KOCAELİ UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOMEDICAL ENGINEERING

DOCTOR OF PHILOSOPHY
IN
BIOMEDICAL ENGINEERING

THE EFFECT OF EXOGENEOUS WILD-TYPE AND A COMPOUND- HETEROZYGOTE MUTANT (Q311R; A371T) PARKIN EXPRESSIONS ON NUCLEAR PROTEOME

BY

ABULA AYIMUGU

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Abula AYIMUGU

Prof.Dr. Murat KASAP
Supervisor, Kocaeli University

Prof.Dr Serdar KÜÇÜK
Jury member, Kocaeli University

Assoc.Prof.Dr. Gürler AKPINAR
Jury member, Kocaeli University

Assoc.Prof.Dr. Bekir ÇÖL
Jury member, Muğla Sıtkı Koçman University

Assoc.Prof.Dr. Mehmet ÖZTÜRK
Jury member, Bolu Abant İzzet Baysal University

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CONTENTS

DEDICATION AND ACKNOWLEDGEMENT i
CONTENTS ii
LIST OF FIGURES iv
LIST OF TABLES vi
LIST OF ABBREVIATIONS vii
ÖZET ix
ABSTRACT x
INTRODUCTION 1
1. LITERATURE REVIEW 3
1.1. Parkinson's Disease (PD) 3
1.1.1. PD overview 3
1.1.2. The Pathology of PD 5
1.2. The Molecular Pathways to PD 6
1.3. Parkin Protein Function 12
1.4. PD and Proteomics Studies 14
1.4.1. Overview of Proteomics studies related to PD 14
1.4.2. *In vitro* cellular models of PD used in Proteomics studies 16
1.5. PD and Cancer 18
1.6. Phosphoproteomics 19
1.7. Objectives 20
2. MATERIAL AND METHODS 22
2.1. Used Materials 22
2.1.1. Chemicals and kits 22
2.1.2. Solutions 22
2.2. Methods 22
2.2.1. Cell culture 22
2.2.1.1. Maintenance of cell lines 22
2.2.1.2. Cryopreservation of cell lines 23
2.2.1.3. Thawing the cells 23
2.2.1.4. Passaging cells 23
2.2.1.5. Cell counting with hemacytometer 24
2.2.2. Optimization of nuclear protein enrichment methods 25
2.2.2.1. Enrichment of nuclear proteins using ReadyPrep Protein Extraction Kit 25
2.2.2.2. Enrichment of nuclear proteins using OptiPrep density gradient centrifugation method 26
2.2.2.3. Enrichment of nuclear proteins using Q-Proteome Nuclear Protein Enrichment kit 26
2.2.3. Protein extraction and proteomics experiments 27
2.2.3.1. Preparation of total cell free extracts 27
2.2.3.2. Determination of protein concentration 27
2.2.3.3. Cleaning of the protein samples 27

2.2.3.4. Isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis 28
2.2.3.5. Image analysis of 2D gels 29
2.2.3.6. Preperation of Sodium dedocyl sulphate-polyacrylamide gels (SDS- PAGE) for western blotting..... 30
2.2.3.7. Western Blotting 30
2.2.4. Phosphoproteomics 31
2.2.4.1. Sample preparation for phosphoproteomics 31
2.2.4.2. Phosphoprotein extraction 31
2.2.4.3. ProQ Diamond phosphoprotein staining 32
2.2.4.4. Sypro Ruby protein staining 32
2.2.4.5. 2DE and Western Blotting analysis for phosphoproteins..... 33
2.2.5. Mass spectrometer analysis..... 34
2.2.5.1. In -gel tryptic digestion 34
2.2.5.2. ZipTip C18 purification for desalting of the peptide solutions..... 34
2.2.5.3. Protein identification by MALDI-TOF/TOF..... 35
2.2.5.4. Statistical analysis..... 35
2.2.5.5. Bioinformatics analysis..... 35
3. RESULTS AND DISCUSSION 36
3.1. Comparison of Nuclear Protein Isolation Methods 36
3.2. Assessment of the WT and the Mutant Parkin Expressions in Enriched Nuclear Protein Fractions 42
3.3. Assessment of Enrichment Levels in Nuclear Protein Extracts Used for Comparative Nuclear Proteome Analysis..... 42
3.4. Comparative 2DE Analysis of the Enriched Nuclear Protein Fractions Expressing Parkin Proteins 43
3.5. Phosphoprotein Analysis of the Protein Extracts Expressing Parkin Proteins 48
3.6. 2D Western Blotting for Verification of the Data Obtained from ProQ-Diamond Stained Gels 52
4. DISCUSSION AND FUTURE WORK..... 55
4.1. Discussion..... 55
4.2. Conclusion 60
4.3. Future Work..... 61
REFERENCES..... 62
SUPPLEMENTARY 79
PUBLISHED PAPERS 85
CURRICULUM VITAE 87

LIST OF FIGURES

Figure 1.1. A schematic diagram showing the molecular paths contribute to PD. 7
Figure 1.2. Pathways of Mitochodrial malfunctions associated with PD pathology 8
Figure 1.3. Pivotal role of chronic oxidative stress in regulating PD progression 9
Figure 1.4. Summary of the effect of PTMs on PD- related proteins 11
Figure 1.5. Working principle of Parkin protein and possible Parkin substrates. 12
Figure 1.6. The structure of Parkin. 13
Figure 3.1. 2D gel electrophoresis analysis of nuclear proteins obtained using Ready Prep Nuclear Protein Extraction kit. 36
Figure 3.2. Determination of histone, lamin, GAPDH and cyclophilin A amounts in the enriched nuclear protein fractions obtained by using Ready Prep Nuclear Protein Extraction kit. 37
Figure 3.3. Extraction of nuclear proteins using density gradient centrifugation method. 38
Figure 3.4. 2D gel electrophoresis analysis of nuclear proteins obtained using differential centrifugation method. 38
Figure 3.5. Determination of histone, lamin, GAPDH and cyclophilin A amounts in enriched nuclear proteins using density gradient centrifugation method. 39
Figure 3.6. Pie chart for demonstration of the enriched protein distribution within the cells 39
Figure 3.7. Determination of histone, GAPDH, Lamin A / C and Cyclophilin A amounts in nuclear proteins enriched with Q-Proteome kit. 40
Figure 3.8. 2D gel electrophoresis analysis of the enriched nuclear proteins obtained using the QProteome nuclear protein isolation method. 41
Figure 3.9. Comparative analysis of nuclear protein enrichment methods. 41
Figure 3.10. Western blot analysis of the WT and the mutant Parkin expressions in nuclear protein extracts enriched from SH-SY5Y cells 42
Figure 3.11. Western blot analysis to demonstrate nuclear protein enrichments in protein fractions isolated from SH-SY5Y cells 43
Figure 3.12. 2DE gel images of SH-SY5Y cells expressing either the wild-type Parkin or the mutant Parkin proteins 44
Figure 3.13. Validation of differentially regulated protein spots 46
Figure 3.14. 2DE gel images used for analysis of the changes occurring at the phophoproteome level 48
Figure 3.15. Changes occurring at the phosphoproteome levels 50
Figure 3.16. 2D Western Blotting for verification of the nuclear proteins 53

Figure 4.1. STRING analysis for the differentially regulated nuclear proteins identified from nucleic acid binding and nuclear insoluble protein fractions. 61

LIST OF TABLES

Table 2.1. Chemicals and kits used in the experiment 22
Table 2.3. SDS-PAGE gel contents 29
Table 2.4. List of primary (1 °) and secondary (2 °) antibodies used in Western blot analysis 31
Table 2.5. Phosphoproteins ProQ Diamond stain procedure 32
Table 2.6. Phosphoproteins Sypro Ruby stain procedure 33
Table 2.7. List of primary (1°) and secondary (2°) AB used in phosphoprotein Western blot analysis. 34
Table 3.1. The list of differentially regulated proteins upon expressions of the wild-type or the mutant Parkin proteins in the nucleic acid binding and the insoluble protein fractions enriched from SH-SY5Y cells. 45
Table 3.2. The list of differentially regulated proteins upon expressions of the wild-type or the mutant Parkin proteins in the nucleic acid binding and the insoluble protein fractions enriched from SH-SY5Y cells. 47
Table 3.3. Phosphoproteins from total cell extracts that were differentially regulated after the WT and the mutant Parkin protein expressions 49
Table 3.4. Phosphoproteins from total cell extracts that were not significantly regulated after the WT and the mutant Parkin protein expressions. 49
Table 3.5. Phosphoproteins that were differentially regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions. 51
Table 3.6. Phosphoproteins that were not significantly regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions. 52
Table 3.7. List of the differentially phosphorylated/dephosphorylated proteins that were differentially regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions 54

LIST OF ABBREVIATIONS

2D	:	Two-Dimensional Gel Electrophoresis (İki Boyutlu Jel Elektroforezi)
6- OHDA	:	6- hydroxydopamine (6-hidrokisdopamin)
ACN	:	Acetonitrile (Asentonitril)
AE	:	Mutant type (Mutant tıp)
AmBic	:	Amonium bicarbonate (Amonyum bikarbonat)
APS	:	Amonium persulfade (Amonyum persulfade)
ARJP	:	Otosomal recessive Juvenile Parkinsonism (Otozomal resesif Juvenil Parkinsonizm)
ATM	:	Ataxia talengiectasia mutated gene (Ataksi talenjiektazi mutastonlu gen)
BSA	:	Bovine serum albumin (Sığır serum albümini)
CBB	:	Coomassie Briliant Blue (Coomassie Briliant Blue)
CHAPS	:	3-[(3-Cholamidopropyl)Dimethyl-Ammonio]-1-Propanesulfonate (3--[(3-Kolamidopropil)Dimetil - Ammonio]-1-Propanesülfanat)
CPEB	:	Cytoplasmic protein extraction buffer (Sitoplazmik protein ekstraksiyon tampon)
CSF	:	Cerebrospinal fluid (Beyin omurilik sıvısı)
DA	:	Dopaminergic (Dopaminerjik)
DDIT3	:	DNA damage-inducible transcript 3 protein S (DNA hasarına neden olan transkript 3proteinS)
DMSO	:	Dimethyl sulfoxide (Dimetil sulfoksit)
DTT	:	Dithiothreitol (Dithiothreitol)
ECHM	:	Enoyl-CoA hydratase (Enoil-CoA hidrataz)
EDTA	:	Ethylenediaminetetraacetic acid (Etilendiamintetraasetik asit)
FBS	:	Fetal bovine serum (Fetal sığır serumu)
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase (Gliseraldehit-3-fosfat dehidrojenaz)
GTP	:	Guanosine-5'-triphosphate (Guanozin-5'-trifosfat)
HMGB1	:	High mobility group protein (Yüksek mobilite grubu proteini)
HNRNPC	:	Heterogeneous nuclear ribonucleoproteins C1/C2 (Heterojen nükleer ribonükleoplroteinleri)
IAA	:	Iodoacetamide (Iodoacetamide)
IBR	:	In-between RING domain (In-between RING bölgesi)
IEF	:	Isoelectric focusing (Izoelektrik odaklama)
IPG	:	Immobilize pH Gradient (Sabit pH Gradyanı)
Kda	:	Kilo Dalton (Kilo Dalton)
LB	:	Lewy body (Lewy cismi)
LRRK2	:	leucine- rich repeat kinase 2 gene (lösin-zengin tekrar kinaz 2 geni)
MALDI-TOF/TOF	:	Matrix Assisted Laser Desorption Ionization - Time of Flight (Matriks destekli lazer desorpsiyon iyonizasyonu-uçuş süresi
MATH	:	Methamphetamine (Metamfetamin)
MC1R	:	Melanocortin (Melanokortin)
MS	:	Mass Spectrometry (Kütle spektrometrisi)

NABPF	:	Nucleic acid binding protein fraction (Nükleik asit bağlayıcı protein fraksiyonu)
NAC	:	Aβ component of amyloid (Amiloid Aβ bileşeni)
NIPF	:	Nuclear insoluble fraction (Nükleer insolübl fraksiyonu)
NUCL	:	Nucleolin (Nucleolin)
PARK2	:	Parkin gene (Parkin geni)
PARP1	:	Poly(ADP-ribose) polymerase 1(Poli (ADP-riboz)polimeraz
PBS	:	Phosphate Buffer Saline (Fosfat tamponlu tuz çözeltisi)
PD	:	Parkinson Disease (Parkinson Hastalığı)
PINK1	:	PTEN-induced kinase 1 (PTEN-indüklenmiş kinaz 1)
PMF	:	Peptide mass fingerprint (Peptid kitle parmak izi)
PRS7	:	26S protease regulatory subunit 7(26S protaz düzenleyici subunit 7)
PTM	:	Post-translational modification (Post-translasyonal modifikasyon)
PVDF	:	Polyvinylidene difluoride (Polivinilidin diflorür)
RBR	:	RING1, IBR and RING2 (RING1, IBR veRING2)
RNS	:	Reactive nitrogen species (Reaktif azot türleri)
ROS	:	Reactive oxygen species (Reactive oksijen türleri)
SD	:	Standard deviations (Standart sapma)
SDS-PAGE	:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Sodyum dodesil sülfat –poliakrilamid jel elektroforezi)
SH-SY5Y	:	Neuroblastma cell lines (Nöroblastom hücre hattı) cell line
SN	:	Substantia nigra (Substantia nigra)
SNCA	:	Alpha-synuclein gene (Alfa-synuclein geni)
SNpc	:	Substantia nigra pars compacta (Substantia nigra pars compacta)
SSP	:	Standart Spot Number (Standart Spot numrası)
TBS	:	Tris-buffered saline (Tris-tamponlu tuz)
TBST	:	Tris-buffered saline, 0.1% Tween 20 (Tris-tamponlu tuz, 0.1% Tween 20)
TCEP	:	Tris (2-carboxyethyl) phosphine (Tris(2-karboksietil)fosfin)
TCTP	:	Translationally controlled tumor protein (Translasyonal olarak control edilen tümör protein)
TEMED	:	Tetramethylethylenediamine (Tetrametilendiamin)
TFA	:	Trifluoroacetic acid (Trifloroasitik asit)
TPI	:	Triosephosphate isomerase (Triosfosfat izomeraz)
Ub	:	Ubiquitin (Ubiquitin)
Ubl	:	Ubiquitin-like domain (Ubiquitin- benzer bölge)
UL	:	Ubiquitin ligase (Ubiquitin ligaz)
UPS	:	Ubiquitin proteasome system (Ubiquitin proteosome sistemi)
WB	:	Western BloT (Western BloT)
WT	:	Wild type (Yabanıl tıp)
α-SYN	:	α-synuclein (α-synuclein)

YABANIL TİP VE MUTANT PAKİN(Q311R; A371T)EKSPRESE EDEN NÖROBLASTOMA HÜCRELERİNDEN ELDE EDİLEN NÜKLEER PROTEİN ÖZÜTLERİNİN PROTEOME ANALİZİ

ÖZET

Parkin 52 kDa'luk E3 ubikitin ligaz aktivitesi gösteren bir proteindir. Parkin ubikitin-proteozom sisteminde rol alarak hücre içi artık ve ömrünü tamamlamış proteinlerin temizlenmesinde rol almaktadır. Parkin üzerinde meydana gelen bazı mutasyonlar erken yaşta ortaya çıkan Parkinson Hastalığına sebep olmaktadır. Bu nedenle Parkin bilim adamlarının dikkatini çekmiş, bu protein ile sayısız çalışma yapılmış ve bu proteinin Parkinson hastalığındaki önemine dair önemli bilgiler elde edilmiştir. Son yıllarda yapılan çalışmalar Parkin'nin bilinen rolünün yanı sıra kanserle olan ilişkisini de ortaya çıkarmış ve Parkin'i kanser çalışmalarının merkezine oturtmuştur. Parkin bir çok kanser türünde delesyona uğradığı için tumor süpresör gen kategorisinde değerlendirilmeye alınmıştır. Hatta yapılan bazı çalışmalarda Parkin'in tumor supresör görevini nasıl yerine getirdiğine dair ipuçları elde edilmiş ve Parkin'nin hücre döngüsünde rol alan bazı proteinleri doğrudan etkilediği gösterilmiştir. Ancak tüm bu çalışmalar Parkin ve kanser arasındaki ilişkiyi detayları ile ortaya koyacak nitelikte değildir. Bu nedenle bu tez kapsamında Parkin ve kanser arasındaki ilişkiyi daha detaylı inceleyebilmek için Parkin ifade eden SH-SY5Y hücrelerinden nüklear proteinleri zenginleştirerek karşılaştırmalı çekirdek proteomu ve çekirdek fosfoproteomu analizleri yaptık. Elde ettiğimiz sonuçlar bize bu güne kadar Parkin'le doğrudan veya dolaylı olarak etkileştiği bilinmeyen proteinlerin varlığını ortaya çıkardı. Tanımlanan bu proteinler ile yaptığımız biyoinformatik analizler sonucunda Parkin'nin tumor süpresör aktivitesini DNA tamir mekanizmalarına verdiği destek üzerinden yapabileceğini gösterdik. Bu tez çalışması Parkinson hastalığı ile kanser arasındaki köprünün kurulması için atılmış bir adım olup elde edilen veriler bu ilişkinin detayları hakkında bizlere ışık tutmaktadır.

Anahtar Kelimeler: 2D Jel Elektroforezi, Çekirdek Proteomu, Fosfoproteom, Kanser, Parkin.

THE EFFECT OF EXOGENEOUS WILD-TYPE AND A COMPOUND-HETEROZYGOTE MUTANT (Q311R; A371T) PARKIN EXPRESSIONS ON NUCLEAR PROTEOME

ABSTRACT

Parkin is a 52 kDa protein with an E3 ubiquitin ligase activity. It plays a significant role in the ubiquitin-proteasome system and acts as a regulator of protein breakdown. Parkin's recognition emanates from its involvement in early-onset Parkinson's disease. In recent years, Parkin was also placed into the center of cancer research due to its tumor suppressor activity. However, the details about how Parkin as a tumor suppressor protein, plays a role in the development or progress of cancers remains unknown. The tumor suppressor activity of Parkin might be carried out by its nuclear form and may not be relevant to its E3 ubiquitin ligase activity. Thus, monitoring the metabolic activity of Parkin in the nucleus should provide clues about the involvement of Parkin in cancer. In here, an effort was placed on determining the changes at the nuclear proteome in the wild-type and a compound-heterozygous mutant Parkin (Q311R and A371T) expressing SH-SY5Y cells. Nuclear proteins were enriched and the changes occurring at the nuclear proteome level upon Parkin expressions were studied using 2D gel electrophoresis coupled with MALDI-TOF/TOF analysis. In addition, changes in phosphorylation levels in total and nuclear protein extracts upon Parkin expressions were also determined using ProQ-Diamond protein stain. A list of differentially regulated proteins that were not previously known to interact or associate with Parkin was generated. The differentially regulated proteins pointed to DNA repair mechanisms and involvement of Parkin and its putative partners in the repair of damaged DNA. The findings documented in here contributed to the current literature and shed some light onto some of the unknowns about PD and cancer.

Keywords: 2D Gel Electrophoresis, Nuclear Proteome, Phosphoproteome, Cancer, Parkin.

INTRODUCTION

The Parkin protein is an E3-ubiquitin ligase which removes improperly folded proteins by sending them to proteosomes for degradation. To date, at least 24 Parkin substrates have been discovered. In cases where Parkin loses its activity, Parkin substrates accumulate in the cells. The group of cells mostly affected by this accumulation is shown to be the motor neurons which are located in Substantia Nigra of the mid-brain. Death of motor neurons, due to complications caused by Parkin mutations, affect control of movements in individuals and ultimately lead to Parkinson's disease (PD). Molecular mechanisms underlining the effect of Parkin mutations are well documented (Kasap, et al., 2017).

In our laboratory within the scope of the TUBITAK project No. 110S310, we demonstrated that the Parkin protein is not only found in the cytoplasm but also localized to the cell nucleus. We have observed that the nuclear Parkin protein is post-translationally modified and the findings obtained are in accordance with the literature (Kasap et al., 2009; Winklhofer 2014; Kao et al., 2009). However, many questions related to the nuclear Parkin protein were not answered at all or partial answers were provided (Alves da Costa C, et al., 2019). Among those questions, two were important to answer; (1) Does nuclear form of Parkin affect the expression of other proteins? (Sriram SR, et al., 2005) (2) Does Parkin carry out its nuclear functions via its E3 ubiquitin ligase activity? (Cookson MR, et al., 2003)

In the early 1990s, epidemiological studies revealed an odd relationship between PD and cancer without providing the molecular details. Various types of cancers were shown to lack Parkin gene or had substantially decreased levels of Parkin expression (Sun XD, et al., 2013). These observations provided the idea that Parkin might be a tumor suppressor protein (Cesari R, et al., 2003). The subsequent studies carried out revealed the presence of several cell-cycle regulation proteins e.g., Cyclin E and D whose levels increase when Parkin was mutated/deleted within the cells. However, the question of how Parkin caused accumulation of those cell cycle proteins

remained unanswered. Unfortunately, the details of Parkin-Cancer relationship is still missing and are needed to be elucidated. In this thesis, to help elucidation of Parkin's contribution to the formation and progress of cancer, we studied the changes on nuclear proteome upon Parkin expression in neuroblastoma cancer cell line (SH-SY5Y). Our findings provided a list of candidate proteins that may play role both in cancer and PD.

1. LITERATURE REVIEW

1. 1. Parkinson's Disease (PD)

1.1.1. PD overview

PD is the second most common progressive neurodegenerative disorder of central nerves system that has complex nature of both non-motor and visible motor symptoms. The first description and clinical symptom of the PD was identified by James Parkinson in *an essay on the shaking palsy* as "paralysis agitans" two hundred years ago. According to James Parkinson's definition, shaking palsy was a nervous disorder characterized by clinical vignettes such as trembling of the limbs at rest, lessened muscular power and a stooped posture associated with a propulsive, festinant gait (Parkinson, 1817). In addition to the original definition of James Parkinson, Jean Martin Charcot redefined the disease by adding muscle rigidity and sensory changes, and substituted the *shaking palsy* with name of Parkinson's disease to commemorate James Parkinson(Goetz, 2002).

PD is a devastating neurodegenerative disorder in developed countries after Alzheimer's disease. An estimation of seven to 10 million people worldwide affected by PD and the incidence of the disease is generally increasing in parallel to the increase in elderly population (Delenclos, et al., 2016). Meta-analysis studies of PD indicate a rising tendency of prevalence in worldwide (Lauren Hirsch, et al., 2016). The incidence rates of the disease also changes on different geographic locations (a prevalence of 1601 per 100,000 in patients from North America, Europe and Australia, and a prevalence of 646 per 100,000 in Asian patients) (Pringsheim, et al., 2014).

PD cannot be defined as the disease of aged people. Approximately four percent of the patients with PD are diagnosed under the age of 50 (Lauren Hirsch, et al., 2016). It was found that men are 1.5 times more vulnerable to have PD than women

(Wooten, et al., 2004). The disease drastically affects patients' quality of life by impeding patient's social life, worsen the financial situation

The clinical diagnosis of PD is based on motor symptoms including rigidity, resting tremor, bradykinesia and postural imbalance and non-motor symptoms such as autonomic dysfunction, neuropsychiatric problems (mood, cognition, behaviour or thought alterations), sensory (especially altered olfactory) and sleep difficulties (Jankovic, 2008). Currently, PD is mainly diagnosed and graded by using the Parkinson's UK Brain Bank criteria, which relies on combined central motor and non-motor symptoms (Postuma, et al., 2015). The pathogenic mechanisms that cause neurodegeneration in PD are not fully known due to the main reason that PD is a multiplex system disorder. In other words, potential risk factors confluence of environmental triggers and genomic defects play pivotal role in the formation of PD (Gasser, 2007). However, up to 50% of the patients diagnosed with PD are falsely diagnosed due to the interference of Parkinsonian-like diseases such as corticobasal degeneration (CBD), multiple system atrophy (MSA), dementia with Lewy bodies (LB), and progressive supranuclear palsy (PSP) (Jellinger, 2003**;** Constantinescu, 2013).

Classical therapeutic interventions consist of pharmacotherapy, deep brain stimulation, and physiotherapy (Cacabelos R., 2017). The pharmacological treatment for PD is focused on restoration of neurotransmitter malfunction in the basal ganglia. Conventional drugs such as dopamine precursors –levodopa, dopamine agonists for symptomatic treatments, monoamine oxidase (MAO) inhibitors, O-methyltransferase (COMT) inhibitors, and other antiparkinsonism drugs are used to halt or reverses the progression of the disease (Cacabelos, 2012**;** Katzenschlager, 2002**;** Cacabelos, et al., 2016**;** Cacabelos, et al., 2017). Recently, neurotrophic factor-based therapies (Tiago Martins Rodrigues, et al., 2014), gene therapy (Sudhakar, et al., 2018), fetal mid-brain transplantation (Olanow, et al., 2003), and stem cell-based therapy (Barker, 2013) are developed to improve and compensate the efficacy of therapeutic treatment. Overall, although PD cannot be cured, the drugs and the approaches used for treatment can relief the symptoms of the disease.

1.1.2. The Pathology of PD

Numerous studies have been undertaken to elucidate pathological mechanism of PD. PD is both a non-motor and a motor disorder. PD-associated neurodegeneration presumably starts up in the prodromal phase which lasts 5–20 years before the onset of the first clinical motor symptoms (Hawkes, 2008). The main pathological sign of PD is the loss of 70% of the dopaminergic neurons in the Substantia Nigra pars Compacta (SNpC) of the mid-brain (Davie, 2008). Lewy bodies which are alpha-synuclein (α-SYN) positive protein- and lipid aggregates are the histological hallmark of the PD. Neuropathological conditions such as genomic defects, epigenetic changes, toxic exposure, oxidative stress, neuroinflammation, metabolic deficiencies; ubiquitin–proteasome system dysfunction and the others pave the way for protein misfolding , aggregation and premature neuronal cell death (Cacabelos, et al., 2017; Rokad, et al., 2016; Toledo, et al., 2013; Irwin, et al., 2017; Wen , et al., 2016; Nussbaum , 2017; Lill , 2016; Xie, et al., 2017).

The genes most commonly associated with PD include α-synuclein gene (SNCA) (encodes α-SYN), PARK8 (encodes LRRK2), Glucocerebrocidase gene (GBA gene) (GBA gene encodes β-Glucocerebrosidase), Park2 (Park2 gene encodes Parkin) , PINK1gene (encodes PTEN-induced kinase 1) , PARK7 gene (encodes DJ-1), VPS35gene (encodes Vacuolar protein sorting-associated protein 35). α-SYN holds the most important clues about the pathology of PD since it can form self-protein aggregates when it is mutated (Han, et al., 1995). Several point mutations were described at the N-terminal region of α-SYN (E46K, A53T, and A30P) and shown to be causing familial PD (Li, et al., 2001; Fredenburg, et al., 2007; Sahay, et al., 2015). Some studies also demonstrated that α-SYN is independently capable of causing PD when SNCA is duplicated or triplicated (Singleton, et al., 2003; Miller, et al., 2004; Chartier, et al., 2015).

In addition to α-SYN, mutations in Parkin (PARK2), PTEN-induced putative kinase 1 (PINK1), Leucine-rich repeat kinase 2 (LRRK2) might contribute to the pathogenesis of familial forms of PD (Lill, 2016; Lardenoije, et al., 2015; Coppedè , 2012; Hernandez, et al., 2016; Hill-Burns, et al., 2016; Chen , 2016; Sandor, 2017). The rare GBA variant N370S is the strongest genetic risk factor for idiopathic forms

of PD (Lill, et al., 2012). A survey of VPS35, a gene encoding vacuole protein sorting protein 35, was shown carry a point mutation (D620N) in a Swiss family, and was strongly correlated with the late onset familial PD (Mohan, 2016). Even though studies associated with PD pathogenesis keep on discovering new genes such as, TMEM230 (Deng, et al., 2016), genetic causes of PD cannot be the only cause of PD.

The cause and progress of PD cannot be explained by a single factor. That assumption contradicts to the multifactorial nature of PD. The joint effects of genetic vulnerability and environmental factors together may lead to PD. For example, neurotoxicants or viruses may enter the body through the nasal cavity or the digestive tract (Hawkes, et al., 2007) and may induce Lewy pathology in susceptible people (Doty 2009; Hawkes, et al., 2009; Reichmann, 2011). Environmental conditions such as pesticides, herbicides and insecticides (mostly toxins) are also attributed as the potential risk factors for PD (Goldman 2013). A population-based case-control study suggested that people who were exposed to metals like manganese, copper, lead, iron, mercury and zinc have the tendency to become PD patients (Gorell, et al., 1999). Exogenous neurotoxins, carbon monoxide, carbondisulfide, hydrogen sulfide, trace elements, cyanide, varnish thinners, organic solvents and nitric oxides might be other risk factors for PD (Adler, et al., 2000).

1. 2. The Molecular Pathways to PD

Molecular studies of the genes associated with PD implicate several pathological metabolic events such as mitochondrial dysfunction, oxidative stress, protein mishandling, and deficits in post-translational modifications which contribute to PD pathogenesis (Figure1.1). I will briefly discuss each of these factors.

Figure 1.1. A schematic diagram showing the molecular paths contributing to PD (Kasap, et al., 2017).

Mitochondrial dysfunction: The initial study to indicate involvement of mitochondrial dysfunction to the pathology of PD demonstrated a decrease in the activity of ubiquinone oxidoreductase (complex I), in the SNpC of the PD brains (Swerdlow, et al., 1996). Recent advances have revealed that mitochondrial dysfunction is a central factor in PD pathophysiology since defects in mitochondrial functions and behaviours closely related to both sporadic and familial PD (Park, et al., 2018). To date several identified genes such as autosomal dominant SNCA, LRRK2 mutations, autosomal recessive Parkin, PINK1, cation-transporting ATPase 13A2 (ATP13A2) mutations as monogenic causes of familial PD were closely associated with the defects in mitochondria (Lill, 2016) (Figure 1.2). In addition, newly discoverd PD associated genes such as VPS35 and coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2) proteins were also found to be associated with mitochondrial function.

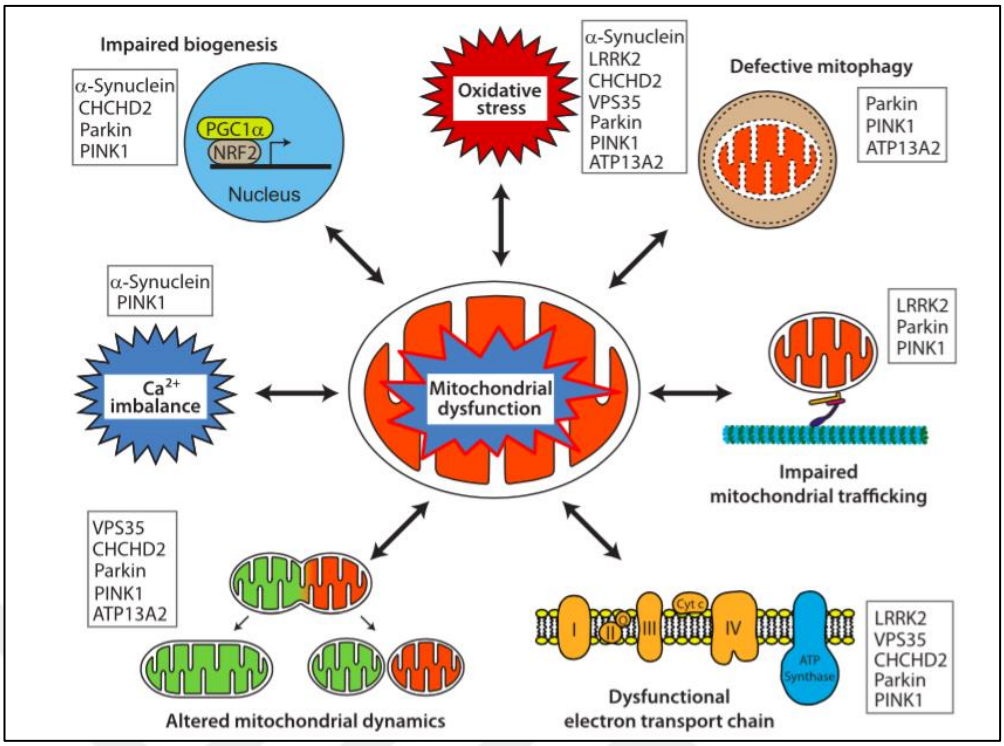

Figure 1.2. Pathways of Mitochodrial malfunctions associated with PD pathology (Jin-Sung Park, et al., 2018)

Defects in mitochondrial biogenesis, increase of reactive oxygen species(ROS) production, mitophagy malfunction, compromised trafficking in mitochondria, electron transport chain malfunction, alteration in mitochondrial dynamics and calcium imbalance may lead to mitochondrial dysfunction It is believed that these dysfunctions of mitochondria contribute to PD pathology. The variety of proteins such as α-SYN, CHCHD2, Parkin, PINK1, ATP13A2, VPS35, LRRK2 are the common causative factors for these interconnected complex cellular pathways which ultimately leads to neurodegeneration.

Oxidative stress**:** Oxidative stress is defined as a cellular damage caused by the imbalance between the level of ROS produced and the ability of a biological system to detoxify the reactive intermediates. It has been found that lipid peroxidation, protein carbonylation and DNA damage are the form of oxidative damage that caused in the nigral dopaminergic neurons (Jenner P., 2003). The causative factors for oxidative damage include high levels of ROS and reactive nitrogen species (RNS) (Dias, et al., 2013). Growing number of evidence indicate that loss of dopaminergic neurons in the midbrain is the result of a cascade of metabolic events triggered by the oxidative damage and mitochondrial dysfunction (Schapira, 2011; Zhu, 2010; Parker, et al., 2008;

Jenner, et al., 2006; Beal, 2005). This indication also advocated by the post-mortem brain analyses in the samples taken from PD patients which provided evidence for oxidative damage (Jenner, 2003; Yoritaka et al., 1996; Floor, 1998; Alam, et al., 1997; Zhang, et al., 1999). The correlation of oxidative stress and loss of dopaminergic neurons also confirmed by the study of animal models using toxins that trigger oxidative stress to imitate motor features of PD (Richardson, et al., 2005; Callio, et al., 2005; Vila, 2003; Perier, et al., 2003; Fukushima, et al., 1997). In fact, a gene called DJ-1 was found to be the causative gene for autosomal recessive form of PD, due to its association with oxidative stress. DJ-1 eliminates mitochondrial ROS and prevents damage to the cells (Andres-Mateos et al., 2007). In overall, studies demonstrated the importance of oxidative stress associated molecular pathways causing neuronal degeneration and identified previously unknown mechanisms leading to PD (Figure 1.3).

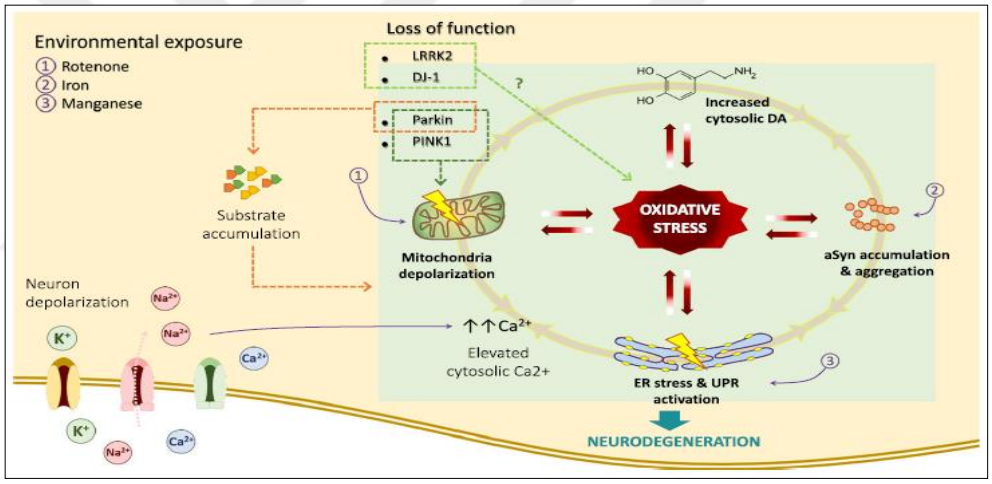

Figure 1.3. Pivotal role of chronic oxidative stress in regulating PD progression (Lesly Puspita, et al., 2017)

The figure 1.3 depicted the major role of oxidative stress in facilitation of PD progression. Oxidative stress can be triggered by mitochondria depolarization, endoplasmic reticulum (ER) stress, α-SYN aggregation and increased level of cytosolic DA. LRRK2, DJ-1, Parkin and PINK1 are the known potentional risk factors for familial cases of PD. Parkin is an E3 ubiquitin ligase which together with PINK1 scavange of damaged mitochondria, monogenetic mutations of LRRK2 leads to vulnerabilty to cellular oxidative stress, DJ-1 is an oxidative stress sensor, environmental exposures such as pesticide rotenone, iron and manganese lead to depolariztion of mitochondria which in turn cause oxidative stress.

Protein mishandling and aggregation: All neurodegenerative diseases share a common feature and accumulate misfolded proteins (Jucker M, 2013). There are varieties of possible reasons of protein accumulations in neurodegenerative diseases. Some proteins may have tendency to self-aggregate in the cells just like α-SYN. On the other hand, mutations in some other proteins contribute to their accumulation in either intra- or extracellular environment. Like some mutations which lead to changes in amino acid sequences, problems with post translational modifications, excessive cellular oxidative stress, and defects in protein synthesis cascade also contribute to PD. In PD, research on protein mishandling and aggregation mainly focused on α-SYN gene mutations.

Parkin on the other hand does not seem to directly affect the protein aggregation process. In fact, in some PD patients caused by Parkin mutations, no Lewy Body formation was observed (Karen M. Doherty, 2013) suggesting that Parkin induced PD has a different route to Parkinsonism. In either case, independent of Lewy body formation, protein misfolding seems to be the main player in PD formation. It appears that the cells cannot deal with the misfolded proteins either because of excess protein misfolding or the problems in misfolded protein handling.

Post-translational modifications(PTM): PTMs play crucial roles in PD pathogenesis, since PTMs bring into variety of changes to proteins which ultimately affect their function, stability, localization and interaction with other proteins (Guerra, et al., 2016; Vijayakumaran, et al., 2015, Rott, et al., 2017; Reimer, et al., 2018; Chakraborty, et al., 2017). The association between PTMs occurring on several PD associated key proteins e.g., α-SYN, PINK1, Parkin, DJ-1 and dynamin related protein 1(Drp1) and LRRK2 has been studied in large extent (Figure 1.4).

α-SYN is found over 90% Lewy bodies protein content and leads to neuronal degeneration, it is phosphorylated at Ser-129 (Zhang, et al., 2015; Reimer, et al., 2018; Karampetsou, et al., 2017; Zhong, et al., 2017). The phosphorylated α-SYN makes dopaminergic neurons more vulnerable to death (Karampetsou, et al., 2017). Further studies also indicated that SUMOylation (binding of different isoforms of the small ubiquitin like modifier protein to target proteins) affect accumulation, exocytosis and degradation of α-SYN (Vijayakumaran, et al., 2015;Rott, et al., 2017; Abeywardana, 2015;Kunadt, et al., 2015). The phosphorylations of PINK1 at Thr-175 and Thr-217

have also been reported to closely related to its association with Parkin (Chakraborty, et al., 2017). The autophosphoryalted PINK1 along with Parkin plays an important role in removal of damaged mitochondria from the cells to keep the cells alive (Jin-Sung Park, et al., 2018). In addition, the control of PINK1 levels intracellularly also administrated by ubiquitylation of PINK1 at Lys-137 (Liu, et al., 2017). Parkin loses its E3-Ubiquitylation activity when phosphorylated at Tyr-143 by c-Abl in an in vivo PD model. Also, when phosphorylated at Ser-94 Parkin gains the ability to control the dopamine release from neurons (Chakraborty, et al., 2017).

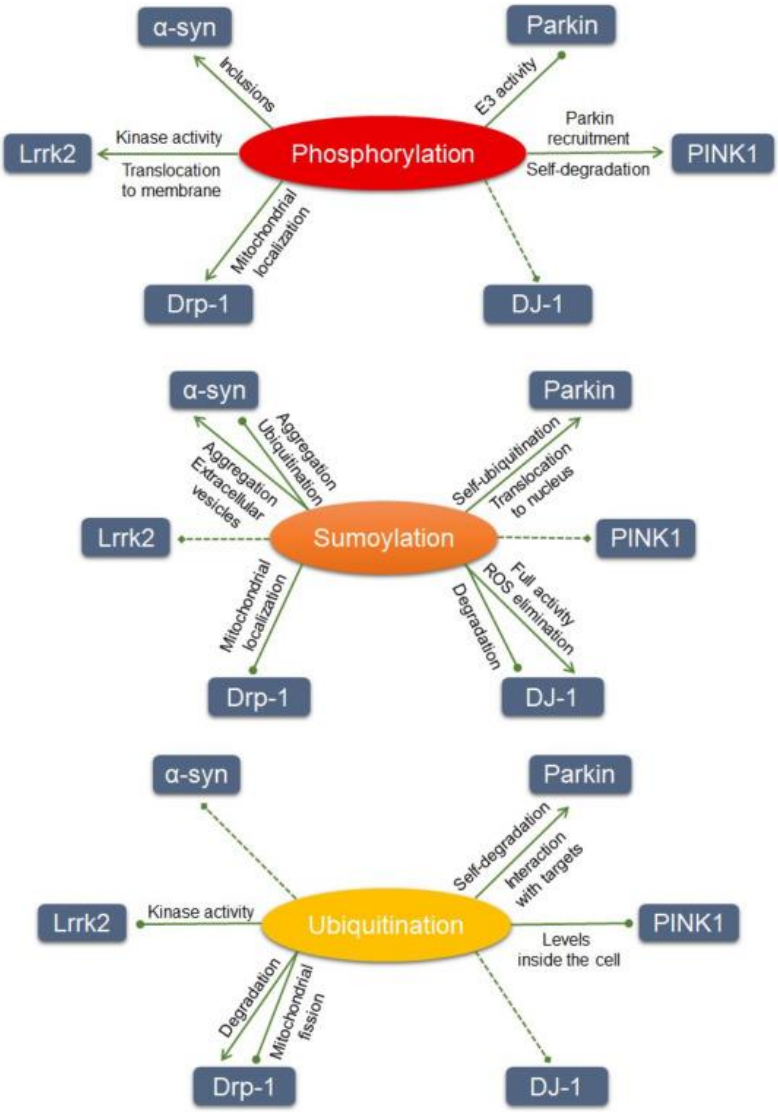

Figure 1.4. Summary of the effect of PTMs on PD- related proteins α-SYN, PINK1, Parkin, DJ-1 and Drp1 and LRRK2. These are the key proteins involve in pathology of PD, while they are also closely associated with PTMs (Stella C.Junqueira, e al., 2018)

It is apparent that PTMs on Parkin and other PD associated proteins play crucial roles in properly maintaining homeostasis of the cells. The question that is still need to explored is that what kind of changes occur in PTM patterns during PD formation and how much of these change are the drivers of the disease process. More effort has to be placed on the dynamics of PTMs to understand the course of PD.

1. 3. Parkin Protein Function

Parkin is encoded by PARK2 gene. PARK2 is located in the 6q 25-27 region of the chromosome and its association was first discovered in a recessive Juvenile Parkinsonism AR (ARJP) patient (Matsumine et al., 1998). Subsequent studies have identified multiple mutations on the Parkin, and some of these mutations have been studied at the molecular level (Djarmati et al., 2004). Parkin is a protein with 465 amino acids and has an E3 ubiquitin ligase (UL) activity (Winklhofer, 2007). Parkin plays an important role in the degradation of its substrates that are improperly folded or damaged by labelling them with ubiquitin (a protein of 8.5 kDa) (Figure 1.5).

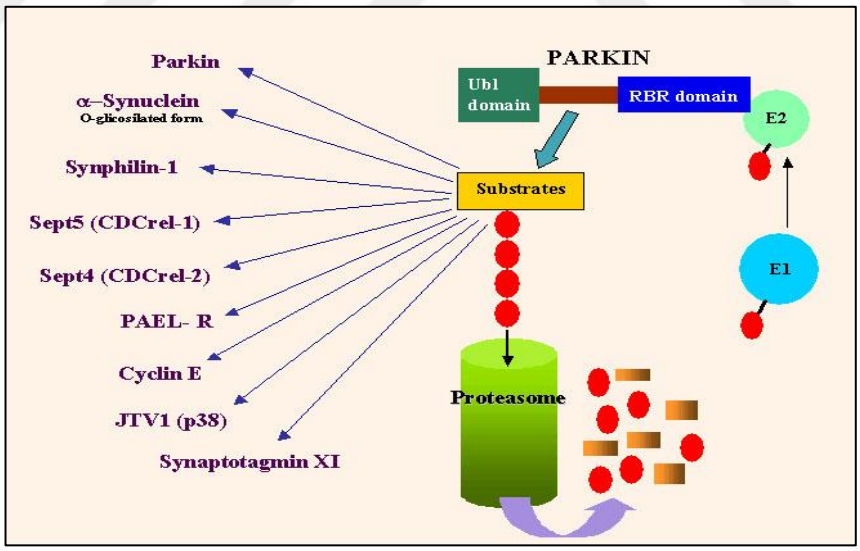

Figure 1.5. Working principle of Parkin protein and possible Parkin substrates (Um et al., 2006) Parkin, consisting of a ubiquitin-like (UBL) domain at its N-terminus and a cysteine-rich RING-IBR- Parkin, consisting of a ubiquitin-like (UBL) domain at its N-terminus and a cysteine-rich RING-IBR-RING motif at its C-terminus, functions as an E3 ubiquitin ligase . Prior studies have reported several substrates of parkin, including CDCrel-1, CDCrel-2, Synphilin-1, Pael-R, a-Synuclein, the p38 subunit of aminoacyl-tRNA synthetase, cyclin E, and polyglutamine protein

Parkin has an ubiquitin-like domain (UBL) at its N terminus and four zinc-coordinating RING-like domains (RING0, RING1, IBR and RING2) (Figure 1.6). More than 120 PD pathology-related mutations have been found in Parkin and these mutations were scattered throughout its domains (Cruts, et al., 2012). The UBL domain (between 1-76 amino acids) at the N-terminal shows 62% of similarity with ubiquitin at the amino acid level (Kitada et al. 1998).

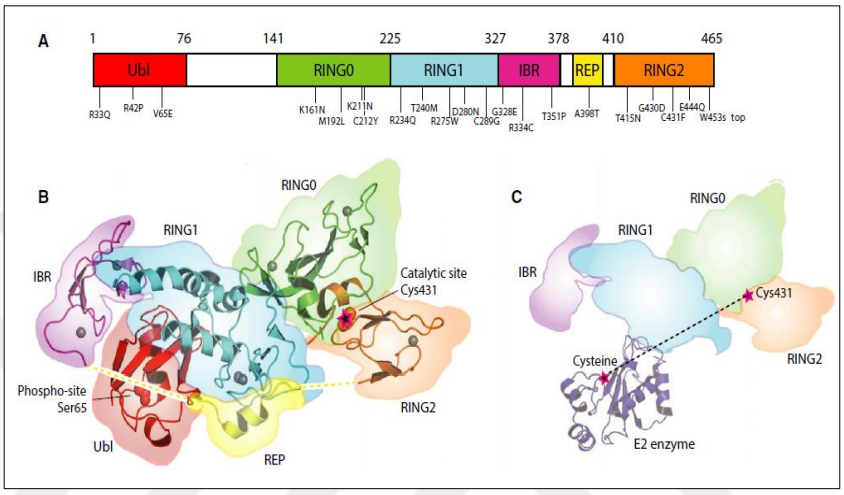

Figure 1.6. The structure of Parkin-(A) Five Parkin domains and PD related mutations. (B) 3D structure of domains of parkin (PDB: 4K95). (C) the structure change occurring on Parkin upon E2 (Marjan Seirafi, et al., 2015)

The UBL and RING0 domains are necessary to regulate the activity of Parkin .The RING0 domain is located in the side-to-side (N-terminus) segment of the RBR (RING1, IBR and RING2) domains, which are connected to the interior. The Parkin suppressor element (REP) is located between the IBR and RING2 domains.

Parkin as an E3 ubiquitin ligase plays vital role foe cells by tagging and targetting the damaged or excessive proteins with ubiquitin for degradation. Unwanted proteins are moved to ubiquitin-proteasome system, where the proteins are broken down. In addition, Parkin involves in the maintenance of mitochondria by helping disposal of the mitochondria that functions improperly. Furthermore, in recent years a stream of studies identified Parkin as a tumor suppressor protein that contributes prevention of a range of cancers (Jiri Bartek & Zdenek Hodny,2014). There is also speculation that Parkin may act as regulator of supply and release of synaptic vesicles from nerve cells, where these synaptic vesicles function as signal transmitters.

1. 4. PD and Proteomics Studies

1.4.1. Overview of Proteomics studies related to PD

Proteomics can be briefly defined as the study of proteomes in cells or in biological fluids and investigate the changes in the proteome level under various physiological conditions. Proteomics encompass a broad range of research fields including identification of novel proteins, their functions, expression levels, cellular localizations, post-translational modifications (PTM), three-dimensional structures, and protein-protein interactions and determination cellular pathways.

The proteomics studies associated with PD focuses on metabolic pathways that may play role in PD pathology and try to discover PD-related biomarkers for diagnostic and prognostic purposes. Serum is the most readily available human sample for proteomics studies. There have been several studies investigated serum protein levels such as the levels of α-SYN, DJ-1, ApoA1 in PD. However, the results of these studies provided no promising data as hoped (Chahine et al. ,2013; Akazawav et al., 2010; Hong et al.,2010; Shi M et al., 2010; Qiang et al., 2013). One of the reasons for the failure is because serum is a complex biological fluid with plenty of proteins. Some of the proteins are too abundant in serum that they shade other proteins that may have diagnostic or prognostic value. To overcome these limitations of serum proteomic studies, some researchers studied serum uric acid levels rather than serum protein profiles (Davis, et al., 1996; de Lau LM ,et al., 2005; Weisskopf, et al., 2007) and found an association between uric acid levels and PD (Morgan,et al., 2010).

Cerebrospinal fluid (CSF) is produced by brain and might be the ideal fluid to observe changes in brain proteome. Tokuda T, et al. had found significantly lower α-SYN levels in CSF samples from PD patients in comparison to the controls (Tokuda, et al., 2006). In addition to α-SYN, CSF proteins such as tau, amyloid beta, beta 2 microglobulin, vitamin D binding protein, APOA2, APOE, BNDF, and IL-8 were studied and found to have a potential in diagnosis of PD (Zhang, et al.,2008). Until the year of 2017, , nearly 3000 proteins have been identified in the human CSF(Sinha,et al.,2009; Guldbrandsen et al.,2014; Magdalinou, et al.,2017).

Human brain tissue has not been the choice in PD studies due to the limitations in availability. However, there are some studies which provided valuable insight into the pathogenesis of PD. For instance, one of the most significant proteome study using the hSNpC reported nearly 1800 PD-associated proteins and provided a new insight into the pathogenic mechanisms of PD (Licker, et al., 2014).

Tissues obtained from animal models are attractive sources for proteomic studies but the results obtained from animal studies may not be applicable to humans. Non-human primates, mammalians other than human and species phylogenetically close to humans are also used as animal models of PD. In general, animal models are created to imitate neurodegeneration in human either by using neurotoxins, or modifying the genetics of the animal. The commonly used neurotoxins are MPTP, 6-Hydroxy DA (6OH-DA) and Methamphetamine (MATH). MPTP is a widely used neurotoxin to create PD animal models and can imitate various pathological features of PD (Zhao, et al., 2007). Researchers have studied DJ-1, PEP-19 and α-SYN by using MPTP treated mice models (Jin et al. , 2005; Skold, et al., 2006; Liu et al., 2008; Kim, et al. , 2009).The most extensive study associated with MPTP treated mice model identified 4895 proteins , of which 270 were associated with dopaminergic pathway (Zhang, et al., 2010). In recent years, alternative model systems such as MPTP-treated monkey model and zebrafish were also used to imitate the complexity of PD (Lin, et al., 2015; Sarath, et al., 2016).

6OH-DA,the first chemical agent identified to have neurotoxic effects on catecholaminergic neurons can be injected unilaterally to the compartments of SNpC to create a PD model called hemiparkinsonian model. The importance of this model is that it provides a within subject control for the studies. In a study performed with 6-OHDA-treated rats 22 proteins were found to be down regulated. Those proteins were associated with neuronal synaptic transmission (Xiong, et al., 2014).

MATH is also a neurotoxin that can be used for creating PD models. MATH causes loss of dopamine by generating significant amount of ROS which causes a drop in complex I activity (Thrash, et al., 2016; Matthew,et al., 2015). In a MATH model, changes in the protein expression profile were determined using 2-DE gel electrophoresis approach and 36 differentially regulated proteins were identified in

the striatum of acute low dose MATH-treated rats (Iwazaki, et al., 2006). The traditional, toxin-based models of PD are acute and rapid, but fail to reflect the true molecular pathology of PD since they cause non-progressive loss of dopaminergic neurons.

Recently, transgenic animal models, lacking PD associated genes, have been created and used to elucidate the pathways contributing to PD pathology. Transgenic mice and Drosophila models expressing a mutant form (A30P mutant) of α-SYN were used to study using proteomics approaches to elucidate how mutations in α-SYN contribute to pathophysiology of PD (Poon, et al., 2005; Xun, et al. 2007a; Xun, et al. 2007b). Similarly, transgenic *C. elegans* model was used to observe changes in over -expressing wild-type α-SYN at proteomics level (Ichibangase, et al., 2008).

1.4.2. *In vitro* cellular models of PD used in Proteomics studies

There are different in vitro models (such as Cell lines, Primary Cell Cultures or Lesion Models) that aimed at to imitate the complex and multifactorial nature of PD. Through these models, the pathogenic mechanism of PD is studied. Primary cell cultures include tyrosine hydroxylase positive neurons (Primary TH neurons), primary cortical and hippocampal neurons and primary human fibroblasts. Primary cell cultures may be considered as the most reliable models to reveal pathologic mechanism of PD since neurons come from the brain. Yet, lack of availability and limited proliferation ability of these neurons prevent them to be widely used.Reports that utilized primary neuron cultures used rodent mesencephalic primary cells and (mainly cortical) primary neurons, stem cells, PC12 cell line, Neuro-2a cell line or MN9D cell line (Helena, et al., 2017).

Neuroblastoma SH-SY5Y human derived cell line, a subline of the SK-N-SH cell line which was established from a bone marrow biopsy of a metastatic neuroblastoma of a 4-year-old female and has undergone three rounds of clonal selection, is an in vitro model widely used in PD research (Biedler, et al., 1978). In general, SH-SY5Y cell line demonstrated the features of moderate activity of dopamine-β-hydroxylase and negligible levels of choline acetyl-transferase, acetylcholinesterase and butyryl-cholinesterase (Biedler, et al., 1978), basal noradrenaline (NA) release (Pahlman, et al., 1984) and display tyrosine hydroxylase

activity (Ross, et al.,1985). Tyrosine hydroxylase has direct association with PD since it can take part in catecholamine synthesis pathway by converting tyrosine to L-dopa which is the precursor of dopamine (DA) (Nagatsu, et al., 1964). The SH-SY5Y cell line can be a good cellular model since this cell line may display a catecholaminergic phenotype and may synthesize both dopamine and noradrenaline (Helena Xicoy, et al., 2017). Ample number of publications was associated with PD have reported experiments performed with the SH-SY5Y cell line. The remaining few studies used cell lines that are not neuronal, such as HEK293, HeLa or glial cells.

Cellular models have advantages of developing pathology more quickly, less costly and offers controlled environment for the experimental studies. These features provide opportunity for larger scale testing in a shorter amount of time. However, they do not represent all aspects of PD since lack of the cellular microenvironment critical to disease development is missing. The findings from cell culture studies should be further validated using animal models.

Previously in our laboratory, SH-SY5Y cells were used as a model to study the effect of expressing wild type and mutant Parkin mutations on the cell proteome (Ozgul et al., 2012). The mutant form of Parkin carried two mutations. One of these mutations (Q311R) was located within the RING1 domain and the other one (A371T) was located within the IBR domain. Some of the differences between the WT and the mutant Parkin proteins e.g., a 10kD decrease in molecular weight of the mutant parkin when it was expressed in HeLa and SH-SY5Y cells were shown (Kasap et al., 2009; Ozgul et al., 2015). To our surprise, however, the mutant Parkin protein displays both *in vivo* and *in vitro* activities and has stability similar to the WT Parkin protein (Ozgul et al., 2015). The mutant Parkin protein was located in the nucleus more than the wild type Parkin protein, remained in the cell longer and was subjected to different type of post-translational modification. The experiments performed in here used the above mentioned cell lines created by Özgul et al. Those cell lines express the wild type and the mutant Parkin proteins.

1. 5. PD and Cancer

Parkin operates as an E3 ubiquitin ligase and attaches ubiquitin moieties onto its substrates (Ozgul et al., 2015). Depending on the number of attached ubiquitin moieties (polyubiquitylation or monoubiquitylation), Parkin substrates can be either directed to proteasomes for degradation or to other parts of the cell to perform various metabolic functions (Nakagawa and Nakayama, 2015).

Mutations observed in Parkin have long been associated with Parkinson's disease (Hampe et al., 2006). This association directed most studies towards understanding the role of Parkin in neurodegeneration processes. Several important features of Parkin were revealed and the mechanisms underlining the role of Parkin in neurodegeneration were mainly elucidated (Barodia et al., 2017; Cookson et al., 2003). During those studies, it was realized that PARK2 is either frequently deleted or its expression is dramatically reduced in a wide range of human cancers (Cesari et al., 2003; Denison et al., 2003a; Denison et al., 2003b; Zanetti et al., 2007). This observation placed an unexpected function on Parkin and marked it as a possible tumor suppressor protein (Fujiwara et al., 2008; Picchio et al., 2004). Several lines of evidence were then presented supporting the notion that Parkin is a tumor suppressor protein. For instance, introduction of intact human chromosome 6 harboring the wild-type Parkin into MCF7 cell line restored their ability to senescence (Negrini et al., 1994; Trent et al., 1990). The intact chromosome 6 harboring the wild-type Parkin altered tumor growth properties and suppressed tumorogenicity. Similarly, *in vivo* expression of Parkin in a lung carcinoma cell line caused consistent reduction in tumor volumes in nude mice (Picchio et al., 2004). Also, Parkin knockout mice lacking exon 3 had displayed enhanced hepatocellular proliferation and developed macroscopic tumors (Fujiwara et al., 2008). In pancreatic cancer, Parkin deletion caused spindle misorientation, chromosomal instability and deregulated growth (Sun et al., 2013).

These observations directed the interest towards understanding the possible role of Parkin in development and progress of various types of cancers. It was especially important to demonstrate that Parkin deletions in cancer cell lines were the drivers causing the development and progress of cancers (Devine et al., 2011). One of the

first molecular evidence demonstrating the direct involvement of Parkin in cancer was provided by Staropoli et al. (2003) who reported that Cyclin E, a cell cycle regulator protein, accumulates in Parkin deficient primary neurons (Staropoli et al., 2003). The counter association between Parkin and Cyclin E was also reported in another study in which c.a. 5000 tumor genomes from 11 different cancer cell types were analyzed (Gong et al., 2014). The researchers demonstrated that Parkin gene was the frequently deleted gene in human cancers and there is a mutually exclusive pattern between Parkin gene deletion and amplification of Cyclin E and Cyclin D. In a more depth functional analysis, cancer specific mutations of Parkin in glioblastoma multiforme, colon and lung cancers were shown to decrease the ability of Parkin to interact with Cyclin E and reduce ubiquination of Cyclin E by Parkin (Veeriah et al., 2010). In addition to Cyclin E, a dramatic increase in CDK6 levels were observed in a breast cancer cell line at mRNA level and the level of increase was Parkin-dose dependent (Tay et al., 2010). Parkin gene was also shown to be the target of p53, a transcription factor playing a pivotal role in tumor prevention (Alves da Costa and Checler, 2011; Zhang et al., 2011). p38, a subunit of aminoacyl tRNA synthase complex is proposed to be the substrate for Parkin (Ko et al., 2005). Reduced ubiquitylation of p38 was observed in Parkin nude mice.

1. 6. Phosphoproteomics

Phosphoproteomics is sub-field of proteomics which is involved in identification, classification and characterization of proteins that contain a phosphate group. Phosphorylation is defined as the addition of a phosphate group to a target amino acid (mostly serine, threonine, or tyrosine residues in eukaryotes) and the reaction is reversible that controlled by two different classes of enzymes, namely protein kinases and phosphatases. Phosphorylation is vital post-translational modification that regulates function, cellular localization and degradation of proteins. Approximately, 30% to 65% of all proteins are phosphorylated and it is speculated that 230,000 different phosphorylation sites are present in human proteome (Vlastaridis, et al., 2017; Cohen, et al., 2002).

In recent years, many human diseases have been found to be closely associated with the phosphorylation of cellular proteins. So far, a phosphoproteome study targeting

the changes occurring in PD has not been carried out. In our laboratory, we tried monitoring the changes occurring in phosphorylation levels upon expression of the wild-type and the mutant Parkin proteins (Ozgul, 2012). However, the experiments did not produce significant data due to the limitations of our laboratory infrastructure and was not published.

A study recently reported that Parkinson's disease-associated mutant LRRK2 phosphorylates Rab7L1 and modifies trans-Golgi morphology (Fujimoto, et al., 2018). In a study published in 2013, the authors discussed the function of α-SYN accumulation which is phosphorylated at Ser129 and proposed that the phosphorylated α-SYN can be therapeutic target for slowing down the development of Parkinson's disease (Sato, et al., 2012).

Studies associated with cancer have concentrated on the alteration of phosphoproteome during tumor progression and speculated that phosphoproteins could be used as biomarkers for cancer diagnostics and therapeutics. Research has indicated that in breast and liver tumors, phosphotyrosine proteomes are different from normal tissues (Da Costa GG, et al., 2006; Haiyu Li, et al., 2009).

1. 7. Objectives

As it is evident from the past and the current literature, it is becoming increasingly apparent that Parkin involves into cell cycle progression and contributes to tumorogenesis as well as the degeneration of the post-mitotic neurons (Devine et al., 2011; Matsuda et al., 2015; Wahabi et al., 2018). Elucidation of the overlapping proteins in cancer and neurodegeneration may open a therapeutic window for both diseases. In here, we studied changes occurring in nuclear proteome of neuroblastoma cells upon the wild-type and the mutant Parkin expressions at nuclear proteome level using 2DE approach coupled with MALDI-TOF/TOF. As we mentioned above, the mutant Parkin protein was previously studied and characterized in our laboratory (Ozgul et al., 2015). This mutant form of Parkin carried two mutations. One of these mutations (Q311R) was located within the RING1 domain and the other one (A371T) was located within the IBR domain. Majority of the cancer-causing mutations was located within these two domains (Veeriah et al., 2010). We, thus, included this double heterozygous mutant to our study. In our

previous study performed in our laboratory, some differences between the WT and the mutant Parkin proteins were shown when the mutant parkin was expressed in HeLa and SH-SY5Y cells (Kasap et al., 2009; Ozgul et al., 2015). To our surprise, however, the mutant Parkin protein displays both *in vivo* and *in vitro* activities and has stability similar to the WT Parkin protein (Ozgul et al., 2015).

Throughout this thesis study, we investigated whether the WT or the mutant Parkin proteins displayed any effect on nuclear proteome and signaling pathways. After enrichment of nuclear proteins from exogenous Parkin expressing and non-expressing cells and performing a 2DE based comparative proteome and phosphoproteome analysis, a list of differentially regulated proteins that were not previously known to interact or associate with Parkin was created. These proteins suggested that nuclear form of the Parkin mainly involves in DNA repair and likely contributes to tumorogenesis via maintenance of DNA in tumor cells.

2. MATERIAL AND METHODS

2.1. Used Materials

2.1.1. Chemicals and kits

Table 2.1. Chemicals and kits used in the experiment

Chemicals	Provider
DMEM Earle's	Biochrome, UK, Catalog no: FG 1445
tetracycline-free FBS	Clontech, US, Catalog no: 631106
penicillin/streptomycin	Biochrome, UK, Catalog no: A 2212
L-Glutamine	Biochrome, UK, Catalog no: K 0282
Trypsin / EDTA	GIBCO, Invitrogen, US, Catalog no: 25200-072
DMSO	Applichem, Germany. Catalog no:A1584
PBS	Biochorome, US, Catalog no: L 1825
OptiPrep Density Gradient Medium -iodixanol	Sigma, US Catalog No: D1556-250 mL, ,
Benzonase	MERCK,Germany, Cat no: 101697
Protease Inhibitor Cocktail	Sigma Aldrich, USA, Cat no: P8340
serine / threonine phosphatase inhibitor Calyculin A	Cell signaling, US, Cat no: 9902
phosphatese inhibitor cocktails 2	Sigma Aldrich, US, Cat no: P 5726
phosphatese inhibitor cocktails 3	Sigma Aldrich, US, Cat no: P0044
The ReadyPrep protein extraction kit (cytoplasmic/nuclear)	Bio-Rad, US, Catalog No: 163-2089
Qproteome Nuclear Protein Kit	QIAGEN, Germany ,Catalog No: 37582
trypsin enzyme	Promega, US, , Cat no: V5280
α-cyano-4-hydroxycinnamic acid	CHCA, Sigma–Aldrich, Cat no: 476870

2.1.2. Solutions

Preparation of solutions used in the experiments were given as supplementary File named SUPPLEMENTARY Table A.

2.2. Methods

2.2.1. Cell culture

2.2.1.1. Maintenance of cell lines

The neuroblastoma cell line (SH-SY5Y) which is already used routinely in our laboratory was used as cellular model in this thesis. SH-SY5Y cells were cultured under standard culture growth conditions. The growth media was DMEM Earle's containing10% tetracycline-free FBS; 100 units/mL penicillin/streptomycin and 2.8 mM L-Glutamine (final concentration). The cells were grown in tissue culture plate

at 37 ºC supplied with 5% CO_2. The culture media were changed every three days when the cell density was reached to ~80% confluency.

2.2.1.2. Cryopreservation of cell lines

In order to freeze the cells, a freezing media containing 70% DMEM (complete media; where the cells had already grown), 20% FBS and 10% DMSO was used. Prior to freezing, the conflueny of the cells has reached to 80-90%. The medium was collected in a separate tube by using 0.2 mikron filter. The cells were washed with sterile PBS. PBS was then removed and 1 mL of 0.25% Trypsin / EDTAsolution was added onto the cells. The culture plate was placed in an incubator at 37 ºC for the trypsin enzyme to work. The cells were monitored under microscope to observe whether they detached from the bottom of the culture plate. The detached cells were then collected by sterile PBS and centrifuged at 500 x g for 10 min at +4 °C. After centrifugation, the upper layer was removed and the cell containing pellet was resuspended in freezing media. The cryovials were kept in a cryo-freezer storage container at -80 ºC for overnight and then transfered to liquid nitrogen tank for storage.

2.2.1.3. Thawing the cells

The cryovial was removed from the liquid nitrogen tank and hold in 37ºC water bath until the sides were thawed but the center remained frozen. The thawed cells were added to the pre-warmed growth media (medium consists of 20% FBS 80% DMEM in gradient form) in a dropwise manner. The cells were then centrifuged at 500 xg for 10 min at +4 ºC. After centrifugation, the liquid phase was removed and the cell pellet was resuspended and seeded in culture plates containing antibiotic free media. The media of the grown cells were then replaced with media containing appropriate antibiotics after overnight incubation.

2.2.1.4. Passaging cells

When the cell confluency reached to ~80%, the cells were passaged to new culture plates. This process was performed as follow; the medium in the culture dish was discarded and the cells were washed with ample amount of PBS.The cells were

detached using 0.25% Trypsin / EDTA and then collected with PBS before centrifugation at 500 x g for 10 min at +4ºC. The upper layer was completely removed and the cells were resuspended in pre-warmed fresh media.

2.2.1.5. Cell counting with hemacytometer

The cells were cultured until they reach 60-90% confluency, then cell counting were performed using a hemacytometer. The protocol used for cell counting was as follow;

- Cultured cells were trypsinized with 0.25% Trypsin / EDTA solution , pelleted by centrifugation and suspended in 4mL of complete media.
- 200 µl of the cell suspension was transfered into a 1.5 mL eppendorf tube.
- 300 µl of PBS and 500 µl of 0.4% trypan blue solution were added to the cell suspension (created a dilution factor of 5) in the centrifuge tube.
- Mixed throughly, allowed to stand for 5 to 15 min and 10 µl of the trypan blue-cell suspension were transferred to a chamber on the hemocytometer touching the edge of the cover-slip by using a micro pipette.
- All the cells (non-viable cells stain blue, viable cells will remain opaque) within the 1mm center square was counted.
- The counting was repeated using the other four corner squares of the hemocytometer.
- Cells concentration per mL were determined using the following calculations

Cells per mL = the average count per square x the dilution factor (our dilution factor was 5) x 10^4

Total cell number = cells per mL x the original volume of fluid from which cell sample was suspended.

2.2.2. Optimization of nuclear protein enrichment methods

2.2.2.1. Enrichment of nuclear proteins using ReadyPrep Protein Extraction Kit

The ReadyPrep protein extraction kit (cytoplasmic/nuclear) is designed to quickly prepare highly enriched fractions of cytoplasmic and nuclear proteins from eukaryotic cells. The fractions were enriched according to the instructions provided by the supplier. In brief;

The cells were transfered (1–5 mL) into a 1.5 mL microcentrifuge tube and washed with PBS three times. 0.05 mL of cell pellet was obtained after centrifugation.

- For each cell pellet, 0.5 mL of ice-cold CPEB (Cytoplasmic protein extraction buffer) was added and the cell pellet was suspended by overtaxing and incubated on ice for 30 min.
- The cell suspension was gently passed (10–20 strokes) through a syringe needle (20 gauge) to lyse the cells without damaging the nuclei.
- The cell lysate was centrifuged at 1000 x g for 10 min at 4°C.
- The pellet (from step e.) which contained the cell nuclei was washed with 0.25 mL of ice-cold CPEB to minimize cytoplasmic protein contamination.
- The nuclear pellet was resuspended in 0.5 mL of PSB and vortexed to solubilize the nuclear proteins. The suspension was centrifuged at a maximum speed of 16000 x g for 20 min at room temperature to pellet the genomic DNA and cell debris. The clarified supernatant was then transfered into a new microcentrifuge tube and labeled as Nuclear Protein Fraction.

2.2.2.2. Enrichment of nuclear proteins using OptiPrep density gradient centrifugation method

The crude nuclear pellet (prepared by Ready Prep Protein Extraction Kit; step of 2. 2.2.1) was resuspended in 25% iodixanol, and placed into an ultracentrifuge tube containing iodixanol gradient (30% and 35%). The centrifugation was performed at 10000 x g for 20 minutes in an ultracentrifuge and the clarified nuclei were then collected as an isopycnic band at the 30%-35% iodixanol interface.

2.2.2.3. Enrichment of nuclear proteins using Q-Proteome Nuclear Protein Enrichment kit

Qproteome Nuclear Protein Kit was used to enrich nuclear proteins. The nuclear protein enrichment protocol was consisted of three steps. The first step involved in seperation of cytosolic proteins. In brief, the cells were incubated in hypotonic lysis buffer (500 µl of Lysis BufferNL supplemented with Protease Inhibitor Solution and 0.1 M DTT) to swell the cells. A detergent solution (25 µl NP) was then added to the swelled cells to achieve the cell lysis. The lysed cells were then centrifuged for 5 min at 10000 x g at 4°C to separate the cytosolic fraction (supernatant) from the pellet containing the cell nuclei. The second step involved in enrichment of the nuclear proteins, called the nucleic-acid binding protein fraction. In brief, the nuclear pellet from the first step was incubated in 50 µl of Extraction Buffer NX1 for 30 min at 4°C. A centrifugation step was performed at 4°C for 10 min at 12000 x g to enrich the nucleic-acid–binding proteins which were found in the supernatant. The pellet contained the nuclear debris along with genomic DNA.

The third step involved in enrichment of the nuclear proteins, called the “insoluble” nuclear protein fraction. Extraction of “insoluble” nuclear proteins was performed by incubation of the nuclear debris with 100 µl of Extraction Buffer NX2 (supplemented with Benzonase, Protease Inhibitor Solution, and 0.1 M DTT Stock Solution) which digested genomic DNA and released nuclear proteins intimately associated with DNA.

2.2.3. Protein extraction and proteomics experiments

2.2.3.1. Preparation of total cell free extracts

When SH-SY5Y cells reached to 80% confluency, the medium was discarded and the cells were washed with ice-cold PBS. The cells were then scraped by using a plastic cell scraper and transfered into a clean 15 mL conical tube and centrifuged at 500 xg for 10 min at +4°C. The precipitated cell pellet was resuspended in 2D sample buffer [1:3 (w/v)] (8 M urea, 2% (w / v) CHAPS, 50 mM DTT, 0.2% 3-10 ampholyte, 0.001% bromphenol blue, 1% of Protease Inhibitor Cocktail. The cells were lysed by adding 0.2 mm stainless steel with a bead beater at 3000 rpm and +4°C for 3 min. The homogenate was then centrifuged for 10 min at 15000 x g at +4°C and the supernatant containing the soluble proteins was transferred into a new tube. To further clarify the supernatant, it was re-centrifuged for 30 min at 20000 x g (at +4°C). The clear supernatant was aliquoted and stored at -80°C after snap-frozen in liquid nitrogen.

2.2.3.2. Determination of protein concentration

Bradford Assay was used to measure total protein concentration in cell-free extracts (Bradford, 1976). In brief, 1 µL of protein extract was mixed with 19 µL sample preparation buffer. The mixture was vortexed with 1mL of 1x Bradford Reagent and allowed to sit for 5 min in the dark. The samples were then measured at 595 nm with Nanodrop. The measured protein concentrations of the samples were determined by comparing with the BSA standards curve prepared before. The BSA standarts were prepared in the same buffer as the unknown protein samples.

2.2.3.3. Cleaning of the protein samples

500 µg of protein containing cell-free extract in 100 µL volume was transferred into a 1.5 mL microcentrifuge tube. The protein sample was precipitated by adding agent 1 and 2, respectively. The mixture was vortexed and centrifuged at 16000 x g for 5 min to form a tight pellet. The pellet was then washed with wash reagent 1 and 2 and incubated at -20°C for 30 min. The tube was centrifuged at maximum speed (> 12000 x g) for 5min to obtained final precipitated protein sample. The pellet was air-

dried at room temprature then resuspended by adding an appropriate volume of 2D sample buffer.

2.2.3.4. Isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis

500 µg protein samples were mixed with 2DE sample buffer to a final volume 200µL. The mixture was slowly loaded into the center of a lane of rehydration/equilibration tray as a continuous line. Protective cover of the IPG strips was peeled using forceps and they were placed onto the mixture positioned with the gel side down. The IPG strips were completely overlayed by adding appropriate volume of mineral oil (~1mL) to prevent evaporation and urea crystallization. Then, the strips were rehydrated under passive condition at 20°C overnight using a PROTEAN IEF device. After rehydration, the IPG strips were transferred onto a focusing tray for IEF. The program was used for 11 cm IPG strips were as follows: 20 min at 250 V with rapid ramp, 2 h at 4000 V with slow ramp and 2.5 h for 4000 V with rapid ramp until a total of 27.000 V/h was reached at 20 °C. When the IEF completed, the IPG strips were stored at -80°C or immediately used for the second-dimension separation.

After focusing, the IPG strips were equilibrated on an orbital shaker and gently shake for 15 min with equilibration buffer I for 30 min and equilibration buffer II for 30 min at room temprature. The equilibration by two buffers (equilibration buffer I contains DTT and equilibration buffer II contains iodoacetamide) ensure that sulfhydryl groups of cysteines were reduced and alkylated.

For the second dimension separation, 12% SDS-polyacrylamide gels (Table 2.3) were casted into Criterion Empty Cassettes. The equilibrated strips were then loaded onto the top surface of the second dimension gel by using a 0.75 mm spacer to gently push the IPG strips. Generation of bubbles was avoided between the strip and the gel, and ensured a good contact. The strips were sealed with agarose overlay solution and solidified for about 10 min. The gel cassettes were placed into Criterion Dodeca system which can run up to 12 gels at once to eliminate gel to gel variation. Tris-glycine electrophoresis buffer (running buffer) was poured into the upper and lower chambers. The gels were run at a constant current of 20 mA for until the dye front

reached to the bottom of the gels (~2 hrs). Fixation of the gels was performed in 40% methanol, 10% acetic acid for at least one hour. The fixative was discarded and the gels were washed three times for 10 min with ultrapure distilled water. The gels were stained in colloidal Coomassie stain for 24 hr by gently rocking at room temperature. For destaining the gells were washed with ultrapure distilled water by gently rocking and if necesary stored for several weeks in 5% acetic acid solution at 4°C until in-gel digestion is performed. Each experiment was performed in triplicate.

Table 2.2. SDS-PAGE gel content

Solutions	Seperating gel (12%, mL)	Stacking gel (4%, mL)
ddH2O	3.3 mL	3 mL
1.5M Tris. HCI pH8.8	2.5	-
0.5M Tris. HCI pH6.8	-	1.25
37.5:1 Acrylamide /Bis	4	0.67
%10 SDS	0.1	0.05
%10 APS	0.1	0.05

2.2.3.5. Image analysis of 2D gels

2D gel images were captured with VersaDoc 4000MP system by using Quantity One software. Spot intensity calibration, spot detection and background substraction were done using by PDQuest 2DE Analysis Software. Stain speckles were filtered and the standardized areas of interest from all gels were warped and matched and the quantity of each spot was normalized by the total valid spot intensity using linear regression model. The statistical significance of image analysis was determined by the Student's *t*-test (statistical level of $p<0.05$ is significant). Gel spots significantly differed in expression (more than 2-fold) were selected and excised from gels using ExQuest Spot-cutter for protein identification. The protein spots detected by the software were inspected using a manual editing tool. Spots that were prone to variation were excluded if the spots were hard to identify by visual inspection. Every-member matching protein spots were selected. Means and standard deviations (SD) were calculated from three independent experiments, and paired Student's *t*-tests were used to assess differences in the average protein abundance between the gels. 2D gel image comparison, protein spot intensities with more than two-fold significant-change ($p<0.05$) in a consistently increased or decreased pattern were

considered differentially expressed. All selected spots were excised using an automated spot cutting tool, ExQuest, and disposed into 96-well plates for identification.

2.2.3.6. Preperation of Sodium dedocyl sulphate- polyacrylamide gels (SDS-PAGE) for western blotting

Protein samples (20 µg) were mixed with sample loading buffer and heated at 95 °C for 5 min and cooled on ice. Sample and were loaded onto a 12% Bis-Tris polyacrylamide gels. The gels were run at RT in a TetraCell vertical electrophoresis buffer tank filled with 1X SDS-PAGE running buffer at 180 V for 15 min until the front dye reached to the end of the gel.

2.2.3.7. Western Blotting

The proteins on the gel were transferred to a nitrocellulose membrane with a Semi-Dry Transfer Cell for 30 min at a constant 25V. Transfered proteins to the membrane were checked using Ponceau S staining before the blocking step. The membrane was then blocked in 5% milk powder in TBST for 1hr at room temperature with sahking. The primary antibody was diluted to the desired concentration in TBST and the membrane was incubated in the primary antibody for overnight at 4ºC. The membrane was then washed with TBST for 3X (changing TBST every 10 mins) and incubated with the secondary antibody diluted to the desired concentration in TBST for 1hr at room temperature. The wash step with TBST was repeated and the membrane was developed using ECL detection reagents and exposure to X-ray film.

Table 2.2. List of primary (1 °) and secondary (2 °) antibodies used in Western blot analysis

1° Antibody	2° Antibody
HistonH3(Cell Signaling Technology, ABD)(1:2000)	Goat IgG Anti-Rabbit HRP Conjugate (Biorad, ABD)(1:20000)
Lamin A/C (4C11): 4777 (Cell Signaling, ABD)(1:2000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:10000)
Glyceraldehyde-3-phosphate dehydrogenase(GAPDH (6C5): sc-32233, (Santa Cruz, ABD) (1:2000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:20000)
Cyclophilin A(PPIA - peptidylprolyl isomerase A (Novus Biologicals, ABD) (1:500)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:20000)
Parkin (H-300): sc-30130 (Santa Cruz, ABD)(1:2000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:20000)
ß-Actin Mouse (Santa Cruz, ABD) (1:1000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD) (1:20000)
Nucleolin (D4C7O): #14574 (Cell Signaling, ABD) (1:2000)	Goat IgG Anti-Rabbit HRP Conjugate (Biorad, ABD)(1:20000)
TCTP Antibody(Translationally controlled tumor protein): #8441(Cell Signaling, ABD) (1:2000)	Goat IgG Anti-Rabbit HRP Conjugate (Biorad, ABD)(1:20000)
Anti-TPI(Triosephosphate isomerase) TIM (H-11): sc-166785 (Santa Cruz, ABD) (1:1000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:20000)
GADD 153 (B-3): sc-7351(Santa Cruz, ABD) (1:2000)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:20000)

2.2.4. Phosphoproteomics

2.2.4.1. Sample preparation for phosphoproteomics

When density of the cells reached ~ 75% confluency, induction was performed by adding tetracycline (Tet, 1µg/mL) to the cell culture to express the WT and the mutant Parkin proteins. A cell culture plate was kept as a control and was not induced with tetracycline. After 24 hours of incubation, the cells were washed 3 times with fresh medium before adding serum free medium. Then, 50 nM serine / threonine phosphatase inhibitor Calyculin was added to the culture plates and the plates were incubated in this medium for 35 min.

2.2.4.2. Phosphoprotein extraction

Nuclear phosphoprotein extraction: the cells were washed with 20 mM HEPES at pH 7.4 buffer and collected by centrifugation at 45000 xg for 10 min at 4°C. The upper layer was discarded and the cell pellet was lysed in a Lysis Buffer NL supplemented

with Protease Inhibitor Solution and 0.1 M DTT containing 1% phosphatese inhibitor cocktails 2 and 3.

Cell free extract preparation for phosphoprotein analysis: cells were lysed in a buffer containing 20mM HEPES at pH 7.4 containing 1% phosphatese inhibitor cocktails 2 and 3. To lyse the cells more efficiently, glass beads were used with the help of a bead beater .The lysed cells were centrifuged at 15000 x g for 30 min at 4°C to clear the supernatant. The supernatant was aliquoted and snap-frozen in liquid nitrogen and stored at -80°C.

2.2.4.3. ProQ Diamond phosphoprotein staining

The preotein were subjected to 2D gel electrophoresis. The gels were then fixed according the procedure summurized in Table 2.3. The fixed gels were then stained with ProQ Dimond phosphoprotein stain.

The gels were visualized using appropriate filters with the VersaDoc MP4000 system.

Table 2.3. Phosphoproteins ProQ Diamond stain procedure

ProQ Diamond	Solutions	Tris-Glycine gel
Step 1: Fixation	50% of Methanol 10% of Acetic Acid	Distilled water fill up to 100 mL, 30min 100 mL, overnight incubation
Step 2: Wash	Ultra pure water	100 mL, 10 min 3 times
Step 3: Staining (in the dark)	ProQ Diamond Stain	60 mL, 90 min stain
Step 4: Destaining (in the dark)	20% of Acetonitrile 50 mM Sodium Acetate pH 4	80-100 mL, 30 min 3 times
Step 5: Wash (in the dark)	Ultra pure water	100 , 5 min 2 times

2.2.4.4. Sypro Ruby protein staining

The ProQ Diomand stained gels were fixed again before Sypro Ruby staining as summurazied in Table 2.4. The stained gels were imaged with Versadoc MP4000 imaging system using appropriate filters.

An *in silico* experiment was created in PDQuest Advance software using the gel images of ProQ Diamond stained gels and Sypro Ruby stained gels. Spots that were regulated in ProQ Diamond stained gels but not in Sypro Ruby stained gels were considered regulated.

Table 2.4. Phosphoproteins Sypro Ruby stain procedure

SyroRuby	Solutions	Tris-Glycine gel
Step 1: Fixation	50% of Methanol 7% of Acetic Acid	Distilled water fill up to 100 mL 30 min
Step 2: Wash	100 mL distilled water	3 times 10 min
Step 3: Staining (in the dark)	Sypro Ruby stain	30 mL overnight stain
Step 4: Destaining (in the dark)	10 % of Methanol 7% of Acetic Acid	Distilled water fill up to 100 mL 30 min
Step 5: Wash (in the dark)	100 mL distilled water	2 2 times 5 min

2.2.4.5.2DE and Western Blotting analysis for phosphoproteins

Proteins prepared for phosphoproteom analysis were subjected to 2-D PAGE as describe above then transferred to PVDF membranes. The western blotting procedure was performed as described above except the antibodies, blocking solution and antibody dilution solutions. After transfer of the proteins to the membrane, 2 mL of Western Blot Blocking Buffer (Protein Free) used to block the membrane at room temperature for 1 hour. The blocking buffer was removed and the membrane was washed thrice with TBS-T for 10 min each. After washing, an antibody mixture was prepared in Western BLoT Immuno Booster Solution 1 according to the dilution rates given in Table 2.5. The membrane was incubated with the antibody mixture at +4 ºC overnight with gentle agitation. After overnight incubation, the membrane was washed thrice with TBS-T for 10 minutes each. The secondary antibody was diluted in Western Blot Immuno Booster Solution 2 and the membrane was incubated in this buffer for 1 hour at room temperature. After incubation, the washing was repeated and the unbound antibodies were removed. The images were taken as described above.

Table 2.5. List of primary (1°) and secondary (2°) AB used in phosphoprotein Western blot analysis.

1° Antibody	2° Antibody
p-Ser (16B4) : sc-81514 , (Santa Cruz, ABD) (1:200)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:10000)
p-Thr (H-2): sc-5267 (Santa Cruz, ABD) (1:200)	Goat IgG Anti-Mouse HRP Conjugate (Biorad, ABD)(1:10000)

2.2.5. Mass spectrometer analysis

2.2.5.1. In -gel tryptic digestion

Protein spots cut by the spot-cutter device in 96-well plates were replaced into 0.6 mL microcentifuge tubes and used for in-gel tryptic digestion. A commercially aviable kit was used. In brief; protein spots were washed with 150 µL of 40% acetonitrile and 60% ammonium bicarbonate (50mM pH 8.0) solution (wash buffer) until the Coomassie stain dye dissapears. The proteins in gel spots were reduced in 50 mM TCEP solution for 10 min at 60°C and then alkylated in 10mM IAA alkylation buffer at room temperature for 1 hour in the dark. After alkylation, the spots were washed twice with washing buffer for 15 min. The wash buffer was then removed and 100% acetonitrile was added onto the spots, allowing dehydration for 15 min. At the end of dehydration, acetonitrile was removed with a pipette and 10 ng modified active trypsin was added to the spots and overnight digestion was performed at 37°C. The supernatants containin the resulting peptides were collected and transferred to clean tubes. Further recovery of the peptides was accomplished by second extraction with 60% acetonitrile/5% TFA. All supernatants were pooled and concentrated by a SpeedVac.

2.2.5.2. ZipTip C18 purification for desalting of the peptide solutions

The dried peptide mixtures from SpeedVac were reconstituted in 10 µL of 0.1% TFA. A reverse-phase ZipTipC_{18} micro column was pre-equilibrated with 50% acetonitrile and washed with TFA. The reconstituted sample was drawn into the tip in order to allow peptide binding, and washed three times with 10 µL of 0.1% TFA to remove any contaminant. The peptides were eluted with α-cyano-4-hydroxycinnamic acid solution in

0.1% TFA/50% acetonitrile as the matrix (1:1, v/v) and spotted (~0.5 μL) directly onto a stainless-steel MALDI target plate.

2.2.5.3. Protein identification by MALDI-TOF/TOF

Protein identification experiments were performed by using ABSCIEX MALDI-TOF/TOF 5800 system. The TOF spectra were recorded in the positive ion reflector mode with a mass range from 400 to 2000 Da. Each spectrum was the cumulative average of 2000 laser shots. The spectra were calibrated with the trypsin autodigestion ion peaks m/z (842.510 and 2211.1046) as internal standards. Ten of the strongest peaks of the TOF spectra per sample were chosen for MS/MS analysis. All of the PMFs were searched in the MASCOT version 2.5. by using a streamLine software, Protein Pilot, with the following criteria: SWISSPROT database; species restriction to H. sapiens; enzyme of trypsin; at least ten independent peptides matched; at most one missed cleavage site; MS tolerance set to ±50 ppm and MS/MS tolerance set to ±0.2 Da; fixed modification being carbamidomethyl (Cys) and variable modification being oxidation (Met); peptide charge of +1 and being monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p<0.05$), were accepted.

2.2.5.4. Statistical analysis

Quantitative results were expressed as means±standard deviation. Two-sample t-tests assuming unequal variances were used for the statistical analyses $p<0.05$ was considered statistical significant.

2.2.5.5. Bioinformatics analysis

Protein-protein interaction (PPI) network of the identified proteins was constructed with the online analysis tool STRING v10.0 (http://www.string-db.org). Classification of the proteins based on biological processes and molecular functions was performed by using a freely available classification system PANTHER (version 11.0 Released 2016-07-15, http://www.pantherdb.org/), NCBI and SWISSPROT databanks. To predict subcellular localization of proteins Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/) databank was used. To determine phosphorylation status of the identified proteins, PhosphSite Plus (https://www.phosphosite.org) was used.

3. RESULTS AND DISCUSSION

3.1. Comparison of Nuclear Protein Isolation Methods

Prior to comparative nuclear proteome analysis, experimental approaches to enrich nuclear protein extracts from SH-SY5Y cells were explored. A commercially available kit from BioRad (Ready Prep Nuclear Protein Extraction kit) was tested for nuclear protein enrichment. This kit allows enrichment of the nuclear proteins by differential centrifugation. To quantitatively assess the degree of enrichment, the enriched nuclear proteins were subjected to 2DE (Figure 3.1).

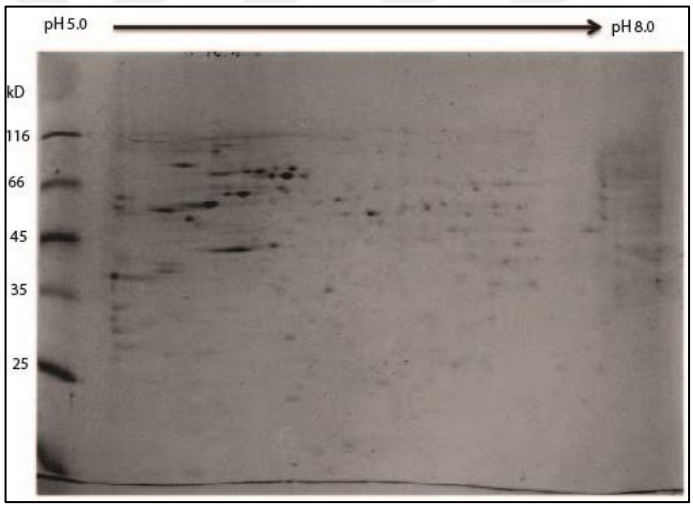

Figure 3.1. 2D gel electrophoresis analysis of nuclear proteins obtained using Ready Prep Nuclear Protein Extraction kit

Ten randomly selected protein spots were cut from a gel and identified by MALDI-TOF / TOF analysis and the intracellular localizations of the identified proteins were determined using SWISS-PROT database or relevant databases (Supplementary Table B). Only two of the identified proteins were localized to nucleus. The rest of the proteins were either cytoplasmic or had no known localization.

The repeated experiments generated similar results indicating that, the enriched nuclear protein extracts obtained by this kit was not enriched at a desirable level. Neverthless, for the sake of verification, enriched nuclear proteins were subjected to

WB analysis and four different antibodies were used to visualize the level of enrichment. Histon-H3 and Lamin were used as the nuclear protein markers, and GAPDH and cyclophilin A were used as the cytoplasmic protein markers (Figure 3.2).

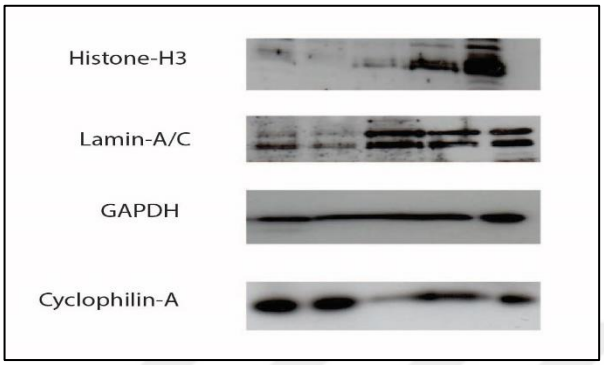

Figure 3.2. Determination of histone, lamin, GAPDH and cyclophilin A amounts in the enriched nuclear protein fractions obtained by using Ready Prep Nuclear Protein Extraction kit

WB results showed that nuclear proteins were partially enriched, and cytoplasmic protein contamination was present in the enriched protein extracts. These results are consistent with the results of 2DE and indicated that the commercial kit failed to enrich nuclear proteins to the desired level.

One of the alternative methods to commercial nuclear protein enrichment kit is the use of density gradient centrifugation method. Density gradient centrifugation is a powerful technique for separating complexes based on their molecular masses. Density gradient centrifugation offers different media options, such as sucrose, Ficoll, Nycodenz, and Iodixanol. Iodixanol was our preferred choice since it does not interfere with the downstream applications and does not need to be removed from the enriched extracts after the procedure is complete. Therefore, iodixanol (trade name OPTI-PREP) was used to enrich the nuclear proteins (Figure 3.3).

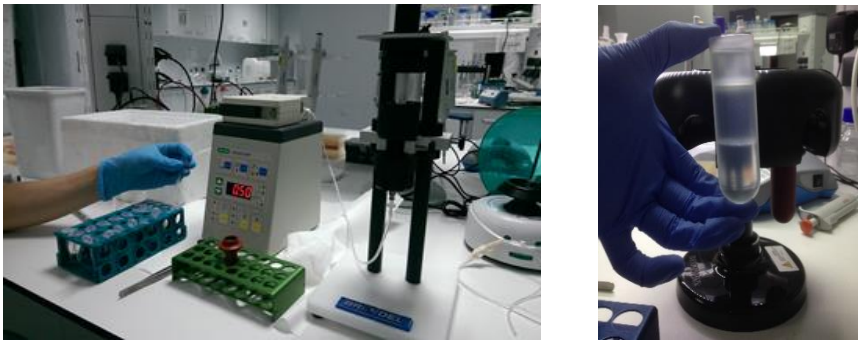

Figure 3.3. Extraction of nuclear proteins using density gradient centrifugation method

Thirty seven fractions were collected to seperate proteins based on their molecular densities. The fractions were then subjected to WB analysis using antibodies against Histon, Lamin A / C, GAPDH and Cyclophilin A. “The results showed that nuclear proteins were predominantly located in between fractions 24 and 31. However, these fractions were also contaminated with the cytoplasmic proteins (Figure 3.5). To assess the level of enrichment, 2DE was performed with fraction 29 and spots were randomly cut and identified by MALDI-TOF/TOF analysis (Figure 3.4) (Supplementary Table C.)

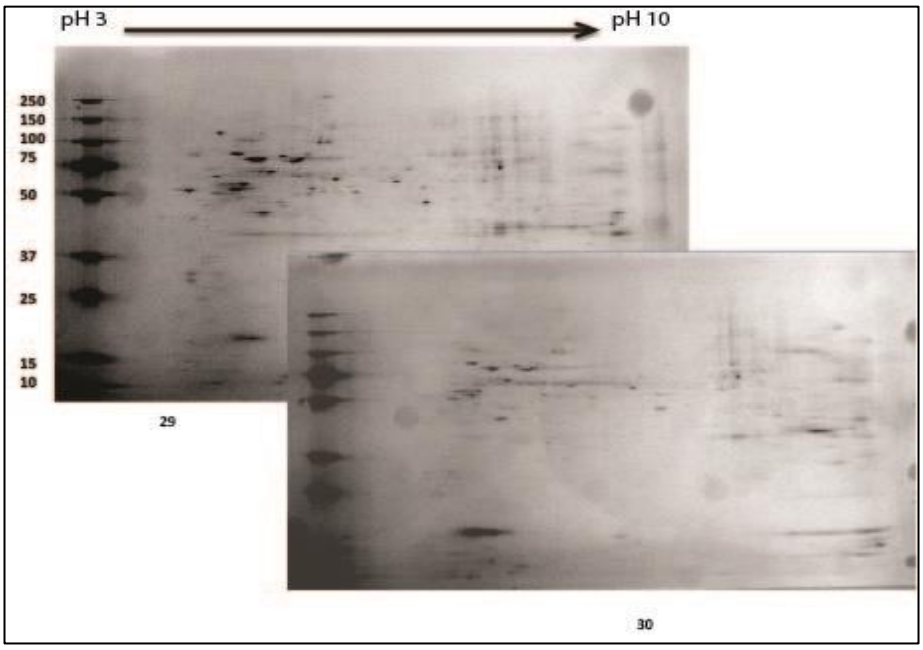

Figure 3.4. 2D gel electrophoresis analysis of nuclear proteins obtained using differential centrifugation method

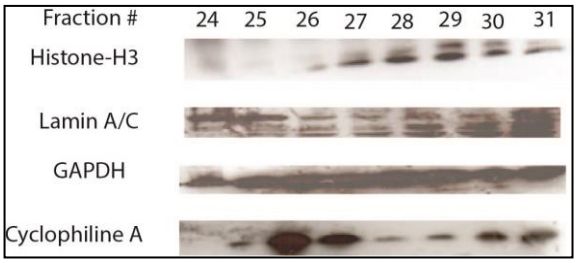

Figure 3.5. Determination of histone, lamin, GAPDH and cyclophilin A amounts in enriched nuclear proteins using density gradient centrifugation method

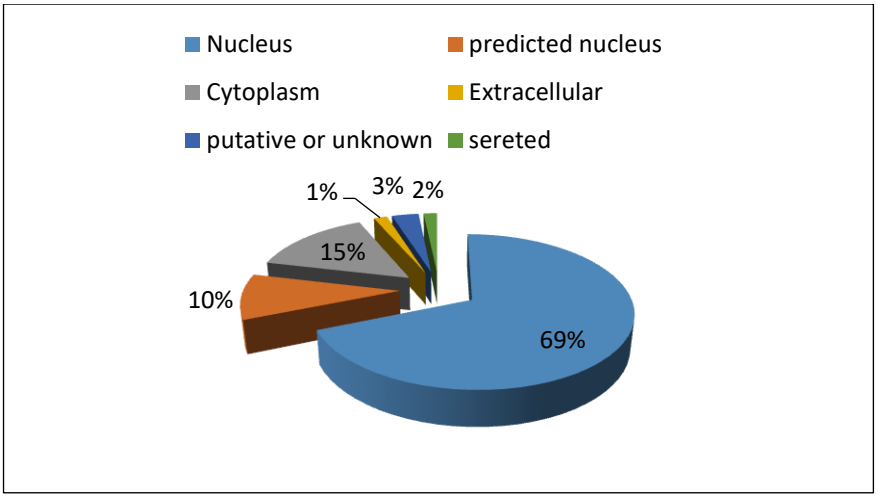

Figure 3.6. Pie chart for demonstration of the enriched protein distribution within the cells. The proteins were enriched using OPTI-Prep density gradiant centrifugation

One of the difficulties we experienced during enrichment of nuclear proteins with density gradient centrifugation was the lack of repetitivness of the experiments. In the repeated experiments, the fractions containing the nuclear proteins were collected at different fractions (sometimes between fractions 24-30 and sometimes between fractşons 26-28) although every single step of the protocole was tidiously performed. Such experimental veraibility would have casued problems during comparative proteome analysis. Therefore, alternative approaches for nuclear protein enrichment were explored.

Q-Proteome nuclear protein isolation kit (Qiagen, USA) was used as an alternative to the differential and density gradient centrifugation approaches. When the instructions were followed, two different nuclear protein fractions were obtained. The first

fraction contained the soluble nucleic acid binding proteins, while the second fraction contained the insoluble nuclear proteins. The enriched protein extracts were then subjected to WB analysis using antibodies against Histon, Lamin A / C, GAPDH and Cyclophilin A (Figure3.7). The nucleic acid binding and insoluble protein fractions were richer in their histone and Lamin A/C contents. In addition, the cyclophilin A level was much lower than the nuclear fractions indicating that the Q-proteome kit provided satisfactory level of enrichment to nuclear proteins. However, GAPDH was detected in the enriched nuclear protein fractions, although the level of GAPDH was much lower in comparison to the cytoplasmic fraction. As we stated previously, GAPDH is not a good cytoplasmic protein marker to demonstrate the level of nuclear protein enrichment.

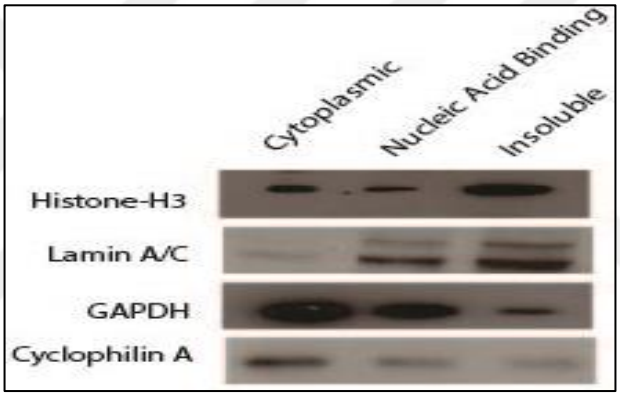

Figure 3.7. Determination of histone, GAPDH, Lamin A/C and Cyclophilin A amounts in nuclear proteins enriched with Q-Proteome kit.

The enriched nuclear protein fractions were subjected to 2DE gel electrophoresis to assess the level of enrichment (Figure3.8). Spots were randomly cut from the gels and identified by MALDI-TOF/TOF analysis. The results demonstrated that 90 % of the identifined proteins in the nucleic acid binding protein fraction were nuclear. Although the insoluble nuclear protein containing fraction had much lower nuclear protein ratio, it was still above 50% indicating that Q-proteome nuclear protein enrichment kit was superior to the other kits/approaches (Figure 3.8) (Supplementary Table D1 and D2).

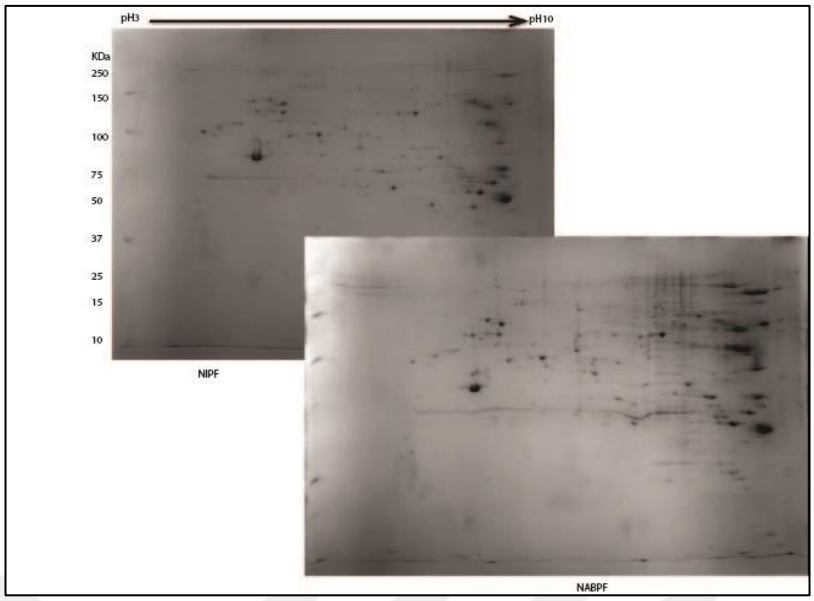

Figure 3.8. 2D gel electrophoresis analysis of the enriched nuclear proteins obtained using the QProteome nuclear protein isolation method

Comparison of the overall results indicated that the Q-proteome isolation method was more successful in enrichment of nuclear proteins than the other tested methods (Figure 3.9).

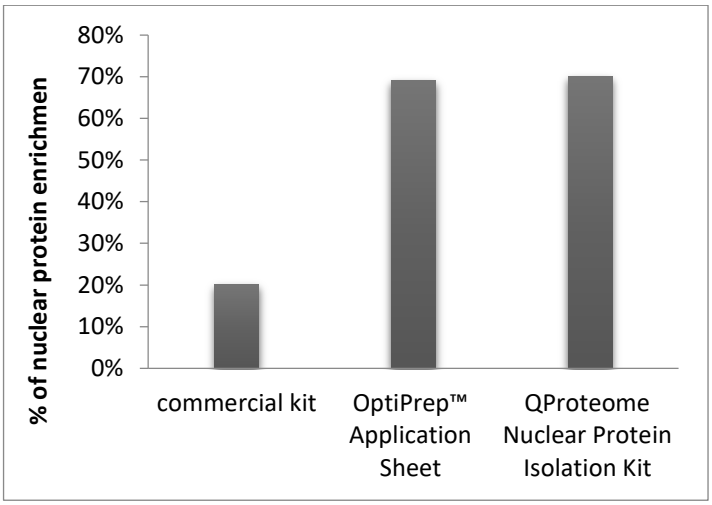

Figure 3.9. Comparative analysis of nuclear protein enrichment methods

3.2. Assessment of the WT and the Mutant Parkin Expressions in Enriched Nuclear Protein Fractions

To carry out comparative nuclear proteome analysis, SH-SY5Y cells expressing either the wild type or the mutant Parkin proteins were used. In addition, SH-SY5Y cells which did not express the Parkin protein was used as the control. Nuclear proteins were enriched from these cell lines using Q-proteome isolation kit. The enrichment experiments were performed in triplicate and the enriched nuclear protein fractions were combined to create pools representing the WT-Parkin+, the mutant Parkin+ and the Parkin- cells. Before running 2DE gels, the protein pools were subjected to WB analysis using anti-Parkin antibody to make sure of the presence of Parkin proteins in induced cell cultures (Figure3.10). Cells which were not induced for Parkin expression lacked the corresponding Parkin bands indicating that comparative proteomics analysis could be carried out with the enriched nuclear protein fractions.

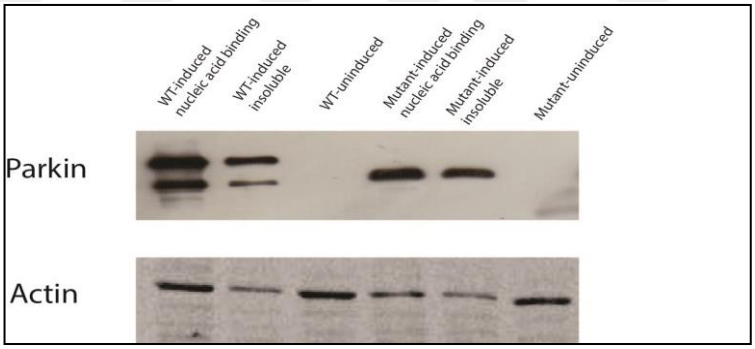

Figure 3.10. Western blot analysis of the WT and the mutant Parkin expressions in nuclear protein extracts enriched from SH-SY5Y cells. The abbreviation stands for, NABPF-Nucleic Acid Binding Protein Fractions ; NIPF - Nuclear Insoluble Protein Fractions. The antibodies used were monoclonal anti-Parkin (sc-30130; Santa Cruz, USA) and monoclonal anti-ß-actin (sc-8432; Santa Cruz, USA)

3.3. Assessment of Enrichment Levels in Nuclear Protein Extracts Used for Comparative Nuclear Proteome Analysis

During enrichment of nuclear proteins, two fractions labeled as the nucleic acid binding protein fraction and the nuclear insoluble protein fraction were obtained.

Both fractions were subjected to western blot analysis to assess the level of nuclear protein enrichments using nuclear and cytoplasmic protein markers (Figure 3.11).

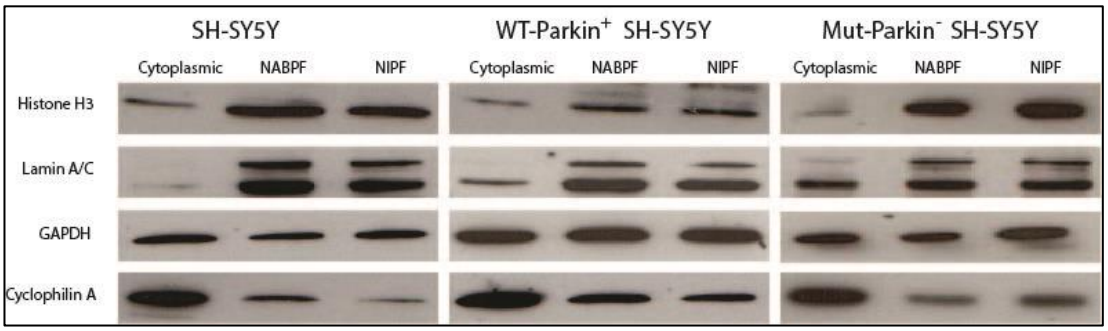

Figure 3.11. Western blot analysis to demonstrate nuclear protein enrichments in protein fractions isolated from SH-SY5Y cells. The abbreviation stands for, NABPF- Nucleic Acid Binding Protein Fractions ; NIPF - Nuclear Insoluble Protein Fractions. The antibodies used were anti-Histon antibody (9715; Cell Signaling Technology, USA), Anti-Lamin A/C antibody (4777; Cell Signaling Technology, USA), Anti-cyclophilin Antibody (5478-M01; Novus Biologicals, USA) and Anti-GAPDH antibody (sc-32233; Santa Cruz, USA)

Significant increases in the levels of two of the nuclear proteins, histone H3 and lamin A/C, were detected in the enriched nuclear protein fractions, while a significant decrease occurred in the level of cyclophilin A, a cytoplasmic protein. Surprisingly, however, there was no notable change in GAPDH levels in the enriched nuclear protein fractions indicating that GAPDH was not a good enrichment marker for the nuclear protein extracts. On the other hand, there was relatively low level of cyclophilin A in the enriched nuclear protein fractions indicating that the enriched nuclear protein fractions might possess cytoplasmic proteins. Quantitative evaluation of the level of enrichment was performed by running 2DE gels from which protein spots were cut and identified. Eighty six percent of the identified proteins localized to the nucleus indicating that a relatively good level of enrichment was achieved.

3.4. Comparative 2DE Analysis of the Enriched Nuclear Protein Fractions Expressing Parkin Proteins

To study the effect of expressing either the WT or the mutant Parkin proteins on nuclear proteome, 2DE gels were generated from both nucleic acid binding and nuclear insoluble protein fractions, prepared from SH-SY5Y cells expressing either the WT or the mutant Parkin proteins (Figure 3.12.).

Nucleic acid binding and nuclear insoluble protein fractions were also included from the control cells that did not express exogenous Parkin proteins. Well-resolved and reproducible 2DE gels were generated and subjected to spot detection. An average of 700 spots per analytical gel was detected. The overall mean coefficient for spot matching was 20% indicating that the protein distribution patterns among the gels were similar. The changes in spot intensities were compared and protein spots significantly differed in expression was identified. The list of the significantly regulated proteins, their respective MALDI scores and their corresponding regulation ratios were presented in Table 3.1.

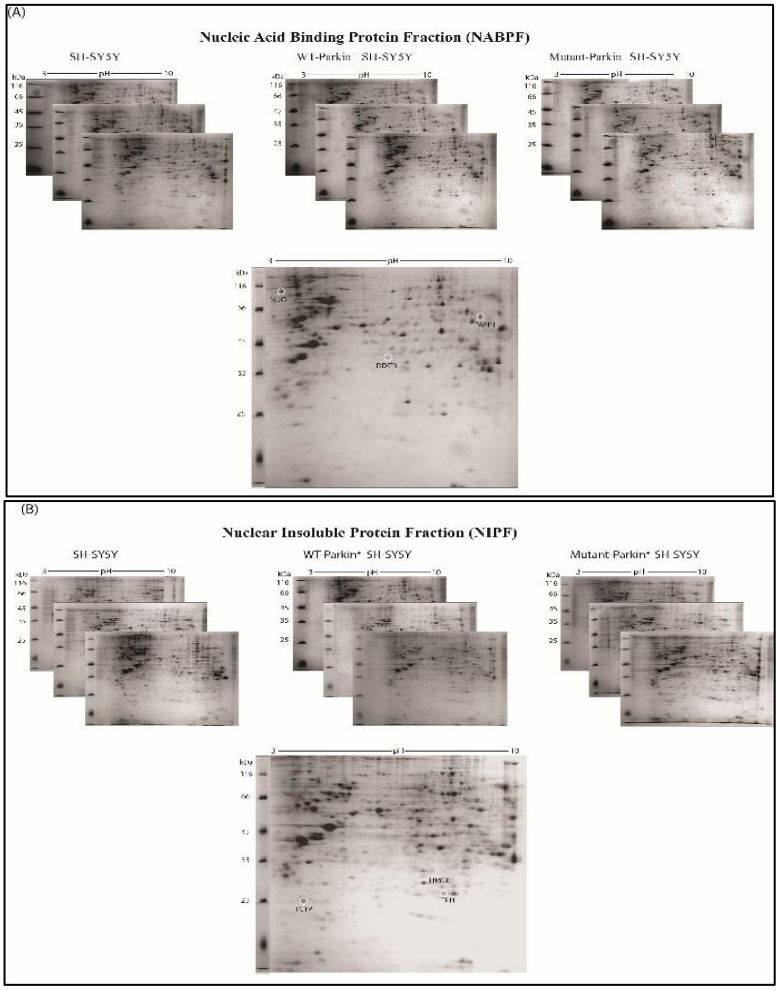

Figure 3.12.2DE gel images of SH-SY5Y cells expressing either the wild-type Parkin or the mutant Parkin proteins. (A) 2DE gel images of the nuclear nucleic acid binding protein fraction enriched from SH-SY5Y cells expressing either the wild-type Parkin or the mutant Parkin proteins. (B) 2DE gel images of the nuclear insoluble protein fraction enriched from SH-SY5Y cells expressing either the wild-type Parkin or the mutant Parkin proteins

Table 3.1. The list of differentially regulated proteins upon expressions of the wild-type or the mutant Parkin proteins in the nucleic acid binding and the insoluble protein fractions enriched from SH-SY5Y cells

Uniprot Accession #	Gene Name	Protein Name	MALDI-TOF/TOF analysis score	Regulation Ratio (WT-Parkin^{+}/WT-Parkin^{-})	Regulation Ratio (Mutant-Parkin^{+}/Mutant-Parkin^{-})	Subcellular location
Nucleic Acid Binding Protein Fraction						
Q5VX85	PARP1	Poly [ADP-ribose] polymerase 1	147	12.64-fold up regulated	9.9-fold up-regulated	Nucleus
P19338	NCL	Nucleolin	109	33.8-fold up regulated	23.51-fold up regulated	nucleus, cytoplasm
P35638	DDIT3	DNA damage-inducible transcript 3 protein	144	19.62-fold down regulated	15.46-fold down regulated	nucleus, cytoplasm
Nuclear Insoluble Protein Fraction						
P09429	HMGB1	High mobility group protein B1	73	15.57 fold up regulated	14.89 fold up regulated	cell membrane, cytoplasm, endosome, nucleus, secreted
P60174	TPI	Triosephosphate isomerase	245	13.13 fold up regulated	17.01 fold up regulated	cytoplasm, nucleus
P13693	TCTP	Translationally-controlled tumor protein	110	12.8 fold down regulation	12.63 fold down regulation	cytoplasm, nucleus

The regulations observed in 2DE experiments were verified by western blot analysis. Four differentially regulated proteins, namely NCL, DDIT3, TPI1 and TCTP were selected. Western blot results agreed with the changes observed in 2DE experiments (Figure 3.13A and B).

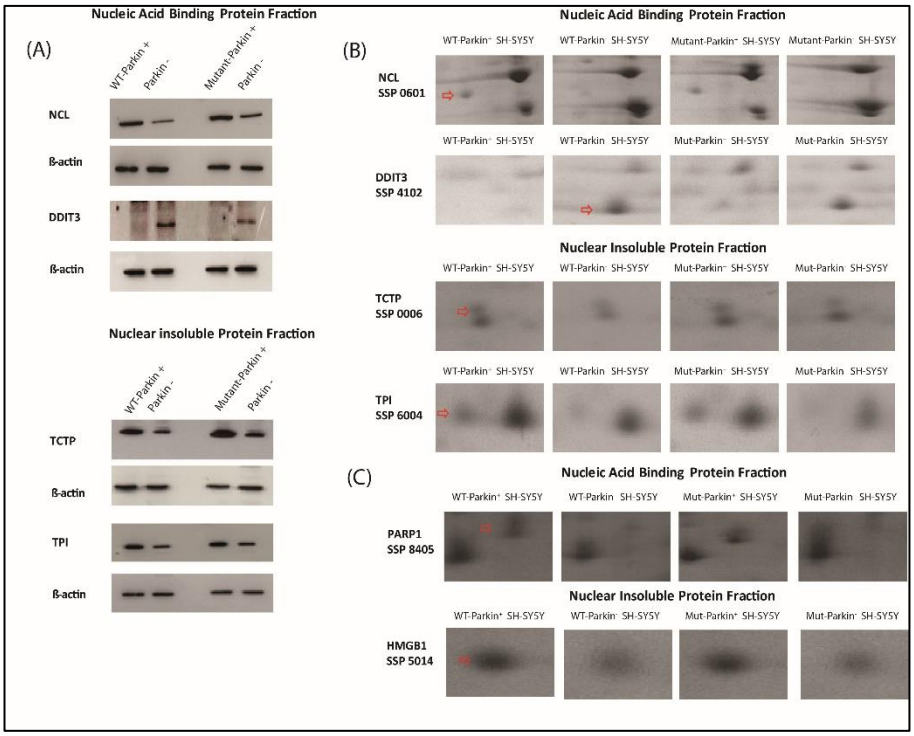

Figure 3.13. Validation of differentially regulated protein spots. (A) Verification of the changes occurred in NCL, DDIT3, TCTP, and TPI levels by western blotting. (B) The close-up images of the validated differentially regulated protein spots generated from the respective the 2DE gels. (C) The close-up images of the invalidated differentially regulated protein spots generated from the respective the 2DE gel

The antibodies used were monoclonal anti-Nucleolin (NCL) (14574; Cell Signaling Technology, USA), monoclonal anti-ß-actin (sc-8432; Santa Cruz, USA), monoclonal anti-translationally controlled tumor protein (TCTP) (8441; Cell Signaling Technology, USA), monoclonal anti-Triosephosphate isomerase (TPI) (sc-166785; Santa Cruz, USA) and monoclonal anti-DNA damage-inducible transcript 3 protein (DDIT3) (sc-7351; Santa Cruz, USA).

NCL was up-regulated in nucleic acid binding fractions enriched from the WT- and the mutant Parkin expressing cells. On the other hand, DDIT3 was down regulated in the nucleic acid binding protein fractions upon synthesis of Parkin proteins. As to the nuclear insoluble protein fractions, TPI1 and TCTP proteins displayed increases in their abundance. Although not verified by western blotting, there were other proteins that were regulated in nucleic acid binding and insoluble nuclear protein fractions, e.i., PARP1 and HMGB1, respectively (Figure 3.13, Table 3.2).

Table 3.2. The list of othern differentially regulated proteins upon expressions of the wild-type or the mutant Parkin proteins in the nucleic acid binding and the insoluble protein fractions enriched from SH-SY5Y cells

Uniprot Accession #	Gene Name	Protein Name	MALDI-TOF/TOF analysis score	Regulation Ratio (WT-Parkin+/WT-Parkin-)	Regulation Ratio (Mutant-Parkin+/Mutant-Parkin-)	Subcellular location
Nucleic Acid Binding Protein Fraction						
P31942	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3	330	Not detected	2.58 fold up regulated	Nucleus
P07910	HNRPC	Heterogeneous nuclear ribonucleoproteins C1/C2	80	4.54 fold up regulated	11.42 fold up regulated	Nucleus, cytoplasm
Nuclear Insoluble Protein Fraction						
P45973	CBX5	Chromobox protein homolog 1	95	3.94 fold down regulated	Not detected	Nucleus
P35232	PHB	Prohibitin	95	2.4 fold down regulated	3.47 fold down regulated	Membrane, mitochondrion, mitochondrion inner membrane, go to nucleus
Q13148	TADBP	TAR DNA-binding protein 43	56	Not detected	2.34 fold down regulated	Cytoplasm, nucleus
Q96J01	THOC3	THO complex subunit	69	2.71 fold up regulated	4.18 fold up regulated	Nucleus
P35998	PRS7	26S protease regulatory subunit 7	47	30.87 fold down regulated	6.98 fold down regulated	Cytoplasm, proteasome, g oto nucleus
P30041	PRDX6	Peroxiredoxin-6	60	2.84 fold down regulated	2.99 fold up regulated	Cytoplasm, lysosome, go to nucleus
Q16629	SFRS7_HUMAN	Splicing factor, arginine/serine-rich 7	35	6.48 fold down regulated	Not detected	Cytoplasm, nucleus
Q15365	PCBP1	Poly(rC)-binding protein 1	97	3.82 fold up regulated	3.76 fold up regulated	Cytoplasm, nucleus
Q92945	FUBP2	Far upstream element-binding protein 2	218	2.08 fold up regulated	2.3 fold up regulated	Cytoplasm, nucleus
P45880	VDAC2	Voltage-dependent anion-selective channel protein 2	113	1.74 fold down regulated	3.37 fold down regulated	Mitochodrian, go to nucleus
P07355	ANXA2	Annexin A2	190	Not detected	2.31 fold down regulated	Basement membrane, extracellular matrix, Secreted, go to nucleus
P63241	IF5A1	Eukaryotic translation initiation factor 5A-1	57	2.75 fold up regulated	Not detected	Cytoplasm, endoplasmic reticulum, membrane, Nuclear pore complex, nucleus
P12004	PCNA	Proliferating cell nuclear antigen	105	2.08 fold up regulated	Not detected	Nucleus

3.5. Phosphoprotein Analysis of the Protein Extracts Expressing Parkin Proteins

Total and enriched nuclear protein fractions were used in 2DE experiments to monitor the changes occurring at the phosphoproteome level (Figure3.15, Table 3.3). The gels were first stained with ProQ-diamond phosphoprotein stain and then with SYPRO Ruby total protein stain. The regulated proteins displayed changes in their levels in ProQ-diamond stained gels but not in SYPRO Ruby stained gels.

The expression of either the WT or the mutant Parkin caused a significant increase in phosphorylation levels of several proteins to significant degrees when total cell-free protein extracts were compared (Figure 7A). These proteins were endoplasmin (ENPL), annexin A5 (ANXA5), peroxyredoxin (PRDX4), enoylCoA hydratase (ECHM) and aldolase A (ALDOA) (Table 3.3). There were other proteins whose levels were also regulated but the regulation ratios were not as significant as the above mentioned proteins (Table 3.4). Therefore, they were not critically evaluated within the scope of this thesis.

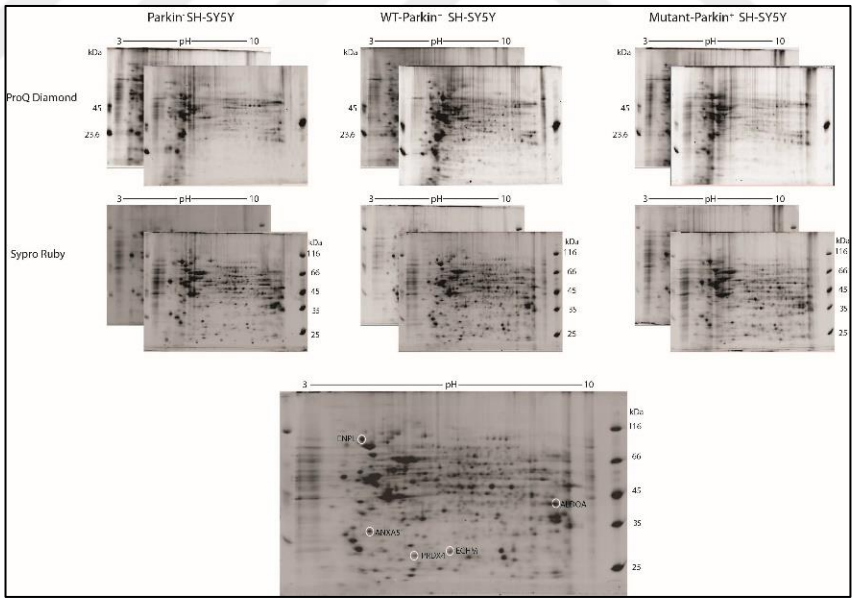

Figure 3.14.2DE gel images used for analysis of the changes occurring at the phophoproteome level. The gels were stained with phosphoprotein stain (ProQ-diamond) and then with SyproRuby total protein stain and imaged with VersaDoc MP4000 using the appropriate filter sets. The cells expressed either the wild-type Parkin or the mutant Parkin proteins

Table 3.3. Phosphoproteins from total cell extracts that were differentially regulated after the WT and the mutant Parkin protein expressions

Uniprot Accession #	Gene name	Protein Name	MALDI-TOF/TOF analysis score	Subcellular Location	Type of phosphorylation listed in Uniprot database	WT/ Control	Mutant / Control
P14625	ENPL	Endoplasmin	150	Endoplasmic reticulum	Phosphoserine, Phosphothreonine Phosphotyrosine	29.5	14.8
P08758	ANXA5	Annexin A5	255	Cytoplasm	Phosphoserine	787.2	382.2
Q13162	PRDX4	Peroxiredoxin-4	283	Endoplasmic reticulum	Phosphoserine Phosphothreonine Phosphotyrosine	26.5	23.2
P30084	ECHM	Enoyl-CoA hydratase.	225	Mitochondria	Phosphoserine,Phosphothreonine	53.4	24.4
P04075	ALDOA	Fructose-bisphosphate aldolase A	163	Cytoplasm	Phosphoserine, Phosphothreonine Phosphotyrosine	25.8	23.8

Table 3.4. Phosphoproteins from total cell extracts that were not significantly regulated after the WT and the mutant Parkin protein expressions

Uniprot Accession#	Gene name	Protein Name	MALDI-TOF/TOF analysis score	Subcellular Location	Type of phosphorylation listed in Uniprot database	WT/ Control	Mutant/Control
P27797	CALR	Calreticulin	123	Endoplasmic reticulum	Phosphoserine Phosphothreonine Phosphotyrosine	2.8	NR
Q86XL3	ANKL2	Ankyrin repeat and LEM domain-containing protein 2	40	Endoplasmic reticulum	Phosphoserine	3.9	NR
P60709	ACTB	Actin. cytoplasmic 1	79	Cytoplasmic. go to nucleus	Phosphoserine Phosphothreonine Phosphotyrosine	4.2	6.5
P07195	LDHB	L-lactate dehydrogenase B chain	175	Cytoplasm	Phosphoserine	4.8	NR
Q13011	ECH1	Delta(3.5)-Delta(2.4)-dienoyl-CoA isomerase. Mitochondrial	161	Mitochondria	Phosphoserine	7.7	2.3
P49450	CENPA	Histone H3-like centromeric protein A	30	Nucleus	phosphoserine	3.9	2.3
P25705	ATPA	ATP synthase subunit alpha. mitochondrial	78	Plasma membrane	phosphoserine	2.8	NR
Q6ZNG1	ZN600	Zinc finger protein 600	66	Nucleus	Phosphoserine Phosphothreonine Phosphotyrosine	6.2	6.1

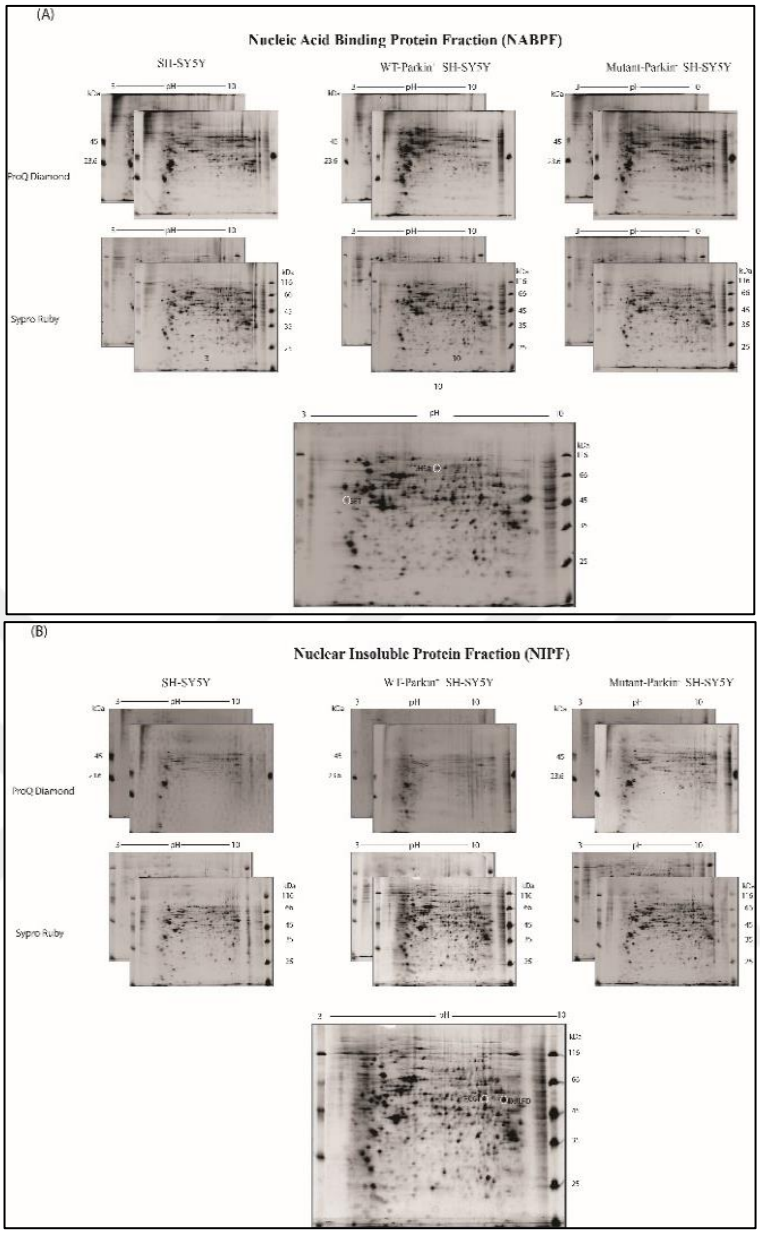

Figure 3.15. Changes occurring at the phosphoproteome levels. (A) 2DE gel images used for analysis of the changes occurring at the phophoproteome level in nucleic acid binding protein fractions. The gels were stained with phosphoprotein stain (ProQ-diamond) and then with SyproRuby total protein stain and imaged with VersaDoc MP4000 using the appropriate filter sets. The cells expressed either the wild-type Parkin or the mutant Parkin proteins. (B) 2DE gel images used for analysis of the changes occurring at the phophoproteome level in nuclear insoluble protein fractions. The gels were stained with phosphoprotein stain (ProQ-diamond) and then with SyproRuby total protein stain and imaged with VersaDoc MP4000 using the appropriate filter sets. The cells expressed either the wild-type Parkin or the mutant Parkin proteins

The changes in phosphorylation status in nucleic acid binding and nuclear insoluble protein fractions after the WT or the mutant Parkin expressions were also determined (Table 3.5). The expressions of the WT and the mutant Parkin proteins caused significant increases in phosphorylation levels of protein SET (SET) and succinate dehydrogenase (DHSA) in nucleic acid binding protein fractions (Figure 3.15 B). There were several other proteins whose phosphorylation levels also changed either upon the WT or the mutant Parkin expressions but the changes were not as prominent and thus will not be discussed within the scope of this thesis (Table 3.6).

Table 3.5. Phosphoproteins that were differentially regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions

Gene Name	Protein Name	MALDI-TOF/TOF analysis score	Subcellular Location	Type of phosphorylated amino acid listed in Uniprot database	WT/ Control	Mutant/Control
Nucleic Acid Binding Protein Fractions						
SET	Protein SET	57	Nucleus	Tyrosine	30.3 fold up regulated	8.9 fold up regulated
DHSA	Succinate dehydrogenase	59	Mitochondria	Tyrosine, Serine, Threonine	7.8 fold up regulated	9.9 fold up regulated
Nuclear Insoluble Protein Fractions						
RCC1	Regulator of chromosome condensation	48	Nucleus	Serine	11.3 fold down regulated	10.4 fold down regulated
DULRD	Serine/threonine-protein phosphatase dullard	33	Nucleus	Tyrosine, Serine, Threonine	11.7 fold down regulated	12.2 fold down regulated

Table 3.6. Phosphoproteins that were not significantly regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions

Uniprot Accession #	Gene name	Protein Name	MALDI-TOF/TOF analysis score	Subcellular Location	Type of phosphorylated amino acid listed in Uniprot database	WT/ Control	Mutant / Control
Nucleic Acid Binding Protein Fractions							
P13693	TCTP	Translationally-controlled tumor protein	131	Nucleus	Phosphotyrosine, Phosphoserine, Phosphothreonine	2,64	Not detected
P05198	IF2A	Eukaryotictranslationinitiationfactor 2 subunit 1	149	Nucleus	Phosphoserine, Phosphothreonine	0,17	Not detected
P14625	ENPL	Endoplasmin	408	Nucleus	Phosphotyrosine, Phosphoserine	4,69	Not detected
Q12874	SF3A3	Splicingfactor 3A subunit 3	108	Nucleus	Phosphotyrosine, Phosphoserine	Not detected	2,41
P13838	UAP56	Spliceosome RNA helicase BAT1	79	Nucleus	Phosphotyrosine, Phosphoserine	2,13	Not detected
P31943	HNRH1	Heterogeneousnuclearribonucleoprotein H	186	Nucleus	phosphoserine	2,08	Not detected
Q07955	SFRS1	Splicingfactor, arginine/serine-rich 1	111	Nucleus	Phosphotyrosine, Phosphoserine	Not detected	5,53
Q12931	TRAP1	Heatshock protein 75 kDa	103	Nucleus	Phosphoserine, Phosphothreonine	Not detected	0,1
Q8WXF1	PSPC1	Paraspecklecomponent 1	38	Nucleus	Phosphoserine	4,14	Not detected
Nuclear Insoluble Protein Fractions							
P06748	NPM	Nucleophosmin	203	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	2,84	2.7 down
P29692	EF1D	Elongation factor 1-delta	57	Nucleus	Phosphoserine, phosphothreonine	Not detected	2.6 down
Q15019	SEPT2	Septin-2	80	Nucleus	Phosphoserine, phosphotyrosine	3.3 down	Not detected

In the nuclear insoluble protein fractions, two proteins chromosome condensation protein (RCC1) and serine/threonine-protein phosphatase dullard (DULRD) displayed changes in their phosphorylation levels (Figure 3.15; Table3.5). For the other phosphorylated proteins, the level of change was not as significant (Table3.6).

3.6. 2D Western Blotting for Verification of the Data Obtained from ProQ-Diamond Stained Gels

To verify the changes occurred in phosphproteome profiles observed in ProQ diamond stained gels, an alternative approach was taken. In this approach two 2DE gels were produced per experiment. One of the gels was stained with ProQ diamond stain, imaged and then re-stained with colloidal Coomassie blue. The other unstained gel was used in the western blotting experiment. The blot was probed with a mixture

of anti-phospho serine and threonine antibodies. The phosphoprotein profiles appeared on the hypersensetive films was then used to cut spots from the Coomassie stained gels (Figures 3.16).

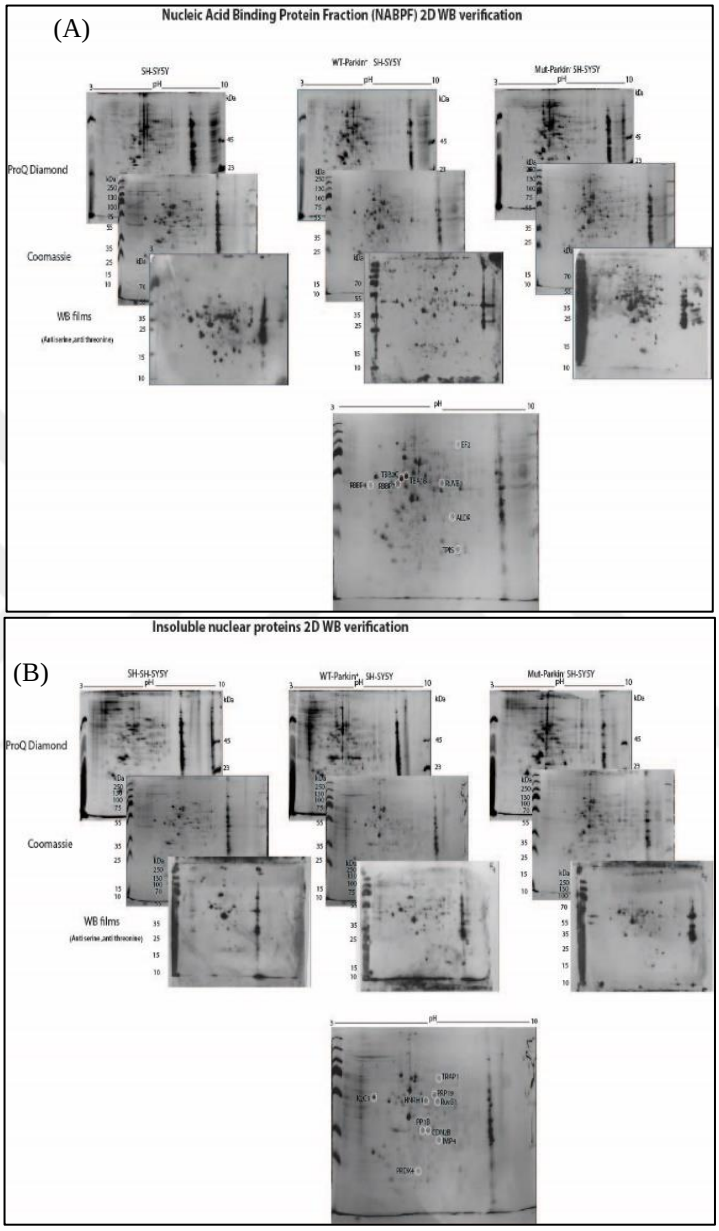

Figure 3.16. 2D Western Blotting for verification of the nuclear proteins. (A) Verification of nucleic acid binding fraction. (B) Verification of insoluble nuclear fraction

List of the differentially phosphorylated/dephosphorylated proteins were given in Table3.7. Eight proteins from nucleic acid binding protein fractions and 9 proteins from nuclear insoluble protein fractions were differentially phosphorylated /

dephosphorylated. And the regulation ratio of all of these identified nuclear proteins in accordance with ProQ diamond stain images.

Table 3.7. List of the differentially phosphorylated/dephosphorylated proteins that were differentially regulated in the nucleic acid binding and nuclear insoluble protein fractions after the WT and the mutant Parkin expressions

Uniprot Accession #	Gene name	Protein Name	MALDI-TOF/TOF analysis score	Subcellular Location	Type of phosphorylated amino acid listed in Uniprot database	WT/ Control	Mutant/Control
Nucleic Acid Binding Protein Fractions							
Q09028	RBBP4_HUMAN	Histone-binding protein RBBP4	113	Nucleus	Phosphoserine	4.36	0.17
Q16576	RBBP7_HUMAN	Histone-binding protein RBBP7	95	Nucleus	Phosphoserine, phosphothreonine	46.87	72.81
P68371	TBB2C_HUMAN	Tubulin beta-2C chain	295	Nucleus	Phosphoserine, phosphothreonine	16.88	6.14
P68363	TBA1B_HUMAN	Tubulin alpha-1B chain	267	Nucleus	Phosphoserine, phosphotyrosine	3.13	3.80
P60174	TPIS_HUMAN	Triosephosphate isomerase	180	Cytoplasm, go to nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	0.29	9.51
P15121	ALDR_HUMAN	Aldose reductase	172	Cytoplasm, go to nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	19.02	150.41
P13639	EF2_HUMAN	Elongation factor 2	81	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	7.01	143.94
Q9Y265	RUVB1_HUMAN	RuvB-like 1	166	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	3.20	2.86
Nuclear Insoluble Protein Fractions							
P04264	K2C1_HUMAN	Keratin, type II cytoskeletal 1	54	Membrane, go to Nucleus	Phosphoserine	2.12	0.003
Q13162	PRDX4_HUMAN	Peroxiredoxin-4	78	Cytoplasm, go to nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	0.11	0.04
P31943	HNRH1_HUMAN	Heterogeneous nuclear ribonucleoprotein H	145	Nucleus	Phosphoserine,tyrosine	1.49	1.77
Q9Y265	RUVB1_HUMAN	RuvB-like 1	121	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	9.01	21.81
Q12931	TRAP1_HUMAN	Heat shock protein 75 kDa, mitochondrial	143	Cytoplasm, go to nucleus	Phosphoserine, phosphothreonine	14.09	15.96
P62140	PP1B_HUMAN	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	40	Nucleus	phosphothreonine	8.02	0.56
P42772	CDN2B_HUMAN	Cyclin-dependent kinase 4 inhibitor B	34	Cytoplasm, go to nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	0.35	0.03
Q96G21	IMP4_HUMAN	U3 small nucleolar ribonucleoprotein protein IMP4	37	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	0	13.84
Q9UMS4	PRP19_HUMAN	Pre-mRNA-processing factor 19	39	Nucleus	Phosphoserine,phosphothreonine,phosphotyrosine	57.03	83.61

4. DISCUSSION AND FUTURE WORK

4.1. Discussion

Parkin localizes to the cytoplasm and the nucleus (Ozgul et al., 2015). The physiological function of cytoplasmic Parkin is studied extensively and known to operate as an E3 ubiquitin ligase to scavenge its substrates within proteosomes (Cookson, 2005). On the other hand, the nuclear form of the Parkin is less studied and there is still much to learn about its physiological function. Research so far demonstrated the involvement of nuclear Parkin in cell cycle regulation, tumor suppression and anti-apoptotosis (Alves da Costa and Checler, 2011; Fujiwara et al., 2008; Staropoli et al., 2003; Tay et al., 2010; Veeriah et al., 2010; Zhang et al., 2011). Parkin also prevents accumulation of damaged mitochondria via mitophagy and prevents mitophagy-associated tumor formation (Chourasia et al., 2015; Salazar et al., 2018). To achieve its tumor-associated function, Parkin has to directly or indirectly interact with several nuclear proteins. For example, Parkin's involvement in cell cycle regulation occurs via Cyclin E, Cyclin D and Myt1 (Bartek and Hodny, 2014; Liu et al., 2018). Similarly, Parkin ensures proper maintenance of mitosis by interacting with Cyclin B1 and Aurora A/B (Lee et al., 2015). The apoptotic function of Parkin requires its involvement with Mcl-1, survivin and follistatin (Carroll et al., 2014; Harrison et al., 2005; Lee et al., 2012). The involvement of Parkin's metabolic reprogramming occurs through its interaction with p53, PTEN, Hif-1α, Myc and PI3K (Liu et al., 2018; Liu et al., 2017; Zhang et al., 2011).

Parkin plays a pivotal role in prevention of various tumors and displays tumor suppressor activity (Devine et al., 2011; Fujiwara et al., 2008; Saito et al., 1996; Staropoli, 2008; Tibiletti et al., 1996). Several key players help Parkin to execute its tumor suppressor function. However, the links to the whole process is still missing. Parkin's tumor suppressor activity is likely to be carried out by its nuclear form. We thus placed an effort on monitoring the changes in nuclear proteome in the wild type and the mutant Parkin expressing cells. Nuclear proteins were enriched from these cell lines to study the changes occurring at the nuclear proteome level. Nucleic acid

binding and insoluble nuclear protein fractions were studied. In the nucleic acid binding nuclear protein fraction, three of the nuclear proteins, NCL, PARP1 and DDIT, displayed significant changes in their expression levels after the WT and the mutant Parkin expressions. The regulations occurring in NCL and DDIT3 were also verified by WB analysis.

NCL is a major nuclear protein expressed by growing eukaryotic cells and involves in several aspects of cellular homeostasis, including cell proliferation, ribosomal biogenesis, signal transduction and apoptosis (Chen and Xu, 2016). NCL also plays a significant part in cancer progression and metastasis by protecting the stability of the genome, limiting the accumulation of DNA damage, regulating apoptosis by elevating Bcl-2 levels and modulating the levels of angiogenic factors (Chen and Xu, 2016; De et al., 2006; Ishimaru et al., 2010; Liang et al., 2013). NCL is identified to be interacting with alpha-synuclein and DJ-1, two critical proteins involved in PD pathogenesis (Caudle et al., 2009). Dramatically reduced NCL levels are observed in human PD brains in comparison to the controls (Caudle et al., 2009). Manipulation of NCL levels in an *in vitro* model of PD resulted in significant alterations in generation of oxidative stress and that was free of mitochondrial comLex I inhibition suggesting a NCL specific changes in oxidative stress and proteosomal pathways (Caudle et al., 2009). Parkin and NCL are multifunctional proteins. They both localized to the nucleus and the cytoplasm, involve in oxidative stress and play roles in cancer and PD. However, there was no report functionally associating these two proteins to a common cellular event. In this regard, this is the first study reporting that modulation of Parkin expression results in changes in NCL levels that suggests a possible link between these two proteins.

DDIT3 is a multifunctional transcription factor, which plays an essential role in the response to a wide variety of cell stresses (Yamaguchi and Wang, 2004). It may induce cell cycle arrest and apoptosis in response to ER stress. A chromosomal aberration involving DDIT3 has been found in a patient with malignant liposarcoma (Rabbitts et al., 1993). A recent publication investigating endoplasmic reticulum (ER) stress linked Parkin and DDIT3 and reported that the turnover of DDIT3 upon tunicamycin induced ER-stress was Parkin-dose dependent (Han et al., 2017).

However, there has been no report linking the nuclear form of DDIT3 to the nuclear form of Parkin.

The last protein that was differentially regulated in the nucleic acid binding protein fraction was PARP1. PARP1 poly-ADP-ribosylate proteins and plays a pivotal role in DNA repair (Ahel et al., 2009; Ray Chaudhuri and Nussenzweig, 2017). The association of PARP1 with Parkinson's disease has already been established (Brundin and Wyse, 2018). The potential of ADP-ribosyl residues to induce formation of pathogenic alpha-synuclein fibers created the idea that PARP1 inhibitors can be used to treat PD (Kam et al., 2018). In fact, several clinical trials proved this correct (Malyuchenko et al., 2015). Our observation of the increase in PARP1 levels upon Parkin expression seems intriguing because Parkin's physiological function does not fit into this frame where it ubiquitylates proteins for scavenging. However, there are studies reported the translocation of Parkin into nucleus upon DNA damage to help in repair (Kao, 2009a; Kao, 2009b). It is likely that Parkin and PARP1 works in coordination during DNA repair.

In the insoluble nuclear protein fractions, three proteins, HMGB1, TCTP and TPI, displayed significant changes in their expression levels after the wild type and the mutant Parkin synthesis. HMGB1 and TCTP are nuclear proteins while TPI is a cytoplasmic protein. The regulations occurring in TPI and TCTP were also verified by WB analysis.

HMGB1 is a multifunctional redox sensitive protein with various roles in different cellular compartments (Yang et al., 2013). In the nucleus, it acts as a DNA chaperone and involves in replication, transcription, chromatin remodeling and DNA repair. HMGB1 has both pro-tumor and anti-tumor activities in tumorogenesis. It can either promote cell survival and or help cell death by regulating multiple cellular events (Kang et al., 2013). In our study, the nuclear form of HMGB1 is up-regulated in parallel to the increase in Parkin levels. Although it is not clear why there is such a change in HMGB1 levels, it is our prediction that Parkin and HMGB1 may work together on the repair of damaged DNA as a part of big protein complex. Like Parkin, HMGB1 may bind different DNA structures without sequence specificity and participates in DNA repair.

TCTP, a multifunctional housekeeping protein, is involved in regulating many fundamental processes, such as cell proliferation, apoptosis, pluripotency, and the cell cycle regulation. Hence, it is not surprising that differential regulation of TCTP levels may lead to tumorogenesis (Koziol and Gurdon, 2012). There is no known direct interaction between TCTP and Parkin despite our observation of the increase in TCTP levels in parallel to the increase in Parkin levels. A co-immunoprecipitation study with TCTP did not list Parkin among TCTP interaction partners (Li et al., 2016). However, an indirect association is still possible between TCTP and Parkin. For example, the mechanism of TCTP-dependent tumorogenesis was postulated to be due to p53 destabilization (Nagano-Ito and Ichikawa, 2012). Parkin also represses expression of p53 by physically interacting with the promoter region of TP53 (Alves da Costa and Checler, 2011). Thus, p53 may form the center node to create a network in which both Parkin and TCTP play roles.

The last protein that was differentially regulated in the insoluble nuclear protein fraction was triose phosphate isomerase (TPI), a cytosolic glycolytic enzyme that functions in catalyzing the interconversion of dihydroxyacetome phosphate to glyceraldehyde 3 phopsphate (Olivares-Illana et al., 2017). It was surprising to see a cytoplasmic protein in our nuclear protein fractions. However, many cytoplasmic metabolic enzymes are known to be relocated into the nucleus, a phenomenon called moonlighting (Boukouris et al., 2016). The nuclear functions of these moonlighting enzymes might be independent of their catalytic activity. So far, several glycolytic enzymes including hexokinase, phosphogluco isomerase and glyceraldehyde triphosphate dehydrogenase were shown to moonlight. To the best of our knowledge, there is no report of TPI moonlighting to the nucleus. Like other glycolytic enzymes, TPI is expected to be upregulated in the cytosol and should favor cancer cell proliferation. The effect of the increase in nuclear TPI levels is not known. The closest study to provide clues about the role of moonlighting TPI was performed with hepatocellular carcinoma cells. The forced expression of TPI slowed tumor growth and decreased tumor weight *in vivo* (Jiang et al., 2017). Furthermore, cell cycle arrest was induced by TPI via altered expressions of beta-catenin, vimentin, p53, p27 and cyclin D1 (Jiang et al., 2017). In our study, we observed an upregulation in TPI levels in the nucleus in parallel to the increase in Parkin levels.

Like TPI, Parkin exerts its effect on cell cycle regulation by interacting with p53, p27 and cyclin D1 (Alves da Costa and Checler, 2011; Gong et al., 2014; Zhang et al., 2011). Thus, there appears to be a cross functional relationship between Parkin and TPI.

Aside from its well-known E3- protein ubiquitin ligase activity, Parkin may play roles in signal transduction by providing a cross-talk between ubiquitination and phosphorylation. Understanding how this cross-talk occurs is important in cell signaling. In this study, we used the fluorescent phophospecific ProQ diamond stain to determine changes in overall phosphorylation status of the soluble proteome between Parkin expressing and non-expressing SH-SY5Y cells. Four phosphoproteins that displayed differential expression pattern were identified. Those proteins were SET, DHSA, RCC1 and DULRD.

SET is a multitasking protein and was shown to stimulate DNA replication activity in an *in vitro* system (Nagata et al., 1995), inhibit Protein Phosphatase 2A [Li et al., 1996], interacts with p35, the activator protein of CDK5 (Qu et al., 2002). DHSA that is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q) (Rasheed and Tarjan, 2018). RCC1is a Guanine-nucleotide releasing factor promotes the exchange of Ran-bound GDP by GTP, and thus plays an important role in RAN-mediated functions in nuclear import (Bischoff and Ponstingl, 1991). DULRD is part of an active phosphatase complex that dephosphorylates and may activate LPIN1 and LPIN2 which are phosphatidate phosphatases that catalyze the conversion of phosphatidic acid to diacylglycerol and control the metabolism of fatty acids at different levels(Wu et al., 2011). There appeared to be no significant functional association among these differentially phosphorylated proteins. We do not know the reason behind increase in the phosphorylation levels of these proteins. Even if that is the case, providing a list of proteins whose phosphorylation levels is affected by Parkin is still a valuable asset for future studies which will aim to discover Parkin associated novel phosphoproteins.

The mutant Parkin (Q311R and A371T) used in this study did not exert any substantial effect on the nuclear or phosphonuclear proteomes. There were several

protein spots whose expression levels were selectively regulated by the mutant but the calculated regulation ratios were not prominent. Therefore, in here, we kept our focus on the proteins whose expression levels have substantially changed when both the WT and the mutant Parkin proteins were expressed. The mutant Parkin was shown to display E3 ubiquitin ligase activity both in vitro and in vivo at a similar level as the wild-type Parkin. It is perhaps better to search for the interaction partners of the mutant Parkin using immunoprecipitation approaches to elucidate the effect of the mutations on Parkin protein.

4.2. Conclusion

Although they are two disparate disorders, the association between PD and cancer is firmLy established. However, majority of the overlapping genes and proteins involved in PD and cancer remains to be discovered. Discovery of shared pathogenic mechanisms may open a therapeutic window for both diseases. Mutations in Parkin are the major drivers of early onset PD and there is evidence that Parkin plays a pivotal role in cancer progression as well. The involvement of Parkin both in PD and cancer, thus, creates a point where the relationship between degeneration of neurons and formation of aggressive cancer types can be studied. By knowing that, two SH-SY5Ycell lines expressing the WT and the mutant Parkin proteins under tetracycline control were used to study changes in nuclear proteome and phosphonuclear proteome. A list of differentially regulated proteins that were not previously known to interact or associate with Parkin was generated. The differentially regulated proteins pointed to DNA repair mechanisms and involvement of Parkin and its putative partners in the repair of damaged DNA (Figure4.1).

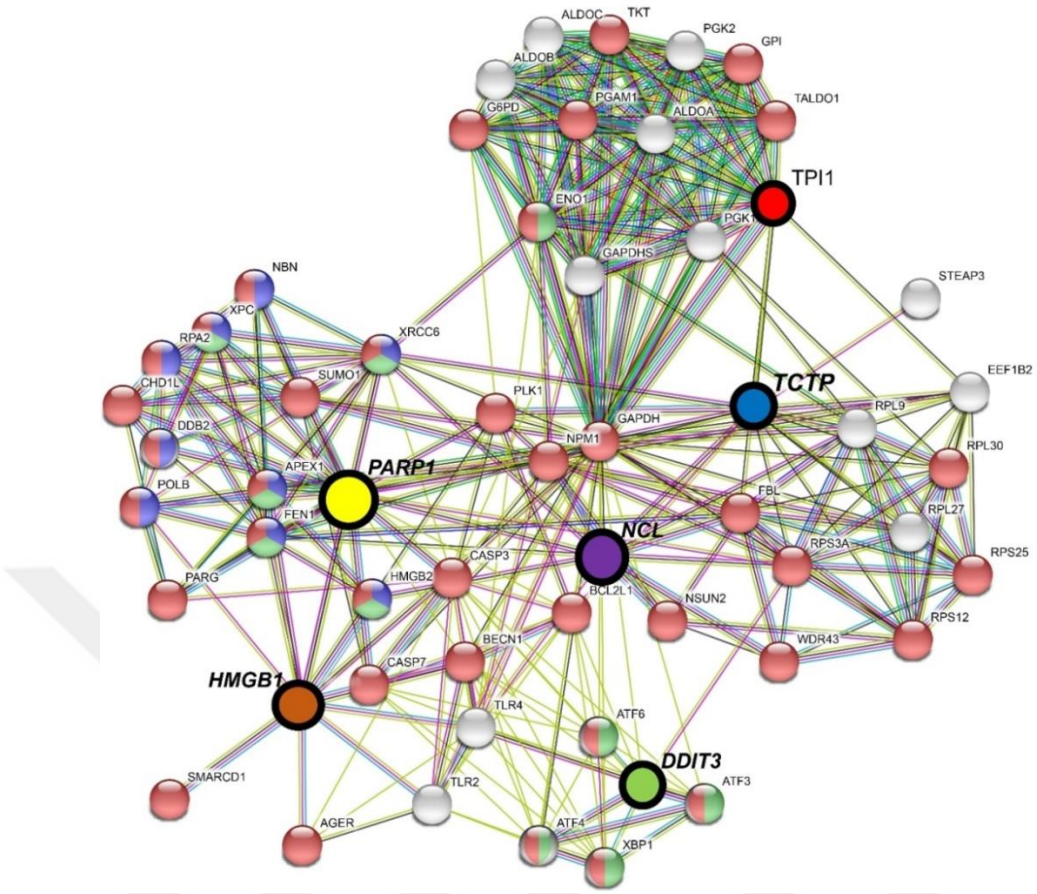

Figure 4.1. STRING analysis for the differentially regulated nuclear proteins identified from nucleic acid binding and nuclear insoluble protein fractions. The colors represent, Pale red- Nuclear proteins; Purple-Cytoplasmic proteins; Grey-Second shell interacting proteins. Green background color. The differentially identified proteins TPI1, TCTP, NCL, PARP1, DDIT3 and HMGB1 were pseudo colored

4.3. Future Work

In this thesis, we have determined significant changes in the levels of several proteins upon the wild-tpye and the mutant Parkin expressions. However, we were not able to answer the question of why the levels of these proteins change upon Parkin expressions. We also do not know whether any of these proteins directly interact with Parkin and behave as the substrates for Parkin's E3 ubiquitin ligase activity. Future work should be carried out to answer these two questions. If direct interactions were discovered, a more complete picture for the involvement of Parkin in cancer would be drawn. Such a picture should provide information about the cross-talk between PD and cancer.

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SUPPLEMENTARY

SUPPLEMENTARY-A

Table . Preparation of solutions used in experiments

Buffer solution names	Final Concentrations	Final volume	Method of Preparation
Tris.HCl pH 8.8	1.5 M	100 mL	Dissolve 18.16 g Tris base in around 80 mL of ddH_2O. Adjust the pH to 8.8 with concentrated HCl. Bring up the volume to 100 mL with ddH_2O.
Tris.HCl pH 6.8	0.5 M	100 mL	Dissolve 6 g Tris base in around 80 mL of ddH_2O. Adjust the pH to 6.8 with concentrated HCl. Bring up the volume to 100 mL with ddH_2O.
Sodium Acetate	3 M	10 mL	2.46 g Sodium Acetate dissolved in 10 mL of ddH_2O.
SDS	10 %	10 mL	1 g SDS dissolved in 10 mL of ddH_2O.
Amonium persulfade (APS)	10%	10 mL	1 g APS dissolved in 10 mL of ddH_2O.
6 X Loading Dye	0,5M Tris-HCl pH 6.8, Glycerol (99.7%), 10 % SDS, ß-Mercaptoethanol, 0.5% (w/v) Bromphenol blue	10 mL	1 mL 0.5 M Tris-HCl pH 6.8, 2mL Glycerol (99.7%), 1.6 mL 10% SDS, 0.4 mL ß-Mercaptoethanol, 0.4 mL 0.5% (w/v) Bromphenol blue added to 2.6 mL of ddH_2O.
Acrylamide/Bisacrylamide	30%	100 mL	29.2 g Acrylamide, 0.8 g Bisacrylamide dissolved in 100 mL, filterd the solution stored at +4 ºC for later use.
SDS-PAGE Running buffer	5X	300 mL	4.5 g Tris, 21.6 g Glycine and 1.5 g SDS dissolved in ddH_2O, 5X buffer diluted to 1X.
SDS-PAGE fixative solution	40% of Methanol, 10 % Acedic acid	100 mL	40 mL of Methanol and 10 mL of Acedic Acid mixed together, filled up to100 mL with ddH_2O.
Western Blot Transfer B uffer	-	100 mL	0.58 gr Tris base, 0.29 gr Glycine, 0.025 gr SDS (0.375 mL of 10% SDS) dissolved in 100 mL of ddH_2O.
TBS-T pH 7.6	25 mM Tris.HCl, 150 mM NaCl, pH 7.2 0.1% Tween20	1L	2.42 gr Tris, 8 gr NaCl, 1mL Tween20 dissolved in 900 mL of ddH_2O. Adjusted pH to 7.6'filled up 1L added 1 mL of Tween20.
Ponceau S staining buffer	0.1% (w/v)	100 mL	0.1 gr Ponceau S and 0.5 mL Acedic acid mixed together, dissolved to 100 mL with ddH_2O.
Blocking solutions (Western Blot)	5%	10 mL	5 g dry milk powder dissolved in 10 mL of TBST filtered .
HyperFilm Developer Solutions	Kodak RP X-OMAT LO		Prepared according to construction of producer. 140 mL ddH_2O, 50 mL solution A, 2 mL solution B, 2 mL solution C mixed together.
HyperFilm Fixation Soltion	Kodak RP X-OMAT LO (Carestream Health, Belgium)	190 mL	Prepared according to construction of producer. 140 mL ddH_2O, 50 mL solution A and solution B mixed together.
2DE-Rehydration Buffer	8 M Urea, 50 mM DTT, 2% (w/v) CHAPS, 0.2% (w/v) Amphylate pH 3-10, 0.001% Bromophenol blue	50 mL	24 gr Urea, 1 gr CHAPS, 0.385 gr DTT, 250 µl Urea. Amphylate (40%) and 0.5 mg Bromophenol blue mixed together, filled up to 50 mL with ddH_2O.
ProQ Dia ond Destaining Solution	50 mM Sodyum Acetate pH 4, 20% Acetontrile	1L	50 mL 1M Sodyum Acetate pH4, 200 mL Acetontrile mixed together and filled up to 1000 mL with ddH_2O.
2D IPG Strip equilibration buffer I	6 M Urea, 0.375 M Tris.HCl pH 8.8, 2 % SDS, 20% Glycerol, 2% (w/v) DTT	250 mL	90.15 gr Urea, 62.5 mL 1.5 M Tris.HCl pH 8.8 5 gr SDS, 50 mL Glycerol ,5 g DTT Mixed together and filled up to 250 mL with ddH_2O.
2D IPG Strip equilibration buffer II	6 M Urea, 0.375 M Tris.HCl pH 8.8, 2 % SDS, 20% Glycerol, 2.5% (w/v) iodoacetic acid	250 mL	90.15 gr Urea, 62.5 mL 1.5 M Tris.HCl pH 8,8 5 gr SDS, 50 mL Glycerol , 6,25 gr iodoacetic acid mixed ad filled up to 250 mL with ddH_2O.
Acetontrile	20%	10 mL	20 mL Acetontrile added 80 mL of ddH_2O and stored at room temperature.
Amonyum Bikarbonat	50 mM	10 mL	40 mg AmBic dissolved in 10 mL of ddH_2O and stored at room temperature.
Collodial CoomassieBrilliant Blue staining	-	1L	1.2 g of 6% CoomassieBrilliant Blue G250 dissolved in 500 mL ddH_2O, add 100 g ammonium sulfate and stir over night at room temprature. Bring up the volume to 700 mL with ddH_2O. Immediately before use add 200 mL methanol and 100 mL ortho-phosphoric acid .

SUPPLEMENTARY-B

Table List of identified nuclear proteins using by MALDI-TOF/TOF (Biorad)

No.	AC no.	Protein Description	Protein Mass	Protein Score	Expect	Matches	pI	Seq. Cov. (%)	Subcellular Location
1	Q0VG73	Putative uncharacterized protein	10756	27	40	5	6.38	26	Unknown
2	Q5VZL5	Zinc finger MYM-type protein 4	172677	21	1.70e+02	11	6.45	5	Cytoplasm
3	P11142	Heat shock cognate 71 kDa protein	70854	93	9.50e-06	7	5.37	9	Cytoplasm, nucleus
4	P11142	Heat shock cognate 71 kDa protein	70854	299	2.60e-26	30	5.37	35	Cytoplasm, nucleus
5	A8MQ14	Putative zinc finger protein	52455	35	6.3	11	8.57	21	Unknown
6	P08107	Heat shock 70 kDa protein 1A/1B	70009	121	1.60e-08	17	5.48	23	Unknown
7	P10809	60 kDa heat shock protein	61016	95	7.0 0e-06	7	5.7	16	Mitichondrian matrix
8	P08670	Vimentin	53619	410	2.00e-37	40	5.06	55	Cytoplasm
9	P06576	ATP synthase subunit beta,	56525	155	6.4e-012	16	5.26	28	Mitochondrian
10	Q5VZL5	Zinc finger MYM-type protein 4	172677	35	6.9	11	6.45	5	Unknown

SUPPLEMENTARY-C

Table. List of identified proteins by MALDI-TOF/TOF(Ultracentrifuge)

No.	AC no.	Protein Description	Protein Mass	Protein Score	Expect	Matches	pI	Seq. Cov. (%)	Subcellular Location
1	P11021	78 kDa glucose-regulated protein	72288	158	3,20E-12	27	5.07	37	Cytoplasm, endoplasmic reticulum
2	P20700	Lamin-B1	66368	185	6.40e-15	30	5.11	38	Nucleus
3	P11142	Heat shock cognate 71 kDa protein	70854	214	8.10e-18	28	5.37	33	Cytoplasm, nucleus, cell membrane
4	P34931	Heat shock 70 kDa protein 1-like	70331	101	1.6e-006	20	5.76	25	Nucleoplasm,cytoplasm
5	P51693	Amyloid-like protein 1	72131	48	0.34	17	5.54	15	Cytoplasm
6	P51693	Amyloid-like protein 1	72131	47	0.37	18	5.54	14	Cytoplasm
7	Q53GI3	Zinc finger protein 394	64216	48	0.36	22	8.14	27	Nucleus
8	O15226	NF-kappa-B-repressing factor	53619	46	0.49	22	8.94	20	Nucleus
9	P20472	Parvalbumin alpha	12051	46	0.53	11	4.98	38	Nucleus
10	O15226	NF-kappa-B-repressing factor	77624	68	0.0034	24	8.94	22	Nucleus

SUPPLEMENTARY-D1

Table D1.1. List of nucleic acid binding proteins identified by MALDI-TOF/TOF analysis (QProteome)

No.	AC no.	Protein Description	Protein Mass	Protein Score	Expect	Matches	pI	Seq. Cov. (%)	Subcellular Location
1	Q92804	TATA-binding protein-associated factor 2N	61793	87	4e-005	16	8,04	30	Nucleus
2	Q13439	Golgin subfamily A member 4	260980	47	0,42	41	5,33	13	Cytoplasm
3	Q96AE4	Far upstream element-binding protein 1	67518	453	1E-41	34	7,18	38	Nucleus
4	O43172	U4/U6 small nuclear ribonucleoprotein Prp4	58412	194	8.1E-16	26	7,71	38	Nucleus
5	P14866	Heterogeneous nuclear ribonucleoprotein L	64092	209	2.60E-17	30	8,46	37	Nucleus
6	Q92945	Far upstream element-binding protein 2	73101	353	1.00E-31	36	6,84	41	Nucleus
7	P14866	Heterogeneous nuclear ribonucleoprotein L	64092	203	1.00E-16	36	8,46	42	Nucleus
8	P14866	Heterogeneous nuclear ribonucleoprotein L	64092	177	4.00E-14	31	8,46	35	Nucleus
9	P14866	Heterogeneous nuclear ribonucleoprotein L	64092	195	6.40E-16	38	8,46	45	Nucleus
10	Q9Y4E5	Zinc finger protein 451	121406	50	0,21	27	6,3	19	Nucleus

SUPPLEMENTARY-D2

D2.1. List of insoluble nuclear proteins identified by MALDI-TOF/TOF analysis (QProteome)

No.	AC no.	Protein Description	Protein Mass	Protein Score	Expect	Matches	pI	Seq. Cov. (%)	Subcellular Location
1	Q09028	Histone-binding protein RBBP4	47626	134	8,10E-10	17	4,74	23	Nucleus
2	Q16576	Histone-binding protein RBBP7	47790	68	0,0029	13	4,89	19	Nucleus
3	Q969G3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	46621	49	0,26	13	4,85	19	Nucleus
4	P07437	Tubulin beta chain	49639	170	2,00E-13	22	4,78	26	Cytoplasm
5	P68363	Tubulin alpha-1B chain	50120	92	1,30E-05	15	4,94	24	Cytoplasm
6	P68363	Tubulin alpha-1B chain	50120	179	2,60E-14	25	4,94	45	Cytoplasm
7	P08670	Vimentin	53619	458	3,20E-42	41	5,06	60	Cytoplasm
8	P08865	40S ribosomal protein SA	32833	117	4,00E-08	6	4,79	16	Nucleus
9	Q7Z7B0	Filamin-A-interacting protein 1	138024	58	0,035	28	8,46	23	Cytoplasm
10	Q13610	Periodic tryptophan protein 1 homolog	55793	114	8,10E-08	15	4,6	13	Nucleus

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CURRICULUM VITAE

She was born in Kashgar in 1980. She completed her primary, secondary and high school education in Kashgar. In 1998, she entered Xinjiang University College of Life Science and Technology and graduated in 2003. Between the years of 2004-2007, she compeleted her master's education in College of Life Science and Technology. She worked as a teacher in Enstitute of Uyghur Traditional Medicine of Xinjiang Medical University from 2009 to 2012. From 2012 until now she is a Phd student in Department of Biomedical Engineering of Graduate School of Natural ad Applied Sciences.