Role of Zinc in Parkin RING2 E3 Ubiquitin Ligase Ubiquitination

By

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Role of Zinc in Parkin RING2 E3 Ubiquitin Ligase Ubiquitination

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Abstract

In common neurodegenerative diseases among older adults, Parkinson's disease (PD) ranks the second after Alzheimer's disease. Symptoms of Parkinson's disease are shaking, slowness of movement, rigidity of the extremities and neck, minimal facial expressions, short-walking steps, stopped posture, and arm swinging. Autosomal-Recessive Juvenile Parkinsonism (AR-JP) is the most frequent cause of familial PD, and results from mutations in the *Parkin* gene. The parkin protein acts as an E3 ubiquitin ligase and plays an important role in the ubiquitin proteasome system (UPS). Parkin's role is to identify and tag proteins that are damaged or no longer needed. Mutations in Parkin represent ~50% of disease-causing defects in AR-JP. It is believed that the E3 ubiquitin ligase activity of the parkin protein may play a protective role in neurodegenerative disorders including Parkinson's, Huntington's, and Alzheimer's diseases. Parkin belongs to a class of multiple RING (**R**eally **I**nteresting **N**ew **G**ene) domain proteins designated as RBR (**R**ING, in **b**etween RING, **R**ING) proteins. RING domains are rich in cysteine amino acids that act as ligands for binding zinc ions. Recent observations of the RING proteins suggest that binding of zinc ions may aid in maintaining their stability and solubility, thereby regulating their function.

The main goal of work described in this thesis was to test the hypothesis that a "zinc switch" regulates the catalytic activity of an isolated RING2 domain of parkin which is responsible for the ubiquitinating activity of parkin. Using a protein construct containing the isolated RING2 domain of parkin, I show, using a combination of centrifugation and autoubiquitination assays, that the catalytic activity of the RING2 domain is strongly inhibited by small amounts of zinc ions in solution. The results support the idea that reversible binding of zinc to a low affinity site on the RING2 domain of parkin regulates the

E3 ligase activity of parkin.

Keywords:

Parkinson’s disease, Parkin, zinc binding, ubiquitination, E3 ubiquitin ligase

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To all the past and present the members of the Richter lab, I really appreciate your support, assistance, and advice during my graduate studies. I could not have done this without all of you, and it was an honor learning so much from many of you and discussing and working together. Especially to Dr. Carolyn Rankin, I would like to show my appreciation for all of her assistance.

This work would not have been possible without the unwavering support and love of my family: my wife Jiwon and my son Brayden. Your encouragement has meant the world to me. Thank you.

Last but not least, I would like to thank my parents. Their support and love have always allowed me to push myself to the fullest, and I would like to express my sincere appreciation to them for believing in me.

Soli Deo Gloria!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

PD	Parkinson's Disease
AR-JP	Autosomal-Recessive Juvenile Parkinsonism
UPS	Ubiquitin Proteasome System
RING	Really Interesting New Gene
HECT	Homologous with E6-associated protein C-Terminus
UbL	Ubiquitin Like domain
IBR	In Between Ring
HSQC	Heteronuclear Single Quantum Coherence
PSD	Post Synaptic Density
Zn-T	Zinc Transporter
siRNA	**S**mall **i**nterfering RNA
IPTG	Isopropylthiogalactoside
TCEP	*Tris*(2-carboxyethyl)phosphine
DLS	Dynamic Light Scattering

Chapter 1. INTRODUCTION

1.1. Parkinson's Disease.

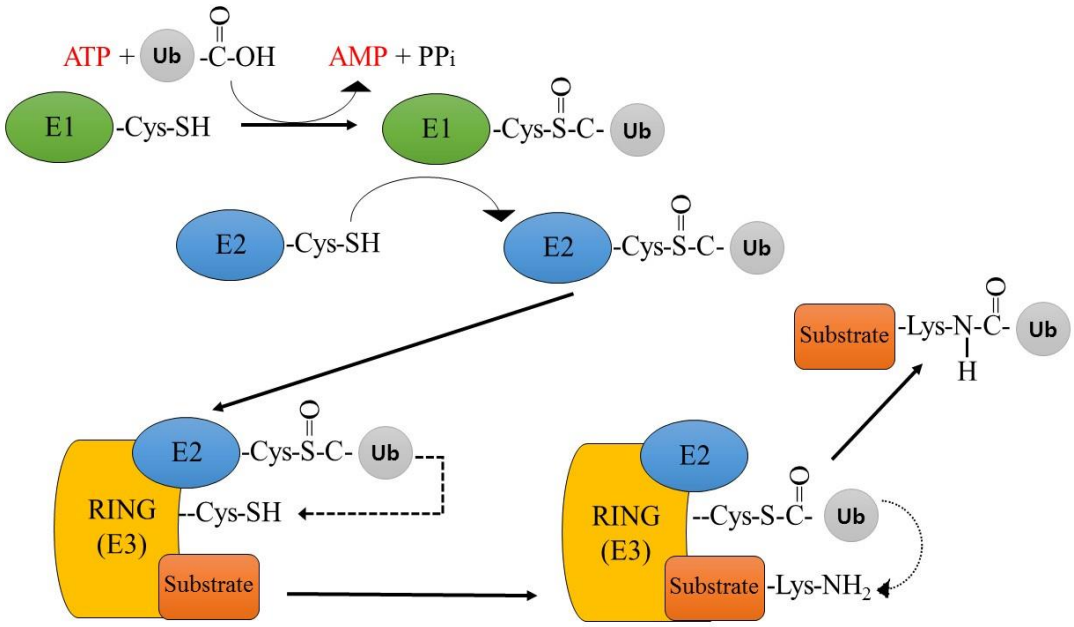
In common neurodegenerative diseases among older adults, Parkinson's disease (PD) ranks the second after Alzheimer's disease. Shaking, slowness of movement, rigidity of the extremities and neck, minimal facial expressions, short-walking steps, stopped posture, and arm swinging are symptoms of Parkinson's disease. These defects in motor function result from the loss of dopaminergic neurons in the substantia nigra. [1] Autosomal-Recessive Juvenile Parkinsonism (AR-JP) is the most frequent cause of familial PD, and results from mutations in the *Parkin* gene. [2][3] A neuropathological hallmark of idiopathic PD is the observation of cytoplasmic insoluble protein aggregates (Lewy bodies) containing α-synuclein. *PARK1*, *PARK4*, and *SNCA* genes encode α-synuclein. [4][5][6]

The *Parkin, or PARK2,* gene that encodes the parkin protein on chromosome 6 (6q25.2-q27) is greater than 500 kb. A 465 amino acid protein with a molecular weight of 51,652 Daltons is translated from the 4.5 kb *parkin* transcript with a 1395 base pair (bp) open reading frame. Parkin is widely expressed in the brain, skeletal muscle, heart, stomach, thyroid, and spinal chord. Mutations in the parkin gene can cause AR-JP wherein the mutated parkin can no longer fulfill its role in the ubiquitin proteasome system (UPS). [2][7][8][9]

1.2. Ubiquitin Proteasome System and Parkin RING2 E3 ligase.

It is the ubiquitin proteasome system (UPS) that is the most crucial pathway for selective protein degradation, and problems of UPS have been reported to occur during neurodegeneration. Ubiquitination has two general functions: (1) the first is to direct the

targeted destruction of tagged proteins by the 26S proteasome (2) the second function is to modulate protein activities, such as subcellular localization, and interfering with protein-protein interactions. [10] Ubiquitin is a 76 amino acids protein with molecular mass of 8.5 kDa. When ubiquitin is conjugated to an internal lysine of another ubiquitin molecule, a polyubiquitin chain is formed with an isopeptide bond. [11] There are three enzymes required for protein ubiquitination through a sequential process; E1, E2, and E3. Ubiquitin is first activated by an ubiquitin-activating enzyme, E1, which leads to a high-energy thioester bond between the E1 active-site cysteine and the carboxyl group of the ubiquitin. [12] Second, ubiquitin is transferred to an E2, ubiquitin-conjugating enzyme, by a transthioesterification reaction. Then, in the hybrid RING/HECT mechanism of Parkin RING2, ubiquitin is transferred to the Cys431 in RING2, and from there to the substrate. This sequential cascade results in ubiquitination of the target protein. **(Figure 1.2.1)** [13]



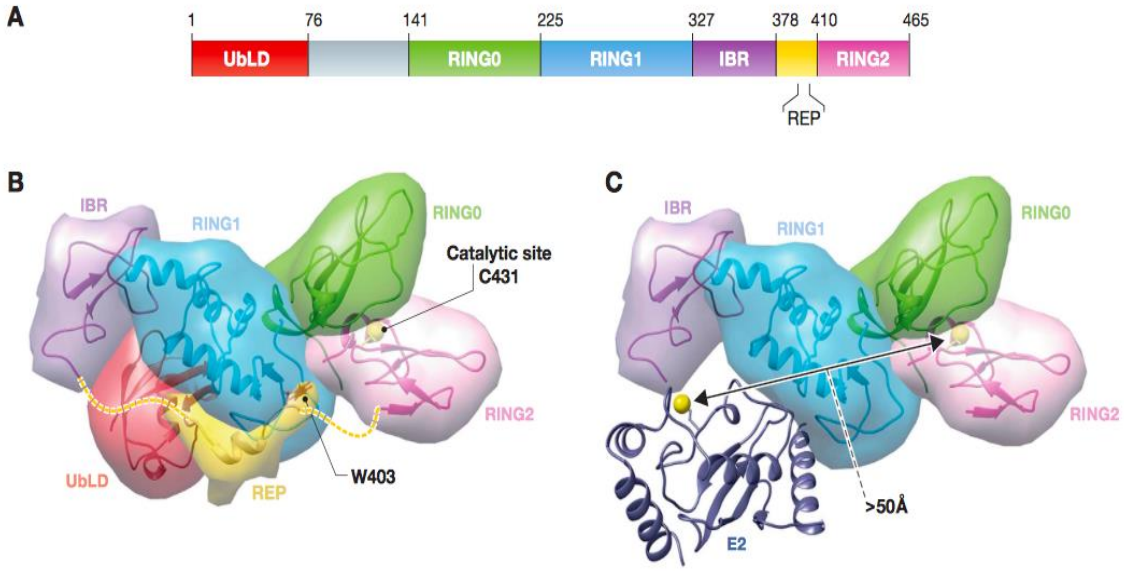
(Figure 1.2.1) The hybrid RING/HECT mechanism of parkin RING2
Ubiquitin is activated by the E1 in an ATP-dependent manner. Subsequently, the ubiquitin is transferred to the E2 through a transthioester reaction. Ubiquitin conjugated E2 interacts with an E3 ligase in order for the ubiquitin to attach to the lysine of the substrate.
E1 (ubiquitin-activating enzyme), E2 (ubiquitin conjugating enzyme), E3 (ubiquitin ligase)

Monoubiquitination is involved in protein sorting, endocytosis, DNA damage response, and epigenetics. [14][15][16][17] Polyubiquitination is involved in proteasomal degradation of aberrant proteins and short-lived proteins such as transcription factor and tumor suppressor P53. [18]

There are three classes of E3 ubiquitin ligase: Really Interesting New Gene (RING), U-box, and Homologous with E6-associated protein C-Terminus (HECT) domain E3 ligase. Parkin is a member of the RBR family of E3s. This subclass of E3 ligases is autoinhibited until activated. [19][20][21][22][23] A recent study indicates that they are hybrid E3s with an E2 binding site in RING1 and a catalytic cysteine residue, C431 in the case of parkin, in the RING2 domain which is responsible for ubiquitin binding. [24] [25]

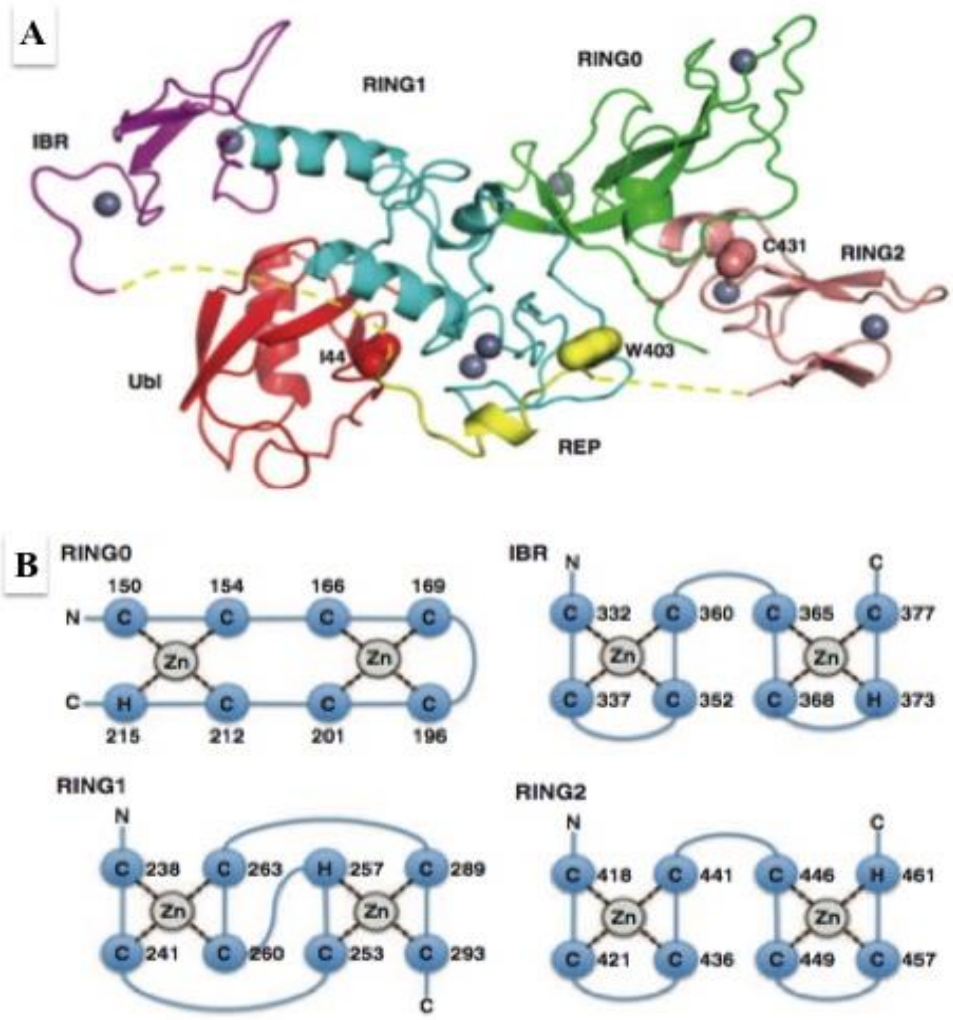
Parkin has five structural domains: Ubiquitin Like domain (UbL), RING0, RING1, In Between Ring (IBR), and RING2. **(Figure 1.2.2 (A))** Each domain (except UbL) binds two zinc (Zn^{2+}) ions. **(Figure 1.2.3 (A), (B))** The UbL domain is involved in a number of protein-protein interactions. For instance, parkin UbL binds to a proteasomal ubiquitin receptor Rpn13/ADRM1. [26] RING0 plays an important role in maintaining parkin in an inactive state. RING0 shares an interface with RING2 and buries C431, rendering it unavailable as a ubiquitin acceptor. **(Figure 1.2.2 (B))** RING0 must be displaced for ubiquitin transfer to occur. Hence, it is deletion of RING0 that leads to an increase in parkin C431 reactivity and autoubiquitination. [20][22][23] As previously mentioned, RING1 has sites for binding two Zn^{2+} ions as well as an E2 binding site. [9] **(Figure 1.2.2 (C))** The IBR domain is situated between the RING1 and RING2 domains, as determined by NMR spectroscopy. It contains two zinc ions, and is proposed to play an important role in stabilizing the overall structure and orientation of the RING domains within the parkin protein. [27] In the full-length parkin assembly, the IBR domain was proposed to act like a bridge that connects the

RING1 and RING0 domains together. [23][28] REP is the repressor element of parkin, which is part of a linker. It includes a two-turn helix that controls the E2 binding face of RING1 **(Figure 1.2.2)**.



(Figure 1.2.2) Schematic and spatial representation of parkin
(A) Primary structures and domains of parkin. (B) Structural representation of full-length parkin. (C) A model of parkin with the E2 UbcH5B/Ube2D2 bound. [9]

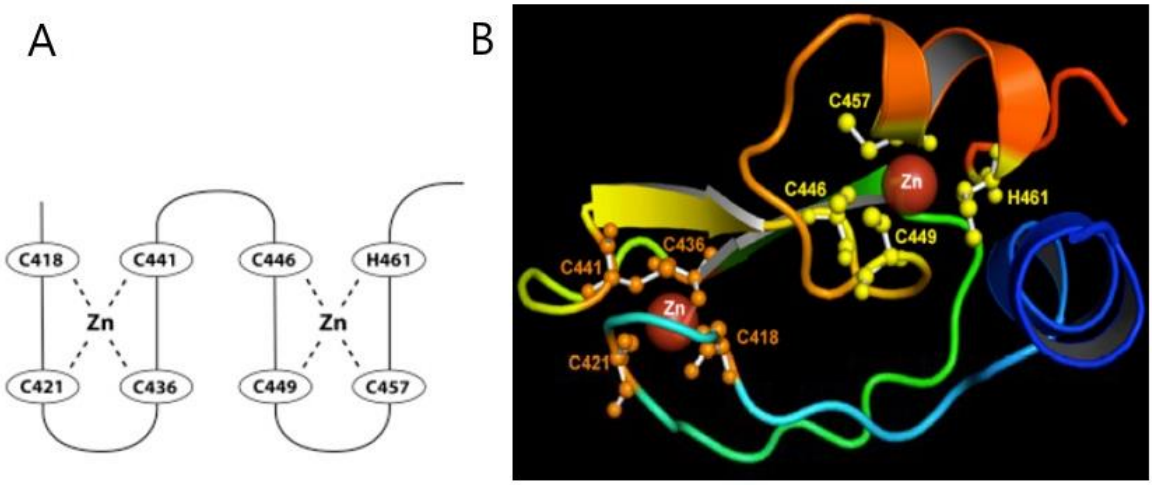
A recent study indicates that the RING2 domain has a similar overall topology to that of the IBR domain that also contains two Zn^{2+} ions. [29][30] Two of the zinc coordinating residues in RING2 (Cys457 and His461) are well conserved in parkin. [23] **(Figure 1.2.3 (B))** The catalytic cysteine, C431, acts as an acceptor for an ubiquitin from bound E2-ubiquitin forming an intermediate E3-ubiquitin which is subsequently transferred to substrate or parkin itself (autoubiquitination). [24] **(Figure 1.2.2 (B), (C))**



(Figure 1.2.3) Full-length structure of parkin
(A) Cartoon representation of the full-length structure. (B) Topology of zinc finger domains in parkin. [23]

As previously mentioned, parkin is an octuple coordinating zinc-binding protein. RING0, RING1, IBR, and RING2 domains each bind two Zn^{2+} ions, and removal of zinc from parkin leads to loss of structure of the protein. [63] In short, zinc binding is required for proper parkin folding. Based on recent studies, C253Y, C289G, and C431F mutations have been shown to decrease parkin E3 ligase activity, and would lead to alter Zn^{2+} binding, which indicates that zinc binding is important for protein function. Particularly, by EDTA addition to the HHARI RING2 domain or the IBR domain, complete unfolding of these domains occurs due to removal of Zn^{2+} ions. [64][27] Note that among the RBR family member HHARI, the

RING2 domain has been known to bind only a single Zn^{2+} ion unlike classical RINGs. Interestingly, the addition of zinc in excess of a 1:1 ratio eventually results in precipitation of the RING2 domain based on ^{15}N-label **H**eteronuclear **S**ingle **Q**uantum **C**oherence (HSQC) experiments with $ZnCl_2$ titration. [64] However, this contrasts with experiments related to instability of parkin C-terminal deletion and chimeric parkin-HHARI proteins: deletion of the three C-terminal residues of parkin RING2 caused the loss of parkin solubility, suggesting that there may be more than one zinc binding to RING2. In addition, parkin can bind a total eight zinc ions; two each for RING0, RING1, IBR, and presumably two by RING2. [27][65][66] To explain this difference, Rankin *et al.* [30] suggested a model that parkin RING2 uses a different set of ligands to accommodate two zinc ions; C418, C421, C436, and C441 coordinate to the first zinc ion while C446, C449, C457, and H461 coordinate to a second zinc ion; Zn site I (4 Cys ligands) and Zn site II (3 Cys and 1 His). **(Figure 1.2.4)** In addition, a recent study indicates that two of zinc coordinating residues in RING2, Cys457 and His461 are indeed involved in binding a second zinc atom in RING2. [23]



(Figure 1.2.4) Newly predicted structural model
(A) Newly predicted structural model: two zinc ions may be bound by RING2. (C) Newly predicted model (cartoon) for the RING2 domain with ligand residues in the zinc binding sites; C418, C421, C436, C441: Zn site I / C446, C449, C457, H461: Zn site II. [30]

1.3. Metal Hypothesis

Considering the relationship between zinc ions and parkin RING structure described in the previous section, zinc and other metals may be an important element that affects protein solubility and activity. Imbalance of metal ions, especially zinc and copper, is thought to play a crucial role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. [31] In the brain, maintenance of zinc and copper homeostasis is very important, and dyshomeostasis of these ions leads to the pathogenic mechanisms of neurodegenerative diseases including Parkinson's disease. [32][33][34][35] Zinc-containing neurons are mainly localized in the limbic and the cortex structures of the brain, so zinc is thought to play an essential role in behavior and emotion. The divalent cation Zn^{2+} is the second most prevalent trace element in the body. For normal functioning of the nervous system, zinc ions are important; sufficient stores are present in synaptic vesicles of glutamatergic neurons, ranging from 10 μM to 100 μM. [36][37]

In Alzheimer's disease (AD), zinc interacts with Abeta (Aβ) which is accumulated inside amyloid plaques. There are suggestions that abnormal interactions with metal ions like zinc and copper can cause Aβ precipitation and toxicity in AD. [38] For example, Zn^{2+} ions induce aggregation of Aβ at pH 7.4 *in vitro*, and this reaction is reversible with chelation [39][40] while Cu^{2+} ions are more effective in conditions representing physiological acidosis (pH 6.6-6.8). [41] To be specific, zinc inhibits γ-secretase activity, which is involved in the generation of Aβ. Besides, Zn^{2+} protects proteolytic cleavage sites on Aβ and thus inhibits the degradation of Aβ with the participation of metalloproteases. [32] In addition, Zn^{2+} induces aggregation of tau-protein and formation of intracellular neurofibrillary tangles, which results in problems of the neuronal transporting system and of cognitive functions. [42][43] Thus,

the imbalance of zinc could be a risk factor of AD development.

In Parkinson's disease (PD), the imbalance of zinc concentration is also implicated in pathogenesis. For instance, an increased level of zinc was found in cerebrospinal fluid and blood serum of patients with PD. [44] Accumulation of cytosolic zinc can be an indication of the degeneration of dopaminergic neurons based on modeling PD *in vivo* and *in vitro* by treatment with MPTP or 6-OHDA. [45] Based on many recent studies, highly increased amount of cytosolic Zn^{2+} ions is common in degenerating neurons of Parkinson's disease, epilepsy, and ischemia. [45][46][47]

Normally, zinc concentration is high at synapses, and bound zinc maintains the organization of the postsynaptic density (PSD). [48][49] During neuronal stimulation, Zn^{2+} concentrations may exceed 100 μM in the synaptic cleft. [50] An influx of zinc ions through postsynaptic glutamate receptors would profoundly affect functions and structures of the PSD. [51] In PSD, zinc transporters have been suggested to play a key and protective role in reducing cellular Zn^{2+} toxicity and in maintaining Zn^{2+} homeostasis. Among the Zinc Transporter (Zn-T) family members, only the ZnT-1 is found in the plasma membrane. [52][53] Interestingly, a recent study indicates that ZnT-1 is the first zinc transporter protein shown to concentrate at the PSD. The expression of ZnT-1 is highest in the cerebral cortex and cerebellum, moderate in the substantia nigra and hippocampus, and lowest in the striatum. **(Table 1.3.1)** [54][55] In these regions, Zn^{2+} ions are released during neuronal activity and are primarily localized to glutamatergic terminals. [56][57] Because a high concentration of Zn^{2+} ions has toxicity, ZnT-1 is strongly needed for zinc homeostasis in neurons although there are metallothioneins that reduce short-term heavy metal toxicity by binding metal ions. [58] According to the ZnT-1 **s**mall **i**nterfering RNA (siRNA) experiment in 2006, an approximately twofold increase in Zn^{2+} ion influx occurred due to a 70% decrease in ZnT-1

protein expression, when ZnT-1 siRNA oligo was transfected. [59]

TABLE 1. Distribution of ZnT-1-Positive Neurons in Various Brain Regions[1]

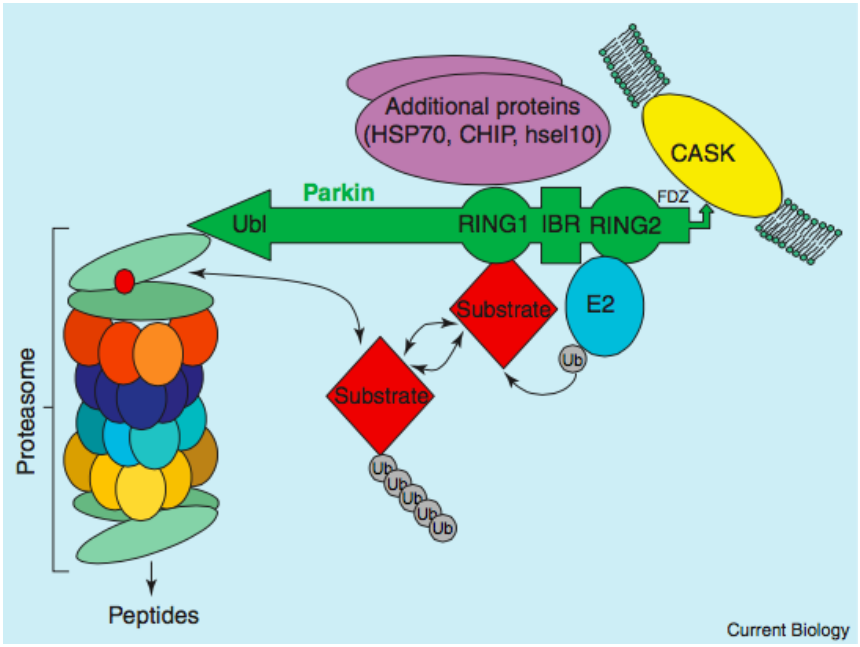
Region	Expression
Telencephalon	
Cortex	
Neocortex	
I	–
II/III	+++
IV	+
V	+++
VI	++
Pyriform	++
Cingulate	+++
Subiculum (dorsal)	+++
Olfactory bulb	
Mitral cell layer	+++
Granule cell layer	+
Glomerular cell layer	++
External plaxiform cell layer	–
Hippocampus	
CA1	++
CA2	++
CA3	+++
Dentate gyrus	
Hilus	+
Granule cells	++
Amygdala	+++[2]
Basal ganglia	
Caudate putamen	++
Globus pallidus	++
Diencephalon	
Thalamus	++
Hypothalamus	
Arcuate	++
Paraventricular	++
Supraoptic	–
Suprachiasmatic	–
Mesencephalon	
Substantia nigra	++
Red nucleus	++
Ventral tegmental area	–
Lateral geniculate nucleus	++
Met- and mylencephalon	
Reticular formation	++
A5 noradrenergic nucleus	++
Cranial nerve nuclei	
Occulomotor	++
Facial	+
Cerebellum	
Cerebellar cortex	
Purkinje cells	++
Granule cells	+
Dentate, interpositus nucleus	+++

[1]–, none; +, <33% of highest expression; ++, <66%; +++, <100%.
[2]Medial amygdyloid nucleus.

(Table 1.3.1) Distribution of ZnT-1-positive neurons in various brain regions [54]

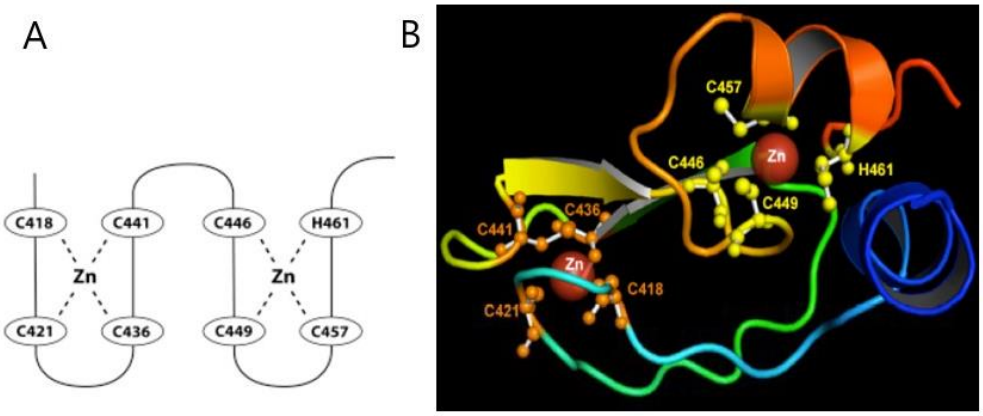
Typically, parkin is localized in the cytoplasm and shows E3 ubiquitin ligase activity. Parkin is also found in synapses and has a generally neuroprotective effect in dopaminergic neurons. For example, parkin binds to endofilina-A in the trafficking of synaptic vesicles.

[60][61] Especially, parkin co-localizes with CASK at synapses in cultured cortical neurons, PSD, and lipid rafts in the brain. **(Figure 1.3.1)** [62] Therefore, when considering the localization of parkin, the importance of Zn-T, and zinc homeostasis in PSD, parkin may be strongly affected by zinc. In other words, if Zn-T has a problem with its function, zinc concentration may increase in PSD, which may affect parkin structure and its E3 ubiquitin ligase activity.



(Figure 1.3.1) Interaction between parkin and CASK membrane protein in neurons [61]

As previously mentioned in the last section, Rankin *et al.* [30] suggested a model that parkin RING2 uses a different set of ligands to accommodate two zinc ions; C418, C421, C436, and C441 coordinate to the first zinc ion while C446, C449, C457, and H461 coordinate to a second zinc ion; Zn site I (4 Cys ligands) and Zn site II (3 Cys and 1 His). **(Figure 1.3.2)**



(Figure 1.3.2) Newly predicted structural model
(A) Newly predicted structural model: two zinc ions may be bound by RING2. (C) Newly predicted model (cartoon) for the RING2 domain with ligand residues in the zinc binding sites; C418, C421, C436, C441: Zn site I / C446, C449, C457, H461: Zn site II. [30]

Based on the above information, a model has arisen in which there may be a transition between bound and unbound Zn^{2+} ions at one of the two zinc binding sites of the RING2, promoting a change in local structure that acts as an allosteric switch affecting the function of the parkin-protein association. [23][30] Therefore, with regard to zinc binding sites, the concentration of zinc, and ratios of zinc within the RING2, zinc may play a pivotal role in the RING2 structure and its activity. Despite the potential importance of zinc binding to the RING2 domain of parkin, the direct effects of different concentrations of zinc on the RING2 activity have not been identified.

In 2013, we published a method of preparing a recombinant protein construct containing the isolated RING2 domain of parkin coupled to the solubility tag, GB1. [68]. Using this construct it was shown that the isolated RING2 domain of parkin is sufficient for E2-dependent E3 ligase activity. It was also shown that one of the two zinc atoms associated with this domain is labile and comes off during desalting; the RING2 with only one zinc bound is fully active for autoubiquitination. It was also observed in that study that high concentrations of $ZnSO_4$ induce RING2 to aggregate. [68]. The aggregating effect of zinc

on RING proteins has also been noted elsewhere. [23][30][64] The fact that the RING2 domain is active with just a single bound zinc ion supports the idea that zinc may act as an allosteric switch that regulates the catalytic activity of the E3 ligase activity of parkin. [23][30][64][68] Here we will use the GB1-RING2 as a model system to study the effects of zinc.

Consequently, the purpose of this thesis is to identify any role of zinc in parkin RING2 E3 ubiquitin ligase ubiquitination based on the new model. My hypothesis is that different ratios of zinc in the RING2 may affect its ubiquitination activity. In particular I hypothesize that binding of zinc to the second, lower affinity zinc binding site on parkin RING2, inhibits the ubiquitination activity.

Chapter 2 MATERIALS AND METHODS

2.1. Protein Expression and Purification.

A linear DNA construct comprised of an N-terminal 6His tag, a GB1 solubilization tag, a TEV protease cleavage site followed by the gene encoding the RING2 domain of Parkin was subcloned into a pET42c vector as described previously. [68] Briefly, one liter of LB containing 1 ml/liter of kanamycin (stock solution, 30 mgs/ml in water) was inoculated with overnight cultures of 50 mL LB. When the OD_{600} was 0.4, the 1 L cultures were incubated (37 °C, 225 rpm). 200 μM of Isopropylthiogalactoside (IPTG) and 100 μM of $ZnSO_4$ were added. Then, protein production was induced for 3 h. The OD_{600} was approximately 1.35 at cell harvest. The cells were pelleted at 4,243 x g for 15 min in Beckman JA-10 rotor, and then the brownish-red colored pellets were weighed and stored at -80 °C until lysis.

Pellets were thawed on ice, and then diluted into lysis buffer containing 50 mM Tris-HCl, pH 8.0 and 500 mM NaCl) (10 mL of lysis buffer per gram of cells) Protease inhibitors (bestatin HCl at a final concentration of 1 μM, leupeptin HCl at a final concentration of 5 μg/mL, and 1,10-phenanthroline at a final concentration of 1 mM) were added to the lysis buffer before use. After dilution of the cells into the lysis buffer, lysozyme (stock solution, 10 mg/mL in 50 mM Tris-HCl, pH 8.0) was added (100 μL/g of cells), then Benzonase was added at 0.5 μL/g of cells. Cells were incubated at room temperature for 30 min. Cell suspensions were sonicated for 12 min using a Branson Digital Sonifier (102C(CE) tip, parameters: 27% amplitude, 2sec. on and 10 sec off). Cells were kept on ice during sonication. Cells were centrifuged at 16,000 x g (Beckman rotor JA20) for 15 min to pellet inclusion

bodies and debris. After determining the protein concentration of the supernatant, glycerol was added to 20% (v/v) and the solution stored at -80ºC until further use.

The GB1-RING2 protein was purified with Qiagen Super Flo NTA resin (Qiagen, Valencia, CA) using a column (1.5cm x 20cm) equilibrated with 25 mM Tris-HCl (pH 8.0). The supernatant was loaded onto the column, and washed with two column volumes of 25 mM Tris-HCl (pH 8.0). The column was washed again with 25 mM Tris-HCl (pH 8.0) containing 10 mM imidazole. Protein was eluted with 300 mM imidazole in 25 mM Tris-HCl (pH 8.0), collected in 2 mL fractions. Protein concentrations were measured by the Bradford method. [67] Prior to storage at -80 °C, glycerol was added to pooled fractions to 20% (v/v).

To remove the imidazole remaining after nickel column purification, the protein was desalted by use of a G25 Sephadex spin column equilibrated with buffer containing 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 250 μM *tris*(2-carboxyethyl)phosphine (TCEP).

2.2. Titration of GB1-RING2 with metal ions.

For high concentration of Zn and GB1-RING2 ratio experiment, protein was added to different concentrations of $ZnSO_4$ in buffer (50 mM Tris-HCl (pH 7.0) and 120 mM NaCl. Zinc and the isolated RING2 protein ratios were from 1 to 7. The concentration of 1:1 ratio was Zinc (54 μM) and GB1-RING2 (54 μM). 2:1 ratio was Zinc (108 μM) and GB1-RING2 (54 μM). 3:1 ratio was Zinc (162 μM) and GB1-RING2 (54 μM). 4:1 ratio was Zinc (216 μM) and GB1-RING2 (54 μM). 5:1 ratio was Zinc (270 μM) and GB1-RING2 (54 μM). 6:1 ratio was Zinc (324 μM) and GB1-RING2 (54 μM). 7:1 ratio was Zinc (378 μM) and GB1-RING2 (54 μM). The total volume was 50 μl

including GB1-RING2. After adding GB1-RING2 into the buffer, each tube was kept for an hour at room temperature. Tubes were then centrifuged at 21,130 x g (Eppendorf centrifuge 5424) for 15 min to compare the protein concentration at the start to the protein concentration of the supernatant at end for measuring the amount that precipitates due to aggregation. Samples were dissolved in PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 1 M urea, and 0.08% (w/v) bromophenol blue) and then applied to 12% (w/w) Bis-Tris gels for fractionation.

For low concentration of Zn and GB1-RING2 ratio experiment, protein was added to different concentrations of $ZnSO_4$ in buffer (50 mM Tris-HCl (pH 7.5) and 120 mM NaCl). The concentration ratio was also from 1 to 7. The concentration of 1:1 ratio was Zn (5.4 μM) and GB1-RING2 (5.4 μM). 2:1 ratio was Zn (10.8 μM) and GB1-RING2 5.4 μM. 3:1 ratio was Zn (16.2 μM) and GB1-RING2 (5.4 μM). 4:1 ratio was Zn (21.6 μM) and GB1-RING2 (5.4 μM). 5:1 ratio was Zn (27 μM) and GB1-RING2 (5.4 μM). 6:1 ratio was Zn (32.4 μM) and GB1-RING2 (5.4 μM). 7:1 ratio was Zn (37.8 μM) and GB1-RING2 (5.4 μM). The total volume was 50 μl including GB1-RING2. After adding GB1-RING2 into the buffer, each tube was kept for an hour at room temperature. Tubes were then centrifuged at 21,130 x g (Eppendorf centrifuge 5424) for 15 min to compare the protein concentration at the start to the protein concentration of the supernatant at end for measuring the amount that precipitates due to aggregation. Samples were dissolved in PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 1 M urea, and 0.08% (w/v)

bromophenol blue) and then applied to 12% (w/w) Bis-Tris gels for fractionation.

For high concentration of $CuSO_4$ and GB1-RING2 ratio experiment, protein was added to different concentrations of $CuSO_4$ in buffer (50 mM Tris-HCl (pH 7.0) and 120 mM NaCl). Cu and the protein concentration ratios were 2:1, 4:1, and 6:1. The concentration of 2:1 ratio was Cu (76 μM) and GB1-RING2 (38 μM). 4:1 ratio was Cu (152 μM) and GB1-RING2 (38 μM). 6:1 ratio was Cu (228 μM) and GB1-RING2 (38 μM). The total volume was 25 μl including GB1-RING2. After adding GB1-RING2 into the buffer, each tube was kept for an hour at room temperature. Tubes were then centrifuged at 21,130 x g (Eppendorf centrifuge 5424) for 15 min to compare the protein concentration at the start to the protein concentration of the supernatant at end for measuring the amount that precipitates due to aggregation.

2.3. Ubiquitination Reactions.

After titration of GB1-RING2 with different zinc ratios (0:1, 3:1, 6:1, 17:1), ubiquitination assays were performed. *In vitro* reactions contained 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1.39 μM UbcH7 (E2), 22 nM UBE1 (E1), 4 mM ATP, 5 μM FLAG-tagged ubiquitin, and GB1-RING2 protein from the zinc titration. Reaction mixtures were incubated for 90 minutes at 37 °C, 5-10 μl of sample was saved every 10 minutes for SDS-PAGE. Samples were dissolved in SDS sample buffer (25 mM Tris-HCl (pH 6.8), 0.8% (w/v) SDS, 3.5% (v/v) glycerol, 1 M urea, 2% (v/v) β-mercaptoethanol, and 0.08% (w/v) bromophenol blue) and then applied to 12% (w/w) Bis-Tris gels for fractionation. The ubiquitination level was measured by quantitation of the

amount of protein present in the major ubiquitinated band by densitometry using ImageQuant TL software, ImageJ, and Photoshop.

Chapter 3. RESULTS AND DISCUSSION

The goal of the work described in this chapter was to define conditions in which we can test the potential role of zinc as an allosteric switch that regulates the catalytic activity of the E3 ligase activity of parkin. To this end, a novel construct described previously (Rankin et al.) that contains only the catalytic RING2 domain of parkin linked to a solubility tag (GB1) was utilized. [68]

3.1. Purification of GB1-RING2.

The pET42c plasmid containing the GB1-RING2 construct was transformed into *E.coli* BL21 cells for expression and the protein over-expressed as described in Chapter 2. After the cell lysate was centrifuged, proteins were purified with Qiagen Super Flo NTA resin (Qiagen, Valencia, CA). Figure 3.1 shows an SDS-PAGE gel of cell extracts at different stages of purification.

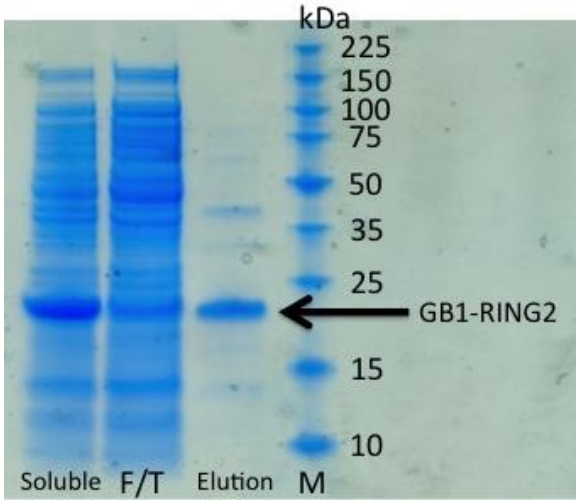


Figure 3.1. Analysis of purified recombinant proteins

The proteins were fractionated by SDS-PAGE. The black arrow indicates GB1-RING2. M, protein marker; Soluble, soluble proteins before Ni-NTA purification; F/T, flow through solution during Ni-NTA purification; Elution, eluted GB1-RING2 protein (18.4 kDa)

3.2. High concentrations of Zn^{2+} effect GB1-RING2 aggregation.

In our initial study of the effects of zinc on the activity and aggregation state of parkin RING2, 1.0 mg/ml of the purified GB1-RING2 construct was titrated with zinc sulfate up to a seven to one molar ratio of zinc to protein. Following the addition of zinc sulfate and incubation for 1 hour, the protein solution was centrifuged to remove insoluble aggregates and the concentration of protein remaining in the supernatant was measured using the Bradford assay. The range of zinc concentrations used was between 54 μM (1:1 zinc to protein ratio) to a final concentration of 378 μM (7:1 zinc to protein ratio). Under these conditions, the concentration of protein in the supernatant decreased substantially and in proportion to the amount of zinc added (Fig. 3.2). The figure3.2 shows that concentration of GB1-RING2 decreased as zinc concentration increased. The data clearly show that zinc strongly induced GB1-RING2 aggregation.

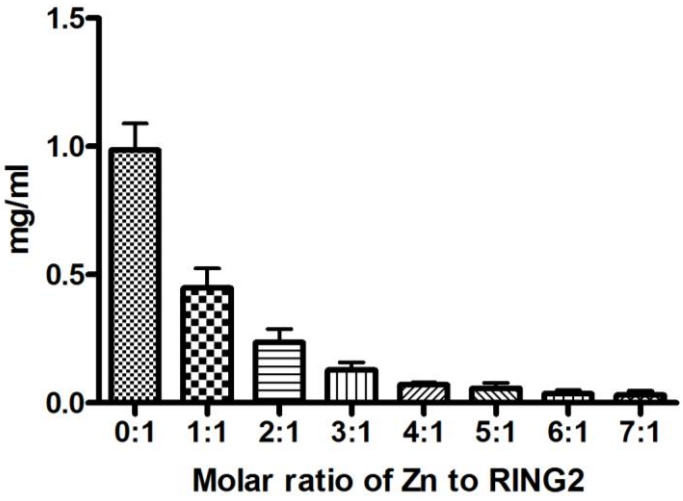


Figure 3.2. High concentration of zinc and GB1-RING2

X-axis: Zinc ratios from 1 (54 μM) to 7 (378 μM). 0:1 is no adding zinc with GB1-RING2 for control. Note that 1:1 ratio is 54 μM of zinc and 54 μM of GB1-RING2. **Y-axis**: concentration of GB1-RING2. GB1-RING2 concentration for control (0:1) was 1.0 mgs/ml.

3.3. Micromolar concentrations of Cu^{2+} also induce GB1-RING2 aggregation.

To examine whether the effect of zinc on RING2 aggregation was specific to zinc or due to a non-specific effect of metal ions, the experiment was repeated with copper in place of zinc. The results are shown in Figure 3.3. In this experiment the concentration of the GB1-RING2 construct used was 0.7 mg/ml with the $CuSO_4$ concentration ranging between 76 μM (2:1 Cu^{2+} to protein ratio) and 228 μM (Cu^{2+} to protein ratio of 6:1). At a ratio of copper to protein of 4:1 or greater, significant amounts of protein aggregation also occurred suggesting that the effect of metal ions on aggregation of the GB1-RING2 construct may not be specific. It is well known that divalent cations such as Cu^{2+} promote the oxidation of thiols to form disulfide bonds. Since the RING2 construct is rich in cysteine residues, the divalent ions may be promoting thiol cross-linking and protein aggregation. If this is the case, using lower concentrations of zinc ions may avoid aggregation while still promoting occupancy of the zinc binding sites on RING2.

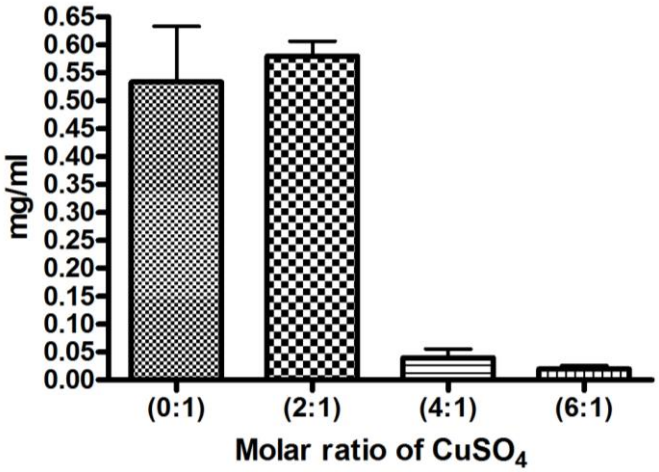


Figure 3.3. High concentration of copper and GB1-RING2

X-axis: Cu^{2+} ratios from 2 (76 μM) to 6 (228 μM). 0:1 is no adding Cu^{2+} with GB1-RING2 for control. Note that 2:1 ratio is 76 μM of Cu^{2+} and 38 μM of GB1-RING2. **Y-axis**: concentration of GB1-RING2. GB1-RING2 concentration was 0.7 mgs/ml.

3.4. Low concentrations of Zn^{2+} do not cause GB1-RING2 aggregation.

To check the effect of lower concentrations of zinc, lower concentrations of the GB1-RING2 construct were titrated with similar ratios of zinc ions. For example, in Figure 3.4, 0.5 mg/ml of GB1-RING2 was used for zinc titration and significant aggregation was not evident until zinc-protein ratios reached 4:1 or greater.

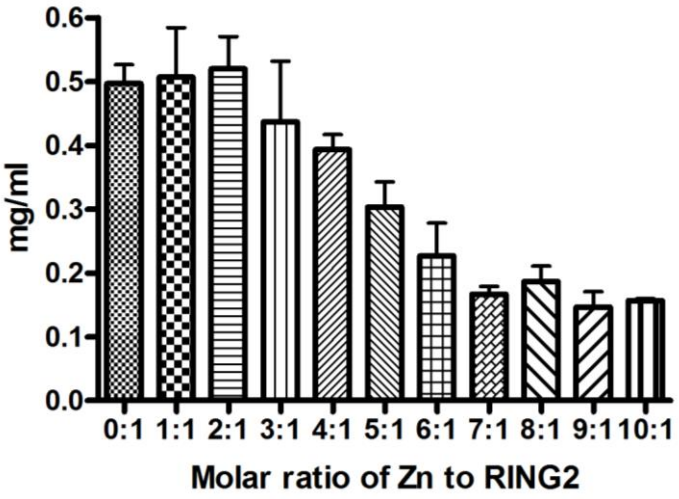


Figure 3.4. Lower concentration of zinc and GB1-RING2

X-axis: Zinc ratios from 1 (27 μM) to 10 (270 μM). 0:1 is no adding zinc with GB1-RING2 for control. Note that 1:1 ratio is 27 μM of zinc and 27 μM of GB1-RING2. Y-axis: concentration of GB1-RING2. GB1-RING2 concentration for control (0:1) was 0.5 mgs/ml.

The effect of lowering the protein concentration and therefore the total zinc ion concentration on protein aggregation was even more evident when the GB1-RING2 concentration was lowered to 0.1 mg/mL (Fig.3.5). In this case the zinc concentration varied between 5.4 μM (1:1) to a final concentration of 37.8 μM (7:1) and under these conditions there was no visible aggregation of the protein. The results were mirrored in the SDS gel shown in Figure 3.6 which shows that the concentration of the protein remains constant up to a ratio of 7 to 1 zinc to protein concentration.

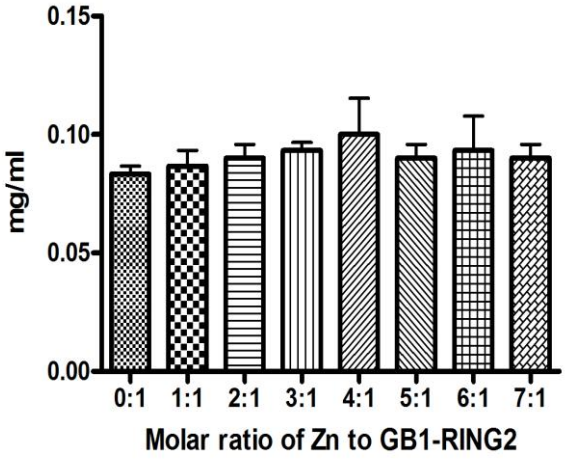


Figure 3.5. Low concentration of zinc and GB1-RING2

X-axis: Zinc ratios from 1 (5.4 μM) to 7 (37.8 μM). 0:1 is no added zinc with GB1-RING2 for control. Note that 1:1 ratio is 5.4 μM of zinc and 5.4 μM of GB1-RING2. **Y-axis**: concentration of GB1-RING2. GB1-RING2 concentration was 0.1 mgs/ml.

After zinc titration with low concentration, protein PAGE was performed. **(Fig. 3.6)** 18.4 kDa of GB1-RING2 band did not decrease as zinc concentration increased. Thus if experiments are performed at protein concentrations of 0.1 mg/mL or less and $ZnSO_4$ concentrations of ~40 μM or less, formation of large aggregates of the protein construct can be avoided. In addition, comparison of samples (from 1:1 to 6:1 ratios) prepared with different concentrations of $ZnSO_4$ by Dynamic Light Scattering (DLS) indicated that $ZnSO_4$ also did not induce formation of smaller aggregates in solution under these conditions, but further investigation is needed. (Data not shown).

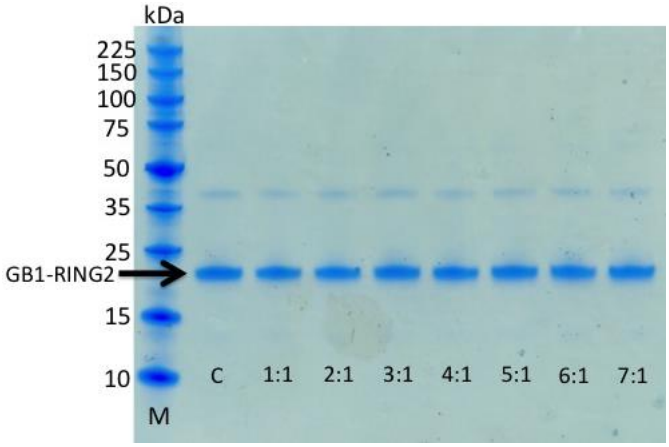


Figure 3.6. PAGE gel of low concentration of zinc titration with GB1-RING2 (Non-reducing gel) (**M**: protein marker, **C**: control without adding zinc)

3.5. The effect of zinc on parkin RING2 E3 ubiquitination ligase activity.

Having established conditions under which addition of $ZnSO_4$ does not cause significant aggregation of the protein, the effect of added zinc on the ubiquitination activity of RING2 was examined. In these experiments zinc was added to the RING2 constructs at different ratios (0:1, 3:1, 6:1, 17:1) and samples were assayed for their ubiquitination activity. The results are shown in Figures 3.7 and 3.8. In Figure 3.7, SDS-PAGE gel was performed on samples taken from the assay mixture every 10 minutes up to 90 minutes. In Figure 3.8 the concentration of the mono-ubiquitinated GB1-RING2, estimated by densitometry of the one-ubiquitin band outlined in red in Figure 3.7, is shown in Figure 3.8 as a function of assay time and zinc concentration. The data indicate a strong inhibition of ubiquitination activity by $ZnSO_4$ at ratios of 3:1 or greater. Note that in Figure 3.7c and 3.8 the data for the 6:1 ratio of zinc to protein appears less inhibited than for that of the 3:1 ratio. This is likely to be the result of the evident heavier load of protein shown in the gel in Figure 3.7c. We will discuss this in the conclusion section.

The effect of added $ZnSO_4$ on ubiquitination activity was further explored in the

experiments shown in Figures 3.9 and 3.10 which directly compare assays performed in the presence of different ratios of $ZnSO_4$ to GB1-RING2. The data once again confirm the existence of a strong inhibition of the ubiquitination activity of parkin RING2 by added zinc ions.

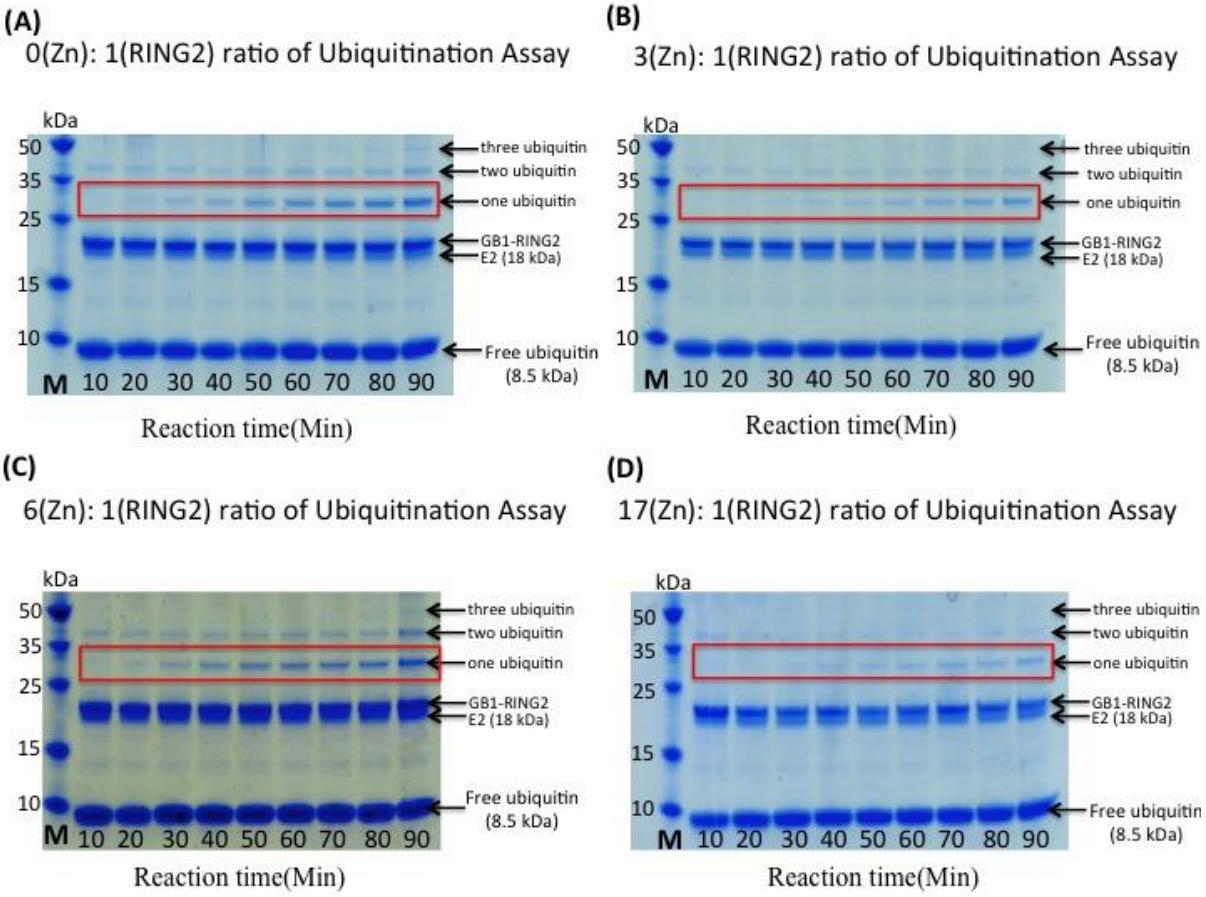


Figure 3.7. PAGE gels of different zinc ratios and ubiquitination activity assays of GB1-RING2**.**
Ubiquitination level was measured by quantitation of the amount of protein present in the major ubiquitinated band (red box) by densitometry using ImageQuant TL software, and is confirmed by Photoshop. (M: protein marker) (A): Zn(0) : GB1-RING1(1) ratio of ubiquitination assay (B) Zn(3) : GB1-RING1(1) ratio of ubiquitination assay (C) Zn(6) : GB1-RING1(1) ratio of ubiquitination assay (D) Zn(17) : GB1-RING1(1) ratio of ubiquitination assay

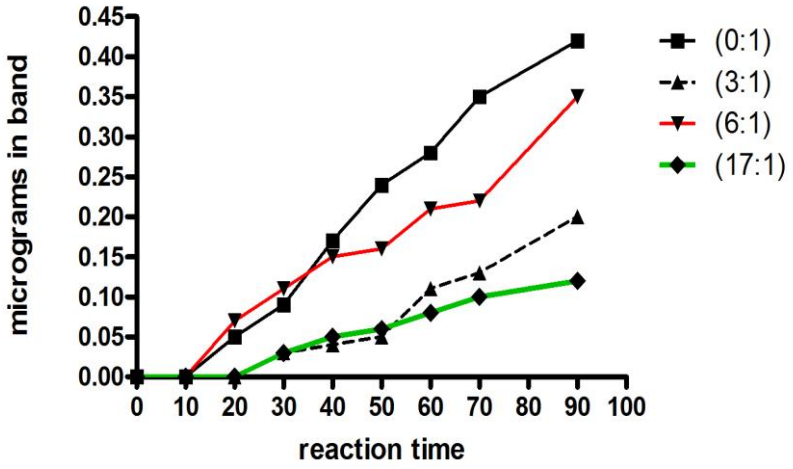


Figure 3.8. Different zinc ratios and ubiquitination activity of GB1-RING2

Ubiquitination level was measured by quantitation of the amount of protein present in the major ubiquitinated band by densitometry using ImageQuant TL software and Photoshop. **X-axis**: reaction time (minute) for ubiquitination assay. **Y-axis**: micrograms of ubiquitinated band.

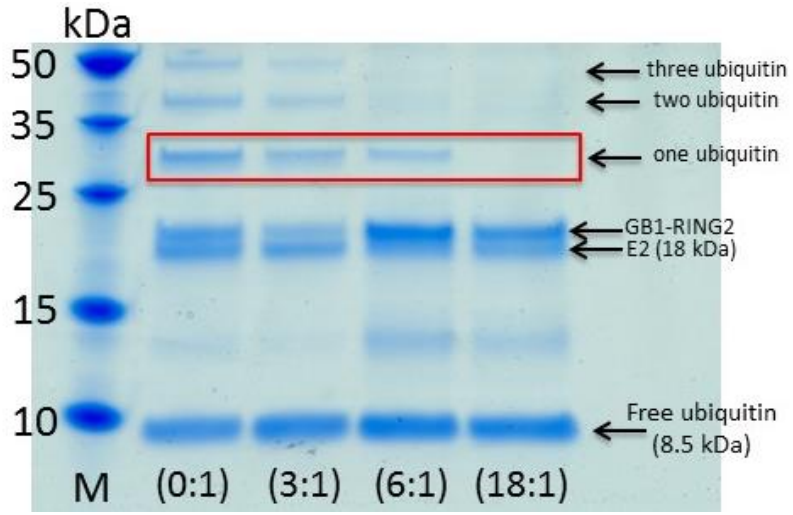


Figure 3.9. PAGE gels of different zinc ratios and ubiquitination activity assays of GB1-RING2 after 90 minutes.

(**M**: protein marker) **(0:1)**: Zn(0) : GB1-RING1(1) ratio of ubiquitination assay for control **(3:1)** Zn(3) : GB1-RING1(1) ratio of ubiquitination assay **(6:1)** Zn(6) : GB1-RING1(1) ratio of ubiquitination assay **(18:1)** Zn(18) : GB1-RING1(1) ratio of ubiquitination assay

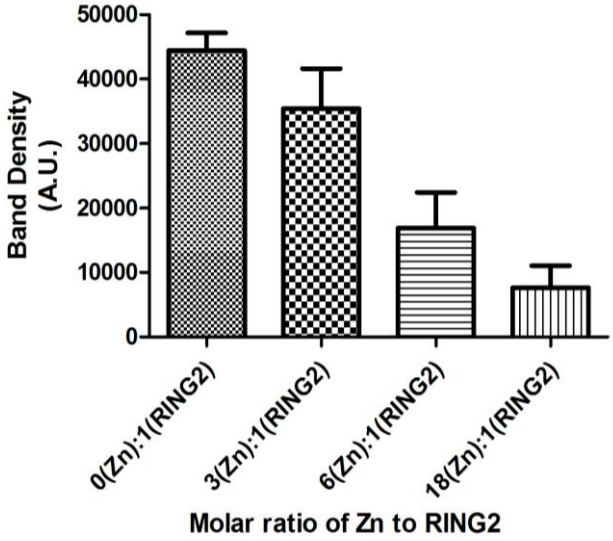


Figure 3.10. Band densities of the one ubiquitin from the PAGE gel (Figure 3-10) by densitometry using ImageQuant TL software and ImageJ.

(0:1): Zn(0) : GB1-RING1(1) ratio of ubiquitination assay for control **(3:1)** Zn(3) : GB1-RING1(1) ratio of ubiquitination assay **(6:1)** Zn(6) : GB1-RING1(1) ratio of ubiquitination assay **(18:1)** Zn(18) : GB1-RING1(1) ratio of ubiquitination assay

3.6. Conclusions and Future Direction

The recombinant protein GB1-RING2 of parkin described by Rankin *et al.* [68] was used in this study. This construct was shown to be active in autoubiquitination and required only one of the two zinc binding sites in RING2 was occupied for this activity. Based on the known properties of parkin and of the isolated RING2 domain [23][68], we hypothesized that the occupancy of one of the two zinc binding sites on RING2, one with an apparently lower affinity, can regulate the autoubiquitinating activity of parkin. In this study, we examined the effect of zinc on the activity of GB1-RING2 domain.

To check the effects of different ratios of zinc to protein, zinc titration was performed at ratios ranging from 0 to 7 mol $ZnSO_4$ per mol RING2. It was observed that the use of higher concentrations of $ZnSO_4$, those above about 60 μM, resulted in significant aggregation of the RING2 construct. This led to the use of lower protein concentrations that

required lower $ZnSO_4$ concentrations to achieve the same ratios. Under these conditions, it was found that the autoubiquitination activity of the construct was highly sensitive to the presence of low concentrations of zinc in the medium. The data shown in Figures 3.7c and 3.8, at a 6:1 ratio of zinc to protein there appears to be less inhibition than for that of the 3:1 ratio. This may be due to the evident heavier load of protein and therefore an experimental artifact. When the experiment was repeated as shown in Figure 9, the inhibitory effect of zinc was much more evident.

Consequently, these results support the idea that reversible binding of zinc to one of the two zinc-binding sites on RING2 regulates the E3 ligase activity of parkin. This may be due to a transition between bound and unbound Zn^{2+} ions at the apparently lower affinity zinc binding site on RING2 that promotes a change in local structure that in turn acts as an allosteric switch affecting the function of the RING2-E2 association.

For the future direction, we will investigate the specificity of the zinc effect by comparing it with the effects of other metal ions such as copper and calcium. Furthermore, we will investigate the role of zinc binding to the second site by using mutations of the known zinc coordinating ligands within that site. Finding the optimum condition for obtaining mono-dispersed GB1-RING2 via a detailed study of the effect of zinc using DLS will also be undertaken.

As previously mentioned in the metal hypothesis, the zinc concentration is high at synapses, and bound zinc helps to maintain the organization of the post-synaptic density. Given our findings, it is likely that the presence of zinc ions at synapses will effect parkin ubiquitination activity, and possibly aggregation, especially if there is a problem with zinc homeostasis. When considering the hypothesis, the results of the experiments described in this thesis may provide an excellent starting point for understanding the role of zinc in parkin

function and in neurological disorders such as AR-JP.

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