# TRANSCRIPTIONAL REGULATION OF USP24 BY NF-KAPPA B

by

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#### ABSTRACT

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases. Impairment of the ubiquitin pathway is believed to play an important role in the pathogenesis of PD. The ubiquitination process regulates the available amount, correct localization, and proper activity of target proteins. This process is carried out under tight regulation not only by enzymes linking ubiquitin molecules onto their targets, but also by deubiquitinating enzymes which remove ubiquitin molecules from the ubiquitinated proteins.

Little is known about the recently discovered deubiquitinating enzyme Ubiquitin Specific Protease 24 (USP24), a product of *USP24* gene which spans a large area (>140,000 bp) on the chromosome 1p32 region. Recent genetic linkage studies indicated that the location of the *USP24* gene is significantly correlated with the disease. These studies imply that USP24 is likely a novel "PARK" protein. As a part of the study to elucidate the participation of USP24 in PD pathogenesis and mechanisms that modulate USP24 activity, we investigated the molecular mechanism of *USP24* gene transcription.

Promoter of the USP24 gene was cloned into pGL3-Basic vector and analyzed by a luciferase based reporter assay system. We identified the transcription starting site(TSS) of USP24 gene and the transcription starting site located at 251 bp upstream of the translation starting site. Expression of the USP24 gene is controlled by a

ii

TATA-box-less promoter.

NF-κB signaling plays an important role in multiple cellular events such as inflammation and programmed cell death. It has been reported that increased NF-κB nuclear translocation occurs in substantia nigra (SN) dopaminergic neurons of PD patients, suggesting that NF-κB signaling is involved in PD pathogenesis. An NF-κB binding site was identified on *USP24* promoter. Overexpression of NF-κB or activation of NF-κB by TNF-α significantly increased *USP24* promoter activity. Mutation of the binding site abolished the regulatory effect of NF-κB on the *USP24* promoter.

In summary, we have identified USP24 gene promoter and our study demonstrates that NF- $\kappa$ B signaling pathway regulates USP24 gene transcription. The results will further our understanding of the regulation of ubiquitin pathway and its involvement in PD pathogenesis.

# **TABLE OF CONTENTS**

ABSTRACT ii
TABLE OF CONTENTSiv
LIST OF FIGURES viii
ACKNOWLEDGEMENTSix
CHAPTER 1: Introduction1
1.1. Overview of Parkinson's Disease1
1.1.1. Brief History of Parkinson's Disease Research1
1.1.2. Epidemiology of Parkinson's Disease1
1.1.2.1. Age and Gender as Risk Factors Associated with Parkinson's
Disease2
1.1.2.2. Environmental Factors and Parkinson's Disease
1.1.2.3. Genetics of Parkinson's Disease
1.1.3. Pathology of Parkinson's Disease4
1.2. Protein Ubiquitination and Deubiquitination
1.2.1 Ubiquitination and Ubiquitin-Proteasome Pathway
1.2.1.1. Ubiquitin
1.2.1.2. Protein Ubiquitination
1.2.1.3. The Ubiquitin-Proteasome Pathway
1.2.1.4 The Ubiquitin-proteasome Pathway Plays Important Roles in
Cellular Activities10

iv

•		
;	1.2.2. Other Regulatory Effects of Ubiquitination	
	1.2.3. Deubiquitination and Deubiquitinating Enzymes	
	1.2.3.1 Subfamilies of Deubiquitinating Enzymes	
	1.2.3.2. Functional Domains of Deubiquitinating Enzymes14	
	1.2.3.3. Biological Significance of Deubiquitinating Enzymes15	
	1.2.4. Involvement of the Ubiquitination Pathway in the Molecular	
	Pathogenesis of Parkinson's Disease16	
	1.3. Ubiquitin Specific Protease 24	
	1.4. Nuclear Factor-Kappa B (NF-κB) Signaling20	
	1.4.1. Components of NF-κB Molecules20	
	1.4.2. Molecular Mechanisms of NF-kB Activation in Cells	
	1.4.3. Biological Functions of NF-κB25	·
	1.4.3.1. NF-κB Signalling and Immunity25	
,	1.4.3.2. NF-κB Signalling and Apoptosis25	
	1.4.4. NF-kB Signalling In the Nervous System and in Neurodegenerative	
	Diseases26	
	1.4.4.1. NF-κB Signalling In the Nervous System	
	1.4.4.2. NF-KB Signalling In Neurodegeneration and Neurodegenerative	
	Diseases27	
	1.5 Rationale and Hypothesis of the Study	
	CHAPTER 2: Materials and Methods	
	2.1. Gene Cloning and Generation of Plasmid Constructs	

v

_ ·		
	2.1.1. Primer Sequences for PCR Amplifications	31
	2.1.2. General Procedures of Molecular Cloning	32
	2.2. Reverse-Transcription-Polymerase Chain Reaction	34
	2.2.1. RNA Extraction	34
	2.2.2. Reverse Transcription	
	2.3. Cell Culture	35
	2.3.1. Culture media	35
	2.3.2. Trypsinization	35
	2.4. Cell Transfection	
÷ .	2.4.1. Lipofectamine 2000 Transfection	
	2.4.2. Calcium Phosphate Method	
	2.5 TNF-α Treatment	
·	2.6. Reporter Gene Transcription Assay	
	2.7. 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5'RI	M-RACE)
	2.8. Immunoblotting	40
	2.9. Electrophoretic Mobility Shifting Assay (EMSA)	43
•	2.10. Data Analysis	44
C	HAPTER 3: Results	45
•	3.1 Characterization of USP24 Gene Coding Sequence.	45
	3.2. Cloning and Characterization of the Human USP24 Promoter	46
		<b>C 11 I</b>

vi

.

REFI	ERENCES	<b>i</b> 5
CHA	PTER 4: Discussion6	51
3	.6. NF-κB Increases Human USP24 mRNA Level	50
3	.5. NF-κB Signalling Regulates Human USP24 Promoter Activity	55
3	.4. USP24 Promoter Contains an NF-κB Binding Site	52
S	H-SY5Y than in HEK293 Cells	51

vii

# LIST OF FIGURES

Figure 1.1. A Three-step Cascade Pathway by Which a Ubiquitin is Attached to a
Protein7
Figure 1.2. Some Members of the NFkB/Rel Family and the IkB Family21
Figure 1.3. Activation of NF-kB24
Figure 3.1. Protein Sequences Alignment of Human USP24 (hUSP24) and Mouse
USP24 (mUSP24) Homologues45
Figure 3.2. Major Domains of the USP24 Protein
Figure 3.3. The Nucleotide Sequence of the Human USP24 Promoter
Figure 3.4. Mapping the USP24 Transcription Starting Site by 5' RLM-RACE49
Figure 3.5. Functional Deletion Analysis of the USP24 Promoter Region50
Figure 3.6. Luciferase Activity was Measured at 48 Hours by a Luminometer
Figure 3.7. Interaction between NF- $\kappa$ B and the NF- $\kappa$ B Binding Element in the Human
<i>USP24</i> Promoter
Figure 3.8. NF-kB Overexpression Increases USP24 Promoter Activity
Figure 3.9. Mutations on the USP24 Promoter Abolish NF-KB's Regulatory Effect57
Figure 3.10. TNF-α Treatment Increases USP24 Promoter Activity
Figure 3.11. NF-кВ Increases Human USP24 mRNA Level60

viii

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ix

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#### **CHAPTER 1: Introduction**

#### **1.1. Overview of Parkinson's Disease**

## 1.1.1. Brief History of Parkinson's Disease Research

The symptoms of PD were fully recognized and documented in 1817, by a British physician named James Parkinson in a short report entitled "*An Essay on the Shaking Palsy (1817)*" (Parkinson, 2002). In the document, Dr. Parkinson described six patients who displayed similar symptoms such as rigidity, tremor at rest, an accelerated gait, and stooped posture. About fourteen years later, the syndrome characterized by these symptoms was named PD, by a French neurologist Dr. Jean-Martin Charcot who added rigidity to Parkinson's excellent clinical description. During the 1950s, the underlying biochemical changes in Parkinson's patients' brains were discovered. Dr. Arvid Carlsson first illustrated the importance of dopamine in neuronal transmission and found that reduced dopamine levels caused PD-like symptoms in animal models (Carlsson, 1959, 2002). These Nobel Prize-winning discoveries led to the use of L-dopa, a molecular precursor of dopamine, as an effective treatment for PD beginning in 1967 (Cotzias, 1968; Hornykiewicz, 2002).

#### **1.1.2.** Epidemiology of Parkinson's Disease

The occurrence of Parkinson's disease is widespread. Among people over 60 years old, the prevalence is about 1% (Nussbaum and Ellis, 2003). Standardized incidence rates of PD, as reported, are 8%-18% per 100,000 person-years (de Lau and Breteler, 2006).

**1.1.2.1.** Age and Gender as Risk Factors Associated with Parkinson's Disease Epidemiological studies have revealed that PD is clearly an age-related disease: 95% of PD patients are above the age of 50 (Van Den Eeden et al., 2003). The prevalence of the disease increases with age, from less than 0.5% of people 60 years old to 4% of people 80 years old (de Lau and Breteler, 2006). The mean age at onset is 61 years, but the disease can occur at juvenile ages or at very late ages of 80 to 90 years (DeStefano et al., 2002). The incidence rate for men is 91% higher than for women (Van Den Eeden et al., 2003)

#### **1.1.2.2.** Environmental Factors and Parkinson's Disease

Research has suggested a relationship between the incidence of PD and chemicals the population is commonly exposed to, such as pesticides, herbicides, and heavy metals (Bocchetta and Corsini, 1986; Hart, 1987; Rajput et al., 1987; Uitti et al., 1989; Gorell et al., 1999; McCormack et al., 2002).

In 1983, a study which investigated four who had accidentally been injected intravenously with drugs contaminated with 1-methy-4-phenyl-1,2,3,6tetrahydropyridine (MPTP) found that dopaminergic neurons of the substantia nigra pars compacta (SNc) were selectively damaged and the subjects showed typical PD symptoms(Langston et al., 1983). This discovery led to the hypothesis that exposure to environmental toxins could result in an increase in the risk of PD development.

Much attention was paid to putative environmental factors that could lead to PD onset including pesticides, herbicides, heavy metals, as well as activities such as farming or living in rural areas. Interestingly, MPTP, as well as other herbicides and pesticides such as paraquats and rotenone are complex I inhibitors that can cause dopamine depletion (Betarbet et al., 2000). However, epidemiological studies generate heterogeneous conclusions, and thus it is unknown whether environmental factors actually contribute to the risk of disease (Lai et al., 2002; Jankovic, 2005; de Lau and Breteler, 2006).

#### 1.1.2.3. Genetics of Parkinson's Disease

Ten regions of the human genome (the *park1-8,10,11* regions) have been implicated in autosomal dominant and autosomal recessive forms of PD. These regions contain several candidate genes potentially involved in the etiology of PD. Only six genes have been identified within the park regions. These are: the *α-synuclein* gene on chromosome 4q21-23(*park1*) (Polymeropoulos et al., 1997), the *ubiquitin C-terminal hydrolase* gene on 4p14(*park5*) (Leroy et al., 1998), the *parkin* (*park2*) gene on 6q25-27 (Kitada et al., 1998), the *DJ-1* gene on 1p36 (*park7*) (van Duijn et al., 2001; Bonifati et al., 2003), the *PINK1* gene on 1p35-36 (*park6*) (Valente et al., 2001; Valente et al., 2004), and the *LRRK2* gene on 12p11.2-q13.1 (*park8*) (Funayama et al., 2002; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Candidate genes on other locations, such as 2q13 (*park3*)(Gasser et al., 1998), 4p15 (*park4*) (Farrer et al., 1999), 1p32 (*park10*) (Hicks et al., 2002), and 2q36-37 (*park11*) (Pankratz et al., 2003), have

not been cloned and identified.

#### 1.1.3. Pathology of Parkinson's Disease

Dopaminergic neurons in the SNc project to the striatum. Symptoms of PD are thought to be caused by the loss of these pigmented dopaminergic neurons (Hirsch et al., 1989). The loss of these cells influences the activity of the neural circuits within the basal ganglia that regulate movement, causing an inhibition of the direct pathway and excitation of the indirect pathway. Since the direct pathway facilitates movement and the indirect pathway inhibits movement, a balance is essential for the normal function of these two pathways, which is interrupted by a loss of dopaminergic cells. Loss of dopaminergic neurons in SNc leads to increased inhibition of the ventral lateral nucleus of the thalamus, which sends excitatory projections to the motor cortex, thus leading to hypokinesia (Dauer and Przedborski, 2003; Schulz and Falkenburger, 2004).

Another feature of PD is the appearance of Lewy bodies in surviving dopaminergic neurons. Lewy bodies were first seen and linked to Parkinson's disease ("paralysis agitans") in 1912 by neurologist Frederic Lewy (1885-1950).

Lewy bodies are eosinophilic cytoplasmic inclusions. The primary structural component of Lewy bodies is  $\alpha$ -synuclein. Immunohistochemical labeling of Lewy bodies using  $\alpha$ -synuclein antibodies shows a dense core surrounded by a halo of 10-nm

wide radiating fibrils. The events leading to the generation of Lewy bodies are still unknown. It is currently under debate whether Lewy bodies are toxic to cells or serve to protect cells from harmful effects of misfolded proteins by sequestering them away from important cellular elements(Dauer and Przedborski, 2003).

#### **1.2. Protein Ubiquitination and Deubiquitination**

#### 1.2.1 Ubiquitination and Ubiquitin-Proteasome Pathway

#### 1.2.1.1. Ubiquitin

Ubiquitin, as its name suggests, is a highly conserved small regulatory protein that is ubiquitously expressed in eukaryotes. First identified in 1975 by Schlesinger et al, (Schlesinger et al., 1975), ubiquitin is a roughly 8.5 kDa peptide that contains 76 amino acid residues and is one of the most highly conserved proteins across species: human and yeast ubiquitins share 96 % sequence homology.

The function of ubiquitin was not known until the early 1980s when its biochemical functions were first elucidated by Aaron Ciechanover, Avram Hershko and Irwin Rose (Hershko et al., 1979; Hershko et al., 1980), who shared the 2004 Nobel Prize in Chemistry based on this work.

#### 1.2.1.2. Protein Ubiquitination

Ubiquitin is covalently conjugated to a protein substrate by an amide bond between the C-terminus of ubiquitin (amino acid residue G76) and the  $\varepsilon$ -amino group of a substrate

lysine residue (Hochstrasser, 1996; Hershko and Ciechanover, 1998). The recognition of the ubiquitin signal by downstream proteins ultimately determines the fate of a host of intracellular proteins.

Ubiquitin is covalently linked to target proteins via a three-step ATP-dependent pathway involving three separate enzymes: E1, E2, and E3. E1 is an activating enzyme which consumes ATP to form a high energy thiolester-ubiquitin intermediate with the carboxyl group of G76, thereby activating the C terminus of ubiquitin for nucleophilic attack. After ubiquitin activation, E2, the ubiquitin-conjugating enzyme, carries the activated ubiquitin by forming a thiol-ester bond with the ubiquitin molecule. Finally, a ligase called E3 transfers the ubiquitin molecule to the lysine residue of its substrate (Hershko et al., 1983; Pickart, 2000, 2001)(Figure 1.1.). E3s are the least defined components of the pathway. However, since they are responsible for the specific recognition of the multitude substrates, they display the greatest variety among its different components (Glickman and Ciechanover, 2002).



Figure 1.1. A Three-step Cascade Pathway by Which a Ubiquitin is Attached to a Protein.

The free carboxyl group of ubiquitin's carboxyl-terminal Gly residue is finally linked through an amide (isopeptide) bond to an  $\varepsilon$ -amino group of a Lys residue, which is on a target protein. Additional cycles can produce a polyubiquitin chain.

The ubiquitin system has a hierarchical structure. A single E1 enzyme can activate ubiquitin for many conjugation reactions by transferring it to different E2 enzymes (Kornitzer and Ciechanover, 2000; Wilkinson, 2000). Most E2 enzymes interact with several E3 enzymes (Voges et al., 1999; Glickman, 2000), and, most E3 enzymes can anchor ubiquitin to more than one downstream target protein. This hierarchical structure is a complicated network of overlapping interactions between its components (Glickman and Ciechanover, 2002).

All known ubiquitination reactions are completed through this three-step mechanism, independent of the downstream signaling pathway of the target (i.e. proteasomal proteolysis, endocytosis) (Pickart, 2000). Studies have revealed distinct patterns of ubiquitination, called polyubiquitination, monoubiquitination, and multiubiquitination (Pickart, 2000; Hicke, 2001). Monoubiquitination and multiubiquitination involve single ubiquitin molecules labeled to one (mono) or more (multi) lysine residues on target proteins, while in polyubiquitination, several ubiquitin molecules form a polyubiquitin chain on the target protein. Additionally, in polyubiquitination, since there are seven internal lysine residues on each ubiquitin peptide, different patterns of ubiquitin chains may also contribute to more diverse codes (Mukhopadhyay and Riezman, 2007).

## 1.2.1.3. The Ubiquitin-Proteasome Pathway

The major role of protein polyubiquitination, as was identified by Ciechanover et al. in 1980s, is to degrade short half-life and defective proteins via the ubiquitin-proteasome pathway (Ciechanover et al., 1980; Glickman and Ciechanover, 2002). This pathway requires that at least four ubiquitin moieties be linked to each other through Lys48-Gly76 isopeptide bonds (Chau et al., 1989; Thrower et al., 2000; Sloper-Mould et al., 2001). These proteins are then degraded by a single enzyme complex called the 26S proteasome. The 26S proteasome complex is a 2.5 MDa molecular machine that contains at least 32 different subunits. These subunits

constitute two major structural units of the complex: a barrel-shaped core complex called the 20S particle and two cap-shaped complexes called 19S regulatory particles.

The 20S particle consists of two  $\alpha$  rings and two  $\beta$  rings, each of which is made up of seven subunits. The two outer  $\alpha$  rings are predominantly structural in purpose, while the two inner  $\beta$  rings are predominantly catalytic(Nandi et al., 2006). In mammals, the  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 subunits in each  $\beta$  ring display protease activity, each with different substrate specificities. The  $\beta$ 1, $\beta$ 2, and  $\beta$ 5 subunits exhibit chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing protease activities (Heinemeyer et al., 1997).

The 19S regulatory particle are located at either end of the barrel-shaped 20s particle and consist of nineteen individual subunits which can be categorized into two assemblies. One is a 10-subunit base that helps the 19s particle bind to the  $\alpha$  rings, while the other is a 9-subunit lid which binds to polyubiquitin chains. Six subunits of the 10-subunit base exhibit ATPase activity which is believed to function in protein unfolding and translocating ubiquitinated protein substrates into the barrel for degradation(Lam et al., 2002; Liu et al., 2006; Sharon et al., 2006).

After a protein has been ubiquitin-marked for proteolysis, it is recognized by the 19S regulatory particle in an ATP-dependent binding step. The central channel of the 20S core particle is narrow and gated by the N-terminal tails of the  $\alpha$ -ring subunits.

Consequently, the interior chamber is at most 53 Å wide and the entrance can be as narrow as 13 Å(Nandi et al., 2006). As a result, substrates entering the barrel must be at least partially unfolded before they enter the core. After the substrate has entered the interior of the 20S particle, it comes in contact with proteolytic active sites and is degraded. (Heinemeyer et al., 1997; Lam et al., 2002).

1.2.1.4 The Ubiquitin-proteasome Pathway Plays Important Roles in Cellular Activities

The ubiquitin-proteasome pathway is required in various cellular functions such as cell cycle control, apoptosis, and transcription regulation.

Mitotic cyclins have one of the shortest life spans of all intracellular proteins. They have a half-life of only a few minutes, after which they are degraded (Lodish, 2004). The degradation of cyclins is triggered by polyubiquitination and processed by the proteasome. This process provides directionality for the cell cycle. For example, exit from mitosis requires dissociation and degradation of cyclin B from the mitosis promoting factor complex(Chesnel et al., 2006). Disrupting this process will arrest cells in late anaphase in vertebrate cells(Surana et al., 1993; Brito and Rieder, 2006).

The ubiquitin-proteasome system is also involved in apoptosis. During apoptosis, increased protein polyubiquitination and E1, E2, and E3 enzyme levels are observed (Haas et al., 1982; Schwartz et al., 1990; Low et al., 1997). Translocation of the

proteasome from the nucleus to outer membrane blebs is also seen during apoptosis(Pitzer et al., 1996). Ubiquitin sequence-specific antisense oligonucleotides can cause a decrease in the proportion of cells displaying the  $\gamma$ -irradiation-induced apoptosis phenotype(Delic et al., 1993). Furthermore, inhibition of the ubiquitin system prevents the apoptosis induced by NGF deprivation in sympathetic neurons(Sadoul et al., 1996). These results suggest that the ubiquitin-proteasome pathway plays a key role in apoptosis.

The ubiquitin-proteasome machinery also plays an important role in gene transcription regulation. The p50 subunit of Nuclear Factor kappa B (NF-kB) is a mature form of NF-kB generated from its precursor p105. The partial proteolysis is carried out by the 26S proteasome complex. During activation of NF- $\kappa$ B signaling, the ubiquitin-proteasome system is responsible for the degradation of NF- $\kappa$ B's inhibitory partner I $\kappa$ B (refer to section 1.4.2.). By regulating the maturation and degradation of transcription factors, the ubiquitin-proteasome pathway is involved in regulating gene transription.

Both *in vivo* and *in vitro* studies have shown that in tumor supressor protein p53 is degraded by the ubiquitin-proteasome system(Ciechanover et al., 1991; Maki et al., 1996). Accumulation of p53-ubiquitin adducts in the cytosol are observed if 26S proteasome function is inhibited(Hershko and Ciechanover, 1998). Degradation of other oncoproteins and tumor suppressors by the ubiquitin-proteasome pathway is

also critical for the normal function and metabolism of cells(Treier et al., 1994; Aberle et al., 1997).

# 1.2.2. Other Regulatory Effects of Ubiquitination

Polyubiquitination associated with the Lys48 linkage normally induces proteolysis, while monoubiquitination or polyubiquitination associated with the Lys63 linkage is usually associated with other downstream target fates. Monoubiquitination and multiubiquitination can subserve a variety of functions, such as endocytosis, histone regulation, and the budding of retroviruses from the plasma membrane (Hicke, 2001).

## **1.2.3.** Deubiquitination and Deubiquitinating Enzymes

Deubiquitinating enzymes are proteases that can specifically cleave ubiquitin molecules off ubiquitin-tagged proteins at the ubiquitin carboxy terminus (D'Andrea and Pellman, 1998). Proteases, a family of protein consisting of 561 members (Puente and Lopez-Otin, 2004), can be divided into five different classes based on their mechanism of catalysis: metallo, aspartic, serine, threonine, and cysteine proteases. The majority of deubiquitinating enzymes are cysteine proteases. The enzymatic activity of cysteine proteases largely relies on the thiol group of a cysteine, which sits in the active site together with an adjacent histidine residue. The histidine is polarized by an aspartate residue. The cysteine, histidine, and aspartate residules form a catalytic triad. During enzyme function, the cysteine makes a nucleophilic attack on the carbonyl group of the peptide bond between the ubiquitin molecule and the target.

As a result, the enzyme forms a covalent intermediate with ubiquitin, and the target protein is released. Reaction of this intermediate with a water molecule causes the release of ubiquitin from the enzyme(Nijman et al., 2005).

## 1.2.3.1 Subfamilies of Deubiquitinating Enzymes

There are five subfamilies of deubiquitinating enzymes(DUBs): Ubiquitin C-Terminal Hydrolases (UCHs), Ubiquitin Specific Proteases (USPs), Machado-Joseph Disease Protein Domain Proteases (MJDs), Ovarian Tumor Proteases (OTUs), and JAMM Motif Proteases. The majority of DUBs reside in the UCH and USP subfamilies (Amerik and Hochstrasser, 2004).

The largest and most diverse subfamily of deubiquitinating enzymes is the USP subfamily. Cysteine proteases of this family contain two short and conserved motifs called Cys and His boxes (Papa and Hochstrasser, 1993), which contain all the catalytic triad residues as well as other residues critical for catalysis.

UCHs were the first deubiquitinating enzymes to be identified. They were originally purified based on their ability to bind to ubiquitin affinity columns (Woo et al., 1995). Like USPs, UCHs contain active sites containing cysteine, histidine and aspartate residues. However, they do not display the USP-conserved Cys and His box domains. In addition, one of the characteristics of UCH family members is their relatively small size and preference in releasing small proteins from ubiquitin conjugation(Amerik and

#### Hochstrasser, 2004).

The number of different E3 enzymes increases with increasing organism complexity, as do their deubiquinating counterparts, DUBs. The large number, varying sizes, and various structural complexities of DUB members suggest that DUBs have diverse substrate specificities(Papa and Hochstrasser, 1993; Wilkinson et al., 1995).

# 1.2.3.2. Functional Domains of Deubiquitinating Enzymes

One of the most important domains of deubiquitinating enzymes is the ubiquitin-associated (UBA) domain. Proteins participating in the ubiquin pathway require a domain to interact with ubiquitin molecules. This is achieved by a modular composition of ubiquitin-binding motifs including UBA domains. The UBA domain is composed of a short motif of about 45 amino acid residues. This motif has been identified in E2, E3, and deubiquitinating enzymes, including mammalian E2-25K, *drosophila* hyperplastic discs protein, and eukaryotic ubiquitin isopeptidase T. UBA domains were found to exist singly or as multiple copies in a tandem arrangement (Hofmann and Bucher, 1996; Buchberger, 2002; Mueller and Feigon, 2002). The 3-D structure of UBA domains is classified as a compact three-helix bundle. This three-helix bundle has an unusually large hydrophobic surface area, which is where DUBs interact(Mueller and Feigon, 2002).

As the UAB domain is a feature of proteins involved in the ubiquitin-proteasome

pathway, the peptidase\_C19 domain is a feature specific to deubiquitinating enzymes. Protein peptidases can be grouped into various clans and families. The peptidase\_C19 domain is a characteristic of proteins that belong to the cysteine peptidase family and the CA clan. A typical cysteine peptidase contains conserved active sites consisting of cysteine, asparagine, and histidine residues, which form a catalytic triad.

# **1.2.3.3. Biological Significance of Deubiquitinating Enzymes**

Gene deletion studies have found no relationship between deubiquitinating enzymes and cell growth or viability in yeast (Amerik et al., 2000). However, research carried out using higher eukaryotes including mammals reveales that USPs and other deubiquitinazing enzymes are involved in various critical cellular processes (Table1).

Deubiquitinating enzymes function at different stages of the ubiquitination pathway. They remove ubiquitin from ubiquitinated proteins, rescuing them from degradation or regulating their localization(Fischer-Vize et al., 1992; D'Andrea and Pellman, 1998). They also trim polyubiquitin chains, editing the polyubiquitination signal(Amerik et al., 1997; Baek et al., 1997). In addition, they remove ubiquitin molecules off target proteins just before the protein encounters the proteasome, facilitating entry into and subsequent unfolding and translocation within the proteasome(Lam et al., 1997; Verma et al., 2002; Yao and Cohen, 2002). The above processes release ubiquitin molecules, which are subsequently recycled back to the ubiquitin pool.

The stability of p53 is highly regulated by the deubiquitinating pathway. This involves its interaction with an enzyme called USP7. Studies have found that different levels of USP7 inhibition can result in distinct degradation fates of p53 (Li et al., 2002a; Cummins et al., 2004).

Deubiquitinating enzymes are also highly involved in chromatin structure modification, and thus in regulation of gene transcription and silencing(Daniel et al., 2004; Yamashita et al., 2004). In yeast, deubiquitination of histone 2B (H2B) by the enzyme Ubp10 is required for telomeric silencing(Emre et al., 2005). In contrast, ubiquitin-H2B conjugates can also be deubiquitinated by Ubp8, which leads to transcriptional activation(Henry et al., 2003; Gardner et al., 2005).

1.2.4. Involvement of the Ubiquitination Pathway in the Molecular Pathogenesis of Parkinson's Disease

The ubiquitin-proteasome pathway has long been found to be involved in neurodegenerative diseases including Parkinson's, Alzheimer's, and Lewy body disease (Lowe et al., 1988; Lowe et al., 1990; Gai et al., 1995). Immunohistochemical staining has revealed that ubiquitin is a component of Lewy bodies and is useful as a diagnostic tool (Kuzuhara et al., 1988; Galvin et al., 1999).

The accumulation of proteins seen in brain matter presenting with Lewy bodies and

the presence of ubiquitin in these accumulations suggest a role for the ubiquitination-proteasome pathway in the pathogenesis of PD (Spillantini et al., 1997). Evidence for this hypothesis is being generated from studies focusing on different components of this pathway, including ubiquitinated substrates, ubiquitinating enzymes, and deubiquitinating enzymes.

Mutations in  $\alpha$ -synuclein proteins, which contribute to the occurrence of familial cases of PD, have been found to be associated with impaired proteasome function. These  $\alpha$ -synuclein mutations can adopt an unusual protein folding pattern that prevents their degradation via the proteasome pathway, even if the proteins are polyubiquitinated(Weinreb et al., 1996; Conway et al., 1998; Bennett et al., 1999). In addition, transgenic mice expressing wild-type human  $\alpha$ -synuclein display dopaminergic loss and formation of inclusions similar to Lewy bodies(Masliah et al., 2000).

Parkin, as its name indicates, is another important molecule involved in PD pathogenesis. *Parkin* was the first gene identified as a contributor to familial parkinsonism. *Parkin* encodes a protein with two RING domains at its carboxy terminus and a ubiquitin-like domain at its amino terminus(Kitada et al., 1998). This structure is suggestive of an E3 (i.e. ubiquitin ligase)-like activity(Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). Specific substrates and functions of the protein are still unknown, partly due to evidence that knockout mice display no

significant change in neuronal inclusions or dopaminergic neuronal loss(Goldberg et al., 2003; Perez and Palmiter, 2005). However, loss-of-function mutation of *parkin*'s counterpart in Drosophila display deficiency in flight muscle function and sperm individualization, indicating a function associated with mitochondria(Greene et al., 2003). This raises the possibility that similar mitochondrial impairment initiates the selective cell loss observed in PD.

Deubiqutinating enzymes have also been found to be involved in PD. Most attention has been focused on Ubiquitin C-terminal hydrolase-L1(UCH-L1). Also called PGP9.5, UCH-L1 is one of the most abundant proteins in the brain, counting for 20% of all proteins in the human brain (Wilkinson et al., 1989; Wilkinson et al., 1992). Interestingly, immunoreactivity for UCH-L1 was found in Lewy bodies, indicating that UCH-L1, like ubiquitin, is a component of Lewy bodies as well(Lowe et al., 1990). Moreover, a missense mutation (Ile93Met) of UCH-L1, which causes a 50% reduction in its enzymatic activity, was discovered in a German family with PD history (Leroy et al., 1998), and dopaminergic neuronal loss has been seen in transgenic mice expressing this mutant protein(Setsuie et al., 2007).

#### 1.3. Ubiquitin Specific Protease 24

The age at which an individual first manifests symptoms of a disease is called Age-at-Onset or AAO. Evidence suggests that the AAO of a disease can be genetically influenced (Daw et al., 1999). Different lines of evidence have shown that

that AAO in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease is genetically controlled (Li et al., 2002b). In particular, genetic studies of PD have found that Apolipoprotein E and the 2q13 region containing the putative park3 gene influence the AAO of Parkinson's disease (DeStefano et al., 2002; Li et al., 2004).

The *park10* region on chromosome 1p32, was recently found to be related to both the risk and AAO of PD (Hicks et al., 2002; Li et al., 2006). Of 30 genes which lie within this region *USP24* has been found to be linked to these findings. The *USP24* gene is located at 55,304,620-55,453,627 on the p arm of Chromosome 1. Based on the predicted sequence in the Ensembl genome database (Ensembl ID: ENSG00000162402), the human *USP24* gene transcript (Ensembl ID: ENST00000294383) is 10,802 bp long and is composed of 68 exons. The shortest exon is 36 bp, while the longest one, containing the 3' UTR, is 2,749bp.

Oliveira *et al.* found that several *USP24* SNPs are significantly related to the AAO of PD(Oliveira et al., 2005). In addition, Li *et al* identified 17 SNPs of the *USP24* gene which contribute to the risk of late-onset PD (Li et al., 2006). These studies suggest that genetic variation in the human *USP24* gene may play an important role in the etiology of PD.

## 1.4. Nuclear Factor-Kappa B (NF-κB) Signaling

NF- $\kappa$ B was first identified as a B cell nuclear factor that binds to a site in the immunoglobulin  $\kappa$  enhancer (Sen and Baltimore, 1986), NF- $\kappa$ B proteins constitute a family of dimeric transcription factors expressed in various tissues and involved in multiple important cellular processes such as cell division, apoptosis, and inflammation (Ghosh et al., 1998).

# 1.4.1. Components of NF-kB Molecules

Subunits that serve as components of NF-κB dimers include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), Rel-B and C-Rel, all of which are characterized by the Rel homology domain (RHD), an N-terminal region of approximately 300 amino acids (Baldwin, 1996). A subfamily of NF-κB proteins, including p65, Rel-B, and c-Rel, consist of proteins with a transactivation domain in their C-termini. In contrast, the p50 and p52 proteins are synthesized as large precursors, p105 and p100(Figure1.2).



#### Figure 1.2. Some Members of the NFkB/Rel Family and the IkB Family

The Rel homology domain is the main characteristic of NF-kB family members. IkB family members have multiple copies of the ankyrin repeats. P105 and P100 contain both the Rel homology domains and ankyrin repeats.

Although the dominant form of NF- $\kappa$ B is a p50/p65 heterodimer, other combinations exist *in vivo* and *in vitro*, such as p65/C-Rel heterodimer, p52/p65 heterodimer, and p50, p52 and p65 homodimers (Urban et al., 1991; Duckett et al., 1993; Siebenlist et al., 1994; Baldwin, 1996). Unlike RelA, RelB, and c-Rel, the p50 and p52 NF- $\kappa$ B subunits do not contain transactivation domains in their C termini, and as a result, p50 and p52 homodimers act as repressors of transcription. However, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB or c-Rel (Li and Verma, 2002). The term NF- $\kappa$ B is most often used to describe the p50/p65 complex since the p50/p65 dimer was the first NF- $\kappa$ B dimer to be identified and is the most abundant in almost all cell types (Karin and Ben-Neriah, 2000).

Except RelB, which is only expressed in certain cell types such as interdigitating dendritic cells (Ryseck et al., 1992), all other NF-κB subunits are constitutively expressed in all cell types (Carrasco et al., 1993).

As a sequence-specific transcription factor, NF-κB has a binding preference characterized by a nucleotide consensus sequence 5'-GGGRNNYYCC-3' (R is purine; Y is pyrimdine, and N is pyrimidine or purine), though different subunits exhibit dissimilar binding affinities(Kunsch et al., 1992).

#### 1.4.2. Molecular Mechanisms of NF-kB Activation in Cells

Prior to activation, NF- $\kappa$ B is sequestered in the cytosol by a family of inhibitory proteins called Inhibitors of kappa Bs (I $\kappa$ Bs). I $\kappa$ B family members include I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , Bcl-3, p100, and p105 (Baeuerle and Baltimore, 1996; Baldwin, 1996), all of which are characterized by several copies (six to eight) of ankyrin repeats (Figure 1.2). The most important and well characterized I $\kappa$ B protein is I $\kappa$ B $\alpha$ .

Activation of NF-kB signaling involves deactivation of IkB (Inoue et al., 1992). Prior

to activation of NF- $\kappa$ B, the I $\kappa$ B $\alpha$  monomer binds to the p65 subunit of NF- $\kappa$ B via its C-terminal ankyrin repeats, which masks the nuclear localization sequence of p65 (Beg and Baldwin, 1993; Baeuerle and Henkel, 1994) and thus keeps NF-kB out of the nucleus. The activity of  $I\kappa B$  is regulated by a kinase called the  $I\kappa B$  kinase (IKK). Through various signals such as tumor-necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1 (IL-1), IKK is activated and phosphorylates two serine residues (serine 32 and 36 of human I $\kappa$ B $\alpha$ ) located in the N-terminus regulatory domain of IκBα (DiDonato et al., 1996; Karin and Ben-Neriah, 2000). Subsequently, phosphorylated I $\kappa$ B $\alpha$  is modified by polyubiquitins at lysines 21 and 22 and undergoes degradation via the ubiquitin-proteasome pathway (Scherer et al., 1995; Baldi et al., 1996). As a result, NF- $\kappa$ B is free to translocate into the nucleus, where it activates transcription of target genes (Figure 1.3). This mechanism of activation makes NF- $\kappa$ B a rapid-acting primary transcription factor whose activation does not need novel protein synthesis and thus can respond quickly to external stimuli. Interestingly, IkBa gene expression is regulated by NF-kB. Newly-synthesized IkBa is able to re-inhibit NF-kB and thus forms an auto feedback loop, resulting in oscillating levels of NF-kB activity (Nelson et al., 2004).



#### Figure 1.3. Activation of NF-ĸB

This is a schematic model of a process in which the NF- $\kappa$ B p50/p65 dimer is activated. After a stimulus activates the IKK activating kinase, IKK phosphorylates I $\kappa$ B at specific serine residues. Phosphorylated I $\kappa$ B is ubiquitinated at two lysine residues and degraded by proteasome pathway. The released NF- $\kappa$ B dimer translocates into nucleus and regulates target gene expression.

NF-κB signaling is activated in response to a wide range of stimuli, including pathogens, stress signals and pro-inflammatory cytokines, such as TNF-α or IL-1. Many pathogens are recognized by specific pattern-recognition receptors on the cell surface. These receptors have evolved to recognize pathogen-derived substances, such as lipopolysaccharide (LPS), peptidylglycans, lipoproteins, and unmethylated bacterial DNA (Kelliher et al., 1998; Imler and Hoffmann, 2000; Ruland et al., 2001; Li and Verma, 2002).

#### 1.4.3. Biological Functions of NF-KB

#### 1.4.3.1. NF-κB Signalling and Immunity

NF-κB is required for the regulation of innate immunity and cellular defense against bacterial invasion (Ghosh et al., 1998), and in particular for the rapid induction of acute-phase defence genes in response to invading pathogens(Gerondakis et al., 1999; Li and Verma, 2002). Aberrant NF-κB activity in mice and humans is associated with susceptibility to microbial infection (Gerondakis et al., 1999; Perkins, 2000). In addition, NF-κB activity is important for the development and maturation of macrophages and neutrophils (Denk et al., 2000). NF-κB signaling is crucial in adaptive immunity as well. Mice lacking individual NF-κB proteins have defects in Band T-cell proliferation, activation, and cytokine production(Li and Verma, 2002).

#### 1.4.3.2. NF-kB Signalling and Apoptosis

NF-κB plays an important regulatory role in cellular apoptosis. It has been found that NF-κB modulates the expression of multiple antiapoptotic or cell survival genes (Mistry et al., 2004; Shishodia and Aggarwal, 2004). Studies using p65 knockout mice reveal that p65 inhibits apoptosis in the liver at embryonic days 14-16 (Van Antwerp et al., 1998). Fibroblast cells generated from p65 knockout mice show reduced

viability in TNF-stimulation experiments while they can be rescued by transient expression of p65, indicating an essential role for NF- $\kappa$ B in preventing TNF-induced cell apoptosis (Beg and Baltimore, 1996). Inhibition of NF- $\kappa$ B using an I $\kappa$ B mutant with strengthened inhibitory ability leads to increased cell death (Liu et al., 1996).

1.4.4. NF-кВ Signalling In the Nervous System and in Neurodegenerative Diseases

#### 1.4.4.1. NF-KB Signalling In the Nervous System

Much evidence supports essential roles for NF- $\kappa$ B signaling in the nervous system. Similar to other organ systems, NF- $\kappa$ B is expressed in all cell types in the nervous system, including neurons, microglia, astrocytes, and oligodendrocytes. NF- $\kappa$ B signalling's upstream mediators such as TNF- $\alpha$  receptor and Fas are also present in neurons and glial cells (Bruce et al., 1996). In addition, neurotrophin receptor p75 has also been found to mediate NF- $\kappa$ B signaling (Carter et al., 1996). Interestingly, electrical activity within neurons and event associated with synaptic transmission are also considered possible neuron-specific stimuli for NF- $\kappa$ B(O'Neill and Kaltschmidt, 1997).

Several genes crucial for neuronal or microglial function are the targets of NF- $\kappa$ B regulation. Products of these genes include TNF- $\alpha$ , IL-6, Bcl-2, manganese superoxide dismutase(Mn-SOD), and inhibitor-of-apoptosis proteins (IAPs) (Mattson and Camandola, 2001).
1.4.4.2. NF-κB Signalling In Neurodegeneration and Neurodegenerative Diseases NF-κB signalling is highly involved in neurodegenerative conditions. In rodent stroke models, NF-κB is activated in CA1 hippocampal neurons of rats following transient global forebrain ischemia (Mattson and Camandola, 2001). Cell culture studies suggest that NF-κB activation can protect neurons against excitotoxic and metabolic insults caused by stroke (Cheng et al., 1994), and hippocampal neurons lacking the p50 subunit of NF-κB display increased vulnerability to excitotoxicity (Yu et al., 1999). In rats with traumatic spinal cord injury, NF-κB activation is observed in neurons surrounding the injury site(Bethea et al., 1998).

Under chronic neurodegenerative conditions, NF- $\kappa$ B activation is also observed. NF- $\kappa$ B can be activated in neurons incubated with amyloid beta peptides. NF- $\kappa$ B is also activated in neurons surrounding early amyloid plaques in humanbrain tissue from patients with Alzheimer's disease (AD) (Kaltschmidt et al., 1997). Immunohistochemical studies reveal increased NF- $\kappa$ B immunoactivity in the hippocampal formation and cerebral cortex of AD patients(Terai et al., 1996). Increased NF- $\kappa$ B activity is highly correlated with increased cyclooxygenase-2 gene transcription in the superior temporal lobe of sporadic AD patients(Lukiw and Bazan, 1998).

Several studies have indicated the importance of NF-kB in PD pathogenesis.

Expression of TNF- $\alpha$  has been found in microglial cells of the substantia nigra in PD patients (Boka et al., 1994). Based on immunohistochemical analysis, Hunot *et al* found that translocation of NF- $\kappa$ B to the nucleus in the dopaminergic neurons of PD patients significantly increased (Hunot et al., 1997). Moreover, it was observed that the NF- $\kappa$ B levels in nigrostriatal dopaminergic regions were significantly higher in PD patients than in controls (Mogi et al., 2007). This evidence suggests that NF- $\kappa$ B is highly involved in PD pathogenesis; however, the identity of genes activated by NF- $\kappa$ B in PD and how they participate in PD pathogenesis remain unclear.

#### 1.5 Rationale and Hypothesis of the Study

PD, like most other neurodegenerative diseases, is believed to be caused by multiple factors, both genetic and environmental. To date researchers have found no single factor that can solely underlie the occurrence of the disease; thus, before we can draw the complete picture of the molecular mechanism of PD we require more detailed evidence.

Although studies have shown that the ubiquitin-proteasome pathway plays a very important role in the molecular pathogenesis of PD, several questions still remain to be answered: how is this pathway involved in the pathology of PD? how many members are participating in it? how do those members interact with each other, and how does impairment of the ubiquitin-proteasome pathway contribute to the pathogenesis of PD?

It is interesting and suggestive that several *park* genes are encoding proteins related to ubiquitination or deubiquitination: parkin has an E3 enzymatic activity (Imai et al., 2000; Zhang et al., 2000),  $\alpha$ -synuclein is targeted to be ubiquitinated when it is phosphorylated (Hasegawa et al., 2002), and UCH-L1 is a ubiquitin carboxyl-terminal hydrolase (Leroy et al., 1998). As details of ubiquitin-involved pathways are being continuously discovered, it is likely that proteins encoded by the corresponding *park* genes, when we consider the network of ubiquitin pathway as a whole, are just the tip of the iceberg. As a result, before we are able to elucidate the involvement of the ubiquitin pathway in the molecular pathogenesis of PD and other neurodegenerative disorders we need to identify more members involved in the ubiquitin pathway.

USP24 gene, as shown in the previous introduction, is a candidate for the *park10* gene. Little is known about the function, localization, or substrates of this USP family member.; however, the significant relationship between USP24 gene and the AAO of PD gives us a hint to focus on this new gene and to investigate the possible roles USP24 protein plays in PD pathology. USP24, as a deubiquitinating enzyme, is crucial for the regulation of cellular processes. Accordingly, its own expression has to be tightly regulated. Any misregulation involved in this process may lead to dysfunction of the ubiquitin pathway network, which may contribute to the occurrence and/or development of PD. As a result, it is essential to elucidate how USP24 gene expression is regulated.

Transcriptional regulation of gene expression is necessary for its protein's spatial and temporal distribution. Transcriptional regulation is mainly achieved by controlling the initiation process of transcription, in which the promoter of a gene plays an important role. As the functions and properties of the protein are currently being investigated we first elucidated the regulation of *USP24* promoter activity. This work will shed light on investigations of the *USP24* gene expression profile and the upstream signalling pathways of USP24.

We hypothesize that human USP24 gene expression is highly regulated at the transcriptional level and that the USP24 gene is a target of an important signaling pathway which may be associated with the pathogenesis of PD.

#### **CHAPTER 2: Materials and Methods**

#### 2.1. Gene Cloning and Generation of Plasmid Constructs

# 2.1.1. Primer Sequences for PCR Amplifications

Primers for cloning were designed to include restriction enzymes sites so that the resulting PCR-amplified fragments could be easily cloned into the multiple cloning sites of vector pGL3-basic (Promega). For *USP24*, ten fragments from 1749 upstream to 102 bp downstream of the translation start site (ATG) were amplified by PCR and inserted in front of the luciferase reporter gene (Luc) in the pGL3-basic expression vector. Primers used to generate different promoter deletion plasmids include: forward,

Sac-1498f: 5'-AAGAGCTCCAGCAGGGGTTTGATGG, Sac-570f:

5'-AAGAGCTCGCAGCGAGGCGTATTCAGG, Sac-504f:

5'-AAGAGCTCGACTGCGGTGCATTTCAGG, Sac-442f:

5'-AAGAGCTCCTAGGATGGGGAGCGGGT, Sac-402f:

5'-AAGAGCTCGGGAAGTCCAACGGGGGTT, Sac-183f:

5'-AAGAGCTCCGGCAGCCCTTTCACCTTA, Sac-89f:

5'-AAGAGCTCGCCTGCCAACGGCGAGCAC, and Sac-37f:

5'-AAGAGCTCGCGGCCCGCCGCCGGGG, and reverse, Xho-370r:

5'-CCGCTCGAGCCCGAGCCTCTCCGAAC, Xho-28r:

5'-CCGCTCGAGCGGGGCCGCGCGCCTCGGCTC, Xho+27r:

5'-CCGCTCGAGCCCTGCGCGCCGCCATGTT, and Xho+149r:

5'-CCGCTCGAGGTTGGCCCCTGCGTTCCTG.

The USP24 promoter region and the inserts of the promoter-luciferase plasmids were sequenced by an automatic fluorescence-based DNA sequencer (ASI PRISM DNA analyzer; Applied Biosystems).

For mutations introduced into the USP24 promoter construct, the primer MutEcoRI-697r has the sequence: 5'-CGGAATTCGGTGCGCCGCGGCTGTAGGC.

For detection of the *USP24* mRNA level, the primers we used are USP24+419f: 5'-GTCCATCCCTTACAAGCGA and USP24+776r:

5'-GCCCAATTCCTTTGAGACA.

#### 2.1.2. General Procedures of Molecular Cloning

2-5 μg of vector plasmid and DNA inserts of interest product were digested with restriction enzymes (New England Biolabs) in 30 μl overnight. Agarose gel electrophoresis was used to separate the desired DNA. 1.5% agarose gels with 0.5μg/ml ethidium bromide were run in 1×TBE buffer (10×TBE: 108 g Trizma, 55 g Boric acid, 40 ml EDTA (0.5 mM/pH 8.0), make up to 1000ml with dH<sub>2</sub>O) at 110V for 30 minutes. The DNA was visualized with UV light and photographed with Kodak Imaging system. Desired DNA was cut out from the agarose gel and isolated with phenol-chlorophorm method. NaAC (final concentration 0.3M) and 2.5 volumes of

ethanol were added to the supernatant collected and incubated at -80 degree Celsius for 30minutes. The DNA were pelleted with top speed (13,000rpm) for 20 minutes at room temperature and washed once with 70% ethanol. DNA was dried and dissolved in 20ul H<sub>2</sub>O and DNA concentration was tested by spectrophotometers. The ligation was usually done with 50-100ng of vector and 100-200ng of insert depending on the size (molar ratio of vector:insert = 1:3).  $1\mu$ l T4 DNA ligase (Invitrogen) was used in 20µl system for cohesive ends ligation at room temperature for more than 1h. The ligated product was transformed into 50-100 $\mu$ l DH5 $\alpha$  competent cells (Invitrogen) by reacting on ice for 30 minutes, 37 degree Celsius for 1 minute and on ice for 5 minutes. The transformation system was then mixed with 200µl LB and shaken for 2 hours at 300rpm and 37 degree Celsius and plated onto ampicillin supplemented LB agar plates. 6 colonies were picked up the next day and inoculated into 1.5ml LB supplemented with ampicillin(60ng/ml). Minipreparation was done with the DNA minipreparation kit from Promega following manufacturer's protocol. The minipreparation was checked with enzyme digestion and confirmed by sequencing. The confirmed plasmid is quickly transformed with 1ul plasmid into 10 $\mu$ l DH5 $\alpha$  by on ice 1 minute, 37 degree Celsiuss 1 minute and ice 1 minute and plated onto the ampicillin plate. The next day, one colony is picked up and inoculated into 8ml LB supplemented with antibiotics and cultured for 8 hours before diluted into 250ml LB. Antibiotic was added right before use with a concentration of 60ng/ml. The culture was continued for 16 hours at 37 degree Celsius and 300rpm before extracting plasmid DNA with the midipreparation kit from Qiagen following the manufacturer's

instructions.

#### 2.2. Reverse-Transcription-Polymerase Chain Reaction

#### 2.2.1. RNA Extraction

Cells were harvested with 0.5mL Tri-Reagent (Sigma, T9424). The sample in Tri-Reagent can be stored at -80 degree Celsius. Cell samples were treated with 0.1mL chloroform and incubated at room temperature for 10 minutes. The mixture was then centrifuged at 4 degree Celsius at 13,000 rmp for 15 minutes. Three phases could been seen after centrifugation. The top aqueous phase containing RNA was collected and mixed with 0.25mL isopropanol. The samples were incubated for 7 minutes at room temperature and then centrifuged at 13,000 rpm for 10 minutes at 4 degree Celsius. The supernatant was removed and the pellet was washed with 0.5mL 75% Ethanol. After centrifuging at 13,000 rpm for 5 minute, RNA pellet was air-dried, dissolved in 30µL DEPC-treated water and incubated in 55°C water bath for 10 minutes. Water used in this process was DEPC-treated and tips and tubes were RNase-free)

#### 2.2.2. Reverse Transcription

cDNA synthesis was carried out with the ThermoScript RT-PCR System kit (Invitrogen, 11146-016) .  $50\mu$ M Oligo(dT) 20 primer, RNA, 10mM dNTP Mix and DEPC-treated water were included in the RNA-primer denaturation reaction.  $5\mu$ g RNA was denatured at 65 degree Celsius for 5 minutes. After that, the cDNA synthesis master reaction mix was added to the reaction. The master mix contained 5× cDNA

Synthesis Buffer, 0.1M dithiothreitol, RNaseOUT<sup>™</sup> (40U/µl), DEPC-treated water and ThermoScript<sup>™</sup>RT (15U/µl). After incubation at 50 degree Celsius for 50 minutes, the reaction was terminated by incubating at 85 degree Celsius for 5 minutes. The remaining RNA templates in the reaction were removed by incubating at 37 degree Celsius for 20 minutes after adding 1µl of RNase H.

#### 2.3. Cell Culture

#### 2.3.1. Culture media

HEK293 (Human Embryonic Kidney cell line), N2a (Mouse neuroblastoma), and SH-SY5Y (Human neuroblastoma) cell lines were cultured in medium shown as below:

Per bottle of medium contains:

500mL	Dulbecco's Modified Eagle Medium (Gibco 11960-069)
5mL	Sodium Pyruvate (Gibco 11360-070)
5mL	Penicillin-Streptomycin (Gibco 15070-063)
5mL	L-glutamine (Gibco 25030-081)
50mL	Fetal Bovine Serum (Gibco 26140-079)

#### 2.3.2. Trypsinization

Trypsinization of all types of cells used in this research follow the same protocol: Medium was removed from the plates. Then cells were washed by room-temperature Hanks Balanced Salt Solution (HBSS) (Gibco 14170-112) and treated with Trypsin-EDTA (Gibco 25200-072). Cells were suspended in fresh culture medium, counted and seeded for transfection. All cell lines were maintained in 37 degree Celsius incubator containing 5% CO<sub>2</sub>.

#### 2.4. Cell Transfection

Cell transfection in this project was done with either calcium phosphate method or with Lipofectamine 2000 (Invitrogen 11668-019).

#### 2.4.1. Lipofectamine 2000 Transfection

Proprietary transfection reagents Lipofectamine 2000 was used in this project for transfection of SH-SY5Y cells in order to achieve higher transfection efficiency.

Cells were seeded one day before transfection and had a 70-80% confluence. DNA was mixed with Opti-MEM I (Gibco 31985-070) at the dilution around 1µg in 100µl. Same amount of Opti-MEM I was mixed with Lipofectamine 2000 at a ratio around 25:1. After 5 minutes at room temperature, the two mixtures were mixed together. After 10 minutes at room temperature incubation, the solution was dropped onto the cultured cells with fresh medium.

#### 2.4.2. Calcium Phosphate Method

Calcium phosphate method was used to transfect HEK293 and N2a cells in this project. Recipes of reagents are shown as below: 0.5M CaCl<sub>2</sub> in 50mL distilled water:

3.675g CaCl<sub>2</sub>-2H<sub>2</sub>O.

2× HEPES-buffered saline solution (HeBS) in 100mL distilled water (PH 7.0):

1.636g NaCl
 1.19g HEPES

•

0.0213g Na<sub>2</sub>HPO<sub>4</sub>, anhydrous

Cells were seeded one day before transfection and had a 70-80% confluence. One hour before transfection, medium was refreshed. Then transfection was done with the following protocol:

1. Mixed 10 $\mu$ g DNA with 125 $\mu$ L 0.5M CaCl<sub>2</sub> to make up to 250 $\mu$ L DNA-CaCl<sub>2</sub> with autoclaved distilled water.

2. DNA-CaCl<sub>2</sub> was added to "bubbled" 2× HeBS solution drop by drop. Rebubble the HeBS after dropping any 100μl DNA solution.

3. DNA-CaCl<sub>2</sub>-HeBS mixture was placed at room temperature for 30 minutes and then dropped to each plate.

4. Culture media were changed after 24 h and cells were harvested 48 h after transfection.

#### **2.5 TNF-**α Treatment

Human TNF- $\alpha$  (Chemicon) powder was dissolved in distilled water at a stock concentration of 100 $\mu$ g/ml and store at -20 degree Celsius. Before treatment, the drug

was diluted into fresh culture media to the final concentrations of 0.1, 1, and 10 ng/ml. Then the old media was changed to fresh media containing the drug.

2.6. Reporter Gene Transcription Assay

The reporter gene transcription assay was carried out with the Promega Dual-Luciferase Reporter Assay system.

48 h after transfection, cells were washed twice with and suspended in cold Dulbecco's Phosphate-Buffered Saline (D-PBS) (Gibco 14190-136). Following a one minute centrifugation at 4000 rpm at 4 degree Celsius, the supernatant was removed and 1× Passive Lysis Buffer was used to lyse the cells. The cell lysis reaction was preceded at room temperature for 30 minutes, followed by vigorous peppiting and 1 min 13,000 rpm centrifugation. After that, supernatant was collected. In order to detect the firefly luciferase activity, 2  $\mu$ L of the cell lysates were mixed with 10  $\mu$ L of luciferase assay reagent II (Promega E1910) and luminescent signal was detected by Luminometer (Turner Designs, TD20/20). The pRL-CMV plasmid expressing the Renilla Luciferase was also included in cell transfection to serve as an internal control and used to normalize the transfection efficiency. To measure Renilla luciferase activity, the addition of 10 µL of Stop & Glo® reagent was followed immediately after the reading of firefly luciferase activity. Firefly luciferase measurement was normalized by Renilla luciferase measurement. Data collected by the luminometer were transferred to a computer during detection.

2.7. 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5'RLM-RACE) The 5' RLM-RACE was performed according to manufacturer's instructions (FirstChoice® RLM-RACE kit (Ambion)). 10µg RNA extracted from SH-SY5Y cells were treated with Calf Intestine Alkaline Phosphatase (CIP) for one hour at 37 degrees Celsius. After that, RNA was purified by phenol:chloroform and then treated by Tobacco Acid Pyrophosphatase (TAP). The TAP-treated RNA was then ligated to a 5' adapter RNA by T4 RNA ligase. The ligation product then underwent reverse transcription to generate RLM-cDNA. Using the cDNA as a template, two nested PCR reactions were carried out to generate the specific 5' RACE products. 5' primers are adapter primers provided in the kit. Sequences of the specifically designed primers are:

USP24: Outer primer: 5' CGGCCTCGTTAATGTCGTTCTTG 3'

Inner primer: 5' CCGCTCGAGATGTGCTGCTCCTCCTCCGATTCC 3'

After the inner PCR products were generated, they were digested and inserted into the pFlagMycHis(c) vector using the BamHI and XhoI sites. The plasmids were then sequenced. The procedures of cloning and sequencing have been described in section

2.1.2.

The PCR reactions for USP24 were carried out with the Phusion Enzyme (NE Biolab). The CIP, TAP, T4 RNA ligase, M-MLV reverse transcriptase and all buffers and reagents used in the 5' RLM-RACE were from the First Choice® RLM-RACE kit (Ambion).

#### 2.8. Immunoblotting

Recipes of buffers are shown as below:

RIPA Lysis buffer:

30ml 5M NaCl, 50ml 1M Tris-HCl pH7.2, 10ml Triton X-100, 10ml 10% SDS,
10g Sodium Deoxycholate to a total volume of 1L with distilled water. (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.05M Tris-HCl, pH
7.2). Supplement with protease inhibitor cocktail Complete (Roche).

SDS sample buffer  $(2\times)$ :

2.5ml 4×Tris-HCI/SDS, pH6.8 (0.1M); 2ml glycerol; 0.4g SDS; 0.31g DTT; 0.1mg bromphenol Blue; Add H<sub>2</sub>O to 10ml and mix. Aliquot to 1ml and store at -80 degree Celsius.

4× Tris-HCI, pH 6.8:

Dissolve 6.05g Trisbase in 40ml  $H_2O$ . Adjust pH to 6.8 with 1N HCI. Add  $H_2O$  to 500ml total volume. Filter the solution through a 0.45um filter and store at 4 degree Celsius up to 1 month.

Tris-HCI/SDS, pH 8.45:

Dissolve 182g Tris base (3.0M) in 300ml  $H_2O$ . Add  $H_2O$  to 500ml total volume. Filter the solution through a 0.45um filter, add 1.5g SDS and store at 4 degree Celsius up to 1 month.

10× Tris-glycine running buffer:

29 g Trizma, 144 g Glycine, 10 g SDS, Make up to 1000ml with dH<sub>2</sub>O.

10× blotting buffer:

30.3 g Trizma, 144 g Glycine, make up to 1000ml with dH<sub>2</sub>O.

 $1 \times$  blotting buffer: Dilute 100 ml  $10 \times$  blotting buffer in 200 ml methanol + 700 ml

dH<sub>2</sub>O.

10× Phosphate buffered saline (PBS):

80 g NaCl, 2 g KCl, 14.4 g Na<sub>2</sub>HPO4, 2.4 g KH<sub>2</sub>PO<sub>4</sub>. Add 800 ml dH<sub>2</sub>O and adjust

pH to 7.4, then make up to 1000ml.

1× PBS-T buffer:

Dilute  $10 \times PBS$  to  $1 \times$  and add 1ml Tween-20 per 1L  $1 \times PBS$ .

Blocking buffer:

LI-COR Blocking Buffer (927-40000), diluted by the same amount of 1×PBS. Stored at 4 degree Celsius.

48 hours after transfection, cells were washed twice with cold PBS and harvested in RIPA-DOC cell lysis buffer containing protease inhibitor. Cell pellets in the lysis buffer were sonicated(Fisher Scientific) and then centrifuged at 13,000rpm for 1 minute. The supernatant can be stored at -20 or -80 degree Celsius.

Before loading, gradient concentration of 0-50µg/ml of BSA (Sigma) was used as protein standard in the test of protein concentration of sample. Then cell lysates were

mixed with same amount of 2×loading buffer and heated at 95 degree Celsius for 5 minutes. The samples were then loaded in 10% Tris-Glycine gels and run at constant current 20mA in Mini-PROTEAN 3 cell (Bio-Rad). PVDF (Polyvinylidene fluoride) membrane (Millipore, IPVH00010) was briefly incubated with methanol and proteins were transferred from SDS-PAGE (Polyacrylamide gel electrophoresis) gel to the membrane by wet transfer system (Bio-Rad, Mini Trans-Blot cell). After 2 hours transfer at 110V, the membrane was incubated in blocking buffer for one hour at room temperature.

After blocking, the membrane was incubated with the primary antibody at 4 degree Celsius overnight. Monoclonal anti-luciferase antibody (Novus Biologicals, Inc, NB 600-307) against the first 258 amino acids of firefly luciferase protein was used to measure firefly luciferase protein expression (1:2000 working dilution in blocking buffer). Internal control  $\beta$ -actin protein was detected by monoclonal anti- $\beta$ -actin antibody AC15 (1:15,000 dilution) (Sigma).

The membrane was washed with PBS-T four times for 5 minutes each time and then incubated with the secondary antibody (1:10000 dilution for goat anti-mouse)(LI-COR) at room temperature for one hour. After rinsing the membrane 4 times for 5 minutes each time, the membrane was put into 1×PBS and then scanned by the Odyssey infrared system (LI-COR) using the 700nm wavelength.

#### 2.9. Electrophoretic Mobility Shifting Assay (EMSA)

Recipes of buffers and gels used in the EMSA away are shown as bellow:

1×TGE running buffer:

25mM Tris

190mM Glycine

1mM EDTA (PH 8.0)

4% native polyacrylamide gel:

For 40 ml mix:

5 ml	40% polyacrylamide stock (Polyacrylamide-BIS ratio = 29:1)							
2 ml	1 M Tris, pH 7.5							
7.6 ml	1 M Glycine							
160 µl	0.5 M EDTA							
26 ml	H2O							
200 µl	10% APS							
30 ul	TEMED							

Gels were poured between glass plates and waited about 1-2 hours to polymerize.

Infrared dye-labeled double-strand DNA oligos were perchased from LI-COR Biosceince. Buffers for incubation of samples are from the LI-COR Infrared EMSA Buffer Kit (829-07910). Each incubation reaction contained:

1µl 10×Binding Buffer (100mM Tris, 500mM KCl, 10mM DTT; pH 7.5),

### $0.5\mu$ l Poly(dI•dC) (1µg/µl in 10mM Tris, 1mM EDTA; pH7.5),

1µl 25mM DTT/2.5%Tween®-20.

In each specific sample, labeled oligonucleotides, non-labeled oligonucleotide competitors, and/or HEK293 cell nuclear extract were added. (For specific combinations and amounts please refer to Chapter 3). Total amount in each sample is 10µl after the addition of water. Samples were incubated in dark at room temperature for 30 minutes. For the supershifting sample, 1ul of antibody (anti-p65 is from Sigma and anti-p50 and -p52 are from Cell Signalling) was added and incubated for additional 20 minutes at room temperature. After the incubation, 1µl or orange dye was mixed to each sample before it was loaded to the gel. The gel ran for 90 minutes at 80V and was scanned by the Odyssey Infrared System (LI-COR) using the 700nm wavelength.

#### 2.10. Data Analysis

Data of agarose gels was collected by the software Image J. Data from western blottings was collected by Licor software.

Statistical analysis in this study was carried out with the software GraphPad Prism 3. Figures were made with the software Canvas 9.

# **CHAPTER 3: Results**

#### 3.1 Characterization of USP24 Gene Coding Sequence.

Alignment of the predicted human and mouse USP24 protein sequences reveals a 97.7% identity, indicating that the *USP24* gene is highly conserved from mouse to human (Figure 3.1).

hUSP24,	1	NESEEEQHTTTLICKGESDEATICKALRIAKNDINEAVALLTNERPGLDYGGYEEMDOG	hUSP24, 144	ILLG	spsaeirrvacoolytleqtdtsahbovokfrofligviltaolpinsptsinerv
mUSP24,	1	NESEEEQHTTTLICKGESDEATICKALRIAKNDINEAVALLTNERPGLDYGGYEEMDOGG	mUSP24, 143	ILLG	Spsaeirpvactolytleqtdtsahbovokfrofligviltaolpinsptsinerv
hU3P24.	61	695999666980566096039886331669639099998748020028280023686	hUSP24, 150	NORL	logomentol royloddint sen gylri spratniede i triunfrennta Bootse
mU3P24,	61	- Papepagapagagagagagagagagagagagagagagagag	mUSP24, 149	NORL	Logomentolroyliddint sen gylri spratniede i triunfeinsta gybtae
hUSF24,	121	ibpetinlyelbsevlyckwsipykresslskollastilarlglsesdencrafnorom	hUSF24, 156	ACWI	llaghlrliktilelogaerrmlosslikfilddflerasri1/NSHSFAssats
mUSF24,	118	Ibpetinlyelbsevlyckwsipykresslskollastilarlglsesdencrafnerom	mUSF24, 155	ACWI	Llaghlrliktilelogaerrmlosslikfilddflerasri1/NSHSFAssaats
hUSP24,	181	deapkkiltssavhkroteihegyvinimleievaer <mark>i</mark> kodpipiollovitkaenfon	hUSP24, 162	C CODE	HPROSTANSRLAAXEVLUMLADSSPENLQIIIKELLUMHHQPUPALCKEPDYLPPU
mUSP24,	178	Deapkkiltssavhkroteihegyvinimleievaerkodpipiollovitkaenfon	mUSP24, 161		HPROSTVMSRLAAXEVLUMLADSSPENLQIIIKELLUMHHQPDPALCKEPDYLPPU
hUSF24.	241	eyhfioiring orniaeufoeginffauspust forefigwuuduunfgelggfraior	nUSP24, 165	DERS	se se froet ruise and a mar a root i word fer et tra opdating ode a radio fer
mUSF24,	238	Eyhfioiring orniaeufgegnfaiseustforernguuduunfgelggraior	mUSP24, 167	DERS	Se se froet ruise and an a root i word fer et radio dating ode a root fer
hUSP24,	301	TRREDIETCANANIĞETCANCERINSKANĞENTDEAITLIĞUNEANERDIKORKIAS	hUSF24, 174	HIME	iski qyyvpenerki frymrkelyyre qodayeff tisi i oqndey i kknor 2 qif kn
mUSP24,	298	TRREDIETCANANIĞETCANCERINSKANĞENTDEAITLIĞUNEANERDIKORKIAS	mUSF24, 173		Iski qyyvpenerki frymrkelyyre qodayeff tisi i oqndey i kyngre qif kn
hUSP24,	361	i peli sa valionepopel v ti voli raddillemiks pepsavansi kevikli bosti s	hUSF24, 180	1 TTQS	n yodchi okocheryerberginalni ovtokorie i sloqvyrgevlegenry yce
mUSF24,	338	i peli sa i kliomepopal vi tvjalridi i lamiks pepsakansi kevikli edatla	mUSF24, 173	3 TTQS	y yydchi okocheryerbergnala lovtokorie i sloqvyrgevlegenry yce
hUSF24,	421	ksynnaidtorildelyensylstalbenitgaqyldrikgiibligsklsldeltkiek	h03P24, 186	1 KCKE	KRITUKRICI KILESVIVI ELMEPSIONESSEGI KIDECI ALEMIMMEPITU SUM
mUSF24,	418	ksynnaidtorildelyensylstalbenitgaqyldrikgiibligsklsldeltkiek	m03F24, 185	6 KCKE	KRITUKRICI KELESVIVI ELMEPSICHESSEGI KUDECI KEEMILMEPITU AGM
hUSP24,	481	IQSQQ3STVIENIHTIIAAABVKINXQQINHIPUIQKSMETESDEVPQKILALIGRIGA	hUSF24, 192	1 ARQI	DSSERUGENGRSVDQGGGGSPRIXVALTENXELVGVIVHSGQANACHYVSFIKDRG
mU3924,	470	IQSQQ3STVIENIHTIIAAAPVKINXQQINHIPUIQKSMETESDEVPQKILALIGRIGA	mUSF24, 191	3 ARQI	DSSSEVGENGRSMDQGGGSPRIXVALTENXELVGVIVHSGQANACHYVSFIKDRG
hUSF24,	541	BADFEIISGNYLIVLWELAKLETLESSILQYALEEHUTIISDAVAVKERIKEYTIKCIE	nUSP24, 198	L CGKC	KAYKENDIYI ESPOINDETLEYEC POSEYRPKYY OQINPYI VARAYMAYNLYD
mUSF24,	539	ERFFERISCHYLDVLWELAKLETLESSILQYALEEHUTIISDAVAVKERVKSYTIKCIE	mOSP24, 197		XAYKENDIYI ESPOINDEILEYEC POSEYRPKYY OQINPYI VARAYMNAYNLYG
hUSP24,	601	DIKEPBERSGLERNERDGYKSGGIRNEGFWNYALRQIHEITRSITKGYYGRGESIIG	hUSF24, 204	1 8730	jõne dat ekkela aaakõeye otsi se seeleti sõõre dadane andotsi talak
mUSP24,	598	DIKEPBERSGLERNERDGYKSGGIRNEGFWNYALRQIHEITRSITKGYYGRGESIIG	mUSF24, 203	5 8730	Sõne datekkela aabõese otsi se seele sõõre dadane undetsi talak
hUSP24,	661	DLKENFEIVKUVTESLIÄCHRLAAAVAGPESESESTIVLERTTIGEVLEAHLEFLAFFLO	hUSP24, 210	1 KGEN	ckolfyjknpartyonyrdenlikfminrdyjosdyfsfylsilkolnatklikhpytfo
mUSP24,	859	DLKENFEIVKUVTESLLACHRLAAAVAGPEGETELTIVLERYTYREVIEAHLEFLAFFLO	mUSP24, 205	8 KGEN	Kkolfyskmpartyonyrddenlikfminrdyyjsdyfsfylsilasinatklikepytfo
hUSF24,	721	layiyiganrakeineolytogdyceldrimofewetkoghdlsody <u>ogo</u> lfkskilrid	hUSP14, 216	1 MANA	ysiqlaiqeleçtyiatekelevoteselatieallskaflanqevettissege
mUSF24,	718	Layiyiganrakeineolytogdyceldrimofewetkogndlsofavqoolfkskilrid	mUSP14, 215	6 MANA	Vsiqlaiqeleçtyiatekelevoteselatieallskaflanqevettissege
hUSP24,	781	SYELTMIGTALFKIPFENVNIGDERLKROGAGLYVEKLEDIEMDELEKTAMESFDESIAN	hUSF24, 223	1 LINI	i filson vrevovavatilsky i dealstycki kelaqilsvila lidkovenikn
mUSP24,	770	SYELTMIGFNLFKIPFENVNLCDERLKROGAGLYVEKLEDVGMDFJWKIAMESFDESIAN	mUSF24, 221	9 LVK1	Vfilson vrevovavatilsky i dealstycki kelaqilsvila lidkovenikn
hUSP14, mUSF14,	841 639	EAIQLIINYSYIMINERIKKESYSIAKKEIADCYIRLSAASSALOSDTLTHAVIRAISMI EAIQLIINYSYININERIKKESYSIAKKEIADCYIRLEAASSALOSDTLTHAVIRAISMI	hUSP24, 225 mUSP24, 221	1 CAQ1 8 CAQ1	YPFLENTE VZKOSI RAGDLLI RESALDENI SELLOASRONO I DZNESAO AREFONE YPSLENTE VZKOSI RAGDLLI RESALDENI SELLOVSRONSO I DZNESKO AREFONE YPSLENTE VZKOSI RAGDLLI RESALDENI SELLOVSRONSO I DZNESKO AREFONE
hUSP24,	901	TATAMPTVATSVQSPYRSTKLVIIERELLLAERYVITIECFISVPRTILPHGASFNGHLI	hUSP24, 234	1 HNT	ayılalırı bolasığıra yaşı korpetisi Arbas Plipinera kalı firekakılır.
mUSP24,	398	TATAMPTVATSVQSPYRSTKLVIIERELLLAERYVITIECFISVPRTILPHGASFNGHLI	mUSP24, 233	S HNT	Ayılalırda yaşıkardı korpetisi Arbas Plipinera kalı firesakılır.
hUSP24,	961	tinytyestkotetveahenetigsyrkiakoisefunigietnosiltynkochilh	hUSP24, 240	1 VME	alredigsilai fenyyysternenystyne spikroletasphelkrupollre il
mUSP24,	950		mUSP24, 233	3 VME	Alredigsilainenyysternenystyni spikroletasphelkrupollredi
hUSF24,	1021	QLGFSDEQILTVRTSCSGTPSGSSADSST3SSSS3GVFSSSYAMEQEKSLPGVVNALVC	nUSP24, 244	1 VIE	de i qvervkev fetengol almensne vdsorce qoʻvke lvtlaqkop aaken fiken
mUSF24,		QLGFSDEQVLTVRTSCSGTPSGSSADSST8SSSSSGAFSSSYAMEQEKSLPGVVNALVC	mUSP24, 243	8 VIE	de i qvervkev fetengli almensne vdsorce qoʻvke lvtlaqkop aaken fiken
hUSP24,	1081	nvfimlyqlanleberitlrvrkillifydfaiqealdqldsigrkkillsef3sqasy	hUSP24, 253	1 SHH	nswavqwlqakmeehiwifqsnverststortfiqriisrqotlayatallarkequg
mUSP24,	1078	nvfimlyqlanleberitlrvrkillifydfaiqealdqldsigrkkillsef3sqask	mUSP24, 253		nowavqwlqkkmeehiwifqsnverststortfqriisrqotlayatallarkequg
hUSP24,	1141	aralsako2H2Faaseilesleretarokatervivilevlääkinfaddomarscaks	hUSP24, 25	1 53N	Gerstranmidraloocsestationerationopp
mUSP24,	1138	apsleako2H2Faassilesleretarokatervivilevlääkinfaddomarscaks	mUSP24, 25	3 53N	Gerstranmidraloocsestationopp
hUSP24, mUSP24,	1201 1199	FCENFLKAGGLELVVNVMCRDEIFSEVDYSTRQUVYSICLCLARFLLVGQIMPTLLDEDL FCENFLKRGGLELVVNVMCRDEIFSEVDYSTRQUVYSICLCLARFLLVGQIMPTLLDEDL			
hUSP24, HUSP24,	1261 1258	TRIGIBALSSRPPRNYSPQTORUMSLIGTYEKSSYRQLGYSDRSSIRVEBIIPAARVAIQ TRIGIBALSSRPPRNYSRQTSRQMSLIGTYEKSSYRQLSYDRSSIRVEBIIPAARVAIQ			
hUSF24, mUSP24,	1321 1310	TME78DETT TVACEMRLSMAAAAGRLDUVGSSOPIKESNALDPAGINDPLSSSOSNESSG TMEABOPTATVACEMRLSMAAAAGRLDUVGSSOPIKESNSLPPAGIRSPLASSOSNESSG			
hUSP24, mUSP24,	1381 1378	SEGEFVALMAGICVRQQSVSTKDBLIAGEALSLIVTCLQLRSQQLAS FVALFCVADFIID SEGEFALMAGICVRQQSVSTKDALIAGEALSLIVTCLQLRSQQLAS FYSLFCVADFIID			

# Figure 3.1. Protein Sequences Alignment of Human USP24 (hUSP24) and Mouse USP24 (mUSP24) Homologues.

Human and mouse USP24 protein sequences are labeled as hUSP24 and mUSP24, respectively. Identical amino acids are highlighted.

Analysis of the USP24 protein sequence, based on the protein domains, families, and functional sites databases PROSITE and PFAM, revealed two major domains of the human USP24 protein, the Peptidase\_C19 Domain and the Ubiquitin-associated Domain (UBA) (Figure 3.2.). As described in section 1.2.3.2., a peptidase\_C19 domain is the catalytic domain of DUBs and UBA domains function to associate with Ubiquitin molecules. These domains characterize USP24 protein as a deubiquitinating enzyme.



#### Figure 3.2. Major Domains of the USP24 Protein

A schematic illustration of the full length USP24 peptide sequence. The UBA domain is located from amino acid 3 to 44. The Peptidase\_C19 domain is spanning from the amino acid 1,689 to 2,043.

# 3.2. Cloning and Characterization of the Human USP24 Promoter

For the human *USP24* promoter, a 1648-bp 5' flanking region of the coding sequence was cloned. A computer software-based analysis of the sequence revealed that this region contains several putative regulatory elements, such as SP1, Oct1, TCF, STAT3, and NF- $\kappa$ B (Figure 3.3.).

-1498:	cagcaggg	gtttgatggg	atgaaaaagt	aaggttagct	ggatttcagg				
-1450:	ttcaggaaga	aaccaggatg	aggacattag	aagatgaact	cagcaagtat				
-1400:	gtagga <u>atag</u>	gcataggaga	tgaactcagc	aagaatgaag	ggatatccga				
OCT-1									
-1350:	atcttgggaa	agggtgggaa	aataacctag	agaactatgg	attgaaggaa				
-1300:	attgaagagg	aaaaatagca	aggacttagg	cccaggacgg	<b>ctttga</b> atgc				
		• •			T CF				
-1250:	ggctcagcac	aaattcgtaa	acttettaaa	acatgatgag	atttttaaa				
-1200:	agtttcatca	gctaccatta	gcgttagaat	attttatatg	tggtccaaga				
-1150:	caatttttct	ccttccaatg	tagcccaggg	aagccaaaag	attggacacc				
-1100:	cttggcttag	atggtggagt	caagggaact	aaagatatga	ccccagtgtg				
-1050:	ccaat <u>gccaa</u>	aggtgagaag	agaa <u>agcaaa</u>	gaaagcaggc	ctttaaagag				
1000.	PAX-5		TCF						
-1000:	gragaggaat	CC tCaaaggt	ggaaagaaac	agggatagga	tgctgcccaa				
_050.	~~~~~~~~	ICF							
-930:	gaggcaggga	caaagaateg	<u>gagccaaa</u> ga E2F	ctgcggtaaa	aatgtgggaa				
-900:	qqtqatqtcc	acqutattca	aacgagtccg	tooccaaaoo	aagtaagagt				
-850:	ctgggtttct	ggaggtttc	atacagagaa	ctqqqattta	cattleagae				
					E2F				
-800:	tggaaaaata	tatttggac	tctcaggggc	tttcagggta	agagttcggt				
-750:	agagttcagt	cagettggeg	gagacaggta	gtcagttaat	gttggaagga				
-700:	aaattcggac	agctgggaat	taatttcaac	gggtttaggg	tgatttcggc				
-650:	aagggctggc	ggggctaggg	tcgagtgtga	atttcgtctt	gggtggtcca				
-600:	cgcgtctggt	ttgggcagga	attetgtgg	gcagcgaggc	gtattcaggc				
-550:	agcgttgcgg	gtgaatctgc	gtgggatgag	ggtggatttc	cttcaggact				
-500:	geggtgeatt	tcaggccggg	gtggaggtgc	ctcacagccg	cggcgca <b>ggg</b>				
-450:	<u>aatteee</u> tet	aggatggggg	agcgggtc <mark>gg</mark>	<u>gegg</u> egeegg	ccagggaggg				
	NF – KB		S	P1					
-400:	gaagtccaac	gggggttcgg	agaggetegg	gggactcgga	cggagagaag				
-350:	ccgggtcgcg	gctgcggagg	ctcggccggc	caaggg <u>attt</u>	ccgcgacgtt				
				ST	AT3				
-300:	cagcgctgcg	agetgegegg	cgctgcggcc	cctcccgtcc	cgggggaacc				
-250:	tgttgccggc	gaaacaggc <u>g</u>	ggcggtggcg	acgatggaga	agcct <u>tcetc</u>				
SP1 E2F									
-200:	<u>geet</u> etgeet	ggctgcacgg	cagccctttc	accttaggca	cgctccgcgg				
-150:	cgcggcccct	ccctccgctc	cttcgtttaa	aacaaacaga	atacgccgcc				
-100:	gccaacccta	gcctgccaac	ggcgagcacg	gtegeeteee	gtgccgcttc				
- 50:	cgggagccga	ggcgcggccc	cdccdcccdd	ggaggaggag	gcggaggggg				
+ 1:	aggaggccaa	catggeggeg	cgcagggetg	ggccgggccg	cagccgcgcc				
+ 51:	tgagegeete	accgcactag	ctcggctcgc	aggacccaag	cccgcaccca.				
+101:	geetggeeeg	getgetgegg	agcacccggg	gcaggaacgc	aggggccaac				
+151:	cräddcacaa	aggeggegeg	gagggtgcgc	cacacaaccc	gccaggcccg				
+201:	ggacccgtag	cttcgccgtg	cgctggccgg	ggcggccagg	aggcccaagc				
+251:	CATEGAATCG	GAGGAGGAG							

# Figure 3.3. The Nucleotide Sequence of the Human USP24 Promoter.

A 1,648bp fragment of the 5' flanking region was isolated from a human genomic library. Putative transcription factor binding sites are underlined in bold. The translation starting site ATG is bold and the adenine serves as +1.

5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5' RLM-RACE) was performed to map the transcription starting site (TSS) of the *USP24* promoter. Compared with other methods of mapping transcription starting sites such as primer extension, nuclease protection assay, or traditional 5' RACE, 5' RLM-RACE has a major advantage in that only authentic capped 5' end of mRNA can be detected. This is fulfilled by removing the 5' phosphate from non-mRNA or incomplete mRNA fragments, which disable them from participating in subsequent ligation reaction. The 7-methylguanosine cap of mRNAs is further removed by a pyrophosphatase that leaves a 5' phosphate group. These phosphate groups make authentic mRNA the only target that can be ligated to an RNA adapter. Finally, the processed RNAs undergo reverse transcription followed by PCR using gene-specific primers and primers homologous to the RNA adapter.

The 5' RLM-RACE product was cloned into a vector and sequenced. The specific procedure was described in section 2.7. As shown in Figure 3.4., 5' RLM-RACE revealed that the TSS of human *USP24* gene is located at 251 bp upstream of the translation starting site. The TSS, an adenine, is marked as +1.



#### Figure 3.4. Mapping the USP24 Transcription Starting Site by 5' RLM-RACE

(A) Agarose gel electrophoresis of the nested PCR product from the RLM-RACE procedure using human SH-SY5Y RNA. Molecular size markers are indicated on the left. (B) Sequence of the 5' UTR region of the *USP24* gene. Locations of the mapped TSS, the translation starting site, and the primers for the inner and outer 5' RLM-RACE PCR are shown in bold or with arrows. (C) Sequencing result of the 5' RLM-RACE product which was cloned into the pFlagMycHis(c) vector. Sequence of the 5' adaptor is shown underlined. The adenine next to the 3' of the adaptor is the TSS of human *USP24* gene.

To determine promoter activity of this 5' UTR region, we cloned different deletions of this 5' flanking region. Locations of the deletions are shown on Figure 3.5.. All deletions were inserted into the pGL3-Basic vector. We transfect these reporter plasmids into a human embryonic kidney cell line (HEK293 cells) and performed

luciferase assays (Figure 3.5.). The construct containing the region from -570-+149 showed the highest promoter activity compared with other constructs, indicating that the region from -570 to +149 contains a functional promoter of the human *USP24* gene. Further deletions of this region, from -504, -442, -402, -183, -89 to -37, showed various levels of promoter activities. However, a 64-bp region from -37 to +27 is required for a basic promoter function. This is consistent with our 5' RLM-RACE assay showing that the transcriptional starting site is located within this region.



#### Figure 3.5. Functional Deletion Analysis of the USP24 Promoter Region

This is a schematic diagram of the USP24 promoter deletion constructs containing various fragments of the 5' flanking region of USP24 gene in the promoter-less vector pGL3-Basic. The firefly luciferase gene (Luc) was used as a reporter gene. The pGL3-Promoter is a non-related promoter serving as a positive control. The pRL-CMV plasmid expressing the *Renilla* luciferase was included in the transfection of each well to serve as an internal control which normalized the transfection efficiency. The numbers represent the endpoints of USP24 promoters. +1 is the TSS.

3.3. USP24 Has a Higher mRNA Level in a Human Neuroblastoma Cell Line SH-SY5Y than in HEK293 Cells

To investigate activities of *USP24* promoters, we transfected the reporter plasmids containing these two promoters into SH-SY5Y and HEK293 cells. Compared with HEK293 cells, a significant increase of promoter activity was detected when the *USP24* promoter construct was transfected into SH-SY5Y cells(Figure3.6., 286.9±14.5 vs. 35.3±0.4, p<0.0001). This result suggests that *USP24* transcription level is higher in SH-SY5Y cells than in HEK293 cells, indicating that *USP24* gene expression is possibly neuronal specific.



#### Figure 3.6. Luciferase Activity was Measured at 48 Hours by a Luminometer.

The pRL-CMV plasmid expressing the Renilla luciferase was used to normalize transfection efficiency. The values, indicating fold of corresponding controls (pGL3-Basic), represent means of the ratios (n = 3). Error bars in this and other following column figures represent standard errors of the means. The symbol "\*" indicates that the p value generated by the Student's t-test (homoscedastic, unpaired, and two-tailed) is smaller than 0.01, which suggests a significant difference between the two groups being compared.

#### 3.4. USP24 Promoter Contains an NF-KB Binding Site

Computer software prediction showed a putative site for NF- $\kappa$ B on the *USP24* promoter (Figure3.3.), which is located from -453 to -444, with the sequence GGGAATTCCC. This prediction could also be confirmed by looking through the entire *USP24* promoter sequence manually: the GGGAATTCCC sequence is homologous to the NF- $\kappa$ B consensus sequence GGGRNNYYCC in which R is any purine, Y is any pyrimidine, and N is any deoxynucleotide. Thus, we hypothesize that

the USP24 gene transcription is regulated by NF- $\kappa$ B and the -453 to -444 region contains the *cis*-acting site.

To verify this putative binding site, we performed the EMSA assay (Figure 3.7.). The labeled USP24p oligo containing the NF-kB binding site showed shifted bands (lane3) after incubated with the nuclear extract of HEK293 cells overexpressing NF-KB p65. These shifted bands are the same as those of labeled NF- $\kappa$ B consensus oligo (lane2). The competition assay using oligos without labeling showed decreased intensity of bands as the concentration of the competitor increases (lane 4 and 5). However, there was little competition effect if we use a high concentration mutated USP24p oligo (lane 6), with two successive G to C mutations at the beginning of the binding site, as a competitor. Anti-p65 antibody, specifically binding to the p65 subunit of NF-kB, resulted in a supershifting band after incubated with the sample (lane 7). A weak supershifting effect can be seen in lane 8, in which anti-p50 antibody was incubated with our sample. Anti-p52 antibody revealed no supershifting effect in the experimental condition (lane 9). In the lane 10, both the shifted and the supershifted bands disappear when high concentration non-labeled competitors exist. The EMSA assay reveals that the USP24p NF-kB binding site has a specific interaction with the NF-kB protein complex in vitro.

No.	1	2	3	4	5	6	7	8	9	10	
NFkB concensus oligo*	+	+	-	-	-	-	-	-	-	-	
USP24Lp oligo*	+	-	+	+	+	+	+	+	+	+	
NFkB concensus oligo	-	-	-	5	50	-	-	-	-	50	
mutated USP24Lp oligo	-	-	-	-	-	50	-	-		-	
HEK293 nuclear extract	-	+	+	+	+	+	+	+	+	+	
anti-p65	-	-	-	-	-	-	+	-		+	
anti-p50	-	-	-	-	-	-	-	+	-	-	
anti-p52	-	-	-	-	-	-	-	-	+	-	
				-							



Nuclear extract used in this experiment were generated from HEK293 cells, which were harvested 48hours after transfection with NF- $\kappa$ B p65 expression vector. Double stranded NF- $\kappa$ B consensus oligo and USP24p oligo (-453--444) containing the putative NF- $\kappa$ B binding site, as indicated with symbol "\*"s, are labeled with infrared label (wavelength 700nm) at the 5' ends of both strands. All one fold oligos used in this experiment have a final concentration (as in the solution loaded to the gel) of 0.005pmol/ $\mu$ l. Folds of competitor oligos used in lane 4,5,6, and 10 are shown as numbers. Sequence of the NF- $\kappa$ B consensus oligo is:

5'-AGTTGAG<u>GGGACTTTCC</u>CAGGC-3'; sequence of the USP24 oligo is: 5'-GGCGCA<u>GGGAATTCCC</u>TCTAGG-3'; and sequence of the USP24 Mutation oligo is: 5'-GGCGCA<u>CCGAATTCCC</u>TCTAGG-3'. (Underlined is the consensus binding site, the putative binding site, or the mutated putative binding site of NF-κB).

# 3.5. NF-kB Signalling Regulates Human USP24 Promoter Activity

To confirm that the *USP24* promoter activity is regulated by the NF- $\kappa$ B signalling, we cotransfected the *USP24* reporter plasmid into HEK293 or N2a cells with either the NF- $\kappa$ B expression vector or a corresponding empty vector pMTF. When NF- $\kappa$ B is overexpressed, *USP24* promoter activity increases significantly, as shown by luciferase assay, both in HEK293 or N2a cells (Figure3.8. A and B). Same cotransfection was performed with HEK293 cells for Western blotting. As Figure3.8.C shows, overexpressing NF- $\kappa$ B causes increased luciferase level, which reflected increased *USP24* promoter activity. NF- $\kappa$ B overexpression resulted in an increase of luciferase activity from 0.217±0.007 to 0.577±0.001 (p<0.001) in HEK293 cells and from 0.208±0.006 to 2.126±0.044 (p<0.001) in N2a cells. NF- $\kappa$ B overexpression also led to increased luciferase protein level from 0.018±0.004 to 0.071±0.004 (p<0.001) in HEK293 cells. β-actin was used as an internal control in the Western blotting.



#### Figure 3.8. NF-KB Overexpression Increases USP24 Promoter Activity

The functional activity of putative NF- $\kappa$ B binding elements was analyzed by dual-luciferase reporter assays in HEK293 (A) (n=4) and N2a cells (B) (n=4). Cells were transfected with *USP24* promoter reporter plasmid (USP24p), either with NF- $\kappa$ B expression vector (solid column) or empty vector pMTF(hollow column). Luciferase measurements generated from control plasmid pGL3-Basic cotransfected with NF- $\kappa$ B or pMTF were also included (control). (C) The Western blotting result (n=3). HEK293 cells were transfected with USP24p and either NF- $\kappa$ B or pMTF. After 48hours they were harvested for Western blotting. Antibody against luciferase and  $\beta$ -actin were used as primary antibody.

To further support the NF- $\kappa$ B's effect, we mutated the NF- $\kappa$ B binding site on the USP24 promoter. The same mutations as in the EMSA assay (i.e. GG to CC at the beginning of the binding site) are introduced into the USP24p reporter plasmid (Figure 3.9.). Cotransfection was performed in HEK293 cells. The mutations result in a significant decrease of USP24p luciferase activity from 0.147±0.005 to 0.076±0.002

(p<0.0001). The data demonstrated that the mutations on the promoter abolished the regulatory effect of NF- $\kappa$ B on the USP24 promoter activity.





(A) Schematic illustration of the mutations introduced into USP24p reporter plasmid. The NF- $\kappa$ B binding site is underlined. (B) Statistics of the luciferase assay result. HEK293 cells were transfected with the wildtype *USP24* promoter reporter plasmid (USP24p) (solid column) or the mutated *USP24* promoter reporter plasmid (USP24p) Mut) (hollow column), together with the NF- $\kappa$ B expression vector or the empty vector pMTF (n=4).

To determine if a signal that can stimulate NF- $\kappa$ B leads to increased USP24 promoter activity, we treated cells transfected with USP24 promoter reporter

plasmid-transfected with TNF- $\alpha$ . As shown in Figure 3.10., TNF- $\alpha$  increased USP24

promoter activity in HEK293 and SH-SY5Y cells (746.6±23.7 (10ng/ml) vs.

 $601.1\pm3.8$  (control) for HEK293 and  $46.4\pm1.1$  vs. $36.0\pm0.3$  for SH-SY5Y, P<0.0001 by ANOVA). Taken together, these data clearly demonstrate that NF-kB regulates the transcription of the human *USP24* gene.





The functional activity of putative NF- $\kappa$ B binding elements was analyzed by dual-luciferase reporter assays in SH-sY5Y (A) (n=4) and HEK293 cells (B) (n=4). Cells were transfected with the USP24 promoter plasmid or the pGL3-Basic plasmid. 24 hours after transfection, TNF- $\alpha$  was added to media at final concentrations shown in the figure. Cells were harvested 24 hours after the TNF- $\alpha$  treatment. Values of the y-axis represent ratio of "USP24 promoter" over "pGL3-Basic" at the corresponding TNF- $\alpha$  doses.

#### 3.6. NF-кВ Increases Human USP24 mRNA Level

To investigate if NF- $\kappa$ B regulates transcription of endogenous *USP24* gene, we performed semi-quantitative RT-PCR with HEK293 cells. Compared with the control which was transfected with the empty vector, HEK293 cells transfected with NF- $\kappa$ B showed a significantly higher level of *USP24* mRNA (180.6±2.28 vs. 100.0±10.8, P<0.01). These data indicate that *USP24* gene transcription is increased by NF- $\kappa$ B overexpression.



#### Figure 3.11. NF-KB Increases Human USP24 mRNA Level

HEK293 cells were transfected with either NF-κB expression vector or empty vector pMTF. Cells were harvested, the mRNA library was extracted, and the reverse transcription was performed as introduced in chapter 2. After the cDNA library was obtained, PCRs using either primer pairs specific to human *USP24*(5' primer: USP24+419f: GTCCATCC CTTACAAGCGA and 3' primer: USP24+776r: GCCCAATTCCTTTGAGACA) or to human β-actin coding sequences were used to amplify corresponding sequences. The band intensity was analyzed by the "Image J" software.

#### **CHAPTER 4: Discussion**

As illustrated in Chapter 3, *USP24* gene transcription is tightly regulated. *USP24* gene transcription is controlled by a TATA-box-less promoter, which has its TSS located at 251 bp 5' upstream of the translation starting site. We showed that *USP24* promoter activity is regulated by NF- $\kappa$ B signalling. We provided evidence that increased *USP24* promoter activity could be observed when NF- $\kappa$ B signalling is activated by its specific activator TNF- $\alpha$ , or when levels of NF- $\kappa$ B p65 subunit are elevated. We further confirmed this signalling pathway by mutagenesis; the activating effect of NF- $\kappa$ B could be diminished by mutations to the NF- $\kappa$ B binding site. The physical interaction of the binding site with the NF- $\kappa$ B molecule was confirmed by EMSA assays.

Although substrates of the USP24 protein and the importance of USP24 in PD pathogenesis is still unclear, our study suggests a possible signalling pathway in which USP24 is involved, the NF- $\kappa$ B signalling pathway. NF- $\kappa$ B plays significant roles in apoptosis and inflammation, both of which are important features of the pathogenesis of neurodegenerative diseases such as PD and AD. This evidence makes *USP24* gene, a downstream target of NF- $\kappa$ B, an excellent candidate involved in apoptosis and inflammation that feature PD and other neurodegenerative diseases.

Worthy of notice is the composition of the NF- $\kappa$ B dimer which binds to the USP24 promoter. Since overexpression of NF- $\kappa$ B p65 significantly increased the USP24

promoter activity, and anti-p65 antibody generated a clear supershifted band in our EMSA assay, we can conclude from our results that the NF- $\kappa$ B p65 subunit is a component of the corresponding NF- $\kappa$ B dimer, however, the other component of the dimer is still unknown. We suggest that the NF-kB p50 subunit is the other component. Firstly, different NF- $\kappa$ B dimers have similar but not identical DNA sequence preferences, making it possible to identify the composition of the NF-kB dimer based on the binding site sequence. The NF-kB binding site on the USP24 promoter is identical to the consensus binding sequence of the NF-κB p50/p65 dimer. In addition, since the p50 subunit is inclined to interact with sequences that are more palindromic (Urban et al., 1991), the NF-kB binding site on the USP24 promoter, with sequence GGGAATTCCC, makes it highly possible that p50 is a component of the dimer which interacts with the USP24 promoter. Secondly, lane 8 of our EMSA result (Figure 3.7.) partially consolidated the assumption that p50 is binding to the USP24 promoter, as a supershifted band was generated when we incubated our binding system with antibodies against p50, but not other subunits such as p52 (lane 9). Thirdly, the p50/p65 dimer is the major form of NF- $\kappa$ B in cells. This evidence provides a strong case for the role of p50 as the other dimer component; however, further evidence, such as overexpression of p50 in our luciferase reporter assay, is required to confirm this suggestion.

Another explanation is that the dimer is a homodimer of p65. This is possible because we have no evidence to exclude this possibility and the supershifting effect of
anti-p65 is much stronger than anti-p50; however, even if this is true in our experimental conditions, it may not accurately represent *in vivo* conditions. For example, the nuclear extract we used in our EMSA contained overexpressed p65 subunits, which increased the chance that p65 formed dimers with itself other than what would truly occur *in vivo*.

As described in section 1.4.4.2., NF-κB is an important responding molecule in multiple cell events such as inflammation, antigen response, and programmed cell death, and is highly involved in neurodegenerative diseases. Specifically, increased NF-κB levels and subsequent translocation was observed in dopaminergic neurons of PD patients. The identification of *USP24* as a responding gene of NF-κB signalling provides a possible downstream pathway related to the cellular events of PD. When NF-κB levels increase or NF-κB signalling is activated, *USP24* expression is up-regulated. This, as can be expected, may increase USP24 protein levels in the cell, resulting in increased deubiquitination of its downstream substrates. Consequently, an elevated level, increased half-life, and altered localization of target proteins may occur, all of which may lead to protein aggregation. Thus, following the identification of the USP24 protein and the discovery of its downstream substrates, the possible roles NF-κB and USP24 play in cellular events of PD can be revealed, and this may pave the way for discovering possible drug development targets.

As we look at the sequence of the USP24 promoter we can also find putative binding

sites of other transcription factors, such as SP1, TCF, and STAT3. Future characterization of these binding sites may contribute to further understanding the transcriptional regulation of *USP24* and its related cellular signalling pathways.

In summary, our study revealed that human *USP24* gene expression is highly regulated at the transcriptional level. Transcription of human *USP24* is controlled by a TATA-box-less promoter. NF- $\kappa$ B signaling, as an important signaling pathway involved in neurodegenerative diseases, can regulate human *USP24* promoter activity via its specific *cis*-acting element on the promoter. Our research may shed light on studies focusing on the relationship of NF- $\kappa$ B signaling, deubiquitinating enzymes, and the pathogenesis of PD.

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