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# **Protein-Protein Interactions between Parkin and Nrdp1**

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## ABSTRACT

Autosomal Recessive Juvenile Parkinson's (AR-JP) is a debilitating disease caused by loss of functions in the *parkin* gene. The parkin protein normally functions as an E3 ligase in the ubiquitination pathway, a cellular process that facilitates the degradation of misfolded proteins. A loss of parkin function results in the accumulation and aggregation of these misfolded proteins, causing cell death of dopaminergic neurons in patients' brains. It has been recently shown that parkin is directly associated with Nrdp1, another ubiquitin E3 ligase. Further evidence indicates that Nrdp1 promotes parkin degradation and modulates parkin's activities on its substrates. We hypothesize that the regulation of interactions between parkin and Nrdp1 may affect the pathogenesis of PD. In this MQP, a yeast two hybrid approach was used to identify the domain(s) of Nrdp1 that binds parkin. Our long term goal is to design peptides against parkin-binding domain(s) in Nrdp1 so that interactions between these two proteins may be intervened. These peptides would have potential therapeutic uses.

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# BACKGROUND

## Parkinson's Disease, Introduction

### *Description and Prevalence in U.S.*

Parkinson's disease (PD) is a neurodegenerative disorder first described by Dr. James Parkinson in 1817 (Parkinson's Disease: Signs and Symptoms, 2005). He characterized the disease as shaky palsy. It wasn't until 1960, with the help of new technologies that changes in the brains of Parkinson's patients were observed (Parkinson's Disease: Signs and Symptoms, 2005). PD affects ~500,000 people in the United States alone, and is the most common movement disorder with about 1% of the population affected over the age of 65, which increases to 4% by age 85 (Giasson and Lee, 2001). PD is a progressive disorder that is characterized by slowed movement, loss of muscle control, and poor balance (*Parkinson's Disease, 2005*).

The cause of the disease is still not completely understood, but it is characterized by the loss of dopamine receptor-containing (dopaminergic) neurons in the *substantia nigra* and the accumulation of Lewy bodies (Zhang *et al.* 2000). The loss of dopamine receptors causes delayed reactions and shakiness. This is brought on by the deterioration of dopaminergic neurons in the substantia nigra. Lewy bodies are the abnormal clustering of proteins that form dense filamentous inclusions in the cytoplasm of the neurons (Giasson and Lee, 2001). Although the underlying cause of the PD is unknown, it has been determined through recent studies that both environmental and genetic factors contribute to the onset of the disease. Rotenone, for example, is used as a pesticide and can cause inhibition of ATP production by disrupting mitochondrial complex 1 (Betarbet

*et al.* 2000), which may destroy dopaminergic neurons. On the other hand, six genes have been recently identified to be directly involved in the pathogenesis of the disease.

### *Current Treatments*

Current treatment options for PD vary depending on the severity and stage of the disease. Early onset of the disease can be treated with a change in diet, exercise, and a combination of drugs to treat tremors. Moderate symptoms are treated with drugs aimed at supplementing the neurotransmitter dopamine to interact with the reduced number receptors in the patients. For long term treatment, dopamine is supplemented, and drugs aimed at increasing the half-life of dopamine in the body are administered (*Parkinson's Disease, 2005*).

## **Autosomal Recessive Juvenile Parkinson's**

### *Description and Prevalence*

Most of Parkinson's disease cases are sporadic. Less than 10% of PD have family history. There are two forms of familial PD, autosomal recessive and autosomal dominant. The form of Autosomal Recessive Juvenile Parkinson's disease (AR-JP) has an onset before age 40, but often earlier than age 20. AR-JP is a very rare form of Parkinson's disease, but the symptoms for AR-JP are typically similar to those of Parkinson's patients, although they have a better response to dopamine treatments. Patients with AR-JP develop neuronal loss as in PD, but do not accumulate Lewy bodies (*Parkinson's Disease, 2005*).

Mutations in the *parkin* gene (discussed below) have been identified to play crucial roles in the onset of AR-JP. Both alleles of the *parkin* gene are mutated in these

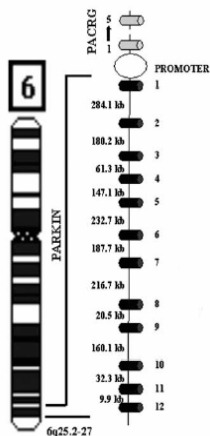
AR-JP patients. Mutant forms of the gene express truncated or inactive forms of the parkin protein that are responsible for about 50% of AR-JP patients with a family history (Krüger, 2004). These mutations are mostly classified by deletion of portions of the gene during transcription (Giasson and Lee, 2001). But missense or nonsense mutations have also been described (see below).

### **Parkinson's Disease, Pathology Mechanism**

*Parkin (discovery, size, structure, location, function)*

The gene parkin linked to AR-JP was first discovered in a Japanese patient (Kitada *et al.* 1998). By using a positional cloning strategy with exon trapping technology and cDNA library screening, Kitada *et al.*, were able to isolate the gene in which exons 3-7 were deleted. Subsequently, they identified a deletion of exon 4 in the same gene to 3 unrelated families with AR-JP, directly linking mutations in this gene to the pathology of the disease (Kitada *et al.* 1998).

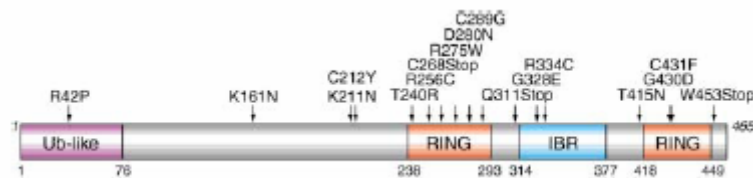
The *parkin* gene is located on chromosome 6 (see Figure 1) and spans 1.5 megabases, making it one of the largest genes in the human genome. It has a total of 12 exons that encode a 465 amino acid protein with a molecular mass of 52 kDa (Giasson and Lee, 2001). The amino acid sequence of parkin has a 62% homology to ubiquitin at the N-terminus (see Figure 2). The carboxy terminus of the protein contains two RING-finger motifs and an in-between RING finger (IBR) domain (see Figure 3) (Giasson and Lee, 2001).



**Figure 1:** The Chromosomal Location of the *parkin* Gene (Mata *et al.* 2004).

Parkin	1	MLVFRFNSSHGFPVEVDSITFQLKEVVAKRQGVHADQLRMIFAGKEL	50
UBIQUITIN HUMAN	1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGKILFDQCRLIFAGKCL	50
UBIQUITIN YEAST	1	MQIFVKTLTGKTITLEVEPSDTIDNVKSKIQDKEGKILFDQCRLIFAGKCL	50
UBIQUITIN SOYBEAN	1	MQIFVKTLTGKTITLEVEPSDTIDNVKAKIQDKEGKILFDQCRLIFAGKCL	50
Parkin	51	RNDWTWQNCDDLQCSIVHIVQRPWRK	76
UBIQUITIN HUMAN	51	EDGRTLSDYNIQKESTLHLVLRRLGG	76
UBIQUITIN YEAST	51	EDGRTLSDYNIQKESTLHLVLRRLGG	76
UBIQUITIN SOYBEAN	51	EDGRTLADYNIQKESTLHLVLRRLGG	76

**Figure 2:** Amino Acid Alignment of Parkin and Ubiquitin Proteins (Kitada *et al.* 1998).



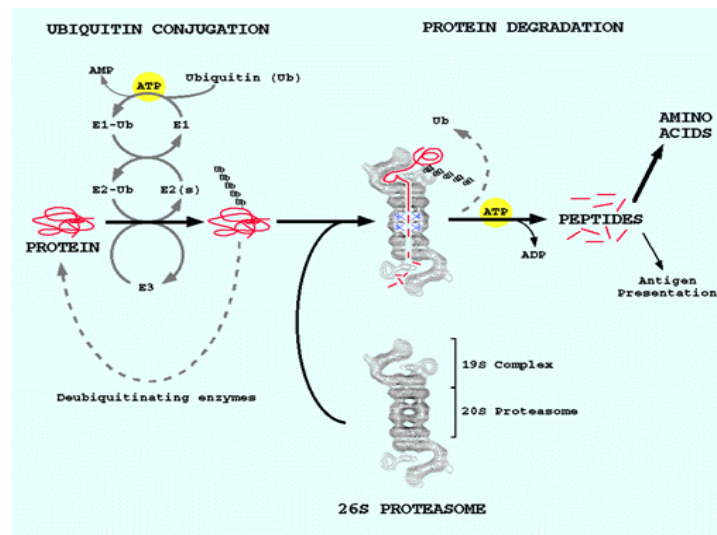
**Figure 3:** Representation of the Parkin protein (Giasson and Lee, 2001). Known parkin point mutations and domains are shown above.

### *E3 (discovery, size, structure, location, function)*

Proteins are degraded in the cell through a natural process called the Ubiquitin Proteasome Pathway (UPP). This pathway is important in the regulation of basic cellular processes, such as removal of misfolded, misfiled, mutated, or old proteins. Ubiquitin (Ub), a 76 amino acid protein, is the primary component in the UPP and is one of the most abundant proteins in the body. It functions as a tag protein, labeling proteins



targeted for degradation. Ub is covalently linked to the substrate by formation of an isopeptide bond between its C-terminus and the amino group of a lysine residue of the target proteins. A polyubiquitin chain is then formed on the protein through the ligation of additional monomers of Ub in successive rounds of ubiquitination. These Ub molecules are added to specific lysine residues on the nearest Ub of the elongating ubiquitin chain. Substrate proteins linked to polyubiquitin chains are recognized for degradation by the proteasome machinery (see Figure 4) (Iechanover, 2001).



**Figure 4:** The Ubiquitination Pathway for Protein Degradation (Goldberg, 2001).

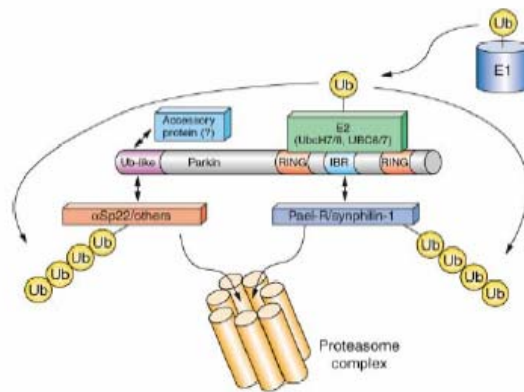
This protein degradation process involves a series of steps, where several enzymes are required to ensure specificity and to activate the Ub complex. The first step involves the ubiquitin-activating enzyme (E1), which activates ubiquitin by forming a linkage between the two. This activated ubiquitin is transferred to ubiquitin-conjugating enzyme (E2), a carrier protein, through the formation of a thiol ester linkage. Next, an ubiquitin-protein ligase (E3) recruits the substrate targeted for degradation, and ubiquitin is transferred to the substrate (see Figure 4). Finally, the proteasome recognizes the

polyubiquitinated substrate, which is degraded into small peptides, and the proteasome releases the reusable ubiquitin (Ciechanover, 2001).

### *Parkin is an E3 Ligase*

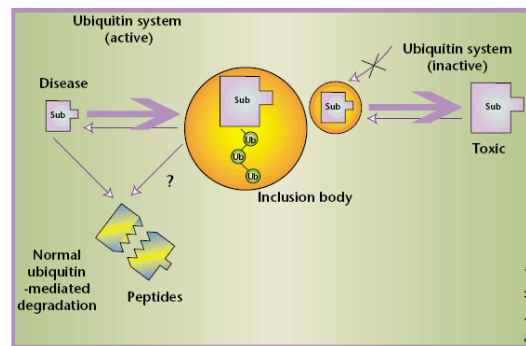
The parkin protein functions as an ubiquitin E3 ligase transferring ubiquitin from E2 to target proteins. It ubiquitinates at least 8 substrates that include  $\alpha$ -synuclein, Pael-R, CDC-rel and synphilin-1, which play a role in synapse formation. It has been proposed that mutations in the parkin protein reduces its ability to ubiquitinate these substrates, resulting in their accumulation and aggregation that eventually lead to the death of nigral neurons in PD patients (Finney *et al.* 2003).

Parkin possesses several distinguished domains, each of which has a specific role. The two typical RING finger domains and the IBR domain at its c-terminus interact with the E2. The N-terminal ubiquitin like domain (UBL) of parkin acts as a binding site for accessory proteins to regulate the levels of parkin expression and the binding of substrates (see Figure 5). The region between residues 77-313 in the parkin protein can be self-ubiquitinated, thus, regulates its own expression levels (Finney *et al.* 2003).



**Figure 5:** The Role of Parkin in the Ubiquitination Pathway (Giasson and Lee, 2001). In this diagram Parkin is represented by the horizontal multicolored cylinder.

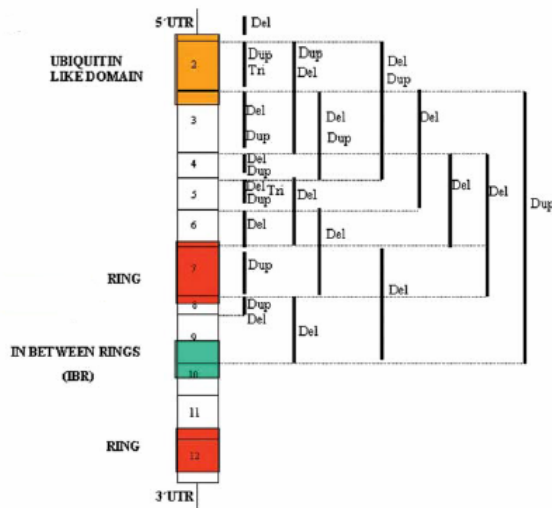
In a normal cell, polyubiquitinated proteins accumulate in inclusion bodies. The formation of inclusion bodies prevents the accumulation of untagged proteins in the cytosol which are toxic to the cell. Although the mechanism is unknown, once the proteins are inside the inclusion body, they are ubiquitinated. In AR-JP patients, when the parkin protein becomes mutated, the ubiquitin system becomes inactive and accumulation of proteins that normally would have been ubiquitinated in the Lewy body accumulate in the cytosol and cause cell death (see Figure 6) (Ciechanover, 2001).



**Figure 6:** Formation of Lewy Bodies in PD Patients (Ciechanover 2001).

### *Parkin Mutations*

Mutations in the *parkin* gene are the most common factor associated with AR-JP. Mutations have been found on both alleles in the majority of patients that cause a loss of function in the protein. Such mutations include deletions of exons, duplication of exons, insertions and deletions of several basepairs, mutations affecting splicing, and point mutations in the C-terminus and RING finger domains (see Figure 7) (Mata *et al.* 2004).



**Figure 7:** Parkin Protein Domain Structure and Known Deletions (Mata *et al.* 2004).

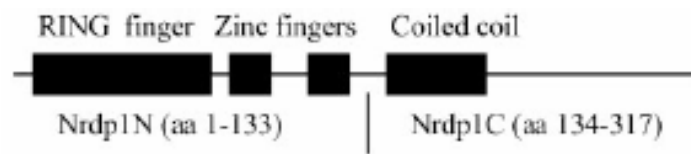
The most common mutation of the *parkin* gene is the deletion of one or several exons. Some deletions result in premature termination of translation, and frameshifts occur due to deletion of exon 3 and 4, or exon 5 resulting in the formation of truncated proteins (see Figure 7). Mutations affecting splicing commonly affect the splice site between exon 5 and 6, and exon 7 and 8. These mutations result in a truncated, altered parkin function (West *et al.* 2002).

Point mutations in the parkin DNA sequence result in amino acid substitutions (see Figure 3, locations shown above the map). These mutations are largely located in the C-terminus of the protein affecting the interaction between E2 and the two RING finger domains. This causes the accumulation of unfolded proteins, which further upregulates parkin mRNA. Overexpression of parkin normally prevents cell death induced by the accumulation of unfolded proteins. Imai *et al.* have shown that under conditions of endoplasmic reticulum (ER) stress caused by the accumulation of unfolded proteins, parkin mRNA and protein levels are upregulated preventing dopaminergic cell death. This shows the normal protective role of the parkin protein (Imai *et al.* 2000).

*Nrdp1 (discovery, size, structure, location, function)*

Neuregulin receptor degradation protein-1 (Nrdp1) was first characterized by Abdullah *et al* (2001). In order to understand the regulation of tyrosine kinases (TK's), Diamonti *et al* (2002) performed Yeast Two Hybrid screens to identify and characterize the proteins that bind TK's. They discovered Nrdp1. They concluded that one possible role of Nrdp1 is in the regulation of overall protein levels in the cell (Diamonti *et al.*2002).

The Nrdp1 gene is located on chromosome 12 and is 3.1 kilobases long. This gene has a protein coding sequence of 951 bps, encoding a 317 amino acid, 36 kDa protein. The protein contains a RING Finger, two Zinc fingers, and a Coiled coil domain (see Figure 8).



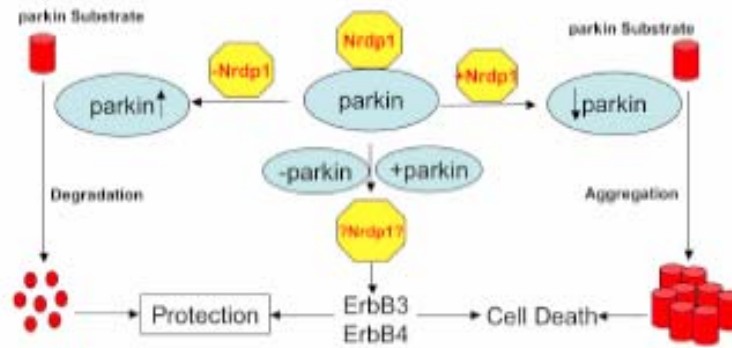
**Figure 8:** Illustration of Nrdp1 Protein Domains (Qiu-Goldberg 2002).

The N-terminus of Nrdp1 contains the RING finger and Zinc finger domains which catalyze the ubiquitination of substrates. The C-terminus contains the substrate binding domain which binds the substrates targeted for ubiquitination. Nrdp1 is expressed in a variety of tissues including the brain, heart, and muscle (Qiu and Goldberg, 2002).

Diamonti *et al* showed that Nrdp1 functions as an E3 ligase, promoting the degradation of several proteins including ErbB3, which is an epidermal growth factor receptor that functions in the regulation of cell growth and differentiation. Research done

by Zhong *et al.* (2005) has shown that Nrdp1 also interacts with parkin in the brain through a yeast two hybrid interaction. This suggests that Nrdp1 plays a role in ubiquitination and subsequent proteosomal degradation of parkin (Zhong *et al.* 2005).

Zhong *et al.* proposed a model for the role of Nrdp1 in the regulation of parkin (see Figure 9). They proposed that increased activity of Nrdp1 is associated with a reduction in cellular parkin levels. This reduction causes an accumulation of parkin substrates and thus resulting in cell death. Decreased activity of Nrdp1 stabilizes parkin and protects the cell.



**Figure 9:** Proposed Mechanism of Nrdp1 in Parkin Regulation (Zhong *et al.* 2005). In this model, overexpression of Nrdp1 (yellow) binds parkin leading to its degradation in the proteasome, and decreased cellular levels (right side), and abnormal aggregation.

## PROJECT PURPOSE

Mutations in the *parkin* gene are responsible for Autosomal Recessive Juvenile Parkinson's (AR-JP) disease. Wild type parkin functions as an E3 ligase in the ubiquitin pathway, which transfers ubiquitin from E2 to the target protein. When a mutation occurs in parkin, its function as an E3 ligase is impaired resulting in diminished proteasome degradation of its substrates. An accumulation of these substrates forms aggregates and subsequently causes cell death. Nrdp1 is an ubiquitin E3 ligase that interacts with the parkin protein. Nrdp1 significantly reduces parkin's levels in the cell, thus the regulation of interactions between parkin and Nrdp1 may affect the pathogenesis of PD.

The goal of this MQP is to investigate which portion of Nrdp1 binds to parkin. Identification of specific motif(s)/domain(s) that is responsible for association between parkin and Nrdp1 may provide important clues on how to design therapeutic peptides that can reduce Nrdp1 from binding and degrading parkin. These peptides will potentially prevent or slow the accumulation of parkin substrates. Therefore, an improvement on the pathogenesis of the disease would be possible.

To identify the domains in Nrdp1 that interacts with parkin, we used the yeast two hybrid assay. Our data indicates that the region containing domains two and three of Nrdp1 specifically interacts with parkin. More research is needed to precisely pinpoint a more defined region of interaction between the two proteins before a specific peptide can be designed.

## METHODS

### Primer Design, PCR, and Amplicon Purification

The cDNA sequence encoding human full length Nrdp1 was obtained from NCBI website accession number NM\_194359. Using the cDNA sequence, PCR primers were designed to amplify full-length, N-terminal, and C-terminal regions of Nrdp1 using program Primer Designer. The primers for amplifying the 549 bp N-terminal fragment are: 5'-CGGAATTCCGGGGTATGATGTAACCCGT-3' and 5'-CCCTCGAGGGGTTGACACTGCGGATTGC-3'. The primers for amplifying the 753 bp C-terminal fragment are: 5'-CGGAATTCCGGTACCTCGGATCATGCGG-3' and 5'-CCCTCGAGGGATTTATCTCTTCCACGCC-3'. The primers for amplifying the 951 bp full length Nrdp1 cDNA are: 5'-CGGAATTCCGGGGTATGATGTAACCCGT-3' and 5'-CCCTCGAGGGATTTATCTCTTCCACGCC-3'. EcoRI and XhoI restriction sites were added to the sense and antisense primers, respectively, to facilitate subsequent subcloning. PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 2 min, and then 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 minutes.

PCR products were extracted with phenol:chloroform and then precipitated at -20°C overnight after adding 1/10 volume of 3M of Sodium Acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Precipitated PCR products were double digested with EcoRI and XhoI for 2 hrs at 37°C. The digested PCR products were then separated on a 2.0% agarose gel run in the presence of ethidium bromide. The fragments with the correct



sizes were purified from 2.0% agarose gels using a gel-extract kit purchased from Qiagen.

### **Preparation of Competent *E. coli* for Transformation**

*E. coli*, strain DH5 $\alpha$ , was plated on a LB plate and was grown overnight at 37°C. Two to three colonies were inoculated into 2 mL of LB media and grown overnight at 37°C. This culture was transferred into 200 mL of LB medium and shaken at 37°C until OD<sub>600</sub>=0.40. After incubation, the 200 mL culture was spun down at 6000 rpm for 5 minutes and the supernatant was discarded completely. The cell pellets were vortexed in 8 mL of an ice cold, sterile solution of 0.1 M RbCl, 30 mM KAc, 50 mM MnCl<sub>2</sub>, and 50% (v/v) glycerol (TF1 buffer). The cells were put on ice for one hour. Cells were centrifuged at 6000 rpm for 5 minutes, the supernatant was completely removed, and cells were resuspended in a 8 mL sterile solution of 10 mM RbCl, 75 mM CaCl<sub>2</sub>, 15% (v/v) glycerol, and 10 mM MOPS (TF2 buffer). The solution was put on ice for at least 15 minutes, and 100  $\mu$ L were aliquoted into cold eppendorf tubes. Tubes were immediately frozen on dry ice and were stored at -70°C.

### **Generation of Constructs for the Yeast Two Hybrid Assays**

Full length Nrdp1 cDNA, N-terminal, and C-terminal digested and purified PCR products were ligated into EcoRI/XhoI digested yeast expression plasmid pGADT7 (Clontech) overnight at 16°C. Ligation products were transformed into competent DH5 $\alpha$  *E. coli* that were prepared by a rubidium chloride protocol (see above) and plated on LB plates supplemented with 100  $\mu$ g/ml ampicillin. Full-length Parkin cDNA in pGBKT7 in *E. coli* was kindly provided by J. Zhou.

### **Screening of Positive Subclones**

Individual ampicillin-resistant *E. coli* colonies were inoculated into 2 mL of LB with 50 µg/ml of ampicillin and grown overnight at 37°C. Plasmid DNAs were purified from cultures using the Qiagen miniprep procedure. The purified plasmids were digested with EcoRI and XhoI, and separated on 1% agarose gels. The plasmid DNAs that contained inserts with the correct sizes were sequenced.

### **The Yeast Two Hybrid Assay**

The Matchmaker Yeast Two-Hybrid System was purchased from CLONTECH. The Y190 lacZ/HIS3 yeast reporter strain was streaked on YPD plates and incubated at 30°C for 4 days. 3-4 colonies of Y190 were transferred into 1 mL of YPD media and vortexed to disperse any clumps. The 1 mL was transferred into 70 mL of YPD media and incubated at 30°C for 16-18 hours at 250 rpm to achieve stationary phase ( $OD_{600} > 1.5$ ). Approximately 50 mL of this overnight culture was transferred into 150 mL of fresh YPD media to reach an  $OD_{600} = 0.2-0.3$  and was incubated at 30°C at 230 rpm until  $OD_{600} = 0.40-0.60$ . Cells were harvested by centrifugation at 1000 x g at room temperature for 5 minutes. The supernatant was discarded, and cells were resuspended in 25 mL of sterile H<sub>2</sub>O. Resuspended cells were centrifuged again at 1000 x g at room temperature for 5 minutes. The supernatant was decanted and cells were resuspended in 1.5 mL of freshly prepared, sterile 1X TE/LiAc.

To test the Yeast Two-Hybrid System, control plasmids, pCL1, pGADT7-T, pGBKT7-53, pGBKT7-Lam, provided by Clontech were used. Three mixtures were performed: pCL1 and pGBKT7-53, pGADT7-T and pGBKT7-53, and pGADT7-T and pGBKT7-Lam. To test the interaction between parkin and Nrdp1, 0.1 µg of purified bait

plasmid (pGBKT7) containing the Gal4 DNA binding domain, 0.1 µg of prey plasmid (pGADT7) containing the Gal4 activation domain, and 0.1 mg of herring testes carrier DNA were mixed with 100 µL of the yeast cell suspension. Eight mixtures were performed: pGBKT7-parkin cDNA and pGADT7-Nrdp1 N-terminus, pGBKT7-parkin cDNA and pGADT7-Nrdp1 C-terminus, pGBKT7-parkin cDNA and pGADT7-full length Nrdp1, pGBKT7-parkin cDNA and pGADT7 (no insert), pGBKT7 (no insert) and pGADT7 (no insert), pGBKT7 (no insert) and pGADT7-Nrdp1 N-terminus, pGBKT7 (no insert) and pGADT7-Nrdp1 C-terminus, and pGBKT7 (no insert) and pGADT7-full length Nrdp1.

600 µL of 40% PEG/LiAc solution was added to each tube, mixed and incubated at 30°C for 30 minutes at 200 rpm. After incubation, 70 µL of DMSO was added to each tube and gently inverted to mix. Tubes were heat shocked for 15 minutes in a 42°C water bath and then chilled on ice for 2 minutes. Cells were pelleted by centrifugation for 5 seconds at 14,000 rpm. Supernatant was removed from each tube, and transformed cells were resuspended in 0.5 mL of 1X TE buffer. Transformed cells were plated on SD –Leu/-Trp media and grown for 4 days for initial selection.

For the colony lift filter β-galactosidase assay, transformed colonies were replated on a new SD –Leu/-Trp plate. A Whatman #3 filter was presoaked in Z-buffer/X-gal solution. A dry Whatman #3 filter was placed over one set of plates to be assayed. The filter was transferred into a pool of liquid nitrogen for 10 seconds. This filter was then placed on the presoaked filter and incubated at 30°C for 30 minutes to 2 hours for the appearance of blue colonies. Interaction between bait and prey proteins (i.e.

parkin and Nrdp1) restores Gal4 function, resulting in expression of the  $\beta$ -galactosidase reporter gene, and the appearance of blue colonies.

For the  $\alpha$ -galactosidase assay, transformed colonies were plated on an SD –Leu/-Trp plate with 100  $\mu$ L of  $\alpha$ -galactosidase. Plates were incubated at 30°C for 4 days. Plates were observed for a blue/white color change. Interaction between bait and prey proteins (i.e. parkin and Nrdp1) restores Gal4 function, resulting in expression of the  $\alpha$ -galactosidase reporter gene, and the appearance of blue colonies.

## RESULTS

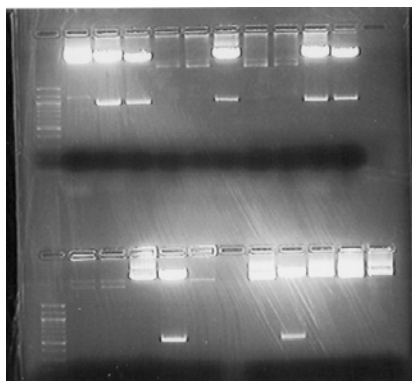
The aim of the project is to identify which region of Nrdp1 binds to parkin. If we can identify this sequence of Nrdp1, it is possible to produce a therapeutic peptide to reduce Nrdp1 from binding and degrading parkin. This potentially will prevent or slow the accumulation of substrates and cause an improvement on the pathogenesis of the disease. To identify the location of the interaction between parkin and Nrdp1, we used the yeast two hybrid screen.

### **Successful Ligation of Nrdp1 PCR Products into Plasmid pGADT7**

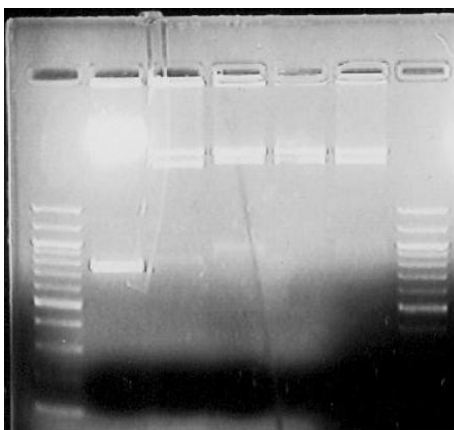
Three kinds of Nrdp1 inserts (full-length, N-terminus, and C-terminus) were amplified from an Nrdp1-containing plasmid by PCR using primers containing EcoRI and XhoI sites at their termini. The amplicons were double digested to produce EcoRI and XhoI termini, and ligated into EcoRI/XhoI cut yeast expression plasmid pGADT7.

### **Selection of Transformed *E. coli* Positives**

In order to confirm the effective subcloning of pGADT7 recombinants in *E. coli*, we transformed *E. coli* with the three ligated plasmids and plated them on 100 µg/ml ampicillin plates. Individual colonies were grown, and plasmids were purified from cultures using the Qiagen miniprep procedure. Figure 10a shows digested pGADT7 containing full-length and the N-terminus of Nrdp1. Verification of the cloning of the C-terminus is shown in figure 10b. Since the band with the correct insert was released from the plasmid, this confirms an accurate subcloning of the three constructs.



**Figure 10a:** TOP portion of gel: Dropout of full length Nrdp1 from digested pGADT7 of subcloned *E. coli* colonies. BOTTOM: Dropout of the N-terminus of Nrdp1 from digested pGADT7 of subcloned *E. coli* colonies.

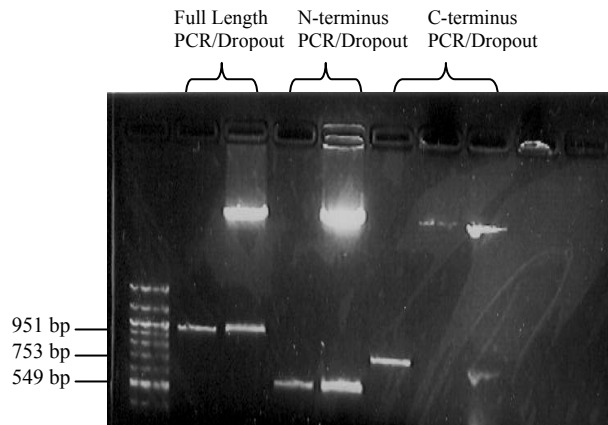


**Figure 10b:** Drop out analysis of the C-terminus of Nrdp1 from digested pGADT7 of cloned *E. coli* colonies.

## Validation of Constructs

To confirm the successful construction of the three yeast expression plasmids, we double-digested the recombinant pGADT7 plasmids with restriction enzymes EcoRI and XhoI for dropout analysis. The original PCR-amplified Nrdp1 fragments, along side the double digested pGADT7, are shown in Figure 11. The N-terminus fragment of Nrdp1, 549 bp in length, contains the ring finger domain and the two zinc finger domains, whereas the C-terminus fragment of Nrdp1, 753 bps in length, contains the two zinc finger domains and the coiled coil domain (see previous Figure 8). Since the PCR fragment corresponded to the dropout in each instance, we were able to conclude that the correct fragment was successfully ligated into the each expression vector. For further

clarification, minipreps of plasmids containing the three Nrdp1 inserts were prepared and sent for sequencing. The data, provided in Appendix A, proves that the desired insert was properly ligated into each plasmid.



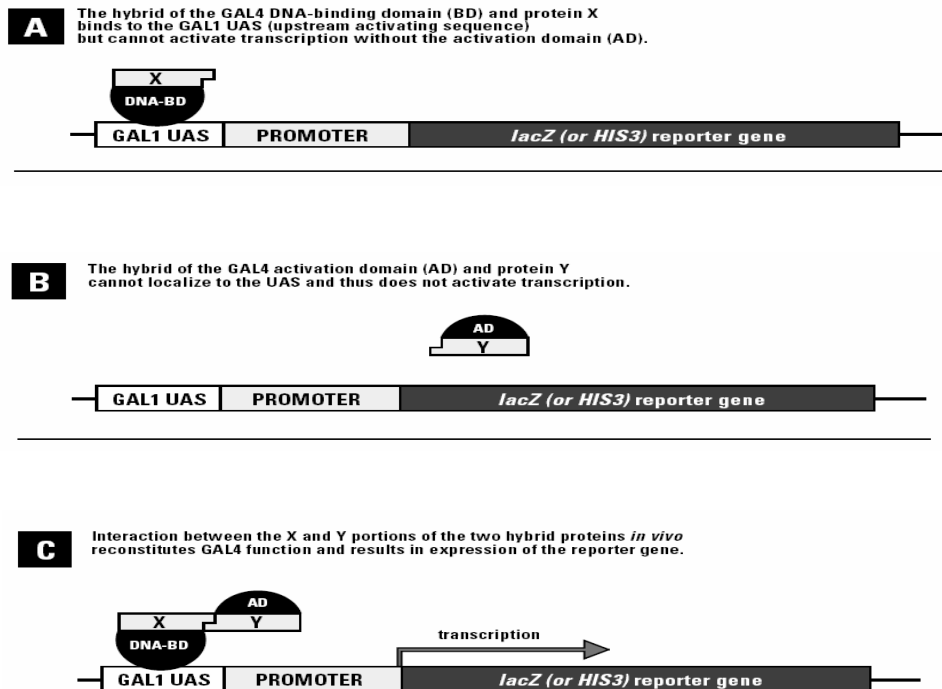
**Figure 11.** Agarose gel demonstrating proper ligation of Nrdp1 PCR fragments into the pGADT7 plasmid. pGADT7 recombinants were double digested with EcoRI and XhoI restriction enzymes. Equal masses of PCR fragment and digested pGADT7 were run on a 2% agarose gel and stained with ethidium bromide.

## Yeast Two Hybrid System

The principle of the yeast two hybrid system is to utilize a growth selection to determine protein-protein interactions. Eukaryotic DNA transcription factors are made of both a DNA binding domain (DNA-BD) and activation domain (AD). The yeast GAL4 transcriptional activator contains both of these domains. The DNA-BD recognizes and binds to the upstream activating sequence of the gene, while the AD interacts with the DNA-BD and other transcription factors to initiate transcription. These two domains are required to activate transcription of the gene (see Figure 12).

In the yeast two hybrid system, two different vectors are used to generate separate fusions of the GAL4 domains to genes encoding proteins that potentially interact. pGADT7 and pGBKT7 encode for the AD and DNA BD, respectively. The two fusion proteins are co-expressed in yeast. If the two non-GAL4 portions of the fusion proteins

interact, the DNA-BD and AD will combine and the GAL4 transcriptional activator will be functional to transcribe the reporter genes (see Figure 12).



**Figure 12:** Schematic of the Yeast Two Hybrid System (Clontech).

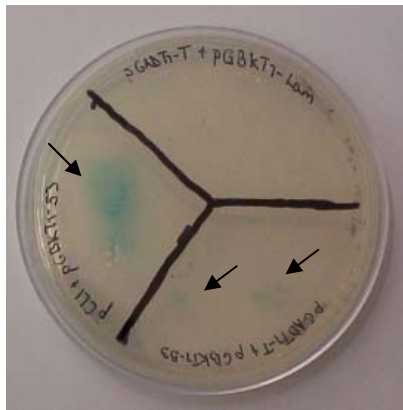
The two plasmids, pGADT7 and pGBKT7, are cotransformed into the yeast strain Y190. Since pGADT7 and pGBKT7 contain selection markers for leucine and tryptophan, respectively, transformants are plated on synthetic dropout (SD) media lacking the amino acids leucine and tryptophan to select for colonies that contain both plasmids. The GAL4 responsive genes consist of MEL1, lacZ, ADE2, and HIS3 reporter genes. Expression of the MEL1 reporter gene is positive for  $\alpha$ -galactosidase activity. Expression of the lacZ reporter gene is positive for  $\beta$ -galactosidase activity, whereas expression of the HIS3 and ADE2 reporter genes does not require addition of histidine or adenine in the media to grow.



### *Utilization of the Yeast Two Hybrid System*

To demonstrate a mastery of the yeast two hybrid assay, the controls provided by Clontech were tested. Three control mixtures, pCL1 and pGBKT7-53, pGADT7-T and pGBKT7-53, and pGADT7-T and pGBKT7-Lam, were co-transformed into yeast. pCL1 provides a positive control for  $\alpha$  and  $\beta$ -galactosidase assays because it encodes the full-length GAL4 protein. pGBKT7-53 and pGADT7-T also provide positive control since they encode fusion proteins between the GAL4 DNA-BD and AD, and murine p53 and SV40 large T-antigen, respectively. Murine p53 and SV40 T-antigen are known to interact strongly. pGBKT7-Lam provides a negative control since it encodes a fusion of DNA-BD with human lamin C, which does not interact with either murine p53 or SV40 T-antigen.

The transformed yeast were plated on a SD –Leu/-Trp plate to select for transformants containing both plasmids. Positive transformants were transferred to a fresh SD –Leu/-Trp plate containing an X-gal solution. As seen in Figure 13, cotransformation of the controls yielded both positive and negative colonies for a  $\alpha$  – galactosidase assay. The blue colonies represent cleavage of the X-gal substrate leaving a water insoluble blue product.

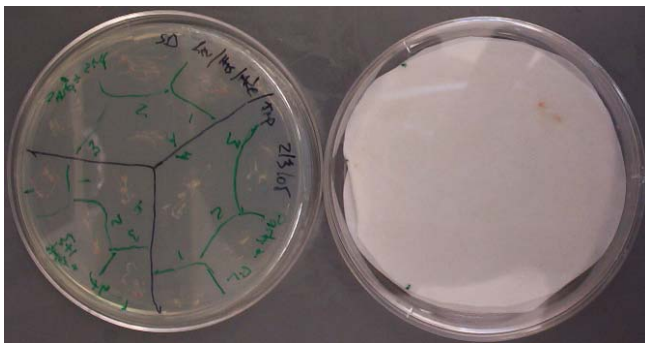


**Figure 13:** Control Plate for Testing the Yeast Two Hybrid System. The arrows denote the formation of blue color for positive colonies.

### *Nrdp1/Parkin Protein-Protein Interactions*

To investigate the binding regions between parkin and Nrdp1, we cotransformed the three constructs, full length, N-terminus, and C-terminus of Nrdp1, into yeast strain Y190. To select for transformants containing both plasmids, yeast were plated on a SD – Leu/-Trp plate. Positive double-transformants were then transferred onto a SD -Leu/-Trp/-Ade/-His plate to select for a positive interaction between parkin and Nrdp1. As seen on the left plate of Figure 14, yeast colonies grew in all three sections (containing: parkin and full length Nrdp1, parkin and N-terminus, and parkin and C-terminus) for this selective medium. From this we can infer that interacting region between parkin and Nrdp1 is most likely located in the overlapping region of the two N-terminal and C-terminal fragments containing zinc finger domains two and three (see Figure 8 in Background).

Illustrated in the right plate of Figure 14 is a colony lift filter  $\beta$ -galactosidase assay. Although yeast colonies grew on the SD -Leu/-Trp/-Ade/-His plate indicating an interaction between the two proteins, no colonies turned blue on the  $\beta$ -galactosidase assay. This indicates a possible weak interaction between the two proteins.



**Figure 14:** Protein-protein interaction between parkin and full-length, N-terminus, and C-terminus of Nrdp1. LEFT: Growth of double plasmid-transformed yeast colonies on a SD -Leu/-Trp/-Ade/-His plate showing growth for all three Nrdp1 constructs tested. This signifies an interaction between parkin and domains two and three of Nrdp1. RIGHT:  $\beta$ -galactosidase assay of yeast colonies on a SD -Leu/-Trp/-Ade/-His plate, showing weak blue staining. This implies a weak interaction between the two proteins.

## DISCUSSION

Autosomal Recessive Juvenile Parkinson's disease (AR-JP) is caused by a mutation in the *parkin* gene. The parkin protein functions as an E3 ligase in the ubiquitin pathway, and loss of parkin function results in cell death. Nrdp1 is a protein that interacts with parkin reducing its stability, activity, and its levels in the cell. Regulating the interactions between parkin and Nrdp1 may thus affect the pathogenesis of AR-JP.

In this project, yeast expression plasmid constructs were successfully generated in pGADT7 containing the full-length, N-terminus, and C-terminus of Nrdp1. The plasmids were transformed into yeast, and tested using the yeast two hybrid system for interactions with parkin. The data demonstrated the interaction between parkin and Nrdp1, and this interaction was effectively narrowed to the region of Nrdp1 containing zinc finger domains two and three (see Fig 8 in Background) since only those domains are common to all three fragments tested. Subsequent to the onset of this project, Zhong *et. al.* (2005) illustrated through westerns, co-immunoprecipitation, and the yeast two hybrid system an interaction between parkin and Nrdp1. Our  $\beta$ -galactosidase assay did not provide blue colonies, so it is reasonable to presume that the interaction between the two proteins is weak, but colonies were observed on plate screenings with appropriate media selections for the interaction.

The identified region of Nrdp1 that interacts with parkin contains two zinc fingers. Little is known on how zinc finger domains regulate protein-protein interactions, but some evidence exists that such domains facilitate the interaction with zinc finger domains in other proteins. This finding has potential for research to examine a further understanding of the mechanism involved. Future experiments would include narrowing

down the interacting region further to possibly a few amino acids. This would include testing whether domain two or three, or domain two and three are necessary for the protein-protein interaction between parkin and Nrdp1. This would lead researchers closer to producing a therapeutic peptide to interfere with Nrdp1 from binding and degrading parkin, and cause an improvement on the pathogenesis of the disease. Efforts have been made to begin this process.

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# APPENDIX A

## A.1 Sequencing data for full length Nrdp1 construct

File: 050408-08\_A15\_Nrdp1-FL-3AD-sequencing.ab1 Run Ended: 2005/4/9 13:11:53 Signal G:500 A:247 C:370 T:334  
Sample: Nrdp1-FL-3AD-sequencing Lane: 64 Base spacing: 14.42 885 bases in 10612 scans Page 1 of 2



## A.2 Sequencing data for N-terminus Nrdp1 construct

File: 050408-08\_I13\_Nrdp1-N-terminus-3AD-sequencing.ab1 Run Ended: 2005/4/9 13:11:53 Signal G:813 A:396 C:640 T:647  
Sample: Nrdp1-N-terminus\_3AD-sequencing Lane: 55 Base spacing: 14.219999 901 bases in 10791 scans Page 1 of 2





### A.3 Sequencing data for C-terminus Nrdp1 construct

File: 050408-08\_M13\_Nrdp1-C-terminus-3AD-sequencing.ab1 Run Ended: 2005/4/9 13:11:53 Signal G:1054 A:533 C:906 T:1006  
Sample: Nrdp1-C-terminus\_3AD-sequencing Lane: 51 Base spacing: 14.42 886 bases in 10613 scans Page 1 of 2

