ret double deficient mice were analyzed at different ages (3–6 month, 12 month and 24 month). Interestingly, parkin and Ret double deficient mice showed an enhanced neurodegeneration of SNpc DA neurons up to 30% compared to 21% in the single Ret-deficient mice at the age of 24 month (Figure 4.2 B). Parkin and Ret double deficient mice for the first time revealed a significant and age-dependent progressive neurodegeneration of SNpc DA neurons indicating the essential role of parkin and Ret in the survival of DA neurons (Figure 4.2 D).

These findings also suggest that, although embryonic compensation could be a reason for no DA cell loss in parkin null mice, these neurons are predisposed to have a stronger response to stress conditions like lack of trophic support. Presumably this situation can be extrapolated to humans where the function of parkin is impaired due to pathogenic mutations. A similar study with another PD-associated protein, DJ-1, demonstrated that aged mice lacking both DJ-1 and Ret exhibit an enhanced degeneration of DA cell bodies, but not axon terminals compared to aged mice that lack only Ret ***(Aron et al. 2010)***. This indicates that the function of DJ-1 in the trophically impaired DA neurons is only confined to the maintenance of cell bodies, whereas parkin's function during trophic impairment affects both cell bodies and the innervations. This could be due to the fact that although both parkin and DJ-1 contribute to the maintenance of mitochondrial integrity, only parkin is capable of regulating the anterograde-retrograde movement of healthy mitochondria between these two neuronal compartments ***(Exner et al. 2012; Schwarz 2013)***. Defective autophagy is observed in PD patients ***(Lynch-Day et al. 2012)***. However, autophagy does not contribute to the progressive degeneration that is observed in parkin and Ret double deficient mice as the levels of the autophagic markers p62 and LC3 were not significantly altered in these mice ruling out the functional role of Ret in regulating autophagy (Figure 4.4) ***(Klein et al. 2014)***. Corroborating our own findings, constitutively active Ret, MEN2B mutant mice revealed no

traces of defective autophagy indicating that Ret function is not targeted to autophagy (Figure 4.14 A & E–G).

5.3 Parkin contributes to the enlarged nigrostriatal DA system during development in MEN2B mutant mice

Several studies have shown the functional role of Ret signaling in the maintenance of DA neurons in the transgenic mouse and the drug (e.g. MPTP, Rotenone) models. Even though Ret signaling does not modulate MPTP-mediated toxicity, it appears to be important for the GDNF mediated neuroprotective and neuroregenerative effect of the nigrostriatal pathway ***(Kowsky et al. 2007; Drinkut et al. 2016)***. MEN2B mutant mice, which express a constitutively active form of Ret, show an increased nigrostriatal DA system and elevated dopamine levels in the ST ***(Mijatovic et al. 2007)***. Recent evidence has shown that the MEN2B mutation protects against the MPTP and 6-OHDA induced DA cell body loss, but not axon terminal loss ***(Mijatovic et al. 2011)***. Based on the above findings and from our own findings stated in the current study, we further investigated the *in vivo* functional cross-talk of parkin and Ret in the establishment of enlarged DA system in MEN2B mutant mice. For this purpose, parkin-deficient MEN2B mice were generated as described (Figure 4.5), and were analyzed at 3–6 months of age as the enlarged DA system in MEN2B mutant mice is a developmental phenotype and ageing is not required. A significant normalization in increased nigrostriatal DA system was observed in parkin-deficient MEN2B mice with respect to MEN2B mutant mice suggesting the importance of parkin in the establishment of DA system and thereby supporting the hypothesis that parkin and Ret function together in maintaining the DA system (Figure 4.2; 4.6 and Meka Thesis 2014).

Significant elevation in the total dopamine levels in MEN2B mutant mice was reported earlier. This increase in dopamine concentration seems to be more prominent in the dorsal ST compared to the ventral ST, corresponding to the fact that most of the SNpc neurons innervate into the dorsal ST ***(Mijatovic et al. 2007)***. Increased accumulation of L-dopa, a precursor for dopamine and an increase in TH activity was observed in MEN2B mutant mice indicating an increase in dopamine levels due to increase in the synthesis of dopamine and not due to the increased number of DA neurons in the SNpc ***(Mijatovic et al. 2008)***. Supporting these findings, total dopamine levels were elevated significantly along with the increase in TH protein levels in the ST of MEN2B mutant mice compared to WT control mice in the present study (Figure 4.10; 4.13 A & E). Moreover, Ret expression was significantly increased in the ST of MEN2B mutant mice compared to WT controls, which was not addressed in the earlier studies (Figure 4.13 A & B). Although deletion of parkin significantly normalized the MEN2B-mediated increase in Ret protein and TH protein levels, this did not affect the elevated dopamine levels in MEN2B mutant mice unlike the normalization that is

observed for DA cell numbers in the SNpc and DA innervations in the ST. This can be explained by the partial normalization of the DA innervations in parkin-deficient MEN2B mice (Meka Thesis 2014). However, deletion of parkin in MEN2B mutant mice significantly improved the turnover of dopamine in MEN2B mutant mice (Figure 4.10 F).

5.4 Behavioral abnormalities in MEN2B mutant mice

Increased synthesis and storage of dopamine levels in the ST of MEN2B mutant mice did not result in increased locomotor activity and their activity was even reduced compared with WT control mice. This is due to the basal extracellular dopamine concentration that is similar to that of WT controls in MEN2B mutant mice ***(Mijatovic et al. 2008)***. Increased locomotor activity in MEN2B mutant mice over control mice was only observed in response to cocaine ***(Mijatovic et al. 2007; Mijatovic et al. 2008)***. Supporting these findings, locomotor activity measured over 10 min in MEN2B mutant mice was similar to that of WT control mice and reduced locomotor activity in MEN2B mutant mice was observed compared to WT controls when measured for 0-5 min and 5-10 min separately (Figure 4.11.1 A–C). This could be due to the increase in DAT levels and its activity in the MEN2B mutant mice, which increases the re-uptake of dopamine in the synaptic cleft ***(Mijatovic et al. 2008)***. However, deletion of parkin failed to rescue this behavior in MEN2B mutant mice (Figure 4.11.1 A–C).

Patients with the condition of pheochromocytoma due to MEN2B mutations evince anxiety-like behavior ***(Archer & Mazzaferri 1999; Lee & Norton 2000; Sawka et al. 2003)***. MEN2B mutant mice at the age of 3–6 months showed no signs of anxious behavior in the open-field test or in the EPM compared to WT control mice (Figure 4.11.1 D; 4.11.2 A–C), as absolute penetrance of the pheochromocytoma phenotype was only shown in MEN2B mutant mice at the age of 6 month ***(Smith-Hicks et al. 2000)***. Based on the time taken to enter the border zone in the open-field test, stretch attend posture in the EPM and time taken to climb down the vertical pole in the pole-test, MEN2B mutant mice rather showed difficulties in initiating movement compared to that of WT control mice (Figure 4.11.1 D; 4.11.2 D & F). The reasoning behind this could be due to the MEN2B mediated alterations caused either in the periphery or in other neuronal populations like motor neurons, where MEN2B is expressed. However, deletion of parkin rescued these behavioral phenotypes to normal levels in MEN2B mutant mice (Figure 4.11.1; 4.11.2), indicating that parkin deletion improves the physiological state of MEN2B mutant mice and the synergistic effect of parkin and Ret is not restricted to the DA system and perhaps extended to other regions where MEN2B is expressed.

5.5 Parkin in the establishment of DA system

In elucidating the role of parkin in regulating the development of enlarged nigrostriatal system in MEN2B mutant mice, it was important to understand at which stage of development this

phenotype is established. During prenatal development, SN DA neurons differentiate and migrate to their target positions. Postnatal development is characterized by contact establishment like extension of axons, terminal differentiation and synapse formation in the ST ***(Voorn et al. 1988; Oo & Burke 1997; Burke 2003; Lyng et al. 2007)***. DA neurons undergo natural cell death (NCD) mostly during first two weeks of the postnatal development period, when a rapid increase in the rate of synapse formation in the ST was observed. The NCD event is biphasic and first takes place at P2 and then again in a second phase at P14 ***(Oo & Burke 1997; Burke 2003)***. Neurotrophic factors promote postnatal survival during NCD by suppressing apoptosis of neurons. GDNF performs this function in the case of DA neurons. Hence, MEN2B and the WT control mice were analyzed soon after the NCD events at P4 and P16 and an increase in the SNpc DA neurons in the MEN2B mutant mice was only observed after the second NCD phase at P16. Interestingly, parkin-deficient MEN2B mutant mice showed a normal number of SNpc DA neurons compared to WT controls at the age of P16 indicating that lack of parkin normalizes the enlarged DA system in MEN2B mutant mice and further supporting the role of parkin in the establishment of DA system (Figure 4.7.2).

5.6 Cellular and biochemical alterations in MEN2B mutant mice are normalized by deleting parkin

Ret is shown to be important for maintaining proper soma size of the DA neurons in the SN, as Ret-deficient mice exhibit a decreased soma area specifically in the SN DA neurons at 3–6 months of age as compared to DCB controls ***(Meka et al. 2015)***. DA neuron soma area seems to be directly proportional to Ret activity, since MEN2B mutant mice showed an increased soma area specifically in the SN DA neurons and deletion of parkin significantly normalized this phenotype in MEN2B mutant mice (Figure 4.8). This phenomenon can be explained by the marked increase in rpS6 phosphorylation in the DA system of MEN2B mutant mice (Figure 4.22.1), as mouse embryonic fibroblasts (MEFs) derived from phosphorylation mutants of rpS6 mice were significantly smaller due to the growth defects compared to their WT control MEFs ***(Ruvinsky et al. 2005; Ruvinsky & Meyuhas 2006)***. As described previously (Figure 4.3), aged Ret-deficient mice showed a decrease in mitochondrial complex I activity implying that Ret function is necessary for the complex I activity. Contradicting these findings, however, complex I activity measured from the SN enriched mitochondrial fractions showed a significant 26% decrease in MEN2B mutant mice without altering total ATP levels. Parkin-deficient mice showed a tendency to have decreased mitochondrial complex I activity and total ATP levels, but these alterations were not significant compared to WT controls. However, deleting parkin, a protein that is involved in the maintenance of mitochondrial quality control restored complex I activity in MEN2B mutant mice and total ATP levels in single parkin-deficient mice (Figure 4.9 A & B). Considering the

oncogenic nature of MEN2B, it has the potential to switch the energy source by upregulating glycolysis and silencing mitochondrial activity in these neurons ***(Gogvadze et al. 2010)***. This can be well explained by the Warburg effect, a phenomenon in which enhanced uptake of glucose which is metabolized by glycolysis and the resulting pyruvate is converted to lactate followed by lactic acid fermentation in the cytosol. Conversely, in normal cells pyruvate is converted to acetyl CoA through the mitochondrial enzyme pyruvate dehydrogenase and acetyl CoA then enters the Krebs cycle ***(Gogvadze et al. 2010; Alfarouk et al. 2014; Alfarouk 2016)***. A recent study showed the ability of parkin to regulate the Warburg effect: loss of parkin activated glycolysis and reduced mitochondrial respiration, leading to the Warburg effect. Importantly, restoring parkin expression reversed the Warburg effect in cells, indicating a potential tumor suppressor function of parkin ***(Cesari et al. 2003; Zhang et al. 2011)***. Supporting the notion that parkin functions as a tumor suppressor, oncogenic MEN2B in the DA system suppressed parkin expression by 44% in the ST of MEN2B mutant mice compared to WT controls (Figure 4.13 A & H). However, deleting parkin restored the mitochondrial complex I activity in the SN of MEN2B mutant mice in the current study, suggesting that presence of parkin is a minimal requirement for MEN2B oncogenic activity or in other words parkin stabilizes MEN2B protein levels (Figure 4.13 A & B).

Besides significant alterations in mitochondrial activity in MEN2B mutant mice, no significant alterations were observed in mitochondrial inner membrane protein subunit, mitofilin (Figure 4.14 A & C) and a master regulator of mitochondrial biogenesis, PGC-1α (Figure 4.14 A & D) indicating that biogenesis and morphology of mitochondria in MEN2B mutant mice are unaffected.

5.7 Parkin-Ret cross-talk is not restricted to CNS in MEN2B mutant mice

Ret signaling is of major importance in the peripheral nervous system, ENS and in neuroendocrine cells (Thyroid C-cells and adrenal gland chromaffin cells) during development, and germline mutations in Ret cause MEN2A, MEN2B, and FMTC syndromes in humans ***(Pachnis et al. 1993; Tsuzuki et al. 1995; Smith-Hicks et al. 2000)***. MEN2B mutant mice carrying the M918T mutation exhibit defects in the male reproductive system and an age dependent penetrance of both C-cell hyperplasia and pheochromocytoma phenotypes. It was shown that newborn MEN2B mutant mice evince bilateral malformation of the adrenal gland morphology ***(Smith-Hicks et al. 2000)***. To analyze whether the synergistic function of parkin and Ret is also found outside the DA system, adrenal gland morphology was studied. My results confirm the previous findings of bilateral malformation of the adrenal glands in MEN2B mutant mice. Incomplete enclosure of the adrenal medulla with the adrenal cortex is evident in MEN2B mutant mice at the age of P14 from my findings (Figure 4.12),

whereas, this morphological alteration was earlier reported in adult homozygous MEN2B mutant mice ***(Smith-Hicks et al. 2000)***. Deletion of parkin partially rescued the adrenal gland morphological alterations in MEN2B mutant mice, as two out of three parkin-deficient MEN2B mice analyzed showed almost complete enclosure of the adrenal medulla with the adrenal cortex (Figure 4.12).

Ret signaling is vital during kidney development: mutations in the tyrosine docking sites of Ret, for example Y1015F in both Ret51 and Ret9 isoforms lead to renal anomalies including supernumerary ureteric buds that fail to separate from the Wolffian ducts and decreased branching morphogenesis ***(Jain, Encinas, et al. 2006; Chen 2009)***. Mice overexpressing Ret exhibit ureteral dilation associated with the urinary tract anomaly vesicoureteral reflux (VUR), which is a retrograde urine flow from the bladder to the ureter contrary to the normal condition ***(Yu et al. 2004; Chen 2009)***. Supporting these findings, MEN2B mutant mice excreted significantly less urine during the open field test compared to WT control mice, whereas, deletion of parkin rescued this abnormality in MEN2B mutant mice (Figure 4.11.1 F). Behavior anomalies in MEN2B mutant mice, like decreased urine excretion and difficulty in initiating movement were completely rescued by deleting parkin in MEN2B mutant mice (Figure 4.11). These findings support the hypothesis that deletion of parkin in MEN2B mutant mice could rescue all the MEN2B-mediated abnormalities. Further experiments are necessary to support the cross-talk of parkin and Ret outside DA system.

5.8 Parkin stabilizes Ret/MEN2B protein levels

Parkin was shown to regulate the internalization and degradation of EGFR by ubiquitinating EPS15, an adaptor protein that is involved in the internalization of EGFR. Furthermore parkin loss was shown to accelerate EGFR endocytosis and its degradation in cells with a reduced EGF-mediated downstream signaling ***(Fallon et al. 2006)***. However, the absolute requirement of EGFR under the physiological and pathological conditions in DA neurons is still obscure. Ret is an RTK and all RTK's appear to be endocytosed in a similar manner. Taken together with our understanding of the function of Ret in the DA system ***(Kramer et al. 2007; Aron et al. 2010)***, we hypothesized that parkin loss could also accelerate Ret endocytosis and degradation in parkin-deficient mice and parkin-deficient MEN2B mice. In agreement with the hypothesis, a significant decrease in Ret protein levels were observed in the striatal lysates of parkin-deficient mice compared to WT controls and parkin-deficient MEN2B mice compared to MEN2B mutant mice (Figure 4.13 A, B & F), suggesting that parkin can regulate Ret protein levels. Conversely, parkin is known to function as a tumor suppressor ***(Cesari et al. 2003; Zhang et al. 2011)***, supporting this notion, parkin protein levels were significantly suppressed in the ST of MEN2B mutant mice compared to WT controls due to the oncogenic nature of MEN2B (Figure 4.13 A & H). Corroborating the *in*

vivo findings that parkin could regulate Ret and MEN2B protein levels, siRNA mediated KD of parkin in SH-SY5Y and MZCRC1 cultures lead to a significant loss of Ret and MEN2B protein levels respectively (Figure 4.17). In humans loss of parkin function is the major cause of PD, pathogenic mutations or truncations lead to inactivation of parkin due to decreased stability or rapid misfolding and aggregation of parkin, respectively ***(Henn et al. 2005; Schlehe et al. 2008)***. However, co-transfection of WT h-parkin but not its pathogenic mutant variants along with MEN2B in HeLa cultures lead to a significant increase in MEN2B protein levels compared to co-transfection of MEN2B along with mCherry (Figure 4.18) suggesting that parkin's function is essential to regulate MEN2B protein levels.

Nurr1 was shown to induce the expression of proteins that are important for neurotransmission, TH **(Sakurada et al. 1999; Schimmel et al. 1999)** and DAT ***(Sacchetti et al. 2001)*** in the DA neurons, including the Ret expression during development ***(Wallén A et al. 2001; Galleguillos et al. 2010)***. In contrast to these findings, although Ret, TH (Figure 4.13 A, B & E) and DAT ***(Mijatovic et al. 2007; Mijatovic et al. 2008)*** expression levels were markedly increased in MEN2B mutant mice, no significant alterations in Nurr1 protein levels are observed in the striatal lysates of these mice (Figure 4.14 A & B). However, analyzing the transcriptional activity of Nurr1 could provide deeper insights into whether the induction of Ret, TH and DAT expression in MEN2B mutant mice are Nurr1 dependent or independent processes.

Ret9 and Ret51 isoforms has dramatic functional and biochemical properties, regarding interaction with the signaling complexes and in there trafficking properties. Ret51 is efficiently transported to cell surface, internalizes faster upon ligand binding and is present in higher amounts on the plasma membrane after the recycling process compared to Ret9 ***(Tsui-Pierchala et al. 2002; Richardson et al. 2012)***. Moreover, both isoforms have different and possibly tissue specific effects as Ret9 is important for kidney and ENS development where Ret51 is dispensable ***(De Graaff et al. 2001)***. No significant loss of Ret9 or Ret51 protein levels were noticed in either parkin-deficient mice compared to WT controls or parkin-deficient MEN2B mice compared to MEN2B mutant mice (Figure 4.13 A, C & D). This could imply that the significant loss of total Ret protein levels that is observed in parkin-deficient mice (22% loss) and in parkin-deficient MEN2B mice (44% loss) compared to their respective controls could be a contribution of both the isoforms (Figure 4.13 A, B & F). However, Ret51 protein levels were significantly lower in parkin-KD conditions compared to control conditions in SH-SY5Y cultures (Figure 4.17 A & C) and Ret9 basal expression levels were below the detection limit in control SH-SY5Y cultures preventing analysis of the effect of parkin-KD on the Ret9 isoform. Our findings show that the function of parkin in regulating Ret protein levels is not restricted to a specific isoform, but rather seems to be general for all the Ret isoforms

(Figure 4.13 A, C & D). Differential activation, maturation and trafficking patterns of Ret9 and Ret51 were addressed in the earlier studies ***(De Graaff et al. 2001; Tsui-Pierchala et al. 2002; Richardson et al. 2012)***, but the differences in basal expression of Ret9 and Ret51 was not reported previously. In the current study we show that Ret51 basal expression is two-fold higher than that of Ret9 in WT control mice (Figure 4.13 A & G). Conversely, MEN2B mutation induced a 94% increase in Ret9 expression, but only a 36% increase in Ret51 expression compared to WT control mice (Figure 4.13 A, C & D). The disparity in the differential induction of Ret9 over Ret51 expression in MEN2B mutant mice compared to WT controls, could explain the enlarged DA system ***(Mijatovic et al. 2007)*** and severity of the oncogenic phenotype in MEN2B mutant mice that was described previously ***(Smith-Hicks et al. 2000)***. As mono-isoformic studies expressing Ret9 or Ret51 in mice demonstrated that only Ret9 is crucial for normal renal and ENS development where Ret51 is dispensable ***(De Graaff et al. 2001; Jain, Encinas, et al. 2006)***. Absence of normal Ret signaling, where Ret9 expression is 2 fold lower compared to Ret51 expression did not alter the development of DA system ***(Kramer et al. 2007)***. Conversely, MEN2B mediated signaling, where Ret9 expression is almost one fold higher to that of WT control mice resulted in the development of an enlarged DA system. Taken together, these results suggest that elevated Ret9 activity could be the only possible explanation for the development of the enlarged DA system in MEN2B mutant mice, which is normalized by deleting parkin.

5.9 Molecular interaction of parkin and Ret

Both our *in vitro* and *in vivo* studies demonstrated that parkin can regulate Ret or MEN2B protein levels (Figure 4.13; 4.17). Parkin, besides polyubiquitinating its target substrates for degradation, it also monoubiquitinate specific substrates like Sept5 ***(Zhang et al. 2000)***, synphilin I ***(Lim et al. 2005)***, DAT ***(Jiang et al. 2004)*** and EPS15 ***(Fallon et al. 2006)***, which are involved in signaling. Parkin also acts as a regulatory switch on the endocytic adaptor protein, EPS15, involved in clathrin-mediated endocytosis of EGFR through interaction and ubiquitination ***(Husnjak & Dikic 2006; Fallon et al. 2006)***. Assuming that parkin is mediating Ret internalization upon ligand binding, I analyzed the interaction of parkin and Ret *in vitro* and *in vivo.* My findings confirmed the physical interaction of both parkin and Ret in SK-N-BE(2) cultures and in the striatal lysates of WT control mice (Figure 4.15.1; 4.15.2). We further analyzed if this interaction is isoform specific or general for all the Ret isoforms. Although the interaction of parkin with Ret51 isoform is evident, due to the low abundancy of Ret9 isoform in the WT striatal lysates, the interaction between Ret9 and parkin seems not conclusive (Figure 4.15.2 A). Parkin regulating Ret protein levels and the physical interaction between parkin and Ret supported our hypothesis that parkin could be facilitating the internalization and turnover of Ret protein.

5.10 Ret/MEN2B degradation and transcription are not controlled by parkin

We observed reduced Ret protein levels in the ST of parkin-deficient mice, parkin-deficient MEN2B mice, in parkin-KD SH-SY5Y cultures and in the HeLa cultures which had been transfected with MEN2B and no parkin. Collectively, these observations point to enhanced internalization leading to degradation of Ret or MEN2B protein. Previous studies examined the turnover of Ret and its isoforms in cell culture using the protein synthesis inhibitor CHX ***(Scott et al. 2005; Richardson et al. 2012; Kales et al. 2014)***. To elucidate the role of parkin in the turnover of Ret, control and parkin-KD SH-SY5Y cultures were treated with CHX for the indicated time points (Figure 4.19.1 A) and Ret decay levels were measured. If parkin is stabilizing Ret protein levels at the degradation level, I expect a more rapid decay of Ret protein in parkin-KD SH-SY5Y cultures, as compared to controls. However, my findings revealed no significant alterations in the decay of Ret protein over the indicated time-points of CHX treatment in the presence or absence of parkin (Figure 4.19.1). To further validate that parkin is not controlling Ret degradation, MEN2B and mCherry or WT h-parkin were co-transfected into HeLa cultures and were treated with either proteasome (MG132) or lysosome (NH_4Cl) inhibitors as shown (Figure 4.19.2 A) ***(Pierchala et al. 2006)***. If parkin is regulating Ret protein levels, blocking proteasome or lysosome mediated degradation should increase MEN2B protein levels irrespective of parkin. Although, there was an increase in MEN2B levels when co-expressed with parkin and not with mCherry, blocking the proteasome or lysosomal degradation systems resulted in no such increase in MEN2B levels when co-expressed with mCherry (Figure 4.19.2). In conclusion, these findings make it unlikely that parkin regulates the degradation of Ret, whereas, the possibility that parkin facilitates Ret internalization cannot be excluded. In 2011, Shin et al. and Castillo-Quan reported that parkin polyubiquitinates the transcriptional repressor PARIS (ZNF746) leading to its proteasomal degradation, thereby de-repressing the expression of PGC-1α and NRF1, resulting in mitochondrial biogenesis and ultimately cell survival. Supporting the above findings, mice overexpressing WT h-parkin showed a significant increase in PGC-1α protein levels by 80% (Figure 4.23 A & K). However, the possibility of parkin transcriptionally regulating Ret is excluded based on the recent findings ***(Meka et al. 2015)*** where Ret mRNA levels were unaltered in parkin-KD SH-SY5Y cultures compared to control conditions (Figure 4.20) leaving the only plausible alternative that parkin might translationally regulate Ret protein levels.

5.11 Parkin complements GDNF/Ret signaling

Phosphorylated tyrosine residues in Ret are docking sites for intracellular signaling proteins. Tyrosine 1062 is common in both Ret9 and 51 isoforms and is a docking site for Shc, ShcC,

IRS1/2, FRS2, DOK1/4/5, and Enigma proteins, which lead to downstream activation of PI3K/Akt, Ras/ERK kinase signaling pathways ***(Santoro et al. 2004; Arighi et al. 2005; Kodama et al. 2005)***. Ligand-dependent activation of Ret tightly regulates cell growth, survival, proliferation, and differentiation, whereas constitutively active MEN2B, which is capable of inducing self-expression (Figure 4.13 A & B), leads to uncontrolled activation of downstream Akt and ERK signaling (Figure 4.22.2 A–G), resulting in tumorigenesis. However, parkin deficiency-mediated loss of Ret in MEN2B mutant mice significantly normalized the oncogenic Ret-mediated activation of Akt and ERK signaling (Figure 4.22.2 A–G) subsequently normalizing the enlarged DA system in MEN2B mutant mice by arresting cell growth and survival. Correspondingly, parkin-KD mediated loss of Ret in SH-SY5Y cultures perturbed the Ret downstream activation of Akt and ERK signaling (Figure 4.21 A & E–J) thereby negatively regulating cell growth and survival in these cultures. Parkin was shown to protect neurons against the pathogenic mutations of DJ-1, α-synuclein, and highly accumulated mitochondrial DNA mutations that could diminish mitochondrial function during ageing ***(Petrucelli et al. 2002; Chang et al. 2014; Pickrell et al. 2015)***. Earlier findings from our group described that DA system phenotypes of aged-Ret-deficient mice are rescued by overexpressing parkin ***(Meka et al. 2015)***. In the present study the possibility of parkin protecting Ret-deficient DA neurons through the activation of both PI3K/Akt and Ras/ERK signaling pathways were described (Figure 4.23 A & E–J). Although previous findings indicated that parkin protects neurons from toxin-induced cell death through Akt signaling ***(Yasuda et al. 2011)***, parkin overexpression was only shown to rescue the MPTP-induced loss of pAkt levels and parkin overexpression per se did not activate Akt signaling. However, I could show that overexpression of parkin explicitly activated Akt signaling (Figure 4.23 A & H–J) which is downstream of Ret. A recent study stated that parkin promotes Akt phosphorylation, but not ERK upon EGFR activation ***(Fallon et al. 2006)***. Conversely, parkin overexpression promoted phosphorylation of both Akt and ERK in our mice (Figure 4.23 A & E–J), indicating that parkin overexpression is beneficial to trophically impaired DA neurons.

5.12 Parkin could regulate Ret translation

Parkin-mediated loss of Akt and ERK signaling in parkin-KD SH-SY5Y and parkin-deficient MEN2B mutant mice imply a negative effect on mTOR signaling. Apart from growth factor-mediated activation of Akt, mTORC2 directly phosphorylates Akt at position S473 which then facilitates mTORC1 activation through S2448 phosphorylation. Activated mTORC1 promotes phosphorylation of rpS6, one of the modulators of protein synthesis through activation of p70 S6K1 (S6K1) ***(Laplante & Sabatini 2009; Hoeffer & Klann 2010)***. However, due to a marked decrease in phosphorylated Akt levels in parkin-KD SH-SY5Y cultures (Figure 4.21 A & H–J) and parkin-deficient MEN2B mice (Figure 4.22.2 A & E–G), the chances for mTORC1

activation and subsequent S6K1 phosphorylation are significantly lower. As a result, I observed significant loss in phosphorylated rpS6 levels in both parkin-KD SH-SY5Y cultures (Figure 4.21 A & B–D) and parkin-deficient MEN2B mice (Figure 4.22.1 A–D), indicating that mTORC1 dependent protein synthesis is affected in this scenario. In accordance with these findings, mice overexpressing WT h-parkin exhibited marked increase in rpS6 phosphorylation levels (Figure 4.23 A–D), where Akt phosphorylation levels were also significantly high (Figure 4.23 A & H–J). Additionally, growth factor mediated activation of Akt can activate mTORC1 signaling by phosphorylating proline-rich Akt substrate of 40 kDa (PRAS40) resulting in dissociation of PRAS40 from mTORC1 and relieving an inhibitory constraint on mTORC1 ***(Wiza et al. 2012)*** thereby promoting the mTORC1 dependent translation initiation. Both activated Akt and ERK can increase the phosphorylation of TSC2 leading to inactivation of TSC1/2 complex and concomitant activation of mTORC1 ***(Laplante & Sabatini 2009)*** thereby promoting translation. Recent evidence suggests that Ras/ERK signaling also regulates assembly of translation preinitiation complexes by phosphorylating rpS6 exclusively at the S235/236 positions through p90 ribosomal S6 kinases ***(Roux et al. 2007)***. However, significant loss in phosphorylated ERK levels and phosphorylated rpS6 levels in parkin-KD SH-SY5Y cultures (Figure 4.21 A & E–G) and parkin-deficient MEN2B mice (Figure 4.22.2 A–D), ascertain a glitch in translation preinitiation complex mediated by Ras/ERK signaling. Whereas, mice overexpressing WT h-parkin exhibited a marked increase in rpS6 phosphorylation levels (Figure 4.23 A–D), where the upstream ERK phosphorylation levels were also significantly high (Figure 4.23 A & E–G) suggesting active Ras/ERK signaling mediated translation in these mice.

Although no specific assays were conducted to state the role of parkin in translationally regulating Ret protein levels, a consistent and significant loss in rpS6 phosphorylation levels as a consequence of compromised upstream Akt, ERK and most probably mTOR signaling in parkin-KD SH-SY5Y cultures (Figure 4.21) and parkin-deficient MEN2B mice (Figure 4.22.1; 4.22.2) strongly suggests impaired mRNA translation. In accordance with these findings, WT h-parkin overexpression compensated for the perturbed Ret signaling by promoting Akt and ERK signaling, leading to increased rpS6 phosphorylation thereby maintaining cell growth, survival, proliferation and differentiation in these mice possibly through PI3K/Akt or Ras/ERK mediated effects on translation. Emphasizing the pertinence of parkin's role in mRNA translation, a recent study illustrated that parkin along with PINK1, regulates localized translation of certain nuclear-encoded respiratory chain components (nRCCs) that are involved in oxidative phosphorylation ***(Gehrke et al. 2015)***. These findings describe the role of PINK1 in recruiting the translationally repressed nRCC mRNAs to the mitochondrial outer membrane, where parkin displaces the translational repressors by promoting monoubiquitination of Pumilio/Pum and hnRNP-F/Glorund thereby activating the

translation of nRCC mRNAs. Further, association of other translational repressors like Dcp1, GW182 and POP2 were also shown to be affected in PINK1 or parkin mutants ***(Gehrke et al. 2015)***. Together, the tantalizing link between parkin and GDNF-Ret signaling raises the possibility that parkin could be translationally controlling the GDNF receptor, Ret, in the DA system of mice.

5.13 Ret nuclear localization

Ligand-based activation of RTK's leads to internalization mostly through clathrin-mediated endocytosis and subsequent activation of downstream targets in signal transduction. Subsequently, these RTK's are degraded and recycled to the cell surface. However, recent evidence also supports the trafficking of RTK's to other intracellular compartments like cytoplasm, mitochondria and nucleus upon ligand-occupied activation ***(Hsu & Hung 2007; Carpenter & Liao 2009; Lemmon & Schlessinger 2010)***. From 20 different subfamilies with a total of 58 RTK's that are identified so far, 18 distinct RTK's were reported to traffic from the cell surface into the nucleus upon ligand binding ***(Carpenter & Liao 2013)***. Supporting the notion of RTK nuclear localization, preliminary results from parkin-mediated Ret internalization experiments indicated a sign of Ret nuclear localization in my study. In agreement with the preliminary findings, sub-cellular fractionation of SK-N-BE(2) cultures confirmed the presence of Ret in the nuclear fraction (Figure 4.16.1 A). These findings were also confirmed in the ventral midbrain of mouse (Figure 4.16.1 B) which is physiologically more relevant. Further characterization of Ret nuclear translocation using the classical surface biotinylation and GDNF treatment, revealed that ligand mediated activation of Ret increases its nuclear localization over time and induces Ret–DNA interaction in the nucleus (Figure 4.16.2), consistent with a potential role for Ret in gene regulation. A recent report, demonstrated Ret localization into the nucleus, where it functions as a tyrosine–threonine dual kinase in regulating a transcriptional factor, Activating Transcription Factor 4 (ATF4) that targets NOXA and PUMA to mediate stress-induced apoptosis ***(Bagheri-Yarmand et al. 2015)***. However, this study reports that ligand-mediated Ret activation is needed for its nuclear transport, whereas in my study Ret nuclear localization was observed with TBS treatment and without ligand activation (Figure 4.16.2) in both the nuclear soluble fraction as well as in the nuclear insoluble fraction. One reason for this observation could be the presence of growth factors in serum that leads to Ret activation in the absence of added ligand. Importantly, GDNF treatment increased the translocation of Ret into nucleus and Ret–DNA interaction, supporting the findings that ligand dependent Ret activation is necessary for its nuclear transport and further gene regulation.

Effects on ATF4 might help to explain my observations with MEN2B mutant mice and parkin. As just mentioned, Ret was shown to repress proapoptotic genes by phosphorylating ATF4

and inhibiting its activity in the nucleus ***(Bagheri-Yarmand et al. 2015)***. Interestingly, in a different study, ATF4 was shown to bind to the parkin promoter and upregulate its transcription during mitochondrial and ER stress via the unfolded protein response (UPR) to promote cell survival ***(Bouman et al. 2011)***. Under severe stress conditions parkin transcription was also found to be repressed by JNK3 via c-Jun, a transcriptional repressor of parkin to suppress cytoprotective pathways ***(Hunot et al. 2004; Brecht et al. 2005; Bouman et al. 2011)***. Together these findings facilitate the understanding of how oncogenic nature of MEN2B could lead to suppressed parkin in MEN2B mutant mice (Figure 4.13 A & H). Conceptually, MEN2B might be phosphorylating ATF4 through its tyrosine-threonine dual kinase activity, thereby negatively regulating ATF4 leading to transcriptional repression of parkin.

5.14 Conclusions

The importance of GDNF-Ret signaling in the DA system and how it is affected during PD pathogenesis were the focus of this study. Here, I show that parkin loss of function directly correlates to diminished GDNF-Ret signaling in the DA system, hastening cell death and leading to overt pathological features of PD. The idea that DA neurons could be desensitized to trophic support due to the disruption of Ret expression resulting from the loss of function mutations of parkin, urges for the co-expression of parkin together with GDNF in disease modifying therapies targeted to the early stages of PD. Further, using parkin and Ret doubly deficient mice for preclinical testing, considering the cell-type specific and progressive degeneration of DA system in these mice, will not only help with finding potential therapeutic compounds that have neuroprotective and restorative abilities, but also to develop novel treatments for PD patients. Alterations in the intricate functions of Ret are implicated in many diseases including cancer. Given that parkin's presence augments Ret activity, parkin could be a potential target in Ret-mediated tumorigenesis.

Together, this study sheds light on the molecular mechanisms, of parkin and Ret cross-talk and how alterations in their signaling might be a cause of degeneration of DA neurons, as observed in PD patients and conversely, how parkin could complement Ret activity and its oncogenic signaling in cancer, suggesting that parkin and Ret signaling pathways might be an ideal target to both life-threatening diseases.

5.15 Future perspectives

To further our understanding of parkin and Ret cross-talk outside the DA system, parkin-deficient MEN2B mice can be examined for their ability to produce copulation plugs as 83% of male MEN2B mutant mice were shown not capable of producing copulation plugs. Further, MEN2B mutant mice depict pheochromocytoma and C-cell hyperplasia phenotypes, whereas

parkin-deficient MEN2B mice could be analyzed for the normalization of these phenotypes. Additionally, tumors of the adrenal glands of MEN2B mutant mice express TH and chromogranin A (CGA) which resembles human pheochromocytomas, and parkin-deficient MEN2B mice can be analyzed for TH and CGA expression ***(Smith-Hicks et al. 2000)*** to confirm the cross-talk of parkin and Ret outside the DA system.

Based on the alterations observed in the key signaling molecules that are known to modulate mRNA translation initiation, parkin was suggested to translationally regulate Ret protein levels in the present study. However, to prove that indeed parkin controls Ret translation, newly synthesized proteins in both control and parkin-KD SH-SY5Y cultures can be metabolically labeled using azidohomoalanine (AHA) and with the help of click-chemistry, reactive azide group of AHA can be covalently linked to an alkyne bearing biotin-tag or a flag-tag. Further, biotin or flag possessing newly synthesized proteins can be purified and analyzed. In principle, the effect of parkin-KD on both general translation and of Ret specifically can be assessed with this technique. Polysome profiling of control and parkin-KD SH-SY5Y cultures could also provide complementary insight into the translational activity in the presence or absence of parkin. In particular, whether parkin is altering ribosome recruitment and/or ribosome density on Ret mRNA can be elucidated by analyzing different fractions collected from the polysome profiling for Ret mRNA using quantitative RT–PCR. Additional mechanistic insight could potentially be gained by examining how the presence of parkin affects polysome association of the translational repressors that re recently described to be substrates for parkin (Pumilio/Pum, hnRNP-F/Glorund, Dcp1, GW182 and POP2) ***(Gehrke et al. 2015)*** by immunoblotting. More generally, the work in this thesis supports a more thorough direct characterization of translational control of Ret by parkin-mediated signaling.

Here I found that parkin can complement GDNF/Ret signaling and both *in vitro* and *in vivo* findings confirm the molecular interaction of parkin and Ret. However, it is important to understand if this interaction is direct or is mediated through other proteins like EPS15. Considering the previous findings that parkin negatively regulates EGFR endocytosis and thereby promoting the EGF/EGFR mediated downstream signaling, through ubiquitination of EPS15 ***(Fallon et al. 2006)***, it seems essential to better understand the importance of parkin in mediating Ret internalization in order to elucidate the role of parkin and Ret molecular interaction in the DA system.

6. References

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