May 2017

Autoinhibition and Activation of Parkin

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Abstract

Mutations in the gene encoding parkin, an E3-ubiquitin ligase, result in 50% of Autosomal Recessive Juvenile Parkinsonism cases. Parkin has been identified as a key mediator of mitochondrial regeneration following oxidative stress, and pathogenic mutations have been shown to impair its ubiquitin ligase activity. Neurodegeneration of dopamine-producing neurons appears to be a downstream consequence of parkin loss-of-function, resulting in early-onset forms of Parkinson’s disease. Although ubiquitination activity is essential for its neuroprotective function, parkin is autoinhibited in its native state by various mechanisms, including its N-terminal ubiquitin-like (UBL) domain. Therefore, the overarching objective of this thesis was to structurally characterize the autoinhibited state of parkin and determine how this inactive structure is modified to a catalytically-competent form.

It was determined that autoinhibited parkin maintains a compact tertiary structure mediated by a tight intramolecular association between the UBL and C-terminal region. A high-resolution NMR strategy was developed and used to identify the binding site of the UBL domain that further revealed allosteric structural changes associated with UBL binding and displacement.

Recently, multiple reports emerged identifying serine 65 phosphorylation of both ubiquitin and parkin’s UBL domain as key inducers of parkin activity. To examine the roles of these phosphorylation signals, several methods were used to generate homogenous S65-phosphorylated ubiquitin and UBL including: chemical modification,
orthogonal translation, and phosphorylation by a catalytically-active kinase. Thermodynamic parameters of ubiquitin and UBL binding to parkin were measured and it was demonstrated how these are significantly altered upon S65 phosphorylation to promote a loss of autoinhibition.

To understand the structural consequences of S65 phosphorylation, the three-dimensional structure of the phosphorylated parkin UBL was solved. Phosphorylation impacts the structure, stability and autoinhibitory association of the UBL domain in parkin. Further, cooperative roles of phospho-ubiquitin and phospho-UBL were established in reorganizing the parkin to an extended structure, allowing its engagement in the ubiquitination cascade.

Finally, to investigate a chemical mechanism of catalysis in activated parkin, a detailed characterization of active site atoms in parkin was performed. Chemical biology approaches were used to generate an activated parkin–ubiquitin intermediate that will provide further mechanistic insight into ubiquitin transfer onto mitochondrial substrates.

**Keywords:** Parkinson’s disease, ubiquitin, parkin, Ub, UBL, RBR, ubiquitin ligase, E3, autoinhibition, PINK1, phosphorylation, nuclear magnetic resonance, neurodegeneration, protein structure, protein dynamics
Co-authorship Statement

Chapter 1

Data in Figure 1.7 was published in Chaugule et al., (2011) EMBO J, and is reproduced here with permission to assist the reader.

Chapter 2

Small Angle X-ray Scattering experiments shown in Figure 2.2 B were collected by R. Julio Martinez-Torres and Andrew Purkiss at the Cancer Research UK London Research Institute. Data analysis for assignment of the RORBR backbone chemical shifts was abetted by Dr. Pascal Mercier, who assigned ~65% of resonances. The crystal structure of UBL–RORBR presented in Figure 2.11 C was determined by Atul Kumar at the Medical Research Council, UK. Thermodynamic parameters presented in Table 2.2 are compiled from experiments collected by Jacob Aguirre, Tara Condos and R. Julio Martinez-Torres (this author contributed the data presented in its raw form in Figure 2.13). HADDOCK structure calculations of pUb on RORBR in Figure 2.14 was conducted by Gary Shaw.

Chapter 3

Synthesis of DBAA reagent was performed by Augusto Mataraz. Mass spectrometry data in Figures 3.4, 3.5, 3.6 and 3.7 were collected at the UWO Biological Mass Spectrometry Laboratory by Paula Pittock. PhosTag gels in Figure 3.4 were conducted jointly with Susanna George, who purified pSer20 and pSer12 Ub variants.
Chapter 4

Phosphorylation time-course experiments in Figure 4.2 were collected with assistance from Michele Rusal, who also optimized conditions for in-vitro UBL phosphorylation. Experiments for pUBL structure determination and validation were collected jointly with Karen Dunkerley. NOE assignments and structure determination of pUBL was conducted by Pascal Mercier, who also prepared Table 4.1. Data presented in Figure 4.4 F, 4.5, 4.6, 4.7, 4.9 and 4.13 were collected jointly with Karen Dunkerley. Figure 4.10 A was prepared by Karen Dunkerley.

Chapter 5

Cloning of Rcat into SUMO vector was performed by Emmy Sun. Mass spectrometry data in Figures 5.6, 5.7, and 5.8 were collected at the UWO Biological Mass Spectrometry Laboratory by Paula Pittock.

Chapter 6

Perspectives are this author’s only.
Dedication

For Jane and Donald Butcher. I know they would be proud.
Acknowledgements

I must first extend my most sincere gratitude to my supervisor, Gary Shaw, for his mentorship over the last number of years. I could not have been more fortunate than to complete this degree under your guidance and I have learned so much from you and the Shaw lab. I thank you for the incredible opportunity I was given many years ago that undoubtedly shaped my life in the most positive way possible.

I thank all those who have mentored and taught me in the lab over the years: Donald Spratt, Steven Beasley, Ben Cook, Pascal Mercier, Liliana Santamaria-Kisiel, Jane Bai, Chee Ng. I also extend my eternal thanks to Kathy Barber, who was always available to help no matter what reagent I needed from the depths of the freezer or whatever piece of equipment decided to break at the most inopportune time (by my own fault or otherwise!). I could not have accomplished this work without your kind guidance and patience. To all my fellow Shaw Lab colleagues: Karen, Tara, Yuning, Aisha, Michele, Alex, Taylor, Chetan, Richard and Julia–thank you all for so many great memories in the lab and many Frosty Fridays that kept me sane and motivated.

I thank my advisory committee members, Stan Dunn and Gilles Lajoie, for constructive advice during our many meetings together. I always appreciated your diverse viewpoints from outside my bubble. I also thank Lee-Ann Briere and Paula Pittock for their expertise and helping my design my experiments and analyze data, no matter how complicated.

Finally, to my family and friends for their unrelenting support during this journey–my parents, Karina, Sam-the-Man and Jawin–you guys are my world. Thank you.
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<tr>
<td>ARJP</td>
<td>autosomal recessive juvenile Parkinson’s disease</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BRcat</td>
<td>benign required for catalysis domain</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBD</td>
<td>chitin binding domain</td>
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<td>CD</td>
<td>circular dichroism</td>
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<td>CSI</td>
<td>chemical shift index</td>
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<td>CSP</td>
<td>chemical shift perturbation</td>
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<tr>
<td>CT</td>
<td>constant time</td>
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<td>Da</td>
<td>daltons</td>
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<tr>
<td>DBAA</td>
<td>α,α′-dibromo-adipyl(bis)amide</td>
</tr>
<tr>
<td>DHA</td>
<td>dehydroalanine</td>
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<td>DJ-1</td>
<td>protein deglycase DJ-1</td>
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<td>DSS</td>
<td>4,4-dimethyl-4-silapentane-1-sulfonic acid</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>DUB</td>
<td>deubiquitinating enzyme</td>
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<td>E1</td>
<td>ubiquitin activating enzyme</td>
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<td>E2</td>
<td>ubiquitin conjugating enzyme</td>
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<td>E3</td>
<td>ubiquitin ligase enzyme</td>
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<tr>
<td>E(X)~Ub</td>
<td>E1, E2 or E3 enzyme with thiolester-bound ubiquitin</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ESI–MS</td>
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<td>FPLC</td>
<td>fast performance liquid chromatography</td>
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<td>HECT</td>
<td>homologous with E6-associated protein C-terminus</td>
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<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
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<td>HHARI</td>
<td>human homologue of Ariadne</td>
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<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
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<td>HMQC</td>
<td>heteronuclear multiple quantum coherence</td>
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<td>HOIL-1</td>
<td>haem-oxidized IRP2 ubiquitin ligase 1</td>
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<td>HOIP</td>
<td>HOIL-1 interacting protein</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HPLC</td>
<td>high pressure/performance liquid chromatography</td>
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<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
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<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>ITC</td>
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<tr>
<td>kDa</td>
<td>kilodaltons (1000 daltons)</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<td>LC–MS</td>
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<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
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<td>MESNA</td>
<td>sodium 2-mercaptoethanesulfonate</td>
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<td>MOPS</td>
<td>3-Morpholinopropane-1-sulfonic acid</td>
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<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PARL</td>
<td>presenilin-associated rhomboid-like protein</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<td>PINK1</td>
<td>PTEN-induced kinase 1</td>
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<tr>
<td>pKa</td>
<td>acid dissociation constant</td>
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<td>ppm</td>
<td>parts per million</td>
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<td>PRE</td>
<td>paramagnetic relaxation enhancement</td>
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<td>pUb</td>
<td>serine 65-phosphorylated ubiquitin</td>
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<td>pUBL</td>
<td>serine 65-phosphorylated parkin UBL domain</td>
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<td>Rcat</td>
<td>required for catalysis domain</td>
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<td>RF1</td>
<td>release factor 1</td>
</tr>
<tr>
<td>RBR</td>
<td>RING1–BRcat–Rcat (originally RING–inBetween–RING)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>R&lt;sub&gt;G&lt;/sub&gt;</td>
<td>radius of gyration</td>
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<td>RING</td>
<td>Really-Interesting-New-Gene</td>
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<td>RORBR</td>
<td>parkin construct spanning RINGO–RING1–BRcat–Rcat</td>
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<td>RMSD</td>
<td>root mean square deviation</td>
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<td>SAXS</td>
<td>small angle X-ray scattering</td>
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<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TOMM</td>
<td>translocase of the outer mitochondrial membrane</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)propane-1,3-diol</td>
</tr>
<tr>
<td>TROSY</td>
<td>transverse relaxation-optimized spectroscopy</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UBA</td>
<td>ubiquitin-associating domain</td>
</tr>
<tr>
<td>UBL</td>
<td>ubiquitin-like domain</td>
</tr>
<tr>
<td>UCH-L1</td>
<td>ubiquitin carboxy-terminal hydrolase L1</td>
</tr>
<tr>
<td>Ulp1</td>
<td>ubiquitin-like specific protease 1</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>VS</td>
<td>vinyl sulfone</td>
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</table>
Chapter 1

Introduction and Background

1.1 Parkinson’s Disease and Hereditary Causes

The symptoms of Parkinson’s disease (PD) were first documented in Western medicine by James Parkinson, a British physician, in his seminal 1817 essay (1). Parkinson termed the disease “Shaking Palsy” and famously defined the malady as follows:

“Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.”

- James Parkinson, 1817

In his essay, Parkinson chronicled six aging patients with what he believed to be a common medical condition, distinct from other trembling disorders. Parkinson illustratively documented the lives of these patients, their physical and mental conditions, in a remarkably emotional style that would be foreign to medical journals today. His work recognized resting tremors, muscle weakness and abnormal posture as common hallmark symptoms in his patients. Importantly, Parkinson recognized the severely progressive nature of this disease that later became synonymous with his name. He stressed the importance of early detection and treatment, and these aspects remain critical to patient prognosis today.

The first biochemical link to PD came nearly 150 years after Parkinson’s essay, largely through the research of Austrian chemist, Oleh Hornykiewicz, who discovered depleted
levels of the neurotransmitter dopamine in post-mortem brains of PD patients (2). This was the first evidence of a link between dopamine and neurodegeneration, which had been suspected for several years. Remarkably, only one year after this finding, Hornykiewicz, along with his colleague Walther Birkmayer in 1961, showed remission of symptoms in 20 PD patients by a single intravenous injection of levodopa, a dopamine analog (3). The magnitude of this discovery was termed “the dopamine miracle” in popular culture and to this date levodopa remains the primary measure for relief of PD symptoms. In the years that followed, impairment of dopaminergic neurons in the substantia nigra section of the midbrain was shown to be responsible for depleted dopamine levels that are causative of PD (4,5). Unfortunately, since these groundbreaking discoveries, remarkably little progress has been made in further unraveling the biochemical origins of PD.

Major advances in PD etiology came near the completion of the human genome project in the late 1990s, when mutations in several genes were linked to PD, often with an early-age onset form of the disease (ie: before 50 years old). The first gene was identified in 1997, termed PARK1, and encodes the protein α-synuclein (6). It was discovered that PD in a family of Italian descent was inherited over generations in an autosomal dominant manner by mutations at the PARK1 locus. The link between α-synuclein and PD represented a breakthrough but raised many questions about the basis of neurodegeneration. This arose from observations that α-synuclein itself is contained within protein aggregates in brains of PD patients, called “Lewy bodies” (7), as well as so-called “amyloid plaques” in the brains of Alzheimer’s patients (8).
Subsequently, Kitada and coworkers discovered a second PD-causing gene locus, \textit{PARK2}, encoding a protein proposed to be involved in the ubiquitin-proteasome system from analysis of its primary sequence (9). They named this protein product “parkin”. In contrast to \textit{PARK1}, disease-causing mutations in \textit{PARK2} are inherited in a recessive manner, causing a disease termed Autosomal Recessive Juvenile Parkinson’s disease (ARJP), which had been described previously (10). The hallmark of ARJP is the extremely early onset of the disease, with dystonia and tremors often beginning before age 30. Although there are exceptions, most studies have found that ARJP patients typically do not have Lewy Bodies characteristic of idiopathic PD, pathologically distinguishing this form of inherited Parkinsonism (11-13).

Beyond \textit{PARK1} and \textit{PARK2}, several other \textit{PARK} genes have been subsequently associated with inherited forms of PD. The best characterized of these genes and their respective protein products are summarized in Table 1.1. Most PD-causing gene products have known enzymatic functions today, with the notable exception of \textit{\alpha}-synuclein, which plays an unspecified role in neuronal synapse transmission. The focus of this thesis will be on the structure and function of the \textit{PARK2} gene product: the ubiquitin ligase, parkin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
<th>Inheritance</th>
<th>Proposed function</th>
<th>Ref</th>
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</thead>
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<tr>
<td>\textit{PARK1}</td>
<td>\textit{\alpha}-synuclein</td>
<td>Dominant</td>
<td>Unknown</td>
<td>(6)</td>
</tr>
<tr>
<td>\textit{PARK2}</td>
<td>Parkin</td>
<td>Recessive</td>
<td>Ubiquitin ligase</td>
<td>(9)</td>
</tr>
<tr>
<td>\textit{PARK5}</td>
<td>UCH-L1</td>
<td>Dominant</td>
<td>Ubiquitin hydrolase</td>
<td>(14)</td>
</tr>
<tr>
<td>\textit{PARK6}</td>
<td>PINK1</td>
<td>Recessive</td>
<td>Kinase</td>
<td>(15)</td>
</tr>
<tr>
<td>\textit{PARK7}</td>
<td>DJ-1</td>
<td>Recessive</td>
<td>Deglycase / chaperone</td>
<td>(16)</td>
</tr>
<tr>
<td>\textit{PARK8}</td>
<td>LRRK2</td>
<td>Dominant</td>
<td>Kinase</td>
<td>(17,18)</td>
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</table>
1.2 Mitochondrial Impairment and Mitophagy in PD

Mitochondrial impairment has long been suspected as causative of Parkinson’s disease. This link first arose in 1976 when Barry Kidston, an American graduate student, developed permanent symptoms of Parkinsonism after intravenously injecting himself with a contaminated batch of 4-propionyloxy-4-phenyl-N-methylpiperidine (MPPP), a synthetic opioid that he (erroneously) synthesized himself. Unbeknownst to Kidston, he in fact had synthesized 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a hydrolyzed byproduct of MPPP. Kidston developed muscle rigidity, weakness and uncontrollable tremors within days of using the drug (19). He was initially admitted to a psychiatric facility and unsuccessfully treated with antipsychotics and electro-convulsion therapy. Only after a doctor tried administering levodopa did Kidston’s condition recover and doctors subsequently diagnosed Parkinson’s disease in this 23 year old male (19).
Today, we know that MPTP is metabolized in the brain to MPP+, a potent neurotoxin that acts by inhibiting complex I of the mitochondrial electron transport chain. Studies in primates showed MPTP-treatment selectively destroys dopaminergic neurons, leading to a disorder with classic symptoms of idiopathic PD (20,21). Brains of these primates also contain inclusion bodies that bear resemblance to Lewy Bodies often observed in post-mortem PD patients, containing aggregates of α-synuclein and ubiquitin (22). Other inhibitors of complex I include the commonly used pesticide, rotenone, which also induces permanent Parkinsonian symptoms in mammals, including humans (23). For unknown reasons, dopaminergic neurons appear particularly susceptible to these mitochondrial toxins. Thus, dysfunction of oxidative phosphorylation can recapitulate Parkinsonism and certain pathological hallmarks of idiopathic PD. These mitochondrial toxins are widely used to this day to reproduce Parkinsonism in animal models for various scientific studies.

Mitochondria are dynamic structures, undergoing large-scale transformations in shape and bioenergetic functions over their lifetimes. Although commonly depicted as bean-shaped structures in illustrations, mitochondria continuously change their morphology by dividing and fusing together in processes termed fission and fusion, respectively (24). Repair of mitochondria following oxidative damage is separately governed by a unique autophagy-like process, termed mitophagy. This pathway selectively recruits autophagy receptors to damaged mitochondria, which results in their engulfment, lysosomal degradation and subsequent regeneration (25,26). This recycling process is stimulated in response to oxidative stress, therefore it is hypothesized that mitophagy is a major mechanism by which mitochondria protect themselves from damage caused by reactive...
oxygen species (ROS), which impair oxidative phosphorylation, amongst other cellular processes.

Induction of mitophagy has been attributed to a complex signaling pathway governed by the protein products of two PD-linked genes, *PARK2* and *PARK6*, encoding the ubiquitin ligase parkin and the Ser/Thr kinase PINK1, respectively. Studies in *Drosophila melanogaster* discovered knock-out flies for either *PARK2* or *PARK6* showed remarkably similar phenotypes, with difficulties flying and extensive damage to dopaminergic neurons (27-29). These studies also showed the two genes function in a common biological pathway, with PINK1 acting upstream of parkin, since overexpression of parkin could rescue PINK1 deficient flies, but not vice-versa (27-29). PINK1 contains a mitochondrial targeting sequence, trafficking the protein to the outer mitochondrial membrane (OMM). Under normal cellular conditions, this targeting sequence is cleaved by the protease PARL (presenilin-associated rhomboid-like protein) and the cleaved PINK1 is degraded by the canonical ubiquitin proteasome system through the N-end rule mechanism (30-33) (see **Figure 1.2 A**). This results in very low levels of PINK1 in healthy mitochondria, despite its constitutive expression. Remarkably, during oxidative stress, inhibition of oxidative phosphorylation or depolarization of the mitochondrial proton gradient, cleavage of PINK1 is inhibited and PINK1 is subsequently stabilized on the OMM of these dysfunctional mitochondria (34,35). This regulatory mechanism makes PINK1 a de facto “sensor” of mitochondrial damage, accumulating selectively on damaged mitochondria. PINK1 subsequently recruits Parkin to the mitochondria and stimulates its activity, allowing parkin to ubiquitinate a multitude of protein substrates, predominantly OMM proteins (36,37) (see **Figure 1.2 B**). Rather than signaling for
Figure 1.2. PINK1 recognizes damaged mitochondria to induce mitophagy.

(A) In healthy mitochondria, PINK1 is constitutively expressed and degraded. PINK1 is localized to the outer mitochondrial membrane (OMM) by its N-terminal targeting sequence. Insertion of the N-terminus through the membrane results in its cleavage by the PARL protease. After cleavage, an N-terminal phenylalanine on PINK1 recruits the ubiquitin-proteasome machinery to degrade PINK1 by the N-end rule.

(B) In mitochondria that are stressed, damaged or depolarized, PARL cleavage is inhibited. This stabilizes PINK1 on the OMM and recruits Parkin. Parkin subsequently ubiquitinates a multitude of OMM proteins, including TOMs (translocases of the outer membrane), MFNs (mitofusins) and VDACs (voltage-dependent anion channels). Ubiquitination of these mitochondrial proteins induce mitophagy. This figure is adapted from Pickerell and Youle, (2015).
proteolytic degradation, parkin-mediated ubiquitination of these substrates appears to induce mitophagy in these damaged mitochondria, leading to their turnover (36,38). These parkin substrates include TOMM70a; mitofusin 1 and 2; VDAC 1, 2 and 3; and hexokinase 1 (37). Notably, the mitofusins are critical regulators of mitochondrial morphology and govern fusion, fission and mitophagy processes, the significance of which is exemplified by the fact that certain mutations in mitofusin 2 are causative of the incurable neurological disorder Charcot–Marie–Tooth disease (24,39). Activation of parkin’s ubiquitin ligase activity is therefore a critical step in initiating mitophagy and maintaining mitochondrial fidelity. Consequently, several PD-linked mutations in parkin that directly or indirectly hinder its ubiquitin ligase activity have been found to impair mitophagy (40-42).

1.3 Ubiquitination and Protein Degradation

A delicate balance between protein synthesis and degradation keeps all living organisms in a constant state of flux. This dynamic nature of cellular metabolism was established in work by Rudolph Schoenheimer, who challenged the historical view that proteins and body tissues were largely static entities. Schoenheimer was one of the first scientists to use chemical isotopes to address biological problems. In the late 1930s, he fed rodents a variety of fatty acids and proteins containing deuterium ($^2$H) and heavy nitrogen ($^{15}$N), to test whether these isotopes were metabolized for energy, or withheld in cells (43,44). His finding that the vast majority of $^2$H and $^{15}$N were incorporated into existing tissues was a
shock to many, who predominantly believed that ingested fats and proteins were used as immediate energy sources. Schoenheimer’s experiments suggested that new proteins were being continuously synthesized in the body. However, since the weights of the animals were unchanged after the experiment, an equivalent amount of protein degradation must have occurred to compensate. It was subsequently established that protein degradation, termed “proteolysis”, actually consumes energy despite the seemingly contradictory fact that peptide bond hydrolysis is exothermic (45).

In the 1980’s the biochemical mechanism of protein degradation began to surface, largely through discoveries by Aaron Ciechanover and Avram Hershko. They found a small protein, only ~8 kDa, was required for ATP-dependent protein degradation (46). The landmark study showed this small heat-stable protein, which they originally named ATP-dependent Proteolysis Factor 1 (APF-1), became covalently linked to protein substrates during the proteolytic cycle. Amazingly, Ciechanover and colleagues already recognized the importance of cysteine residues in the protein’s function at this early stage, as proteolysis was significantly inhibited by N-ethyl-maleimide, a thiol-blocking reagent (46). Experiments performed shortly after showed APF-1 was in fact, the same protein that was previously found attached covalently to histones through an isopeptide bond with its C-terminus (47,48). The protein was renamed ubiquitin (Ub) owing to its ubiquitous expression and high degree of sequence conservation across eukaryotes (48). It was recognized that Ub-bound proteins were ultimately degraded by proteolytic machinery, although the precise details of this mechanism took several years to establish.
1.4 E1, E2, E3 and DUB enzymes

We now know that Ub transfer is a coordinated cascade between multiple different enzymes, each with a role to specifically deliver the Ub molecule onto a particular substrate protein. Classically, these ubiquitinated substrate proteins are destined for degradation by the proteolytic machinery, although it is now recognized that ubiquitination also governs many other signaling pathways in the cell. The canonical ubiquitination pathway is shown graphically in Figure 1.3 A and can be described as follows. Firstly, Ub is “activated” by a ubiquitin-activating enzyme (E1), in an ATP-dependent step (49). Hydrolysis of ATP by the E1 is coupled to adenylation of the Ub C-terminus, resulting in subsequent transfer of Ub onto the catalytic cysteine of the E1 (50). The linkage between the catalytic cysteine of the E1 and carboxy C-terminus of Ub is a thiolester bond, containing sufficient free energy to drive subsequent enzymatic steps. Secondly, the E1~Ub conjugate associates with a ubiquitin-conjugating enzyme (E2), which accepts Ub from the E1 enzyme by means of thiolester transfer reaction onto its own catalytic cysteine (49,51,52). Thirdly, the E2~Ub conjugate associates with a ubiquitin ligase enzyme (E3), which catalyzes the final ligation step of Ub onto an amino group of a substrate protein, resulting in an isopeptide bond between the substrate and carboxy C-terminus of Ub (47,49). This process can repeat several times, adding consecutive Ub molecules by means of a polyubiquitin chain. The Ub-substrate linkage is maintained until the protein is degraded by the proteolytic machinery, or Ub is removed by a deubiquitinating (DUB) enzyme that catalyzes the removal of Ub, the reverse reaction of ubiquitin ligases (49).
Figure 1.3. Proposed mechanisms of ubiquitination by E3 ubiquitin ligases.

(A) The canonical ubiquitination pathway is shown, as described in the text. The final step in the pathway, catalyzed by E3 ubiquitin ligases, can be achieved by multiple mechanisms. These are shown below for (B) RING-type, (C) HECT-type and (D) RBR-type ubiquitin ligases, highlighting the functional differences between each family of E3.
This simplistic representation of the ubiquitination pathway overlooks many of the complexities that enable ubiquitin to act as a signal for so many fundamental cellular processes. The completion of the human genome in particular demonstrated the critical hierarchical structure of the pathway. While there are only two E1 enzymes in humans, there are dozens of E2 enzymes and thousands of E3 enzymes (53). In this manner, the ubiquitin-conjugating machinery gains specificity as the pathway proceeds. E1 and E2 enzymes have relatively conserved protein folds and active site structures, while E3 enzymes are more unique and employ various functional mechanisms. E3 enzymes frequently contain accessory domains or are themselves part of larger multi-domain proteins. This hierarchical structure enables remarkable substrate specificity, with E3 ligases ultimately determining the fate of Ub.

E3 ubiquitin ligases are diverse in structure and functionality, but can generally be grouped into a few major families. The first family, the Really-Interesting-New-Gene (RING) E3s comprise the vast majority of E3 enzymes in the genome. These ubiquitin ligases contain a zinc-binding RING domain, which coordinate two Zn\(^{2+}\) ions in a cross-brace fashion (54). These enzymes harbor conserved structural features that allow them to act as scaffolds for E2–Ub conjugates. RING E3s also generally have affinity for specific substrates, bringing them in close proximity to the E2–Ub conjugate. This allows for direct transfer of Ub from E2 onto substrate (Figure 1.3 B). In contrast, a second family, the Homologous with E6-associated protein C-Terminus (HECT) E3s catalyze ubiquitination through a HECT–Ub covalent intermediate. This occurs by transfer of Ub onto a catalytic cysteine in the HECT domain, resulting in a thiolester bond (55) (Figure 1.3 C). Subsequently, Ub is transferred from the HECT domain onto a substrate lysine.
While RINGs and HECTs comprise the vast majority of ubiquitin ligases, other smaller families of E3s exist, including the “U-box” and “RBR” ubiquitin ligases, which will be described in detail below (56,57).

The functional consequence of protein ubiquitination is dictated by several factors, including: (i) The substrate tagged with the ubiquitin moiety; (ii) The specific lysine residue modified on the substrate; and (iii) The nature of the ubiquitin modification, whether a single Ub or a polyubiquitin chain is formed. Multiple ubiquitin molecules can be conjugated together in a polymeric chain through one of seven lysine residues in Ub (K6, K11, K27, K29, K33, K48, K63) or the N-terminal amino group (Figure 1.4 A). The innumerable potential structures of these polyubiquitin chains bestow the specificity of the ubiquitin signal. For example, K48-linked polyubiquitin adopts a relatively compact structure, while K63-linked polyubiquitin is significantly more extended (Figure 1.4 B–C) (58,59). Further, due to a lack of intramolecular contacts in K63-linked polyubiquitin, these ubiquitin linkages can adopt a wide range of relative conformations, adding an additional level of diversity to the polyubiquitin signal. In general, K48-linked polyubiquitinated substrates are destined for proteolytic degradation by the proteasome, the classic interpretation of ubiquitination. Conversely, K63-linked chains govern other cellular processes including DNA-damage repair in the nucleus and organelle trafficking in the cytosol. K6, K11, K27, K29 and K33 polyubiquitin linkages are less well-studied, but have also generally been associated with non-proteolytic roles (53). Therefore, the signaling possibilities of the ubiquitin system extend far beyond the original proteolysis functions originally described by Hershko, Ciechanover and colleagues.
Figure 1.4. Different polyubiquitin linkages bestow unique structural features.

(A) Structure of human ubiquitin, highlighting the N-terminal amino group and seven lysine residues (coloured red) that are able to form polyubiquitin linkages with the C-terminus of another ubiquitin moiety.

(B) Lysine 48-linked di-ubiquitin crystal structure, PDB: 1AAR. The proximal Ub contributes the amino group in the linkage, while the distal Ub is linked by its C-terminus. The proximal Ub is oriented as in (A).

(C) Lysine 63-linked di-ubiquitin crystal structure, PDB: 2JF5. The proximal Ub is oriented as in (A).
Once the ubiquitin signal has served its purpose, the ubiquitin moiety is removed from substrates and recycled by deubiquitinating (DUB) enzymes. These are a family of approximately 100 hydrolase enzymes that specifically cleave the peptide bond at the junction between the C-terminus of ubiquitin and the substrate. Thus, DUB enzymes frequently counteract the degradative signal conveyed by E3 ubiquitin ligases and have a stabilizing effect, often increasing levels of substrate proteins. Most DUBs catalyze deubiquitination using a cysteine protease mechanism, employing a catalytic triad of a cysteine, histidine and a third charged or polar residue (60). The histidine residue is aligned and polarized by the charged residue, raising its pKa such that it can deprotonate the catalytic cysteine’s thiol group. The resulting thiolate anion then performs a nucleophilic attack on the peptide bond, resulting in its hydrolysis.

Catalytic mechanisms of DUB enzymes have been well-studied using irreversible ubiquitin probes that bind to the active site cysteine in a non-reversible manner, trapping the covalent intermediate (61-63). An interesting observation from these studies is that active site histidine residues of DUB enzymes are often mis-aligned in the absence of substrates (64-66). That is, the orientation of the histidine residue is such that it cannot form a Cys-His thiolate-imidazolium ion pair to initiate catalysis. Substrate binding is often necessary for DUB enzymes to rearrange the active site residues into a catalytically-competent conformation. It has been speculated that this regulatory mechanism prevents undesirable oxidation of the active site cysteine or engagement of other cysteine-reactive molecules in the cell (67,68). DUBs recognize the C-terminal tail of ubiquitin, which is linked to a substrate or another ubiquitin molecule in a polyubiquitin linkage (69,70). Therefore, different DUB enzymes show remarkable specificity for hydrolyzing specific
polyubiquitin linkages, allowing them to relay targeted biological signals. These features have made DUB enzymes attractive therapeutic targets in pharmaceutical research (71).

1.5 RBRs: A Novel Class of Autoinhibited Ubiquitin Ligases

A small family of E3 ligases exists known as “RBR” ubiquitin ligases, comprising only about a dozen of 1000+ E3 ligases contained in the human genome (Figure 1.5). The best characterized members of the RBR E3 ligase family are parkin, which is mutated in hereditary forms of Parkinson’s disease; HHARI (human homologue of Ariadne), which is most closely related to parkin by primary amino acid sequence; and HOIP (HOIL-1-interacting protein) and HOIL-1 (haem-oxidized IRP2 ubiquitin ligase 1), both of which are members of the linear ubiquitination chain assembly complex (LUBAC) that specifically catalyzes linear polyubiquitin chains, linked through the N-terminal amino group of Ub.

RBR was historically an abbreviation for RING-inBetween-RING, recognizing their shared composition of three consecutive protein domains, originally thought to be multiple RING domains that cooperated to catalyze ubiquitin ligation (57). This assumption was based on the observed ubiquitin ligase activity of these cysteine-rich enzymes that presumably coordinated Zn$^{2+}$ ions like traditional RING domains. However, recent advances in our knowledge of these enzymes, including through this work, render this nomenclature invalid. Today, we know RBR proteins contain only one RING
domain, while the other domains adopt a zinc-coordinating fold that is completely distinct from a RING (72-74). Mechanistic studies also revealed that RBR ubiquitin ligases do not catalyze Ub transfer directly from E2 to substrate, like traditional RINGs. Instead, RBRs employ a catalytic thiolester intermediate with Ub analogous to the HECT mechanism of ubiquitin transfer (75-77). Because of the mixed characteristics of both RING and HECT ubiquitin ligases, this has led to RBRs being described as “RING/HECT hybrids” (75).

To reduce ambiguity in communication and scientific reports, a new nomenclature has been proposed for the RBR domain triad, maintaining the familiar RBR abbreviation (Figure 1.5) (78). First, the most C-terminal domain harbors the catalytic site, will be named $R_{cat}$, and is absolutely Required for catalysis. Secondly, the domain directly upstream adopts a similar structure to $R_{cat}$ but lacks a catalytic site, and is therefore a Benign $R_{cat}$ ($BR_{cat}$). Finally, the most N-terminal domain is the only true RING domain and retains its historic name $RING_1$. It is interesting that, despite their covalent mechanism of ubiquitin transfer, the active sites of RBR proteins do not resemble those of HECT E3s. Similarly, the $RING_1$ domain in RBRs actually lacks several hallmarks of other RING proteins that enable them to recruit E2 enzymes (78). These observations suggest that RBRs employ a novel mechanism of ubiquitin transfer. Consistent with this idea, there are no examples of isolated $R_{cat}$ or $BR_{cat}$ domains in other proteins, suggesting the triad of $RING_1$–$BR_{cat}$–$R_{cat}$ always cooperate together in nature.

In addition to the “RBR” domain triad, most RBR ubiquitin ligases contain an assortment of different accessory domains upstream or downstream in sequence from the RBR catalytic module (Figure 1.5). Parkin and HOIL-1 both contain N-terminal ubiquitin-like
Figure 1.5. Domain architecture of RBR ubiquitin ligases.
Domain architecture of ten selected members of the RBR family of E3 ubiquitin ligases. The common RBR domains; RING1, BRcat and Rcat; are aligned and shown in magenta, grey and dark green, respectively. Other notable accessory domains found in these proteins are also shown and include Ubiquitin-like domain (UBL), Ubiquitin-associating domain (UBA), Ubiquitin-interacting motif (UIM), Linear-determining domain (LDD), and Glu/Asp rich stretches (Acidic). This figure is adapted from Spratt et al., 2014.
domains (UBLs), which bear high sequence and structural similarity to ubiquitin and subsequently recruit other ubiquitin-associating machinery (79-81). HHARI and its close relative, ARI2, both contain an extended C-terminal region named the Ariadne domain. Dorfin likewise harbours a C-terminal region of similar size eponymously termed the Dorfin domain. HOIP contains a ubiquitin associating (UBA) domain in its N-terminus that recruits its associating E3 partner, HOIL-1 during linear polyubiquitin chain formation (81). HOIP also contains multiple other zinc-finger domains that confer its linear ubiquitin chain specificity (82). Parkin contains an additional zinc-binding domain outside the RBR module, originally termed RING0 due to its sequential arrangement directly upstream of RING1 (83).

One hallmark of RBR ubiquitin ligases that has recently become apparent is the autoinhibited nature of their ubiquitin ligase activity, first demonstrated for parkin in 2011 (84). Subsequent studies have shown this autoinhibited characteristic also exists in other RBRs, although occurring through completely distinct mechanisms. For example, the Ariadne domain associates with the Rcat domain to suppress ligase activity in HHARI and ARI2 (85), while HOIPs UBA domain maintains an autoinhibited state in the absence of HOIL-1 (76,77). This suggests that RBR proteins must be activated by some extraneous signal to carry out their full ubiquitination potential. The structural mechanism for autoinhibition and release of this state, specifically as it relates to parkin, will be the major focus of this dissertation.
1.6 Parkin

Parkin is the most studied member of the RBR family of ubiquitin ligases, due to its direct causative link with inherited forms of PD (9). Mutations in the \textit{PARK2} gene, which encodes parkin, are causative of ARJP, an early-onset form of PD. The nature of these mutations are extremely diverse, including exon rearrangements, deletions, and dozens of unique missense and nonsense mutations (86) (\textbf{Figure 1.6 A}). The translated product of the \textit{PARK2} gene is 52 kDa (465 residues), with the RBR module found at the C-terminal end of parkin. Directly upstream of RING1, parkin contains a fourth zinc-coordinating domain, originally termed RING0, discovered by limited proteolysis experiments (83). This name is a misnomer, as we now know this domain is not a RING, and more closely resembles an NZF-like zinc finger fold (87). Due to historical connotations—and because, BEATLES—this domain name will be amended to RINGO. Parkin also harbors a ubiquitin-like (UBL) domain at its N-terminus, followed by a ~75 residue linker with little predicted structure. The UBL shares 30% sequence identity and ~60% sequence similarity with ubiquitin (\textbf{Figure 1.6 D–E}). Available structures of the isolated parkin UBL and ubiquitin show the overall folds of both proteins are indistinguishable, however differences in the primary sequence of the UBL in parkin render it significantly less thermodynamically stable than ubiquitin, and more prone to unfolding (88,89). The only other region of parkin with a known three dimensional structure is the Benign Rcat (BRcat), solved in solution as the isolated domain with an unstructured N-terminal linker preceding the structure (72). The BRcat coordinates two Zn$^{2+}$ ions through a linear zinc-finger-like fashion and are indispensable to the structure of the domain. At the beginning of this dissertation, no other structures of parkin were available and it was unclear how
Figure 1.6. Parkin domain map and existing three-dimensional structures.

(A) Domain map for parkin. Residue numberings and domain boundaries are shown beneath. Selected disease-causing mutants are shown above. (B) 1.7 Å resolution crystal structure of the UBL domain from mouse parkin. Secondary structure elements are labelled. (C) Solution NMR structure of the BRcat domain from human parkin. (D) 1.8 Å resolution crystal structure of human ubiquitin, oriented the same way as (C). (E) Sequence alignment of ubiquitin and parkin’s UBL, highlighting regions of similarity.
the UBL and BRcat domains contributed to the overall structure or function of full-length parkin.

Pathogenic mutations in parkin are found throughout each of the 5 structural domains of the protein but tend to cluster to the N and C-termini (90) (Figure 1.6 A). Despite extensive research, there appears to be no unifying mechanism for how pathogenic parkin mutations induce neurodegeneration. Many pathogenic substitutions in parkin are found at cysteine residues. Native mass spectrometry experiments have shown Parkin coordinates a total of 8 structural Zn\(^{2+}\) ions using its many cysteine residues (35 cysteines in total, 7.5% of sequence composition) as zinc-coordinating ligands, suggesting protein misfolding as a rationale for loss-of-function in these mutations (83). Additionally, certain substitutions in the UBL and BRcat domains (R42P, T351P) have also been shown to cause protein unfolding, leading to subsequent proteasomal degradation (72,89,91). In contrast, many other substitutions (including, but not limited to: K48A, G328E, R334C, T415N, G430D) retain their native fold, but are apparently functionally perturbed (72,73,83,88). Unfortunately, the vast majority of these parkin mutations lack a biochemical explanation for their deleterious effects.

The best-described role of parkin is at the mitochondria where its ubiquitin ligase activity stimulates autophagic recycling of damaged mitochondria through a specialized process termed mitophagy (36). Proteomic studies showed that parkin acts to ubiquitinate a range of substrates on the outer mitochondrial membrane (37), presumably recruiting mitophagy machinery. Proteomic analyses of parkin-dependent polyubiquitin chains in mitochondria showed parkin preferentially synthesizes K48 and K63-linked polyubiquitin
chains on OMM proteins to stimulate mitophagy (92). Screening of E2 enzymes *in vitro* and *in vivo* showed specific E2 enzymes appear to be used by parkin, specifically to induce mitophagy. These included UbcH5a/b, UbcH7 and the Ubc13/MMS2 heterodimeric E2 enzyme (93,94). Beyond its role in mitophagy, parkin has also been shown to play a role in endoplasmic reticulum-associated protein degradation (ERAD). Parkin ubiquitinisates misfolded forms of proteins at the endoplasmic reticulum, including the parkin-associated endothelin receptor-like receptor (Pael-R), that results in its degradation (95). Importantly, Pael-R is a component of Lewy bodies and its degradation by parkin is inhibited by certain PD-causing mutations in parkin (95-97). Parkin may also play a role in cellular immunity by mediating resistance to intracellular pathogens, including *Mycobacterium tuberculosis* and *Salmonella enterica* through an autophagy-like pathway (98).

Biochemical studies using native parkin were sparse in the literature prior to 2011, owing to practical difficulties studying untagged, purified forms of parkin. Expression and purification of recombinant parkin is challenged by the absolute necessity of protein folding around 8 structural Zn\(^{2+}\) ions, dispersed throughout its 4 C-terminal domains. To circumvent these challenges, N-terminal solubility tags were regularly employed to aid in protein folding and improving solubility both *in vitro* and *in vivo*. Commonly used N-terminal tags included maltose-binding protein (MBP), glutathione S transferase (GST), green fluorescent protein (GFP), HA and FLAG tags. These studies identified numerous putative parkin interacting proteins and ubiquitination substrates (for an exhaustive list, see ref (78)). Unfortunately, it has been demonstrated that these N-terminal tags have significant implications on the structure, stability and enzymatic activity of parkin, calling
in to question the biological relevance of most of these observations (84,99). N-terminal tags decrease the thermal stability of parkin and artificially stimulate its ubiquitin ligase activity, increasing the likelihood of false-positive results from experimental assays (Figure 1.7 A) (99). It was proposed that this occurs by remodeling parkin from its native conformation to a more active form. Therefore, it is essential to study parkin in a native state in vitro and in vivo without N-terminal tags.

Indeed, many studies using endogenous or untagged forms of parkin were unable to observe robust ubiquitin ligase activity, in contrast to experiments using tagged parkin (100-103). A landmark study in 2011 by Chaugule and colleagues used purified human parkin and showed the protein actually lacks detectable ubiquitin ligase activity in its native state, even when excesses of all other ubiquitination machinery (E1, E2, Ub and ATP) were present (Figure 1.7 B) (84). Astonishingly, it was shown that parkin’s ubiquitin ligase activity is robustly stimulated simply by deleting the N-terminal UBL domain, suggesting the UBL suppresses enzymatic activity in native parkin (84). It was rationalized that this occurs through an association of the UBL with the catalytic RBR module of parkin, suppressing its activity. Consistent with this idea, addition of excess UBL domain to in-vitro ubiquitination assays showed a dose-dependent inhibition of parkin’s ubiquitin ligase activity (Figure 1.7 C). The experiments suggested that native parkin is autoinhibited, mediated by an intramolecular association of the UBL domain (Figure 1.7 D). The structural basis of this autoinhibition and how parkin becomes modulated to an activated state remained unknown.
Figure 1.7. Ubiquitination assays with untagged parkin showed the UBL domain suppresses ubiquitin ligase activity.

All data in this figure are reproduced, with permission from Chaugule et al. (2011). Parkin ubiquitination assays are shown, containing E1 (Uba1), E2 (UbcH7), E3 (various parkin species), His-tagged ubiquitin and ATP. Reactions were incubated at 37°C for 1 h and then quenched with SDS gel-loading buffer and boiled. Samples were run by SDS-PAGE and visualized by Western Blot or Coomassie staining as labelled. (A) N-terminal tags increase parkin’s ubiquitin ligase activity. (B) Deletion of the UBL (∆UBL) increases parkin ubiquitin ligase activity. (C) Dose-dependent inhibition of parkin’s ubiquitin ligase activity by an excess of UBL domain. (D) Domain layout of parkin, proposing an intramolecular association of UBL with the C-terminal RBR region of parkin.
1.7 Scope of Thesis

Although ubiquitin ligase activity is essential for its neuroprotective function, parkin is autoinhibited in its native state, most notably by its N-terminal UBL domain. At the start of this thesis, the only available structures of parkin were of the N-terminal UBL and the BRcat, both solved as the isolated domains (72,104). No information existed about the orientation of these structures with respect to the other domains of parkin. Further, it was unclear how the three RBR domains, which had not been structurally characterized, cooperate together to confer ubiquitin ligase activity.

The objectives of this thesis were:

1) Identify the mechanism by which the UBL domain maintains an autoinhibited structure to suppress parkin’s ubiquitin ligase activity.

2) Determine how the autoinhibited structure of parkin is modulated to a catalytically-competent state.

3) Assess how pathogenic mutations in parkin affect its activation and catalysis.

4) Rationalize a chemical mechanism of Rcat-mediated ubiquitination for parkin

To address these goals, a comprehensive biophysical characterization of autoinhibited parkin was undertaken. A high-resolution NMR strategy was developed to study isotopically-labelled human parkin in solution for the first time. These data were used to identify the intramolecular binding site of the UBL domain and further revealed allosteric structural changes that accompany UBL association and dissociation. Thermodynamic
parameters of both UBL and Ub binding were determined to rationalize how parkin’s inhibitory domain (UBL) and substrate (Ub) cooperate to regulate autoinhibition.

While this study was underway, multiple reports emerged identifying serine 65 phosphorylation of both Ub and parkin’s UBL as key inducers of parkin activity. To examine the roles of these phosphorylation signals, several methods were used to generate S65-phosphorylated Ub and UBL including: chemical modification, orthogonal translation in *E. coli*, and phosphorylation by catalytically active forms of PINK1. To understand the structural consequences of phosphorylation, the three-dimensional structure of S65-phosphorylated parkin UBL was determined. Further, the roles of phospho-ubiquitin and phospho-UBL were examined in reorganizing the structure of parkin to an activated state.

Finally, to investigate a chemical mechanism of catalysis in activated parkin, a detailed characterization of active site atoms in parkin was performed, using primarily NMR spectroscopy. Chemical biology approaches were used to generate an activated parkin–ubiquitin intermediate that will provide further mechanistic knowledge into ubiquitin transfer from parkin onto target mitochondrial substrates. Since all RBR ubiquitin ligases harbour conserved catalytic machinery, these studies likely extend our knowledge across the family of RBR ubiquitin ligase. Specifically with respects to parkin, this work provides valuable insight into the induction of the mitophagy pathway, which has key implications for cellular fidelity and neurodegenerative diseases, including PD.
1.8 References


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Chapter 2

Disruption of the Autoinhibited State Primes Parkin for Activation and Catalysis†

2.1 Introduction

Parkin belongs to the family of E3 ubiquitin ligases, known as RING1–BRcat–Rcat (RBR) ubiquitin ligases (3,4). This family of E3 ligases catalyze ubiquitination using a RING-HECT hybrid mechanism, employing both an E2-recruiting RING domain and a covalent catalytic intermediate with ubiquitin (5). Members of the RBR family are autoinhibited by unique accessory domains (see Figure 1.5) (6-8). Specifically, parkin contains an N-terminal Ubiquitin-like (UBL) domain that resembles parkin’s substrate, ubiquitin, in sequence and structure (see Figure 1.6). Native parkin is largely inactive but studies have shown deletion of the UBL domain dramatically increases parkin’s ubiquitin ligase activity (see Figure 1.7) (6,9). It was hypothesized this occurs through an intramolecular association of the UBL with parkin’s C-terminal region to suppress catalytic activity. At the beginning of this study, the structural basis of UBL-mediated autoinhibition was unknown. Only structures of the isolated UBL and BRcat were available (10,11), providing little insight into how the UBL regulates catalytic activity or how parkin recruits ubiquitin or E2 enzymes. To understand how the inhibitory and

† Data presented in this chapter has been published. It is reproduced here, with permission from:


catalytic domains function in parkin, an extensive biophysical and biochemical characterization of parkin was carried out in solution. It was shown that the tertiary structure of native parkin is dependent on the interaction of the UBL domain with the C-terminus to maintain a compact, globular structure. Using a new isotopic labelling strategy, amide resonance assignments were determined for the RORBR domain of parkin and these were used to characterize the autoinhibitory association of the UBL by NMR spectroscopy, complemented by high-resolution X-ray crystal structures. It was discovered that extensive intramolecular contacts in parkin convey allostERIC changes throughout the structure that are influenced by the UBL domain. Further, the effects of serine 65 phosphorylation of ubiquitin and parkin’s UBL was studied, a proposed activating signal for parkin (12-16), and it was found the role of the UBL is to maintain autoinhibition in the absence of this phosphorylation signal. Phospho-ubiquitin binding induces an allostERIC displacement of the UBL domain, revealing a ubiquitin recognition site used to assist recruitment of the E2–ubiquitin conjugate. Together, the data establish a framework for understanding the cycle of parkin inhibition and activation.

2.2 Materials and Methods

2.2.1 Cloning, Protein Expression and Purification

The DNA fragment encoding the human park2 gene was inserted into a pET SUMO expression system vector (Invitrogen). UBL, ΔUBL, RORBR, RINGO–RING1 and BRcat–Rcat constructs were then generated using a modified site directed mutagenesis
protocol (17). PCR reactions were then subjected to extensive DpnI digestion overnight at 37 °C. The following day, plasmid DNA was purified using a PCR clean-up kit (Bio Basic Inc) and 10 µL of plasmid product was transformed into competent MM294 cells and incubated on antibiotic-treated LB-agar plates overnight at 37 °C. Multiple colonies (four to six) were selected, cultured and their plasmid DNA sequenced to verify the desired mutagenesis.

All human parkin constructs (full-length parkin, UBL, ∆UBL, RORBR, BRcat–Rcat) were expressed as His-SUMO fusion proteins in *E. coli* BL21(DE3) cells (see Figure 2.1). Cells were grown at 37 °C in either LB or minimal media (described in detail below) supplemented with 500-700 µM ZnCl₂. Cells were induced at an OD₆₀₀ of 0.8 with 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (or 25 µM IPTG for full-length parkin) and incubated overnight at 16 °C. Cells were harvested in lysis buffer (50 mM Tris, 500 mM NaCl, 0.5 mM TCEP, 25 mM imidazole, pH 8.0), lysed by an EmulsiFlex-C5 homogenizer (Avestin), ultracentrifuged at 41,000 rpm (124,000 x g) and proteins purified by Ni²⁺ affinity using a HisTrap FF column on an AKTA FPLC (GE Healthcare). The column was extensively washed with wash buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, pH 8.0) and proteins eluted with elution buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 500 mM imidazole, pH 8.0). Fractions containing the protein of interest were pooled, Ulp1 protease was added to cleave the SUMO tag (~1:50 ratio Ulp1:parkin) and dialyzed overnight at 4 °C against wash buffer. After cleavage was complete, proteins were passed over the HisTrap FF column again in wash buffer and the flow through was collected, containing proteins of interest. Proteins were concentrated and subjected to size exclusion chromatography on a HiLoad Superdex 75 for a final purification step.
Drosophila Melanogaster parkin constructs (RORBR, BRcat, Rcat) were expressed as GST-fusion proteins in E.coli BL21 codon+RIL cells. Cells were grown in either LB or minimal media supplemented with 500-700 µM ZnCl$_2$. Cells were induced at an OD$_{600}$ of 0.8 with 0.5 mM of isopropyl b-D-1-thiogalactopyranoside (IPTG) (or 25 µM IPTG for full-length parkin) and incubated overnight at 16 °C. Cells were harvested in lysis buffer (25 mM Tris, 150 mM NaCl, 3 mM DTT, pH 7.5) and lysed by an EmulsiFlex-C5 homogenizer (Avestin), ultracentrifuged at 41,000 rpm (124,000 x g) and proteins purified by GST affinity on a GSTrap FF column on an AKTA FPLC (GE Healthcare). Proteins were eluted from the GST column using a high pH gradient elution buffer (25 mM Tris, 150 mM NaCl, 3 mM DTT, pH 10), followed by addition of 10% (v/v) neutralizer buffer (1 M Tris, pH 7.0). Fractions containing the protein of interest were pooled, TEV protease was added to cleave the GST tag (~1:50 ratio TEV:parkin) and proteins were dialyzed overnight against lysis buffer at 4 °C. After cleavage was complete, proteins were passed over the GSTrap FF column again in lysis buffer and the flow through was collected, containing proteins of interest. Proteins were concentrated and subjected to size exclusion chromatography on a HiLoad Superdex 75 for a final purification step.

2.2.2 MOPS Minimal Media

MOPS minimal media was prepared as either a 10X stock solution (for H$_2$O growths) or a 1X solution (for D$_2$O growths).

To prepare 1 L of 10X MOPS stock solution in H$_2$O: 83.7 g MOPS and 7.2 g tricine were added to a sterile flask and 500 mL autoclaved H$_2$O was added. The solution was basified
to pH 7.4 with ~20 mL of 10 M KOH. Next, 10 mL of 10 mM FeSO$_4$*7H$_2$O (freshly made) was added, causing the media to turn a yellow colour. 0.48 g of K$_2$SO$_4$, 5 µL of 1 M CaCl$_2$, 1.06 g MgCl$_2$*6H$_2$O and 29.22 g NaCl were then added. Finally, 10 mL of a 1000X micronutrient stock (standard trace elements used with M9 media) was added and the final volume was brought to one liter with autoclaved H$_2$O. The solution was filter sterilized (0.2 µm filter), removing much of the yellow colour, and aliquoted and stored at -20 °C until use. To prepare 1X media from 10X stock, 100 mL of 10X MOPS was added to 900 mL autoclaved H$_2$O along with 3 mL of 0.132 M K$_2$HPO$_4$. Glucose and ammonium chloride (either isotopically enriched or natural abundance) were added along with the appropriate antibiotic for the expression plasmid. For expression of parkin proteins containing zinc, 1.2 mL of a 0.5 M ZnCl$_2$ solution was always added to the media before induction.

To prepare 900 mL (1 kg) of 1X MOPS media in 100% D$_2$O: 7.54 g MOPS and 0.65 g tricine were added to a sterile flask and 500 mL D$_2$O was added. The solution was pH’d to 7.4 with KOD (potassium deuteroxide). Next, 0.9 mL of 10 mM FeSO$_4$*7H$_2$O (freshly made in D$_2$O) was added. 0.04 g of K$_2$SO$_4$, 0.5 µL of 1 M CaCl$_2$ (in D$_2$O), 0.09 g MgCl$_2$*6H$_2$O, 2.63 g NaCl and 0.07 g of K$_2$HPO$_4$ were then added. 0.9 mL of a 1000X micronutrient stock (standard trace elements used for M9 media but in D$_2$O) was added and the final volume was brought to one liter with D$_2$O. The solution was filter sterilized (0.2 µm filter) and used immediately. $^{13}$C/$^2$H-glucose (2 g) and $^{15}$N-ammonium chloride (1 g) were added as powder along with the appropriate antibiotic for the expression plasmid (antibiotics were separately prepared in D$_2$O for deuterated growths). For
expression of parkin proteins containing zinc, 1.2 mL of a 0.5 M ZnCl$_2$ solution (also prepared in D$_2$O) was always added to the media before induction.

To prepare 150 mL of 70% D$_2$O MOPS media: 15 mL of 10X MOPS stock solution, 1.5 mL of 0.132 M K$_2$HPO$_4$, 1.5 mL of 40% glucose, 1.5 mL of 10% NH$_4$Cl and the appropriate antibiotic (all in H$_2$O) were added to 82 mL 70% D$_2$O and 48 mL 100% D$_2$O.

For deuterated growths, cells were transitioned into 100% D$_2$O as follows. *E. coli* BL21(DE3) cells were freshly transformed and plated on LB-agar plates with the appropriate antibiotic. Once colonies were visible on the plates, 4 mL starter cultures were prepared in LB media and allowed to reach saturation at 37 °C. Saturated cultures were used to inoculate 150 mL 70% D$_2$O MOPS media to an OD$_{600}$ of 0.05 (150 mL of 70% D$_2$O MOPS media was sufficient for 2 L growths). 70% D$_2$O cultures were grown at 37 °C with shaking until an OD$_{600}$ of 0.8-0.9 was reached (avoiding senescence). Cultures were then centrifuged at 4000 x g (without cooling) and resuspended in 1-2 mL of pre-warmed 100% D$_2$O MOPS media. Resuspended cells were then added to the final 100% D$_2$O media until an OD$_{600}$ of ~0.04 was reached and allowed to grow at 37 °C until induction with IPTG at an OD$_{600}$ of ~0.8. Induction was at 16 °C for 20-24 hours.

### 2.2.3 Analytical Ultracentrifugation

Sedimentation velocity experiments were performed using a Beckman Coulter XL-A analytical ultracentrifuge equipped with an An-60 Ti rotor. Samples at 2-20 µM protein concentration were extensively dialyzed into 25 mM Tris HCl, 50 mM NaCl and 0.5 mM TCEP pH 8.0 before experiments. Samples (380 µl) and matching buffer blank (400 µL)
were prepared in a double sector cell (1.2 cm) with quartz windows. All experiments were conducted at 45,000 rpm at 20 °C with 30+ minutes for temperature equilibration. Velocity cells were scanned at equal intervals (60 scans total) and the absorbance at 280 nm measured with three replicates averaged per scan. Data were processed by c(s) distribution and analyzed using the program SEDFIT v12.1 (18). Non-linear regression was used to determine the frictional ratio \( \frac{f/f_0} \) for each species as well as the sedimentation coefficient corrected to 20 °C. All data were fit to an rmsd equal or less than 0.008 in SEDFIT.

2.2.4 Isothermal Titration Calorimetry

All isothermal titration calorimetry experiments were performed using a NanoITC (TA Instruments) at 25°C. Experiments were completed 2–3 times using freshly prepared proteins in 50 mM HEPES, 50 mM NaCl and 250 µM TCEP at pH 7.5. Proteins in the cell and syringe were extensively dialyzed in the same buffer before experiments. In all cases, concentrated RORBR was injected into UBL variants in the cell. The optimal concentrations of protein in the experiments were determined to be: 300 µM RORBR parkin titrated into 30 µM UBL, 650 µM RORBR titrated into 60 µM UBL\textsuperscript{S65E}, 500 µM RORBR titrated into 35 µM pUBL. Concentrations of RORBR and UBL were verified by absorbance at 280 nm when unfolded in guanidine HCl. The extinction coefficients used were 11,000 M\(^{-1}\)cm\(^{-1}\) for unfolded UBL and 47,900 M\(^{-1}\)cm\(^{-1}\) for unfolded RORBR. All data were processed and analyzed in NanoAnalyze v3.1.2 (TA instruments) to determine best fit values for \( K_A \), \( \Delta H \) and \( N \). \( \Delta S \) was calculated according to eq (1).

\[
\Delta G = -RT \ln K_A = \Delta H - T \Delta S
\]

\[
\Delta S = R \ln K_A + (\Delta H/T)
\]  

eq (1)
2.2.5 NMR Spectroscopy

All NMR experiments were collected at 25°C on a Varian Inova 600 MHz NMR spectrometer equipped with a triple resonance cryogenic probe and z-field gradients. All NMR data were collected in 25 mM HEPES pH 7.0, 100 mM NaCl, 0.5 mM TCEP, 10% D\textsubscript{2}O, 200 μM DSS as an internal reference and 200 μM imidazole as an internal pH indicator. All data were processed using NMRPipe (19) and analyzed using NMRViewJ (20).

NMR experiments for RORBR were all collected using a sensitivity-enhanced \textsuperscript{1}H-\textsuperscript{15}N TROSY pulse sequence without \textsuperscript{15}N decoupling (21,22). The \textsuperscript{1}H spectral window was 8000 Hz centered on 4.780 ppm and the \textsuperscript{15}N spectral window was 1700 Hz centered on 118.100 ppm. An acquisition time of 64 ms and a 1.7 s recycling delay were used in all cases. Triple resonance experiments (HNCA (23), HN(CO)CA (24), HN(CA)CB (25), HN(COCA)CB (24), HNCO (23), HN(CA)CO (24)) for assignment of the RORBR backbone were conducted using \textsuperscript{1}H-\textsuperscript{15}N TROSY-based pulse sequences with deuterium decoupling for fully deuterated proteins (26).

Heteronuclear NOE experiments (27) were performed using 500 μM \textsuperscript{2}H/\textsuperscript{13}C/\textsuperscript{15}N-labelled RORBR. Proton saturation was achieved through a 5-s irradiation following an 11-s relaxation delay. The equivalent non-saturated experiment contained a 16-s relaxation delay. Long recycling delays were essential due to the long T1 relaxation times of amides in deuterated RORBR, which must be completely recovered for accurate NOE measurement (28,29). Both saturated and non-saturated experiments were conducted in
duplicate, collected in an interleaving manner. NOEs were measured in NMRViewJ (20) and plotted as the average NOE ± standard error.

Interaction experiments were conducted using $^2\text{H}/^{12}\text{C}/^{15}\text{N}$-labelled RORBR and $^{13}\text{C}$-labelled Ub or UBL (or phosphorylated/phosphomimetic variants). $^1\text{H}-{^{15}}\text{N}$ TROSY-HSQC spectra were collected to monitor chemical shifts of backbone amide in RORBR as described above. $^1\text{H}-{^{13}}\text{C}$ HMQC spectra (30) were collected to monitor chemical shifts of side chain methyl groups in Ub or UBL. Amide chemical shift perturbations were quantified according to eq (2).

$$\text{CSP} = \sqrt{0.2\Delta\delta N^2 + \Delta\delta H^2} \quad \text{eq (2)}$$

Where $\Delta\delta N$ represents the change in nitrogen chemical shift (in ppm) and $\Delta\delta H$ represents the change in proton chemical shift (in ppm).

Paramagnetic relaxation enhancement (PRE) experiments were used to confirm the orientation of the pUb molecule with respect to the RORBR protein. Briefly, $^2\text{H}/^{12}\text{C}/^{14}\text{N}$-labelled cysteine-substituted Ub (L8C or K48C) was expressed in minimal media, purified and phosphorylated using *Pediculus humanus* GST-PINK1 as described in Chapter 3. Proteins were spin-labelled with 3-(2-iodoacetamido)-PROXYL (Sigma Aldrich) by incubating each protein with 10 molar equivalents of spin label for 2 h at room temperature and then dialyzed exhaustively at 4°C. ESI-MS confirmed quantitative (> 95%) phosphorylation and successful incorporation of spin label (~90 and ~60% spin label incorporation for L8C and K48C, respectively). $^1\text{H}-{^{15}}\text{N}$ TROSY-HSQC spectra were
collected monitoring 400 μM $^2\text{H}/^{13}\text{C}/^{15}\text{N}$-labelled RORBR before and after the addition of 1.2 molar equivalents of each nitroxide-labelled pUb variant. Identical spectra were collected following the addition of 10 molar equivalents of fresh ascorbic acid to quench the nitroxide spin tag. PRE factors ($I/I_0$) for RORBR were determined in NMRViewJ (20) by measuring peak intensities with the active and quenched nitroxide spin tag.

2.3 Results

2.3.1 Expression and purification of parkin constructs

At the time this study was conceived, few biochemical experiments had been performed with untagged, human parkin owing to the protein’s poor solubility without an appropriate affinity tag. His or GST-fusions of full-length parkin produced relatively small quantities of soluble recombinant protein with protease degradation evident even under expression at 16 °C (31). It was determined that recombinant expression of human parkin in *E. coli* was markedly improved using an N-terminal His-SUMO(small ubiquitin-like modifying protein)-fusion tag. Interestingly this tag folds into a stable protein with similar structure to parkin’s substrate, ubiquitin, and parkin’s own UBL domain. Using site directed mutagenesis, over 6 parkin constructs were generated employing an N-terminal His-SUMO tag (Figure 2.1 A): Full length parkin (1-465), UBL (1-76), ΔUBL (77-465), RORBR (141-465), BRcat–Rcat (321-465), and Rcat (410-465). While UBL, Rcat and BRcat could all be expressed as isolated domains, RINGO and RING1 domains were only soluble as part of longer fragments (Figure 2.1 B). This
Figure 2.1. Overview of parkin constructs used and their recombinant purification.

(A) Domain map of parkin constructs used in this study. Residue numberings denote construct boundaries.

(B) SDS-PAGE gels showing representative affinity purification of each construct. An asterisk (*) represents the final product used for experiments. Note: RINGO-RING1 construct was not soluble and could not be purified.
suggested RINGO and RING1 may have an indispensable position in the overall structure in parkin, central to the protein folding. All constructs were soluble in LB and minimal media (as described in section 2.3.3) supplemented with 0.5-0.7 mM ZnCl$_2$ and protein induction only after cells had been cooled to 16 °C. The SUMO-fusion protein is easily removed from parkin after purification by the SUMO-specific protease, Ulp1, to generate native, untagged protein. The use of untagged parkin in all experiments was essential given the known influence of affinity tags on parkin’s E3 ligase activity (9). All the above constructs were purified to homogeneity using gel filtration chromatography as a final purification step prior to all experiments.

2.3.2 Parkin maintains a compact structure mediated by the UBL domain

To understand the parkin structure in the context of the full-length protein, analytical ultracentrifugation was used to study its overall shape in solution. Under the conditions used, full-length parkin sediments as a monomer with a sedimentation coefficient of 4.1 S (Figure 2.2 A). Interestingly the frictional coefficient, f/f$_0$, which reports the offset in shape of a molecule relative to a perfect sphere, increased from 1.38 to 1.53 in ∆UBL–parkin, where the UBL domain is removed. This suggests parkin adopts a globular structure dependent on the presence of the UBL domain. This observation was also observed by small-angle X-ray scattering (SAXS) data collected by our colleagues at the University of Dundee. The results show removal of the UBL domain leads to an extended conformation of parkin, with a larger radius of gyration (32.0 Å; D$_{max}$ 110 Å) than the 8-kDa larger full-length protein (29.0 Å; D$_{max}$ 95 Å) (Figure 2.2 B, C). These results indicate the molecular envelope of ∆UBL–parkin has a more extended shape, and that in the presence of the UBL domain parkin adopts a more compact conformation. The most
Figure 2.2. Parkin maintains a compact tertiary structure mediated by the UBL.  
(A) Sedimentation velocity experiments of parkin, ΔUBL, and BRcat–Rcat. All data were analyzed using the Lamm equation and fit to a c(s) distribution. Sedimentation coefficients, corrected to 20 °C and in H₂O, were determined to be 4.1 S for full-length parkin, 3.5 S for ΔUBL and 2.2 S for BRcat–Rcat. Fitted frictional coefficients (f/f₀) were calculated to be 1.38 for full-length parkin, 1.53 for ΔUBL and 1.31 for BRcat–Rcat.  
(B) Superposition of scattering data (left) and distance distribution (right) plots from purified human parkin samples, wild-type (red) and ΔUBL-parkin (blue). The plots show the quality of the data and the radius of gyration.  
(C) Representative and averaged ab initio models of wild-type parkin (red, left) and ΔUBL (blue, right). Two views for each protein are shown rotated 90° about the x axis.  
(D) ¹H-¹⁵N HSQC spectra of UBL domain alone (left spectrum) and after addition of 3 molar equivalents unlabelled ΔUBL (right spectrum). The significant broadening of UBL resonances reports an interaction with ΔUBL, forming a higher molecular weight species.
logical rationale for these findings is that the UBL interacts with the C-terminal region of parkin through an unstructured linker from residues 77-140, as was suggested by Chaugule and colleagues (6). To confirm this, $^1$H-$^{15}$N HSQC spectra of $^{15}$N-labelled UBL were acquired by NMR spectroscopy. Given the small molecular weight of the UBL domain (~8.5 kDa), a spectrum is obtained where all backbone amide correlations are readily observed (Figure 2.2 D, left). Conversely, the equivalent sample of $^{15}$N-labelled UBL with an excess of unlabelled ΔUBL produced a severely broadened spectrum, with few resonances visible at equivalent contour levels (Figure 2.2 D, right). The result is indicative of an interaction of UBL with ΔUBL, forming a higher molecular weight species with attenuated T2 relaxation time, resulting in the broadened spectrum.

Since the NMR data suggested an intramolecular interaction between UBL and ΔUBL, we also wondered if other intramolecular interactions might exist within the RINGO–RING1–BRcat–Rcat domains. At the time of the study, human parkin could not be expressed in minimal media due to poor protein solubility, presumably due to misincorporation of the eight Zn$^{2+}$ ion cofactors required for protein folding (31). As a substitute, the Rcat, BRcat–Rcat and RORBR domains were expressed from Drosophila melanogaster (fly) parkin in minimal media to acquire NMR spectra. The analogous domains in fly parkin were significantly more soluble in E. coli and sufficient protein was purified to conduct NMR experiments. Comparison of HSQC spectra for Rcat and BRcat–Rcat show the resonances in Rcat overlap nearly perfectly in the tandem domain, suggesting the two domains do not interact in parkin (Figure 2.3 A). This was further supported by NOE data showing the 26-residue linker between the two proteins is unstructured (PDB: 2M48, BMRB: 18990). Conversely, the spectrum of RORBR showed...
Figure 2.3. BRcat and Rcat are remote in parkin but interact with RINGO and RING1
(A) Superposition of $^1$H-$^{15}$N HSQC spectra for fly parkin BRcat–Rcat (black contours) with spectra for the individual BRcat (pink) and Rcat (blue) domains. The alignment of resonances in the tandem protein indicates these domains do not interact together. (B) Superposition of $^1$H-$^{15}$N HSQC spectra for RORBR (red contours) and BRcat–Rcat (teal contours). The large number of chemical shift changes indicates that RINGO–RING1 interact with BRcat–Rcat. Residue assignments in BRcat and Rcat that undergo the largest changes in chemical shift are indicated.
many chemical-shift changes were observed in both BRcat (including D367, V380, V381 and N384) and Rcat (including G447, A457, W465 and C475) domains in the context of RORBR (Figure 2.3 B). This indicates that the environments of these residues in BRcat and Rcat are changed by the presence of the RINGO and RING1 domains. The interpretation of the data is that the RINGO and/or RING1 domains are interacting with the BRcat–Rcat structure forming a more compact arrangement in native parkin.

2.3.3 Development of a high-resolution NMR strategy for human parkin

While the results in Figure 2.3 represented the first report of NMR spectra of parkin’s C-terminus encompassing all four zinc binding domains, the quality of the data precluded a high-resolution analysis of protein interactions in parkin. The NMR spectra suffer from a lack of resonance intensity, broad linewidths, and significant overlap in both $^1$H and $^{15}$N dimensions. This arises from several factors including limited sample concentration and the relatively large molecular weight of RORBR (~37 kDa). In NMR spectroscopy, molecules larger than about 20 kDa experience significantly slower molecular tumbling and decreased transverse (T2) relaxation times, the major source of signal attenuation in protein samples (32). This manifests as increased (broad) linewidths and often-undetectable resonance signals. To overcome these challenges, we sought to design a minimal media that could produce a significantly increased fraction of soluble human parkin, and that could be broadly applied to different isotopic labeling schemes for NMR.

As previously mentioned, human parkin constructs comprising the C-terminal zinc binding domains were largely insoluble in M9 minimal media, by far the most widely used minimal media used for isotopic labeling (Figure 2.4). Compared to LB media,
Figure 2.4. Solubility of human parkin RORBR in different expression media.
SDS-PAGE showing solubility of overexpressed His-SUMO-RORBR (indicated by black arrow) from E. coli BL21(DE3) cells grown in LB media, M9 minimal media, and MOPS minimal media. Gel samples were taken directly before induction with IPTG (uninduced), 16 hours after induction with IPTG (induced), and following cell lysis and ultracentrifugation to separate soluble and insoluble fractions.
parkin RORBR expressed in M9 minimal media was almost completely insoluble, despite similar overall protein expression levels. Therefore, other minimal media compositions were explored. While M9 minimal media is buffered by high concentrations of phosphate, alternate culture media can be designed using other buffering agents, for example, with 3-(N-morpholino)propanesulfonic acid (MOPS) to maintain the media buffering capacity. A medium using MOPS buffer was developed by Neidhardt and colleagues in 1974 and was initially targeted towards $^{32}$S-labelling of proteins and DNA (33). Initial trials using this recipe were promising in attaining soluble parkin RORBR from minimal media. It was suspected the enhanced solubility was from improved protein folding and zinc incorporation in RORBR domains due to significantly less phosphate available to deplete zinc in the insoluble form of zinc(II) phosphate. Therefore, the Neidhardt recipe was modified to further reduce the phosphate concentration to minimal levels necessary for $E.coli$ growth and an excess of zinc chloride was added (Table 2.1). In the modified recipe, hereafter referred to as “MOPS minimal media”, human RORBR-parkin solubility was dramatically improved (Figure 2.4). Further, $^{13}$C-glucose and $^{15}$N-ammonium chloride could be used as the sole carbon and nitrogen sources to obtain isotopically labelled protein for NMR studies.

While employing the MOPS minimal media significantly increased protein concentration in our NMR samples ($\geq$500 $\mu$M), this does not address the unfavourable T2 relaxation properties of such a large molecule. The problem of rapid T2 relaxation in large proteins is often overcome by deuteration ($^2$H incorporation) of all non-exchangeable protons in the protein backbone and sidechains (34,35). This seeks primarily to reduce proton-proton dipolar coupling in the sample by replacing most protons in the molecule with deuterium.
Table 2.1. Composition of MOPS minimal media.
Final chemical composition of MOPS minimal media compared to standard M9 minimal media. An identical amount of carbon/nitrogen sources (as glucose/NH₄Cl) and trace micronutrients (including B, Mo, Co, Cu, Mn) were supplemented to both media.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>MOPS</th>
<th>M9</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>0.4 mM</td>
<td>—</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>—</td>
<td>22 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>—</td>
<td>42 mM</td>
</tr>
<tr>
<td>MOPS</td>
<td>40 mM</td>
<td>—</td>
</tr>
<tr>
<td>Tricine</td>
<td>4.0 mM</td>
<td>—</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.5 mM‡</td>
<td>0.5 mM‡</td>
</tr>
<tr>
<td>NaCl</td>
<td>50 mM</td>
<td>8.6 mM</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>276 µM</td>
<td>—</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>—</td>
<td>2 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>523 µM</td>
<td>—</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.5 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>10 µM</td>
<td>1 µM</td>
</tr>
</tbody>
</table>

‡ Supplemental zinc is added specifically for parkin expression and is not required for cell growth.
Therefore it was attempted to express RORBR–parkin in MOPS minimal media generated in 100% D$_2$O and using $^{15}$N-ammonium chloride and $^2$H/$^{13}$C-glucose as the sole nitrogen and carbon sources. It was determined that E. coli can successfully culture in the MOPS minimal media in 100% D$_2$O with a growth time from inoculation to an OD$_{600}$ of 0.8 at 37 °C on the order of 10 hours (Figure 2.5 A). Further, overexpressed RORBR was properly folded and could be purified to homogeneity by standard affinity purification procedures and gel filtration chromatography (Figure 2.5 B). NMR spectra of perdeuterated RORBR clearly show the increase in spectral quality when compared to the protonated spectrum (Figure 2.5 C, D). Dozens of amide resonances that were undetectable in the protonated spectrum are readily observed in the deuterated spectrum. The well-dispersed nature of the deuterated spectrum also confirms the protein remains well-folded and adopts significant secondary structure in solution. In large, deuterated proteins, spectral resolution can be enhanced by employing a Transverse Relaxation Optimized Spectroscopy (TROSY) pulse sequence (21). TROSY experiments select for only the slowest relaxing (most narrow) component of an NMR multiplet signal, discarding the faster relaxing components that only serve to broaden the final signal. While the TROSY effect is theoretically most pronounced at magnetic field strengths of 900 MHz–1 GHz, significant benefits were observed when spectra of perdeuterated RORBR were collected with a TROSY-HSQC pulse sequence at 600 MHz (Figure 2.5 E). The spectra show increased resolution in the $^{15}$N dimension and more equalized peak intensities across the spectrum.

Next, triple resonance experiments were collected with the goal of assigning the protein backbone of RORBR. $^1$H-$^{15}$N-$^{13}$C-correlated HNCA, HN(CO)CA, HN(CA)CB,
Figure 2.5. MOPS minimal media can be employed to produce perdeuterated parkin for TROSY-NMR experiments.

(A) Growth curve of *E. coli* in MOPS minimal media in 100% D₂O, supplemented with ²H/¹³C-glucose and ¹⁵N-ammonium chloride. Cell density was measured by absorbance at 600 nm. The marked arrow represents the time of induction with 0.7 mM IPTG and temperature reduction to 16 °C. (B) SDS-PAGE of purification of ²H/¹³C/¹⁵N-labelled RORBR parkin. The final lane represents the purified protein used for NMR experiments. (C-E) ¹H-¹⁵N correlated NMR spectra of parkin RORBR showing significant improvement of spectral intensity upon perdeuteration (D panel) and spectral linewidth upon application of a TROSY pulse sequence (E panel).
HN(COCA)CB, HNCO and HN(CA)CO experiments were all obtained using a TROSY-HSQC pulse sequence and deuterium decoupling optimized for large, perdeuterated proteins (Figure 2.6 A-F) (26). Using sequential backbone assignment techniques, over 95% of non-proline amide resonances in RORBR–parkin could be assigned. This provided atomic probes evenly distributed throughout the entire structure of parkin that can be used to define protein interaction sites, dynamics and conformational changes in the protein backbone.

The amide assignments were used to measure $^1$H-$^{15}$N heteronuclear NOEs, which provide valuable information on flexibility and dynamics of the protein backbone (27). In these experiments, two $^1$H-$^{15}$N-correlated spectra are collected where one experiment contains a 5 second proton saturation period prior to the pulse sequence and the other spectrum contains a delay of equivalent length. Amides that undergo rapid motions (on the ps to ns timescale) show decreased intensity in the proton-saturated spectrum. Analysis of heteronuclear NOE values across the RORBR backbone showed significant rigidity within all 4 zinc-binding domains, with NOE values near 0.7 (Figure 2.7). Notably, the entire BRcat domain exhibited decreased NOE intensity, indicative of overall greater flexibility in this domain. However, the smallest NOEs were observed throughout the “tether” linking BRcat and Rcat domains, suggesting this is the most flexible region of the protein. Interestingly, the tether domain appears to be separated into two regions, separated by a significantly less flexible section from residues E395-E404. The data show RORBR–parkin adopts significant tertiary structure, with increases in backbone flexibility observed in BRcat and tether regions.
Figure 2.6. TROSY triple resonance experiments for RORBR backbone assignment. 
$^1\text{H}$$^\text{13}$C projections of three-dimensional NMR experiments used for RORBR backbone assignment. The nitrogen spectral window for all experiments was 103.5-131.5 ppm. All experiments were collected using a TROSY pulse sequence and deuterium decoupling. The experiments shown are as follows: (A) HNCA, (B) HN(CO)CA, (C) HN(CA)CB, (D) HN(COCA)CB, (E) HNCO, (F) HN(CA)CO.
Figure 2.7. Backbone dynamics in parkin RORBR.

Heteronuclear NOE analysis for parkin RORBR. (A) The values plotted are the average NOE of two independent experiments (indicated by error bars) at 600 MHz. Only resolved resonances in the $^1$H-$^{15}$N HSQC spectrum were selected for analysis. The average NOE for data within 1 SD of the mean was 0.71, indicated by a dashed line. Decreased NOE values in the BRcat and tether domains are indicative of increased mobility in the protein backbone. (B) Representative 1D proton slice, exemplifying the differences in amide resonance intensity for 4 residues in the non-saturated (black) and saturated (red) experiment pair.
It will be addressed here that during the first year of this study, three independent research
groups published crystal structures of the RORBR domain from human and rat parkin, all
released in May 2013 (36-38). The three crystal structures were all in remarkable
agreement, with no major differences between structures. Therefore, for all further
discussions of the free RORBR structure, the higher resolution, 1.6 Å human RORBR
structure by Riley and coworkers is referenced (PDB: 4I1H, Figure 2.8) (37). As the
earlier experiments had predicted, the RORBR domain adopts a relatively compacted
shape, with extensive intramolecular contacts between the four zinc-binding domains.
The Rcat and BRcat domains are positioned opposite each other, separated by the long
“tether” spanning residues V380-T415. In all crystals, the tether contains significantly
less electron density, reflected in high temperature(B)-factors, which preclude the
observation of structure in most of this region. However, the tether adopts a 2-turn helix
in all structures from residues E395-R402, which makes extensive contacts with the
RING1 domain and is well defined, reflected by lower B-factors in these atoms. This is in
remarkable agreement with the heteronuclear NOE data for RORBR that showed
flexibility in the tether, with a significant gain of structure from residues E395-E404
(Figure 2.7). Also in agreement with this data is the increased flexibility of the BRcat
domain, which is the least defined zinc-binding domain across all structures. Multiple
loops in this domain are unobserved in the different crystal structures, suggesting a lack
of rigidity. While an atomic structure of RORBR was unlikely to be solved \textit{ab initio} by
NMR owing to the inability to collect NOE experiments on a deuterated protein, the
crystal structures provided a remarkable opportunity to use the NMR assignments to
uncover dynamics, interaction surfaces and conformational changes that cannot be
Figure 2.8. Structure of human parkin RORBR.
Rendering of the 1.6 Å crystal structure of human parkin RORBR (Riley et al., 2013, PDB: 4I1H). Two views are shown, rotated 180° around the x-axis. The crystallized fragment (residue 141 to C-terminus) lacks the UBL domain. Domains are labelled and coloured according to the scheme in Figure 2.1. Linkers where electron density was not observed are shown as dashed lines. The eight zinc atoms in the structure are represented as grey spheres.
revealed by crystallography. In this manner, NMR spectroscopy is highly complementary to X-ray crystallography when examining these high-molecular weight systems.

2.3.4 Autoinhibitory association of the UBL domain with RORBR

The RORBR backbone resonance assignments with the complementary RORBR crystal structure allowed for detailed identification of protein interaction sites using chemical shift perturbations from NMR experiments. Atoms near an interaction site will experience a change in their chemical environment upon ligand binding, which will be reported by a chemical shift change. Given the strong inhibitory role of the UBL on parkin activity (6), we sought to determine the UBL-binding interface that maintains autoinhibition in parkin. NMR interaction experiments were conducted by adding $^{13}\text{C}$-labelled UBL domain to $^2\text{H}/^{12}\text{C}/^{15}\text{N}$-labelled RORBR and $^1\text{H}-^{15}\text{N}$ TROSY-HSQC spectra were collected as well as $^1\text{H}-^{13}\text{C}$ HMQC spectra. The selected isotopic-labelling scheme allows parallel data experiments from both the RORBR (observing amide chemical shifts) and UBL (observing methyl chemical shifts) perspectives while preserving spectral quality by deuterating the RORBR component. The HSQC spectrum of RORBR undergoes significant chemical shift changes upon addition of 1 and 3 molar equivalents UBL domain, consistent with a tight interaction. Slow and intermediate chemical shift exchange was observed in RING1, BRcat and tether domains upon UBL binding (Figure 2.9). Significant chemical shift changes, often beyond detection in the bound state, were observed in the region connecting RING1 and BRcat (helix “H3”, residues I298-G329). Notably, UBL binding does not seem to influence chemical shifts in the Rcat or RINGO domains, suggesting these domains do not contribute to the autoinhibitory UBL interaction.
Figure 2.9. RORBR interaction with the UBL observed in trans.

(A) Selected regions from 600 MHz $^1$H-$^{15}$N TROSY HSQC spectra showing 200 µM $^2$H/$^{13}$C/$^{15}$N-labelled RORBR (black contours) and following the addition of 300 µM of the UBL domain (red contours). Residues shown that undergo chemical shift changes are F277 (RING1), Y312 and Y318 (helix “H3”) and G385 (tether following BRcat domain and not visible in the crystal structure). (B) Chemical shift perturbation plot for RORBR domain following addition of UBL. CSP was calculated as $CSP = \left[ \Delta\delta_{HN}^2 + 0.2\Delta\delta_N^2 \right]^{0.5}$. Grey bars indicate resonances that broadened or shift beyond assignment in the bound species. A dashed line (0.62) represents the mean CSP + 1.5 standard deviation.
To determine the complementary interaction interface on the UBL domain, changes in the \(^1\text{H}^{13}\text{C}\) HMQC spectrum of UBL were monitored upon binding of RORBR. It has been demonstrated that methyl resonances in an HMQC-type experiment are optimized for the TROSY effect exploited in the \(^1\text{H}^{15}\text{N}\) experiments described previously (30). Therefore, methyl resonances were used as probes to monitor the association of UBL and RORBR, which form a 46 kDa protein complex. The HMQC spectrum of UBL shows numerous slow exchange chemical shift changes as the protein is saturated with RORBR (Figure 2.10). Methyl groups from residues I2, L41, I44, A46, L50, L61, I66 and V70 show large chemical shift changes, reporting their involvement in formation of the UBL–RORBR complex. Many of these residues localize to the \(\beta\)-sheet face of the UBL domain, including the hydrophobic patch (I44, A46, V70).

Mapping the chemical shift perturbations observed in UBL and RORBR upon their association provided an interaction surface on each of the two molecules, which have known crystal structures (10,37). As stated above, the UBL interaction surface involves its hydrophobic patch, as supported by numerous methyl chemical shift perturbations in this region (Figure 2.11 A). Complementary interacting residues mapped on to the RORBR structure were localized to one half of the structure, with extensive chemical shifts observed in RING1, BRcat and tether domains and no significant chemical shifts in RINGO or Rcat domains (Figure 2.11 B). However, the RORBR interaction surface was less conclusive, as there appeared to a larger interface area than could be explained by UBL binding alone. This suggested there might be a conformational change that accompanies UBL binding that manifests as additional chemical shift changes.
Figure 2.10. UBL interaction with RORBR observed *in trans*. Selected methyl regions for 600 MHz $^1$H-$^{13}$C HMQC spectra of 150 µM $^{13}$C-labelled Ubl domain alone (black contours) and in the presence of 1.5 molar equivalents $^2$H/$^{12}$C/$^{15}$N-labelled RORBR (red contours). Based on the crystal structure, chemical shift perturbations for A46, L61 and L66 face the tether region following the BRcat domain where A383-A390 were not observed indicating these residues in the UBL domain transiently interact with this region.
Figure 2.11. The autoinhibitory interface is observed in solution and in crystallo.

(A) RORBR surface representation with UBL interaction surface highlighted as observed by solution NMR experiments. Yellow represents amide chemical shift perturbations \( \geq 1.5 \) standard deviations above the mean (See also Figure 2.8). Red represents amide chemical shifts that are shifted or broadened beyond reassignment in the bound species.

(B) UBL surface representation with RORBR interaction surface highlighted as observed by solution NMR experiments. Yellow represents methyl chemical shifts that are significantly perturbed (\( \geq 1.5 \) standard deviations above mean) upon RORBR binding.

(C) 1.8 Å crystal structure of autoinhibited human parkin (PDB: 5C1Z). Residues 84-144 in the flexible “linker” were deleted to assist with crystallization. Each domain is labelled as in Fig 2.1. The eight zinc atoms in the structure are represented as grey spheres.
To investigate the UBL–RORBR interaction surface further, our colleagues at the University of Dundee crystallized a construct of parkin where the flexible linker connecting UBL and RORBR domains (residues 84-144) was deleted to assist with crystallization (construct termed UBLRORBR). An X-ray crystal structure of UBLRORBR was solved and refined to 1.8 Å resolution (PDB: 5C1Z, Figure 2.11 C). Residues 354 and 357–360 in the BRcat domain were missing in the density, as are residues 383–390 and 406–413 in the tether, similar to what was observed in isolated RORBR crystal structures described previously. The position of the UBL domain, located between the RING1 and BRcat domains, is in excellent agreement with the solution NMR data (Figure 2.11 A, B). The interface formed between the UBL domain and RORBR buries ~2,150 Å² accessible surface area. The primary contact is between the UBL and RING1 domains, where the core interface is stabilized by hydrophobic interactions mediated by I44 and V70 of the UBL domain and L266, V269 and T270 of RING1. Interestingly, the UBL lies adjacent to the tether region that is unresolved in the crystal structure. The NMR data showed chemical shift changes occur in this region including A383, S384 and G385, indicating these residues interact with the UBL at least transiently.

Because the NMR data also suggested there may be a conformational change as a consequence of UBL binding, the UBLRORBR structure was compared with the truncated RORBR structures. The global RORBR structure in the autoinhibited (UBL-bound) form is surprisingly consistent with structures of the free RORBR, with RMSDs between 1.4 Å (compared to rat RORBR, PDB: 4K7D) and 1.8 Å (compared to human RORBR, PDB: 4I1F). Interestingly, when the structures are superimposed using the RINGO/Rcat domains as the reference point (which exhibited negligible chemical shift
changes upon UBL binding), the flexibility of the other domains is more pronounced (Figure 2.12 A). In particular, the RINGO/RING1 interface widens in the absence of the UBL domain as the BRcat domain swings ~12 Å away from its position when the UBL domain is attached. This movement propagates from the RINGO/RING1 interface through a long, bent helix comprising residues K299-G329 (termed “helix H3”), which is the site of numerous large chemical shift perturbations that were observed in the NMR interaction experiments. Close examination of this interface in UBLRORBR with that in RORBR reveals a number of changes (Figure 2.12 B). A salt bridge formed by R234 and E404 pinning the domains together is absent in the RORBR structure, but present when the UBL domain is bound. A hydrogen bond between H227 and E300 is observed in the presence of the UBL domain, but not in the RORBR structures. In the UBL-bound structure, the side chains of H302 and E300 are facing into the interface, while in the RORBR structures, these residues are flipped out ~180° away from the interface (Figure 2.12 C). This latter conformation is observed in all RORBR structures, suggesting this is a consequence of the absence of the UBL domain, rather than an artifact of crystal packing. These subtle, but significant conformational changes upon UBL binding provide an explanation for the chemical shift perturbations that could not be reconciled by the UBL binding site alone. The UBLRORBR crystal structure and complimentary NMR data therefore suggest a conformational hinge in parkin centered near the RING0/RING1 interface that is regulated by the UBL.

2.3.5 S65 phosphorylation modifies UBL and Ub interactions with parkin

Numerous studies published in 2012-2014 discovered that parkin’s ubiquitin ligase activity is dramatically stimulated by the kinase PINK1. The first site of phosphorylation
Figure 2.12. The RINGO/RING1 interface is a hinge.

(A) Overlay of autoinhibited UBLRORBR parkin (PDB: 5C1Z, 1.8 Å crystal structure) and RORBR (PDB: 4I1H, 1.6 Å crystal structure lacking the UBL domain). Removal of the UBL causes a hinge opening at the RINGO/RING1 interface that results in a swing of the BRcat domain. 

(B) Overlay of the RINGO/RING1 interface from UBLRORBR (coloured) and RORBR (grey). Green dashes show distances in Å between residues in UBLRORBR, and red dashes show distances between residues in RORBR.

(C) Comparison of the hinge in UBLRORBR and RORBR.
was found to be serine 65 in parkin’s UBL domain (12,13). However, subsequent studies showed that parkin’s substrate, ubiquitin, is also phosphorylated by PINK1 at the equivalent serine 65 position and this also dramatically increases parkin’s ubiquitination activity (14-16). It was hypothesized that phosphorylation of the UBL weakens the autoinhibitory association with RORBR, as serine 65 faces the tether in the UBLRORBR structure. However, a rationale of how the analogous phosphorylation of ubiquitin would also induce parkin activity was unclear. To examine the effects of serine 65 phosphorylation on the association of UBL with RORBR, isothermal titration calorimetry was used to measure thermodynamic properties of the interaction in trans. Phosphorylation of serine 65 in the UBL (referred to as “pUBL”) weakens the affinity of the RORBR interaction by an order of magnitude (K_\text{D} of 3.8 vs 31 \mu\text{M} for UBL and pUBL, respectively) (Figure 2.13). This was observed as significantly less binding enthalpy in the ITC isotherms when the UBL was phosphorylated (\Delta H of -44 vs -15 kJ/mol for UBL and pUBL, respectively). As expected from the crystal structures, pUBL interacts exothermically with RORBR with negative enthalpy (-15 kJ/mol) and small entropy (+33 J/mol*K) changes consistent with new ionic and hydrophobic interactions formed and minimal overall structural change (Table 2.2). The data also show that UBL binding to RORBR is enthalpically driven regardless of whether the phosphomimetic (UBL^{S65E}) or pUBL is used, indicating the phosphomimetic UBL and pUBL proteins likely have similar binding modes to RORBR. This suggests that S65 phosphorylation of parkin increases ubiquitin ligase activity by weakening the autoinhibitory association of the UBL.
Figure 2.13. Phosphorylation of the UBL weakens the autoinhibitory interaction.
Representative isothermal Titration Calorimetry (ITC) experiments showing binding of RORBR to (A) UBL, (B) UBL$^{S65E}$ and (C) UBL phosphorylated at Ser65 (pUBL). Best-fit dissociations constants and binding stoichiometry are indicated.
# Table 2.2. Thermodynamic properties for parkin activation

All values derived from Isothermal Titration Calorimetry experiments at 25 °C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activator</th>
<th>$K_D$ (µM)</th>
<th>$N$</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (J/mol*K)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. R0RBR parkin with UBL and Ub</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R0RBR</td>
<td>UBL</td>
<td>3.8 ± 1.3</td>
<td>0.98</td>
<td>−44</td>
<td>−44</td>
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<tr>
<td></td>
<td>UBL (S65E)</td>
<td>5.3 ± 1.2</td>
<td>0.98</td>
<td>−46</td>
<td>−54</td>
</tr>
<tr>
<td></td>
<td>pUBL</td>
<td>31 ± 17</td>
<td>1.04</td>
<td>−15</td>
<td>+37</td>
</tr>
<tr>
<td></td>
<td>Ub</td>
<td>67 ± 1.0</td>
<td>1.05</td>
<td>+31</td>
<td>+184</td>
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<tr>
<td></td>
<td>Ub (S65E)</td>
<td>6.6 ± 0.1</td>
<td>1.10</td>
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<td>+261</td>
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<tr>
<td></td>
<td>pUb</td>
<td>0.016 ± 0.002</td>
<td>0.93</td>
<td>+32</td>
<td>+261</td>
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<td><strong>B. Full-length parkin with UBL and Ub</strong></td>
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<tr>
<td>Parkin</td>
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<td>N.O. ‡</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Ub</td>
<td>45 ± 9.0</td>
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<tr>
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<td>+293</td>
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<tr>
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<td>pUb</td>
<td>0.021 ± 0.006</td>
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</table>

‡ N.O., binding not observed
To observe whether a similar effect is observed when ubiquitin is phosphorylated, thermodynamic properties were measured for Ub, Ub^{S65E} and phosphorylated Ub (pUB) in a similar manner. Remarkably, the affinity of Ub for RORBR was enhanced by over 4000-fold upon phosphorylation of S65 (Table 2.2). In contrast to UBL binding, the binding of pUb to RORBR is an endothermic binding event marked by large positive enthalpy (+32 kJ/mol) and entropy (+261 J/mol*K) changes. The large positive entropy change signifies that the pUb–RORBR interaction is driven by an increase in disorder in the system, likely a result of a loss of structure in parkin that accompanies pUb binding. Interestingly, a previous three-dimensional structure of the BRcat domain (PDB: 2JMO, comprising residues 308-384) shows little structure in the region that contains helix H3, the site of several chemical shift changes upon RORBR interaction with pUb (11). This is in contrast to the RORBR crystal structure (Figure 2.8) that shows a well-defined, albeit bent helix that interacts with the first Zn^{2+}-binding site of the BRcat domain and the RING1 domain. Together, these data show that pUb binding to the RINGO/RING1 hinge interface results in a concomitant loss of structure near helix H3 and the BRcat domain.

The significant thermodynamic differences between pUBL and pUb interactions with RORBR suggest the two molecules use different binding mechanisms. Therefore, to understand how pUb binds to RORBR chemical shift perturbation experiments were performed using TROSY NMR experiments as described previously for UBL. Addition of pUb to RORBR results in a cluster of residues with significant chemical shift changes including Y149, C150, K151 and G152 (in RINGO); R275, F277, V278, D280, Q282, G284, Y285 and S286 (in RING1); and I306, G308, E309, Y312 and N313 (helix H3). These residues surround the hinge at the RINGO/RING1 interface that was modified by
UBL binding and suggested the hinge region is a pUb-binding site. To determine the orientation of pUb with respect to parkin, paramagnetic relaxation enhancement (PRE) experiments were collected with a nitrooxide spin label engineered at positions L8 or K48 in pUb. This was accomplished by mutagenesis of L8 or K48 to cysteine, followed by cysteine modification with an alkyl halide-linked spin label. Spin-labelled pUb species were then added to RORBR and TROSY-HSQC experiments were collected. Resonances were heavily suppressed for atoms within ~20 Å of the unpaired electron radical, owing to the distance dependence ($r^{-6}$) of the PRE effect (39). In this manner, the PRE factor (ratio of peak intensity with or without an unpaired electron radical) can be used to define molecule orientation and obtain long-range distance restraints beyond the ability of NOEs. When an unpaired electron spin label was present at the L8 position in pUb, many resonances localized to the BRcat domain were attenuated, suggesting the probe lies close to these residues when pUb is bound (Figure 2.14 A). A similar experiment with a spin label at the K48 position in pUb produced a different result, with PRE effects broadly observed across RINGO, RING1 and helix H3 (Figure 2.14 B). These restraints, along with the chemical shift perturbations allowed us to generate a model of pUb bound to RORBR using HADDOCK (40). In the model, pUb sits across a V-shaped cavity formed at the RINGO/RING1 hinge (Figure 2.14 C). A basic triad, consisting of K151-H302-R305, surrounds pSer65 of pUb. The pUb orientation is governed by the $\beta_1$–$\beta_2$ loop in pUb that interacts with residues on helix H3 and residues in the adjacent BRcat domain. The C-terminal tail of pUb including residues V70 and L71 runs parallel to helix H3. In addition, residues in the $\beta_3$–$\beta_4$ region of pUb (I44, A46) intercalate between strands $\beta_{16}$–$\beta_{17}$ and helix H3. Of note, the pUb-binding site is remote from the UBL site on the opposite side of RING1, suggesting that pUb may activate parkin in an allosteric manner,
Figure 2.14. PRE experiments define the pUb binding site in RORBR.
A nitroxide spin tag was incorporated into unlabelled pUb at positions Leu8 (A) or Lys48 (B). Each pUb species was then added to $^2H/^{15}N$-labelled RORBR and TROSY HSQC spectra were recorded. For each position, two identical spectra were collected with an active or ascorbate quenched spin tag. Relative peak intensities in the quenched/active spin tags were plotted as a function of residue. A representative spectrum and $^1H$ trace showing the paramagnetic effect in pUb-L8$^*$ is shown. (C) NMR restraints were used to drive pUb molecular docking to RORBR using HADDOCK. The 20 lowest energy structures for phosphorylated ubiquitin bound to parkin are shown. PDB coordinates for pUB (4WZP) and RORBR parkin (4I1F) were used as starting structures.
rather than by acting as canonical ubiquitin substrate. This idea was further supported by ITC data that showed full-length parkin with its UBL domain phosphorylated (pParkin) binds pUb approximately 8x more strongly than unphosphorylated parkin (Table 2.2).

An allosteric mechanism could suggest pUBL and pUb cannot be bound to parkin simultaneously. In order to test this hypothesis, a competition experiment was performed and monitored by NMR spectroscopy. Unlabelled RORBR parkin was titrated into $^{13}$C-labelled UBL$^{S65E}$ and monitored in $^1$H-$^{13}$C HMQC spectra such that signals from both the unbound and bound forms of UBL$^{S65E}$ were visible. For example, changes in the positions of A46 and L61 of the UBL domain show a clear chemical shift upon binding with RORBR (Figure 2.15 B). Upon addition of Ub$^{S65E}$, the signals for the bound form of UBL revert back to those of the unbound UBL state (Figure 2.15 C). The experiment shows that phosphomimetic Ub binding displaces the bound UBL$^{S65E}$ domain from parkin. A reciprocal experiment from the perspective of $^{13}$C-labelled Ub$^{S65E}$ produced the same effect, showing UBL$^{S65E}$ is unable to bind an activated RORBR–Ub$^{S65E}$ complex, despite similar dissociation constants for UBL$^{S65E}$ and Ub$^{S65E}$ binding to the RORBR module (Table 2.2). Together, these experiments suggest that simultaneous binding of phosphorylated UBL and phosphorylated ubiquitin to RORBR is not possible. Further, the data suggest the binding of phosphorylated Ub releases the UBL from parkin, consistent with the proposed allosteric loss of structure to the near helix H3 and BRcat domains that would disrupt the UBL-binding site. This would also indicate that activation of parkin through pUb binding would prevent re-engagement with the UBL domain until the pUb is released.
Figure 2.15. Allosteric release of phosphomimetic UBL by phosphomimetic Ub.

(A) $^1$H traces of $^{13}$C-labelled UBL$^{S65E}$ spectra as shown beneath in panels B and C.

(B) Selected regions of $^1$H-$^1$C HMQC spectra for 150 µM $^{13}$C-labelled UBL$^{S65E}$ (black contours) following the addition of one molar equivalent of unlabelled RORBR parkin. Approximately 83% of UBL$^{S65E}$ is bound to RORBR parkin (orange contours) based on a Kd of 6 µM. The arrows show the position of the bound $^{13}$C-labelled UBL$^{S65E}$ signal upon RORBR binding.

(C) The same sample and regions following the addition of one molar equivalent unlabelled Ub$^{S65E}$ shows the disappearance of most bound UBL$^{S65E}$–RORBR species and re-appearance of unbound UBL$^{S65E}$ (red contours), indicating Ub$^{S65E}$ is able to displace UBL$^{S65E}$. 
2.4 Discussion

Historically, parkin was thought to be constitutively active due to its apparent ubiquitin ligase activity in cells (3,4). The discovery of UBL-mediated autoinhibition (6) propelled efforts to uncover the structural role of the UBL and activators of parkin activity. Here, NMR and X-ray crystallography were used to determine the UBL-binding site on RORBR, resulting in the first high-resolution structure of this important autoinhibitory interface in human parkin (Figure 2.11). An important observation from the structure of autoinhibited parkin is that the catalytic cysteine (C431) in the Rcat domain remains in a similar environment as in truncated parkin structures lacking the UBL domain (36-38). The NMR chemical shift perturbation experiments showed minimal changes for C431 or to residues at the RINGO/RING2 interface near the catalytic site, confirming this region of parkin does not undergo any large structural change that might expose C431 (Figure 2.9). However, close inspection of the NMR data and crystal structures revealed a hinge at the RINGO/RING1 interface that is allosterically regulated by the UBL association (Figure 2.12). Binding of the UBL to RORBR propagates a structural signal through helix H3 to the phosphoubiquitin-binding site at the RINGO/RING1 hinge. Thus, the increased E3 ligase activity observed upon phosphoubiquitin binding likely results from enhanced recruitment of the E2~Ub conjugate following release of the UBL domain, rather than a change in structure at the catalytic site. This would explain why UBL uses its I44-patch surface to interact with parkin, as this interaction interface on RORBR could presumably accommodate a substrate ubiquitin in the context of an E2~ubiquitin conjugate.
The development of the NMR strategy described here to study human parkin represents a breakthrough in our ability to observe interactions and conformational changes in the context of the entire RORBR module rather than the isolated domains contained therein. The RORBR backbone assignments obtained from TROSY triple resonance experiments provide atomic probes covering the whole protein that can serve to identify binding sites for E2 enzymes, mitochondrial substrates and other regulators of parkin activity. Importantly, the data showed the RORBR molecule observed in crystal structures is representative of the structure in solution, as supported by the heteronuclear NOE experiments (Figure 2.7). The NMR assignments were also used to determine the binding sites of the UBL and pUb on the RORBR surface of parkin. It is remarkable that pUb and UBL occupy completely distinct binding sites on parkin, despite the high structure and sequence conservation between these two molecules (62% sequence similarity). Interestingly, both pUb and UBL heavily invoke their β1-β2 loops for binding to RORBR and this section is the most dissimilar between the two proteins, suggesting a role of structural specificity in this region.

At present, the most compelling activator of parkin is the Ser/Thr kinase, PINK1, functioning upstream of parkin in cellular function (41-43). Isothermal titration calorimetry experiments discovered opposing thermodynamic effects of UBL and Ub phosphorylation at Ser65, which cooperate to relieve UBL-mediated autoinhibition (Table 2.2). Importantly, UBL (and pUBL) binding is enthalpically driven, while pUb binding is favoured entropically. Phosphorylation of the UBL weakens its affinity (~10-fold) for RORBR, while phosphorylation of ubiquitin dramatically enhances its affinity (~4,000-fold) for a pUb-binding site that is centered around a basic triad that
accommodates the phosphoserine group (Figure 2.14). Using a competition experiment, it was showed that binding of phosphorylated ubiquitin releases the UBL domain from RORBR (Figure 2.15). This occurs through translation of an allosteric signal through helix H3 to the UBL binding site. The release of the UBL domain from RORBR would presumably enhance the accessibility of serine 65 in UBL to be phosphorylated by PINK1 to promote the maintenance of this released state. This is consistent with observations that, while parkin can be phosphorylated by PINK1 in the absence of phosphoubiquitin, the addition of pUb enhances parkin phosphorylation, suggesting that parkin bound to pUb is a better substrate for PINK1 than parkin alone (44). The release of the UBL domain therefore primes parkin for catalysis by revealing a binding site for an E2-ubiquitin conjugate to engage the E3 ligase, and subsequently transfer ubiquitin to a mitochondrial substrate. The structures determined here of autoinhibited (UBL-bound) and primed (pUb-bound) parkin provide important insights into parkin regulation and activation and will be key to designing potential therapeutics to perturb these conformational states.

2.5 References


Chapter 3

Generation of Ser65-Phosphorylated Ubiquitin-Like Proteins†

3.1 Introduction

It has recently been shown that phosphorylation of serine 65 in the ubiquitin-like (UBL) domain of parkin stimulates its ubiquitin ligase activity (2,3). Subsequently, phosphorylation of ubiquitin (Ub) at the analogous serine 65 position was also discovered to stimulate parkin ubiquitination (4-6), which was shown to function through an allosteric mechanism in Chapter 2. The kinase responsible for these phosphorylation events is the PARK6 gene product, PTEN-induced kinase 1 (PINK1) (2-6). To further identify the structural basis of this phosphorylation signal, we sought to produce preparative amounts of ubiquitin and parkin UBL proteins stoichiometrically phosphorylated at serine 65. Such biophysical studies had remained elusive since recombinant forms of human PINK1 display little intrinsic kinase activity, and PINK1 itself undergoes extensive post-translational processing in mammals that regulates its activity (2,7-12). Generation of homogenously-phosphorylated proteins by their respective kinases is often challenging due to promiscuity of catalyzed phosphorylation sites, and the fact that in-vivo scenarios rarely merit total phosphorylation of biological targets. Consequently, several chemical biology techniques to generate custom phosphorylated proteins have been described to circumvent these challenges.

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Chemical conversion of cysteine residues to phosphocysteine has been largely pioneered by Benjamin Davis’ research group. This method proceeds by conversion of a target cysteine residue to an electrophilic dehydroalanine (DHA) intermediate, which can be subsequently modified by a variety of nucleophiles to mimic post-translational modifications (13-15). This method has been successfully applied to generate structural mimics of glycosylation (14), acetylation (15), and phosphorylation (16,17) modifications in proteins, providing unique biological insights to these post-translational modifications in their respective systems. The major drawbacks to this technique include the relatively harsh conditions required during the chemical conversion and the dependency on a solvent-accessible cysteine residue in the protein for modification.

A second method to generate custom post-translational modifications is by genetic code expansion in *E. coli* by selective re-assignment of the TAG stop codon, first explored by Peter Schultz’s lab. The use of an orthogonal translation system with a desired tRNA/tRNA synthetase pair recognizing the UAG codon in mRNA can introduce non-canonical amino acids, including phosphoserine, at any desired position in a protein (18,19). The use of this system in *E. coli* with release factor 1 (RF1) deletion, eliminating cellular recognition of UAG as a stop signal, was highly toxic to the cells by disrupting termination of endogenously expressed mRNA with UAG codons (20). Subsequently, bacterial strains have been created in which all genomic UAG codons have been recoded to UAA, significantly improved cell fitness and protein expression (20-22). Expressed phosphoproteins, however, are frequently contaminated by incorporation of natural amino acids as a result of near-cognate decoding at the UAG locus, limiting the use of this method when pure phosphoproteins are required (21).
Finally, for serine 65-phosphorylation specifically, the recent discovery that certain insect orthologues of PINK1 are constitutively active kinases provided an avenue for overcoming the latent activity of human PINK1 (23). The primary sequence of PINK1 in *Drosophila melanogaster* (fruit fly), *Tribolium castaneum* (red flour beetle) and *Pediculus humanus* (body louse) contain variable-length insertions throughout the kinase domain which apparently increase the intrinsic kinase activity in these insect PINK1 orthologues relative to the human protein (23). However, this discovery was largely made in the context of PINK1 self(auto)-phosphorylation and of peptide substrates and therefore the utility of these insect kinases with regards to phosphorylating ubiquitin or parkin directly had not been robustly demonstrated.

In this work, the aforementioned methods were applied to generate serine 65 phosphorylated ubiquitin (pUb) and UBL (pUBL) from parkin. Serine 65 phosphorylated proteins were successfully obtained by orthogonal translation and using a recombinant form of *Pediculus humanus* PINK1. When generating pUBL by the orthogonal translation strategy, a previously undocumented “codon-skipping” phenomenon was serendipitously discovered, where the ribosome underwent apparent +3 frameshifting. Ultimately, the method yielding the most phosphorylated protein was found to be through use of a catalytically active insect PINK1, which is easily amenable to isotopic labelling strategies, enabling its use in structural studies. The advantages and disadvantages of each technique are discussed with regards to generating phosphorylated proteins.
3.2 Materials and Methods

3.2.1 Chemical modification of cysteine to phosphocysteine

A cysteine residue was substituted for the position of serine 65 in Ub and parkin UBL using a modified site-directed mutagenesis protocol (24). Additionally, in parkin UBL, the C59S and C59L substitutions were introduced in a similar manner. Proteins were overexpressed in *E. coli* BL21(DE3) cells grown in LB media. Cells were grown at 37°C until an OD$_{600}$ of 0.6 was reached at which point cells were cooled to 16°C and induced with 0.5 mM IPTG for 16 hours. Cells were harvested and resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, 0.5 mM TCEP, 25 mM imidazole, pH 8.0). Cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin), ultracentrifuged at 41,000 rpm (124,000 x g) and proteins purified by Ni$^{2+}$ affinity using a HisTrap FF column on an AKTA FPLC (GE Healthcare). Affinity captured proteins were eluted from the column with elution buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 500 mM imidazole, pH 8.0). Fractions containing the protein of interest were pooled, protease (TEV for Ub; Ulp1 for UBL) was added to cleave the His tag (~1:50 ratio protease:protein) and dialyzed overnight at 4°C against lysis buffer. After cleavage was complete, proteins were passed over the HisTrap FF column again in lysis buffer and the flow through was collected, containing proteins of interest. Proteins were concentrated and subjected to size exclusion chromatography on a HiLoad Superdex 75 equilibrated with 50 mM HEPES, 50 mM NaCl, pH 8.0 for a final purification step and to remove TCEP.

For cysteine modification to dehydroalanine, $\alpha,\alpha'$-dibromo-adipyl(bis)amide (DBAA) was synthesized as described in (16) and obtained as a solid. DBAA (500 µmol, 151 mg,
250 equiv.) was added as a solid to purified Ub or UBL proteins (2 μmol, 20 mg, 1.0 equiv.). The cloudy suspension was incubated at 37 °C with shaking for 3 hours. The mixture was then centrifuged at 40,000 rpm (or syringe filtered with a 0.2 μm filter) to remove precipitated proteins and insoluble DBAA and proteins dialysed overnight against 50 mM HEPES, 100 mM NaCl, pH 8.0 to remove soluble DBAA. The following day, sodium thiophosphate tribasic hydrate (33 mmol, 6.0 g, 16,500 equiv.) was added gradually as a solid (in about 5 intervals over 25 mins). The final pH of the reaction after sodium thiophosphate addition was between 10.5 and 11. The reaction mixture was then incubated at 37 °C with shaking for 4 hours. Following incubation, the cloudy reaction mixture was spun down and put into dialysis against 50 mM HEPES, 100 mM NaCl, pH 8.0 to remove excess sodium thiophosphate. Proteins were then subjected to LC-MS analysis to assess the reaction products.

Mass spectrometry was performed on a Quattro Micro mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode with an Agilent 1100 HPLC used for LC gradient delivery. Reverse phase chromatography was performed using a C18 1 x 150 mm column. Briefly, proteins were injected into the column in 95 % buffer A (H₂O + 0.1% formic acid), 5% buffer B (acetonitrile + 0.1 % formic acid) at 0.1 mL/min. Over 30 mins, a gradient buffer change to 5% buffer A, 95 % buffer B was performed and eluent injected in-line into the mass spectrometer. The mass survey range was set to 600-1600 m/z. Cone voltage for electrospray ionization was 30V and source temperature was 80 °C. Calibration was performed with a myoglobin standard, with mass error of 0.5 Da.
3.2.2 Dissolving and refolding of synthetic pUBL peptide

A synthesized pUBL peptide was obtained from ChemPeptide (Shanghai, China) as a lyophilized solid (10 mg peptide). For rapid dilution refolding, 1.8 mg peptide was dissolved in 2 mL of 2 M guanidine hydrochloride in refolding buffer (50 mM KH$_2$PO$_4$, 100 mM KCl, 100 mM arginine, 100 mM glutamine, 1 mM EDTA, 1 mM DTT, pH 7.5). This 100 µM peptide solution was slowly added (0.5 mL/hour) dropwise from a fine-needle syringe, into a stirring 50 mL solution of cold refolding buffer on ice. After addition, the 4 µM peptide solution contained approximately 75 mM guanidine hydrochloride. To remove residual guanidine hydrochloride, the solution was dialyzed overnight against 2 L of refolding buffer. The following day, the dialysis buffer was changed to 25 mM KH$_2$PO$_4$, 25 mM KCl, 1 mM EDTA, 1 mM DTT, pH 7.5 to remove arginine and glutamine.

3.2.3 Expression of pUb and pUBL in release-factor deficient E. coli

Recombinant proteins were expressed in E. coli ΔRF1 (C321.ΔA.exp, Addgene 49018) (22). The 1st generation pSer system was on the plasmid pKD-pSer1 (Addgene 52054) that expresses SepRS, EFSep and 5 copies of the tRNASEp expression cassette. The Ub and UBL expression plasmid was constructed using a T7 promoter, T7 terminator, and a cloning site (NdeI/BamHI) in an ampicillin-resistant backbone derived from pUC18. A modified quick-change mutagenesis protocol (24) was used to insert in-frame TAG codons and DNA sequencing confirmed the correct sequence of the plasmids. pKD-pSer1 and Ub/UBL expression plasmids were freshly co-transformed into E. coli ΔRF1 cells and plated on LB-agar plates with 25 µg/mL kanamycin and 100 µg/mL ampicillin. Single colonies were picked to inoculate 10 mL starter cultures in LB (with 100 µg/mL...
ampicillin and 25 µg/mL kanamycin), which were grown, shaking overnight at 37 °C. Once saturated, the starter culture was used to inoculate 1 L of LB media supplemented with ampicillin (100 µg/mL), kanamycin (25 µg/mL), and O-phospho-L-serine (2.5 mM, Sigma-Aldrich). Cultures were grown at 37 °C until OD_{600} was 0.6. An additional 2.5 mM O-phospho-L-serine was added to cultures at this point (5 mM total O-phospho-L-serine) and the incubation temperature was reduced to 16 °C. Protein expression was induced at OD_{600} of 0.8 by addition of 1 mM IPTG and continued for 18 hours at 16 °C. Cells were harvested and proteins purified by Ni^{2+} affinity exactly as described in section 3.2.1 except that 50 mM NaF and 2 mM NaVO_{3} were included in the lysis buffer as phosphatase inhibitors. For some Ub variants, the His tag was retained to differentiate Ub chains by molecular weight. It has been established that the presence or absence of an N-terminal His tag has no effect on the interaction of the Ub substrate with E1, E2 or E3 enzymes involved in the parkin ubiquitination pathway (25).

Purified Ub or UBL proteins were separated on a 1 mL HiTrap SP-XL column using an AKTA FPLC system (GE Healthcare). Prior to purification, samples were dialysed overnight in buffer A (20 mM MES, pH 6.0). Cation exchange chromatography was carried out at a flow rate of 0.3 mL/min with a 1 hour gradient of 0-100% buffer B (20 mM MES, 200 mM NaCl, pH 6.0). Phosphorylated protein species were collected in the unbound (flow-through) fractions while the salt gradient eluted the non-phosphorylated species.

Mass spectrometry was performed both before and after ion exchange chromatography. Samples were prepared for MS by overnight dialysis in 15 mM ammonium acetate to
remove salt adducts. Whole protein ESI-MS was performed on a Q-Tof Micro mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode (+0.1% formic acid). Samples were prepared for Tandem ESI-MS/MS by digestion with trypsin or Glu-C protease as follows. Briefly, 25 µg of protein in 100 µL was reduced with DTT (5 mM final concentration) at 70 °C for 15 minutes. Samples were then alkylated with iodoacetamide (30 mM final concentration) at room temperature for 45 minutes in the dark. Alkylation was quenched with DTT (30 mM final concentration) for 30 minutes at room temperature. Protease digestion was performed using a 1:40 ratio of protease to sample at 37 °C overnight. Samples were evaporated by centrifugal speedvac and stored at -20 °C until mass spectrometry was performed.

Ubiquitination assays were adapted from a previous study (26) and contained 0.1 µM E1 (Uba1), 1 µM E2 (UbcH7), 1 µM E3 (Parkin), 5 mM ATP, 5 mM MgCl2, 50 mM HEPES pH 7.4, and a 20 µM final concentration of Ub and/or pUb in a 20 µL final reaction. Reactions were initiated by addition of ATP and incubated at 30 °C for 1 h. Reactions were quenched with SDS-loading buffer (containing 50 mM EDTA) and subjected to standard SDS-PAGE and western blotting procedures using anti-parkin primary antibody (kind gift from Dr. Michael Schlossmacher, University of Ottawa) and fluorescent-labeled secondary antibody (Mandel Scientific). Blots were visualized using the Odyssey imaging system (Li-Cor biosciences).

The E2-loading assay was adapted from a previous study (27) with modifications as follows. The reaction contained 0.5 µM E1 (Uba1), 20 µM E2 (UbcH7), 20 µM Ub or pUb variants, and 10 mM ATP in reaction buffer containing 50 mM HEPES and 100 mM
NaCl at pH 7.0. Reactions were initiated by addition of ATP and incubated at 37 °C for 10 minutes. Samples were quenched with SDS non-reducing loading buffer (containing 50 mM EDTA). The reaction products were separated by SDS-PAGE and visualized by Coomassie staining.

### 3.2.4 Phosphorylation of Ub and UBL by *Pediculus humanus PINK1*

GST-tagged *Pediculus humanus* PINK1 (128-C) was expressed in *E. coli* BL21(DE3) cells in LB media. Cells were grown at 37 °C until an OD$_{600}$ of 0.8 was reached, at which point overexpression was induced with 0.5 mM IPTG and the incubation temperature lowered to 16 °C for 18 hours. Overexpressed proteins were purified on a GSTrap FF column (GE Healthcare) in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT at pH 7.4) and eluted with freshly prepared elution buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT and 10 mM glutathione, pH 8.0). Following elution, glutathione was removed from PINK1 by two 2L dialysis changes against 50 mM Tris, 50 mM NaCl, 3 mM DTT, pH 10, followed by a final 2L dialysis against 50 mM Tris, 50 mM NaCl, 3 mM DTT, pH 7.5.

For *in-vitro* phosphorylation, purified GST-PINK1 (10 µM) was incubated at 25 °C with either Ub (500 µM) or UBL (500 µM) and phosphorylation initiated by addition of MgCl$_2$ and ATP to a 10 mM final concentration. The reaction was monitored to completion using Phos-Tag SDS–PAGE as described below. Following the reaction, GST-PINK1 was removed from the reaction using a GSTrap FF column, collecting the flow-through fractions containing pUb or the pUbl domain. A final purification step by gel filtration
was performed on a HiLoad Superdex 75 equilibrated in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.0, to remove residual GST-PINK1 and to remove excess ATP.

Phos-tag acrylamide (Wako Pure Chemicals Ltd., Japan) was purchased and gels were prepared according to manufacturer’s instructions (28,29). 12 % polyacrylamide gels were prepared with 50 µM Phos-tag reagent and 0.7 mM ZnCl₂ embedded in the gel. Protein samples for Phos-tag SDS-PAGE were always prepared in buffers without phosphate or EDTA, including the SDS gel-loading buffer. Empty lanes in gels were filled with equivalent buffer blank samples. Gels were run under constant current at 25 mA per gel with the gel tank submerged in an ice bath until the dye front had left the gel. Phos-tag gels were always imaged with Coomassie blue stain.

3.3 Results

3.3.1 Synthesis of phosphorylated Ub and UBL by chemical modification

Insertion of a phosphate group at the position of residue 65 in Ub and parkin’s UBL domain was first attempted by chemical modification of cysteine through a reactive dehydroalanine (DHA) intermediate. A cysteine substitution (S65C) was inserted at residue 65 in ubiquitin and in parkin’s UBL domain. While ubiquitin harbors no cysteine residue in its native sequence, parkin’s UBL contains one partially-accessible cysteine (C59) that could form undesired side products during cysteine modification. However, a C59S substitution in parkin UBL was largely insoluble, suggesting a more non-polar role for C59 in the context of the UBL structure. Therefore, a C59L/S65C double mutant was
generated for modification of parkin UBL, obtaining overexpression and solubility of this double mutant comparable to the wild-type protein.

Modification of cysteine 65 to dehydroalanine in Ub and parkin UBL was performed using α,α′-dibromo-adipyl(bis)amide (DBAA) (Figure 3.1 A), proceeding through an alkylation-elimination reaction mechanism. In the proposed reaction scheme, alkylation occurs rapidly at 37 °C, while longer incubation periods (up to 3 hours at 37 °C with shaking) promote cyclization and elimination of the DBAA adduct to form dehydroalanine (14). Reaction of parkin UBL\textsuperscript{C59L/S65C} with DBAA over 3 hours resulted in elimination to dehydroalanine, observed as a loss of 33 Da by electrospray ionization mass spectrometry (ESI-MS) (Figure 3.2 A-B). Roughly 60% conversion to UBL–DHA was achieved, estimated by peak intensities in the mass spectra. The presence of UBL–DHA was also corroborated by successful addition of 2-mercaptoethanol to generate a thioether. The insertion of an electrophilic dehydroalanine group at position 65 in parkin UBL can therefore allow for subsequent modification with a variety of nucleophiles to generate a custom thioethers. Unexpectedly, the same reaction conditions of DBAA with Ub\textsuperscript{S65C} repeatedly produced less than 10% dehydroalanine product, remaining largely unmodified upon reaction with DBAA. To eliminate the possibility of cysteine oxidation, Ub\textsuperscript{S65C} was reacted with Ellman’s reagent (5,5′-dithiobis-2-nitrobenzoic acid) and underwent the expected color change to a bright yellow solution, confirming the presence of reduced cysteine. This dissimilarity in chemical reactivity suggested there is a difference in the structure or accessibility of residue 65 in Ub and parkin UBL. In an attempt to enhance the reactivity of cysteine 65 in Ub\textsuperscript{S65C}, the organic solvent dimethylformamide was added to the reaction with DBAA. This however, further
Figure 3.1. Reaction scheme to generate dehydroalanine and phosphocysteine modifications.

(A) A cysteine substitution is inserted at the desired phosphorylation position. The reduced cysteine is modified with DBAA over 3 hours to generate dehydroalanine. The alkylated and cyclized reaction intermediates are shown. B) The dehydroalanine species is subjected to nucleophilic attack by sodium thiophosphate, generating a phosphocysteine residue. The phosphocysteine modification differs from phosphoserine by only one atom (O to S).
Figure 3.2. Chemical modification of UBL to generate UBL–phosphocysteine.
Raw ESI-MS data collected during chemical modification of parkin UBL to insert a phosphate group at residue 65. The +8 charged ion species is shown in all cases with identical m/z range shown along the x-axis. The expected m/z for each intermediate is shown at the bottom, along with the corresponding molecular mass of the protein. (A) Starting material, purified UBL domain with C59L/S65C substitution. (B) After modification with DBAA, generating a dehydroalanine intermediate (loss of 33 Da). (C) After modification with sodium thiophosphate, generating a phosphocysteine residue (gain of 113 Da relative to DHA species). (D) Following cation exchange chromatography to separate UBL–SPO$_3$ from unreacted products.
hindered elimination of DBAA, resulting in a +138 Da contaminant corresponding to Ub adducts with cyclized DBAA.

To generate the phosphoserine mimic, UBL–DHA was reacted with a large excess of sodium thiophosphate (NaSPO$_3$) over 4 hours to generate UBL phosphocysteine (UBL–SPO$_3$) (Figure 3.1 B). Ideal reactivity of thiophosphate occurred when the tribasic salt was added as a solid to UBL–DHA, consequently raising the pH of the reaction to ~10.5. Although most thiols would be partially nucleophilic around neutral pH, the –2 charge on the thiophosphate ion presumably raises the third pKa of the thiol and hinders its ability to function as a nucleophile. Thus the high pH of the reaction enhances deprotonation of the thiol to accelerate the reaction. Unfortunately, even under these optimal conditions, only ~50% conversion of UBL–DHA to UBL–SPO$_3$ was obtained (Figure 3.2 B-C). Increasing the concentration of sodium thiophosphate to a 25,000-fold excess relative to UBL–DHA was unable to force the reaction to completion, similar to attempts in a previous study (17). Notably, cation exchange chromatography was able to separate unreacted products, leaving the desired UBL–SPO$_3$, as detected by ESI-MS (Figure 3.2 D). However, the significant loss of material over the reaction and subsequent purification precluded the use of this purified protein for structural studies.

3.3.2  Refolding of a synthetic phosphorylated UBL peptide

In an attempt to increase the quantity of phosphorylated parkin UBL for biophysical experiments, a lyophilized Ser65-phosphorylated UBL (pUBL) was synthesized and obtained from a commercial source. Unlike lyophilized ubiquitin, which is highly soluble, lyophilized pUBL was insoluble in aqueous buffers. Changing the ionic strength or pH of
aqueous buffers did little to enhance solubility of the pUBL peptide. We resorted to
dissolving the pUBL peptide in 2 M guanidine hydrochloride, solubilizing an unfolded
form of the protein. To refold the peptide, a refolding buffer containing 0.1 M arginine
and glutamine was used, as arginine is an effective protein-stabilizing agent owing to its
guanidinium side chain group (30,31). Diffusion-driven dialysis into the refolding buffer
overnight was unable to refold pUBL, resulting in protein precipitation. A more
successful result was obtained using a rapid dilution strategy, where the dissolved peptide
in 2 M guanidine hydrochloride was slowly added drop-wise to a stirring solution of
chilled refolding buffer. This resulted in a clear, dilute protein solution (4 µM pUBL)
with only 75 mM guanidine hydrochloride. Overnight dialysis into the same refolding
buffer removed the majority of residual guanidine hydrochloride without any protein
precipitation, leaving a clear protein solution in 0.1 M arginine/glutamine refolding
buffer. Further dialysis to remove arginine/glutamine, however, resulted in precipitation
of pUBL. This suggested that arginine/glutamine was solubilizing a non-native structure
of pUBL, which was only realized upon removal of arginine. This pUBL sample was
subjected to SDS–PAGE analysis to assess the integrity of the protein. In contrast to other
UBL variants, the synthetic pUBL peptide displayed heterogeneity by SDS–PAGE,
observed as a smeared band with apparent degradation or contamination of the peptide
with lower molecular weight species (Figure 3.3 A). Subsequent analysis of the peptide
by ESI-MS confirmed a heterogenous sample of peptide masses, with multiple ion
species observed in addition to the expected pUBL ion series (Figure 3.3 B). This
heterogeneity likely results from complications of synthesis of such a long peptide by
traditional stepwise peptide synthesis techniques. It is possible that a synthesis approach
employing native chemical ligation might have been more successful, as the relatively
Figure 3.3. Refolding of a synthetic Ser65-phosphorylated UBL peptide.
Custom synthesis of a Ser65-phosphorylated UBL peptide was performed by ChemPeptide Limited (Shanghai, China). (A) SDS-PAGE of UBL variants showing heterogeneity of the synthetic peptide. Native UBL, UBL S65E, and pUBL generated by orthogonal translation are shown for comparison. (B) Raw ESI-MS of synthetic peptide dissolved in 50/50% (v/v) H₂O/acetonitrile. The observed parent ions expected for ionization of pUBL (molecular weight 8904 Da) are labelled.
short C-terminus of pUBL encompassing the phosphorylation site (residues 65-76) could presumably be synthesized separately with higher purity.

3.3.3 Generation of phosphorylated Ub and UBL by orthogonal translation reveals codon skipping

To generate Ser65-phosphorylated Ub and parkin UBL, an orthogonal translation system was used whereby phosphoserine is encoded into the DNA sequence of the protein by a TAG codon, normally reserved as a stop codon in *E. coli*. DNA sequences encoding Ub and parkin UBL, with a TAG codon at residue 65 (and terminating stop codons assigned as TAA), were cloned into a pUC-18 plasmid. The 1st generation pSer system in a pKD-pSer1 plasmid (Addgene #52054) was co-transformed into *E. coli* ∆RF1 along with the pUC-18 plasmid. Overexpression of the two plasmids with IPTG in the presence of exogenous O-phospho-L-serine allowed for incorporation of phosphoserine at the UAG codon position. Protein products terminated at residue 65 were not observed, confirming a reassigned role for the UAG codon. Following affinity purification, Ub variants were analyzed by ESI–MS to determine the identity of overexpressed proteins.

For ubiquitin, two major mass peaks were observed corresponding to two full-length protein products: one with the expected mass of pSer65 ubiquitin (pUb) and one with Gln65, an apparent mistranslation product (Figure 3.4 A). Notably, no product corresponding to native Ub (with Ser65) was observed, confirming serine incorporation was abolished and dephosphorylation of pUb does not occur under the conditions used for purification. The protein mixture was further purified using cation-exchange chromatography, which showed the two well-defined peaks, consistent with the two mass
Figure 3.4. Generation of genetically-encoded pUb variants by orthogonal translation.

(A) Whole protein ESI-MS (deconvoluted) Ub protein expression products with Gln or pSer at position 65. (B) Cation-exchange chromatography shows separation of phosphorylated and non-phosphorylated Ub protein products. (C) Tandem MS/MS of trypsin-digested pUb confirms the protein identity and incorporation of phosphoserine at position 65. (D) Phos-tag SDS–PAGE showing ion-exchange fractions of orthogonally translated pUb variants (S65, S20 or S12). The gels show separation of mistranslated products (lanes 1-2) from pure phosphorylated protein (lanes 3-4).
peaks observed (Figure 3.4 B). The identity of pUb was confirmed by tandem ESI–MS/MS (Figure 3.4 C) and by Phos-tag gels (28,29), which retard phosphorylated proteins so that a band-shift separates unphosphorylated Ub from pUb (Figure 3.4 D). The results confirm unambiguously the purification of pUb at a yield of ~11 µg/L from E. coli ΔRF1 cultures. This method to generate pUb was successfully extended to produce Ub phosphorylated at other putative phosphorylation sites, Thr12 and Ser20 (Figure 3.4 D).

To produce parkin UBL phosphorylated at Ser65, identical expression and purification steps were used as with ubiquitin variants. pUBL and Gln65-UBL were readily identified, however ESI–MS revealed a third and unexpected protein species. A protein mass corresponding to UBL entirely lacking the amino acid encoded at residue 65 (UBL Δ65) was observed as a third major species (Figure 3.5 A). This previously unknown interpretation of the UAG codon was verified by tandem ESI–MS/MS, identifying the trypsin digested peptide (VQNCDLDQQ64I66VHVQPRPWR), and confirming deletion of the amino acid at position 65 and formation of a peptide bond between Gln64 and Ile66, apparently skipping the codon encoding residue 65 (Figure 3.5 C–E). Because we suspected this might have resulted from a contaminating plasmid lacking the TAG locus, the UBL expression plasmid was freshly transformed and 10 independent clones were re-sequenced, showing no evidence for deletion in the DNA sequence. Two of these independent clones were re-expressed and purified with the pSer system in E. coli ΔRF1. The UBL Δ65 codon skipped product was robustly reproduced as observed by ESI–MS (Figure 3.6 A–C). Fascinatingly, other phosphoproteins produced using the pSer system did not show evidence for this codon skipping. The notable exception to this was Ub\(^{pSer7}\),
Figure 3.5. Codon skipping as a byproduct of pUBL orthogonal translation.

(A) Whole protein ESI-MS (deconvoluted) indicates UBL protein products with Gln or pSer at position 65. Interestingly, a protein mass corresponding exactly to the deletion of one Ser residue (relative to native UBL) is observed.

(B) Cation-exchange chromatography shows separation of phosphorylated and mistranslated protein products.

(C) Tandem MS/MS of purified phosphorylated UBL, confirming phosphoserine incorporation at residue 65.

(D) Tandem MS/MS of purified mistranslated UBL, confirming glutamine incorporation at residue 65.

(E) Tandem MS/MS of purified mistranslated UBL, confirming the "skipping" of residue 65 during orthogonal translation. The resulting protein is native UBL, but lacks residue 65.
Figure 3.6. Reproducible codon skipping at position 65 in UBL by orthogonal translation.

Full protein ESI-MS (deconvoluted) for all translated UBL products with TAG at position 65. The codon skipped (∆65, lacking residue 65) protein appears reproducibly in 3 independently-transformed and re-sequenced clones (A-C).
which showed evidence for the same codon-skipping phenomenon, albeit to a smaller extent than was observed with pUBL. This observation proves codon-skipping was not an anomaly of the UBL parent plasmid, and indicates that in release-factor 1 deficient *E. coli*, the UAG codons can be skipped or bypassed by the ribosome to yield proteins with a single deleted residue.

### 3.3.4 Phosphorylation of Ub and UBL by insect orthologues of PINK1

We were intrigued by recent reports that insect orthologues of PINK1 display constitutive kinase activity *in-vitro* (2,23). While human PINK1 is deficient for kinase activity, we wondered whether these insect forms could specifically phosphorylate serine 65 in human forms of ubiquitin or parkin’s UBL. The highest solubility of recombinant PINK1 was achieved with a GST–fusion of *Pediculus humanus* PINK1 lacking the N-terminal transmembrane domain (128–C). Expression of this construct in *E. coli* BL21(DE3) yielded on average 5 mg of PINK1 per litre of culture in LB media. Purified GST–PINK1 was used to phosphorylate ubiquitin and parkin UBL the reaction monitored by Phos-tag SDS–PAGE (28,29). Remarkably, complete phosphorylation of ubiquitin and UBL at room temperature was observed on the order of minutes as observed by Phos-tag SDS–PAGE, reflected by retarded migration of a band corresponding to the phosphorylated species (Figure 3.7 A–B). Following removal of PINK1 and excess ATP, the integrity of these phosphorylated proteins were assessed by ESI–MS. The mass spectra clearly showed one main species corresponding to the mass of phosphorylated UBL (Figure 3.7 C). Optimization of the reaction conditions revealed that phosphorylation by GST–PINK1 proceeds to completion within minutes using ratios of PINK1:UBL as low as...
Figure 3.7. Complete Ser65-phosphorylation of Ub and parkin UBL by *Pediculus humanus* PINK1.

(A–B) Coomassie-stained Zn$^{2+}$ Phos-Tag SDS-PAGE showing phosphorylation of (A) parkin UBL or (B) ubiquitin by *Pediculus humanus* PINK1 (GST tagged). The slower migrating band represents the phosphorylated protein species. (C) Mass spectrum of pUBL after phosphorylation and subsequent removal of GST-PINK1. Raw and deconvoluted spectra are shown. MW$_{\text{calculated}}$: 8,904 Da (8,824 Da + PO$_4^-$).
1:50. The yields obtained of recombinantly expressed PINK1 are therefore sufficient for large-scale preparations of pUb or pUBL on the scale of tens of milligrams.

To assess whether the generated Ser65-phosphorylated proteins could stimulate parkin, ubiquitination assays were conducted to monitor parkin’s ubiquitin ligase activity. Substitution of Ub with pUb\textsuperscript{kinase} (phosphorylated by the kinase PINK1) \textit{in-vitro} greatly enhanced autoubiquitination activity of parkin, observed by an increase in parkin species modified with progressively longer ubiquitin chains (Figure 3.8 A, lanes 1-3). Higher activity was observed with a 10:90 ratio of pUb:Ub rather than 100% pUb, further supporting an allosteric role for pUb as was proposed in chapter 2. The enhanced activity observed with a low pUb:Ub ratio was also consistent with reports from other research groups (32,33). However, in contrast to these reports, definitive incorporation of pUb into polyubiquitin chains was observed when pUb was the sole ubiquitin source, confirmed by differential SDS–PAGE migration using his-tagged Ub and tagless pUb (Figure 3.8 A, lanes 2-3), although incorporation of pUb occurred to a lesser extent over the timecourse of these experiments. In order to assess the integrity of the orthogonally-translated pUb (pUb\textsuperscript{orthog}) described in section 3.3.3, the same ubiquitination assay was conducted in the presence of 10% or 100% pUb\textsuperscript{orthog} generated in \textit{E. coli} \Delta RF1 (Figure 3.8 A, lanes 4-5). Essentially the same level of autoubiquitination activity was observed whether pUb\textsuperscript{kinase} or pUb\textsuperscript{orthog} were used, suggesting the pUb proteins generated by orthogonal translation are representative of ubiquitin phosphorylated by the kinase PINK1.

Previous reports (6,32,33) and the data presented in Figure 3.8 A corroborate that pUb is an inefficient substrate for ubiquitination relative to native Ub. While it is possible that
Figure 3.8. Designer phosphorylated proteins can be used in biological assays.

(A) Parkin auto-ubiquitination assays with unmodified Ub (His-Ub), PINK1 phosphorylated Ub (pUb\text{\textsuperscript{kinase}}) and orthogonally translated phosphorylated Ub (pUb\text{\textsuperscript{orthog}}) were visualized by western blot. All reactions contained Uba1 (E1), UbcH7 (E2), full-length parkin (E3), ATP and different Ub variants. The % stoichiometry of each Ub variant is shown above each lane.

(B) E2 (UbcH7)-loading assay with Ub and orthogonally translated pUb variants. The reaction at t=0 shows only free E2 and Ub reactants. Following a 5 minute reaction (after addition of ATP), the appearance of an upper band indicates formation of the E2~Ub or E2~pUb conjugate.
parkin’s catalytic activity is reduced when pUb is used as a substrate, it is also conceivable that pUb is a less competent substrate for E1 or E2 enzymes acting upstream of parkin. To explore this possibility, the ability of the E2 enzyme UbcH7 to engage in a Ub conjugate with phosphorylated Ub was tested. As observed by non-reducing SDS–PAGE, pUb was less readily converted into an E2–Ub conjugate when compared to native Ub (Figure 3.8 B). In addition to pUb<sup>pSer<sub>65</sub></sup>, we also tested the ability of another pUb variant generated by orthogonal translation, pUb<sup>pSer<sub>20</sub></sup>, to form an E2–Ub conjugate. While both forms of phosphorylated Ub were hindered in their ability to form an E2–Ub conjugate, the effect was slightly more pronounced with pUb<sup>pSer<sub>65</sub></sup> (Figure 3.8 B). The results show that processing of phosphorylated ubiquitin is hindered at the E1 or E2 enzyme stage, supporting observations that parkin ubiquitination is less efficient with phosphorylated ubiquitin.

### 3.4 Discussion

In this work, we set out to generate ubiquitin and UBL proteins homogenously phosphorylated at serine 65. Complete stoichiometry of the phosphorylated state is essential for biochemical studies, given the known effects of pUb stoichiometry on parkin activity (32-34). Further, the use of true phosphorylated proteins was sought, rather than the more accessible aspartate or glutamate phosphomimetics, as these mimetics are not proven to recapitulate pUb in vivo (34). Although these natural amino acids are negatively charged, the carboxylate side chains of aspartate and glutamate are poor phosphomimetics, harbouring a –1 charge rather than –2, and possessing planar geometry
compared to the tetrahedral phosphate group. These deficiencies of phosphomimetics was further evidenced in Table 2.2, where the parkin-binding properties of Ub/UBL phosphomimetics were significantly altered from the truly-phosphorylated counterparts. The generation of homogenous Ub and UBL variants possessing phosphoserine (or phosphocysteine) was demonstrated here by 3 independent methods. A summary of the investigated techniques is shown in Table 3.1.

Chemical modification of cysteine through the dehydroalanine intermediate is a versatile method to introduce an assortment of protein modifications, including phosphate groups, onto proteins. We found that success of this method is heavily dependent on accessibility and chemical environment of the cysteine residue to be modified. For example UBL\textsuperscript{S65C} was much more amenable to oxidative elimination than Ub\textsuperscript{S65C}. This may be due to weakened thermodynamic stability in UBL relative to Ub (35), that presumably increases the relative accessibility of partially buried amino acids, including residue 65. Further, Ub possesses a glutamic acid residue (E64) directly preceding residue 65, while UBL possesses a glutamine (Q64). This additional negative charge in Ub could further hamper modification at residue 65 by DBAA or the nucleophilic thiophosphate ion. Despite the relatively high stability of ubiquitin-like proteins, we found the harsh conditions required for reaction with DBAA and sodium thiophosphate result in substantial loss of soluble protein over the course of the modification, precluding its use in our structural studies.

Orthogonal translation by recoding of \textit{E. coli} UAG codons was successfully used to incorporate phosphoserine at residue 65 in both ubiquitin and parkin UBL. By this method, pure phosphoproteins were obtained on the order of \(\sim 10 \, \mu g\) per litre of LB media.
<table>
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<tr>
<th>Method</th>
<th>Yield</th>
<th>Potential contaminants</th>
<th>Isotopic-labelling possible?</th>
<th>Practicality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical modification of cysteine</td>
<td>&lt;40 % starting concentration, surface accessibility dependent</td>
<td>DBAA adducts, Unmodified DHA</td>
<td>Yes</td>
<td>Technically challenging</td>
</tr>
<tr>
<td>Synthetic peptide refolding</td>
<td>Very protein-dependent</td>
<td>Peptide synthesis by-products, misfolded aggregates</td>
<td>Not feasible</td>
<td>Protein-dependent</td>
</tr>
<tr>
<td>Orthogonal translation of UAG codon</td>
<td>~10 µg/L in LB media</td>
<td>Mistranslated products</td>
<td>Yes</td>
<td>Facile, but laborious</td>
</tr>
<tr>
<td>Catalytically-active kinase</td>
<td>&gt;99 % starting concentration</td>
<td>Added kinase</td>
<td>Yes</td>
<td>Facile</td>
</tr>
</tbody>
</table>

Table 3.1. Comparison of methods to generate Serine 65-phosphorylated Ub and UBL
culture. While these quantities of protein preclude most structural techniques, further advances in this field continue to improve the yields of phosphoproteins closer to the realm required for X-ray crystallography and NMR spectroscopy (36,37). Importantly, this study reinforces that caution must be taken when employing phosphoproteins generated by this method, as mistranslation at the UAG locus contaminates the overexpressed proteins (20,21). Remarkably, in pUBL$^{pSer65}$ and pUb$^{pSer7}$ variants, phosphoserine insertion competed not only with near-cognate translation from glutamine but also with codon skipping. In both cases, protein products resulting from codon skipping include a peptide bond between the residues encoded immediately before and after the UAG codon. It is proposed that the ribosome underwent a +3 frameshift leading to a single in-frame amino acid deletion and a novel interpretation of the UAG codon. It is unclear why the incidence of this phenomenon varies between the different proteins tested in this study, although it is conceivable that mRNA sequence determinants surrounding the UAG codon confer a preference for codon skipping over near-cognate translation. Aside from an obvious comparison to ribosome frameshifting (38), the only other precedent for mRNA skipping is from recent work in mitochondria of the yeast Magnusiomyces capitatus. In this case, the ribosome bypasses ~100 nucleotide intragenic untranslated regions (UTR) in the mRNA and forms a peptide bond between the two residues encoded on either side of the UTR (39). Notably, pUb and pUBL could still be separated from the mistranslated products using cation exchange chromatography to yield pure phosphoprotein for in-vitro experiments, as shown by mass spectrometry and Phos-tag gels.
Undoubtedly, this study shows the most efficient way to generate serine 65 phosphorylated proteins is using a catalytically active form of insect PINK1, first identified by the Muqit laboratory (23). Pure phosphoproteins were obtained by this method on the order of tens of milligrams, sufficient quantities for structural studies. Importantly, the isolated expression of ubiquitin and UBL permit their isotopic labeling for techniques such as NMR spectroscopy. Further modifications could conceivably add affinity tags, paramagnetic ions, or fluorescent tags for a variety of biochemical applications. The main inconvenience of this method, the contamination of the protein sample by the added PINK1, is easily overcome by use of a GST–PINK1 fusion protein that can be reclaimed by affinity purification after the phosphorylation reaction. Despite initial worries of promiscuity from using a hyperactive kinase, we were pleasantly surprised by the remarkable specificity of *Pediculus humanus* PINK1 for serine 65. Mass spectrometry data for both ubiquitin and parkin UBL show only one phosphorylation site and little evidence for subsequent modifications, consistent with results in other studies (2,5,6,32). This is impressive since ubiquitin, in particular, harbours a phosphorylatable threonine 66 residue that is actually more solvent accessible than serine 65 but is apparently not phosphorylated by PINK1. Further, while the primary sequence of ubiquitin is essentially unchanged between *Pediculus humanus* and humans (99% identity), there is significant sequence divergence between *Pediculus humanus* and human parkin in the UBL domain (30% identity). Nonetheless, *Pediculus humanus* PINK1 can effectively phosphorylate human parkin UBL on a timescale similar to ubiquitin. This method for generating preparative amounts of phosphorylated ubiquitin and parkin will therefore be essential for further unraveling the roles of these phosphorylation signals in the cell.
3.5 References


Chapter 4

Structure of phosphorylated parkin UBL domain and insights into PINK1-orchestrated activation†

4.1 Introduction

Post-translational modifications are sophisticated biological “switches”, relaying molecular signals to govern cellular processes and respond to external stimuli. Multiple studies show parkin activity is stimulated through phosphorylation by the kinase PTEN-induced kinase 1 (PINK1) in response to mitochondrial oxidative stress (2,3). This in turn facilitates parkin-mediated ubiquitination of several proteins at the outer mitochondrial membrane and signals the turnover of damaged mitochondria through the mitophagy pathway (4-6). Some mutations in parkin cause its dysfunction, leading to an accumulation of mitochondrial damage that appears to be especially detrimental in neurons (7).

Parkin belongs to the RBR subfamily of E3 ubiquitin ligases (8) that function through a unique catalytic mechanism employing both a RING domain and a covalent thiolester intermediate (9). These enzymes are structurally autoinhibited in their native states by unique accessory domains (10-12), indicating that RBR ligases must be activated in order to carry out their full ubiquitination potential. Specifically, parkin contains an N-terminal ubiquitin-like (UBL) domain shown to inhibit Ub ligase activity (10). Three-dimensional

† Data presented in this chapter has been published. It is reproduced here, with permission from:
structures of parkin show the UBL domain associates with its C-terminal (RORBR) region through both ionic and hydrophobic interactions, blocking the proposed E2 recognition site (see Chapter 2, (13,14)).

PINK1 stimulates parkin activity through phosphorylation of both Ub and parkin’s UBL domain at an equivalent serine 65 position in sequence and structure (2,3,15-18). While each phosphorylation event can increase parkin activity independently, maximal activity is obtained when both parkin and Ub are phosphorylated (15,16). Phosphorylation of parkin at S65 decreases its affinity for RORBR while simultaneously increasing its affinity for phosphorylated ubiquitin (pUb) (see Chapter 2, (13,14,19,20)). Three-dimensional structures show this results from an optimization of a pUb-binding site, remote from the UBL site (see Chapter 2). Further, binding of pUb allosterically induces a conformational change, activating parkin’s activity (see Chapter 2, (13,14,20,21)).

Although the functional effect of PINK1 on parkin activity is well reported, the molecular interpretation of the phosphorylation signal, especially in conjugation with pUb, is less clear. In this work, we present the solution structure of PINK1-phosphorylated UBL (pUBL) from human parkin. A significant loss of thermodynamic stability results from changes to the UBL hydrophobic core, remodeling its secondary structure near the phosphorylation site. Changes to surface electrostatic potential from the phosphoserine group in pUBL disrupt its autoinhibitory association with the C-terminus of parkin. Further, it is shown that phosphorylation of both UBL and Ub are needed to form an extended structure where the E2-binding site is unprotected, allowing its recruitment during the ubiquitination cascade.
4.2 Materials and Methods

4.2.1 Protein Expression and Purification

Human parkin (1-465) and UBL domain (1-76) were overexpressed as His-SUMO fusion proteins in *E. coli* BL21(DE3). Disease-state mutations and the UBL-I44A mutation were introduced using a modified site-directed mutagenesis protocol (22). Cells were grown in LB or minimal media (supplemented with 500 µM ZnCl₂ for full-length parkin) at 37 °C until an OD₆₀₀ of 0.6 was reached at which point cells were cooled to 16 °C and induced with 0.5 mM IPTG for 16 hours. Cells were harvested and resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, 0.5 mM TCEP, 25 mM imidazole, pH 8.0). Cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin), ultracentrifuged at 41,000 rpm (124,000 x g) and proteins purified by Ni²⁺ affinity using a HisTrap FF column on an AKTA FPLC (GE Healthcare). Affinity captured proteins were eluted from the column with elution buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 500 mM imidazole, pH 8.0). Fractions containing the protein of interest were pooled, Ulp1 protease was added to cleave the His-SUMO tag (~1:50 ratio protease:protein) and dialyzed overnight at 4 °C against lysis buffer. After cleavage was complete, proteins were passed over the HisTrap FF column again in lysis buffer and the flow through was collected, containing proteins of interest. A final purification step by gel filtration was performed on a HiLoad Superdex75 pre-equilibrated in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP pH 7.0. Proteins for phosphorylation time-course experiments were aliquoted, flash-frozen and stored at -80 °C until use. Proteins for structural experiments were always prepared fresh and used immediately after purification.
GST-tagged *Pediculus humanus* PINK1 (128-C) was expressed in *E. coli* BL21(DE3) cells in LB media. Cells were grown at 37 °C until an OD$_{600}$ of 0.8 was reached, at which point overexpression was induced with 0.5 mM IPTG and the incubation temperature lowered to 16 °C for 18 hours. Overexpressed proteins were purified on a GSTrap FF column (GE Healthcare) in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT at pH 7.4) and eluted with freshly prepared elution buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT and 10 mM glutathione, pH 8.0). Following elution, glutathione was removed from PINK1 by two 2L dialysis changes against 50 mM Tris, 50 mM NaCl, 3 mM DTT, pH 10, followed by a final 2L dialysis against 50 mM Tris, 50 mM NaCl, 3 mM DTT, pH 7.5.

### 4.2.2 Phosphorylation of Parkin and UBL domain

For phosphorylation time-course experiments, purified GST-PINK1 was used to phosphorylate parkin and UBL mutants as follows. Proteins were diluted in 50 mM Tris, 50 mM NaCl, 3 mM DTT, pH 7.5 to obtain a PINK1:parkin ratio of 1:2(1:20) for full-length parkin(UBL domain) in a total volume of 200 µL. Phosphorylation reactions were initiated by addition of 20 mM MgCl$_2$, 10 mM ATP, pH 7.5 and allowed to proceed at 25 °C. The final concentration of parkin in reactions was 1 µM. At the specified time intervals, 10 µL of sample was quenched with 3x SDS-loading buffer (containing 100 mM EDTA) and boiled at 95 °C for 5 minutes. Samples were then subjected to SDS–PAGE (one 15-lane gel per time-course reaction).

Unstained gels were cut to include the band corresponding to parkin or UBL. Proteins were then transferred onto a shared nitrocellulose membrane (0.2 µm pore size) using a
semi-dry iBlot2 apparatus (Invitrogen). The transfer voltage was as follows: 20 V for 1:00 min, 23 V for 4:00 min, 25 V for 1:00 min. The membrane was then rinsed with water, blocked with 10X BSA blocker (Pierce) in TBS for 1 hour at room temperature. The membrane was rinsed with TBS + 10% aqueous Tween20 (TBST) and incubated with primary antibody (anti-pSer65 parkin, Ubiquigent Cat. No. 68-0056-100) overnight at 4 °C. The next day, the membrane was rinsed with 3-4 washes of TBST, incubated with a fluorescent secondary antibody (anti-sheep Dylight 680nm, Pierce Cat No. SA5-10058) for 45 minutes and washed again 3 times with TBST. Membranes were scanned by Odyssey Imaging system (LiCor) and fluorescence intensity measured at 700 nm.

Fluorescence intensity was plotted against time for each parkin variant, demonstrating the formation of phosphorylated parkin. The formation of pParkin can be described chemically as in eq (1), or kinetically through a second order rate law eq (2). Since the concentration of ATP is present at a 10,000-fold excess and largely unchanged over the reaction, the process was simplified as pseudo-first order eq (3) to yield the rate equation in terms of pParkin concentration eq (4).

\[
\text{Parkin} + \text{ATP} \rightarrow \text{pParkin} + \text{ADP} \quad \text{eq (1)}
\]

\[
\frac{-d[\text{Parkin}]}{dt} = k[\text{Parkin}][\text{ATP}] \quad \text{eq (2)}
\]

\[
\frac{-d[\text{Parkin}]}{dt} = k[\text{Parkin}] \quad \text{eq (3)}
\]

\[
\int_{0}^{t} \frac{-d[\text{Parkin}]}{[\text{Parkin}]} = \int_{0}^{t} kt
\]
\[
\ln \frac{[\text{Parkin}]_t}{[\text{Parkin}]_0} = -kt
\]

\[
[\text{Parkin}]_t = [\text{Parkin}]_0 e^{-kt}
\]

if \([\text{Parkin}] + [\text{pParkin}] = [\text{T}], \quad \{[\text{T}] - [\text{pParkin}]\}_t = \{[\text{T}] - [\text{pParkin}]\}_0 e^{-kt}
\]

and \([\text{pParkin}]_0 = 0, \quad \{[\text{T}] - [\text{pParkin}]\}_t = [\text{T}] e^{-kt}
\]

\[-[\text{pParkin}]_t = [\text{T}] e^{-kt} - [\text{T}]
\]

\[
[\text{pParkin}]_t = [\text{T}] - [\text{T}] e^{-kt}
\]  

**eq (4)**

Non-linear regression was performed to determine \(k \pm \text{SE}\) for each parkin variant. A two-tailed T-test to assess for statistical variation from wild-type parkin was performed. All curve fitting and statistical tests were conducted in GraphPad Prism 6.

For preparative-scale structural experiments, purified GST-PINK1 was used to phosphorylate parkin and UBL as follows. Proteins were dialysed into 50 mM Tris, 50 mM NaCl and 1 mM DTT pH 7.5. A PINK1:parkin ratio of 1:2(1:30) was used to phosphorylate full-length parkin(UBL domain). Phosphorylation was initiated by addition of 20 mM MgCl\(_2\), 10 mM ATP, pH 7.5 and allowed to proceed at 25 °C. The reaction was monitored to completion using Phos-Tag SDS-PAGE and confirmed by mass spectrometry. Phosphorylated parkin species were further purified by a second GST affinity capture and gel filtration chromatography to remove GST-PINK1.
4.2.3 Circular Dichroism

Thermal and chemical denaturation experiments were performed using a JASCO J-810 CD spectropolarimeter equipped with a Peltier temperature control. Data points were collected as an average of 3 replicate scans. A 1 mM path length cuvette was used. For all experiments, purified protein samples were dialysed against 3 changes of 20 mM KH$_2$PO$_4$ pH 7.0 at 4 °C to remove chloride ions. Protein concentrations were verified by measuring absorbance at 280 nm in 6 M Guanidine HCl. For thermal denaturation experiments, the temperature was increased from 5 °C to 85 °C at a rate of 10 °C per hour. For chemical denaturation experiments, proteins were aliquoted into solutions of freshly prepared urea at various concentrations and allowed to reach equilibrium overnight at 4 °C. CD spectra were collected the following day at 5 °C. All data were fit using GraphPad Prism to determine denaturation midpoint as previously described (23,24). Briefly, the free energy difference ($\Delta G_{\text{u H2O}}^{\text{H2O}}$) between folded and unfolded forms of the protein and the slope of the unfolding transition ($m$) were determined by nonlinear regression for the dependence of the observed CD ellipticity ($Y_{\text{obs}}$) as a function of urea concentration ([D]) according to eq (5) and (6) (25).

\[ K_u = e^{\frac{\Delta G_{\text{u H2O}}^{\text{H2O}} + m[D]}{-RT}} \quad \text{eq (5)} \]

\[ Y_{\text{obs}} = \frac{Y_f + Y_u K_u}{1 + K_u} \quad \text{eq (6)} \]
The baselines prior to \((Y_f)\) and following \((Y_u)\) the unfolding transition were also fit to the linear relationships shown in eq (7) and (8) where \(m_f\) and \(m_u\) are the slopes of the pre- and post-unfolding transition and are the intercepts for the folded and unfolded states, respectively.

\[
Y_f = Y_f^0 + m_f[D] \quad \text{eq (7)}
\]

\[
Y_u = Y_u^0 + m_u[D] \quad \text{eq (8)}
\]

### 4.2.4 NMR Spectroscopy

All NMR data were collected on a Varian Inova 600 MHz NMR spectrometer equipped with a triple resonance cryogenic probe. All experiments were performed in NMR buffer (25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.0, 10\% (v/v) D\(_2\)O) at 25 °C unless otherwise specified. 200 µM DSS was used as an internal reference and 300 µM imidazole was used as an internal pH indicator (26). Interaction experiments of (p)UBL and RORBR parkin were performed as previously described in Chapter 2.2.5. All data were processed using NMRPipe (27) and analyzed using NMRViewJ (28).

### pUBL Structure Determination

Triple resonance experiments for assignment were collected with \(^{13}\)C/\(^{15}\)N-labelled UBL samples at concentrations of 200-600 µM. Backbone and sidechain atom assignments were obtained from HNCA (29), HNCACB (30), CBCA(CO)NH (31), HNCO (29), C(CO)NH (30), HCCH-TOCSY (32,33) and (HB)CB(CGCD)HD (34) experiments. \(^{15}\)N-NOESY and \(^{13}\)C-NOESY experiments were collected to define distance restraints. Histidine tautomers and protonation states were determined by C\(\delta\) chemical shifts and N\(\varepsilon\)
chemical shifts in $^1$H-$^{13}$C HSQC and $^1$H-$^{15}$N HMBC experiments (35,36). Structures were calculated in CYANA using manual and automatic NOE assignments and dihedral ($\phi, \psi$) angle restraints from TALOS+ (37,38). TALOS+ restraints were not used for residues 56-66, allowing this region of the structure to be driven solely by NOEs. The standard CYANA protocol was used with eight cycles of structure generation and refinement (100 structures per round). The final 50 structures were water refined using a modified force field in XPLOR-NIH (39). The 25 structures with the lowest NOE energies were chosen as representative of the calculation. SSP scores were calculated using CA and CB chemical shifts (40). CSI scores were calculated using CA, HA and CO chemical shifts (41).

Amide temperature coefficients

UBL and pUBL temperature coefficients were determined by collecting $^1$H-$^{15}$N HSQC spectra of 200 $\mu$M $^{15}$N-labelled UBL and pUBL from 6 °C to 40 °C in 5 °C increments. After each temperature change, samples were equilibrated for at least 30 minutes and the final temperature verified by the chemical shift of water referenced to DSS (42). For each residue, $\Delta \delta_{NH}$ was plotted against $\Delta T$ and the slope of the line (temperature coefficient) determined by linear regression and visualized in GraphPad Prism 6. Plots of temperature coefficient vs chemical shift deviation were generated according to Andersen et al. (43).

Hydrogen-deuterium exchange

Hydrogen bonds were verified by hydrogen-deuterium exchange in $^1$H-$^{15}$N HSQC spectra. A PD-10 desalting column (GE Healthcare) pre-equilibrated with NMR buffer in 100% D$_2$O was used according to manufacturer’s instructions. The earliest fractions
(containing D$_2$O exchanged protein but not H$_2$O buffer) were immediately loaded into NMR tubes and HSQC spectra re-collected identically (~20 minutes after exchange).

**pKa measurements**

$^1$H-$^{15}$N HSQC and $^1$H-$^{13}$C HSQC experiments of $^{13}$C/$^{15}$N-labelled UBL and pUBL at 200 µM were collected over a range of pH 4.7-8.8. The chemical shift of imidazole was used to verify the final sample pH in the spectrometer (26). To calculate pKa of titrating residues, $^1$H chemical shifts of residues over the pH range were plotted and fit to a modified Henderson-Hasselbalch eq (9):

$$\delta_{obs} = \frac{(10^{pH-pKa})\delta_A + \delta_{HA}}{1 + 10^{pH-pKa}}$$

Where $\delta$ represents the chemical shift of the deprotonated ($\delta_A$) or protonated ($\delta_{HA}$) state.

**$^3$J$_{HNHA}$ Coupling Constants**

$^3$J backbone coupling constants for $^{13}$C/$^{15}$N-labelled pUBL (500 µM) were determined by quantitative J-correlation (44). The HNHA experiment was collected in three dimensions (HN, N, HA) with a $^1$H-$^1$H coupling period of 25 ms. Peak intensities were measured in NMRViewJ (27) and $^3$J coupling constants determined according to eq (10) (45).

$$I_{ratio} = -\tan[\pi \cdot J \cdot 0.025] \tan[\pi \cdot J \cdot 0.025]$$

Where $I_{ratio}$ is the ratio of intensities of the cross-peak and diagonal signals for a given proton resonance.
Heteronuclear NOE

$^1$H-$^{15}$N heteronuclear NOEs were measured using $^{15}$N-labelled UBL or pUBL (500 µM) according to the method of (46). Proton saturation was achieved through a 3 s irradiation period following a 3 s recycling delay. The equivalent non-saturated experiment contained a 6 s recycling delay. Both saturated and non-saturated experiments were collected in duplicate in an interleaving manner. Peak intensities and heteronuclear NOEs were determined in NMRViewJ (27).

T2 Relaxation Experiments

T2 relaxation times of $^{15}$N-labelled full-length parkin and $^{15}$N-labelled UBL were determined by arraying the CPMG period and measuring the envelope of signal intensities at 8.75–9.25 ppm in the first increments of $^1$H-$^{15}$N T2 spectra. Two-dimensional $^1$H-$^{15}$N HSQC spectra were then collected with a 30 ms T2 delay period ($2 \times \tau_{1/2}$ of full-length parkin) as a measure to distinguish short vs. long T2 species (47). The concentration of $^{15}$N-labelled parkin was 200 µM. The concentration of $^{15}$N-labelled pParkin was 150 µM with or without an excess of unlabeled pUb.

4.2.5 Analytical Ultracentrifugation

Sedimentation velocity experiments were performed on a Beckman XL-A analytical ultra- centrifuge equipped with an An60-Ti rotor. Double sector cells (1.2 cm) with quartz windows were filled with 380 µL sample and 400 µL reference buffer. All data were collected in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP pH 7.0 at 20 °C with 60+ minutes for temperature equilibration. Experiments with isolated UBL were performed at 55,000 rpm. Experiments with full-length parkin and pUb-bound parkin were performed
at 45,000 rpm. Cells were scanned at equal intervals (60 or 90 scans total) and absorbance measured at 280 nm. Data were processed by c(s) distribution analysis in SEDFIT v15.01 (48), accounting for equilibration and rotor acceleration times. All data were fit to an RMSD <0.005 in SEDFIT. Partial specific volume ($v_{\text{bar}}$) was 0.719 mL/g, viscosity ($\eta$) was 0.0103 Poise, and density ($\rho$) was 1.0043 g/mL. Sedimentation coefficients observed in buffer were corrected for 20 °C in water, and frictional coefficients ($f/f_0$) were calculated from $S_{20,w}$ and known molecular weights of all species as follows:

$$f = \frac{M \left(1 - v_{\text{bar}} \cdot \rho \right)}{N_A \cdot S_{20,w}} \quad \text{eq (11)}$$

$$f_0 = 6 \cdot \pi \cdot \eta \cdot \sqrt[3]{\frac{3 \cdot M \cdot v_{\text{bar}}}{4 \cdot \pi \cdot N_A}} \quad \text{eq (12)}$$

Where $v_{\text{bar}}$ is partial specific volume in mL/g, $\eta$ is viscosity in units Poise, $\rho$ is density in g/mL, $M$ is molecular weight in g/mol, and $N_A$ is Avagadro’s number: $6.022 \times 10^{23}$ mol$^{-1}$.

### 4.2.6 PINK1-activated parkin models

Randomized models of the flexible linker region in parkin (residues 77-142) were generated using the Ensemble Optimization Method (v2.1) (49) using coordinates for pUb-bound parkin (5CAW) and pUBL as rigid bodies. 200 structural models were generated with native-like CA angles for disordered proteins. Shell models were generated for all 200 structures using residue-level primary models in HYDROPRO (50). The atomic elements radius (AER) was set at 4.8 Å for all simulations. Viscosity ($\eta$), and density ($\rho$) values were set to 0.01 and 0.99, respectively for 20 °C in water. Partial
specific volume was unchanged from SEDFIT analysis. Calculated radii of gyration and sedimentation coefficients were extracted for analysis. Visual representation of all 200 structures was done in PyMOL.

### 4.3 Results

#### 4.3.1 Molecular determinants of parkin phosphorylation and disease-state mutants

As described in Chapter 3, a catalytically-active insect orthologue of PINK1, from *Pediculus humanus* (body louse), was employed to phosphorylate parkin at serine 65. Despite differences in primary sequence around the phosphorylation site between human parkin and *P. humanus* parkin (Figure 4.1 A), full phosphorylation was obtained of human parkin and ubiquitin using this insect form of PINK1. The most conserved regions in the UBL domain are in the $\beta$-sheet face containing the hydrophobic patch, a common recruitment site for ubiquitin-interacting motifs (UIMs) (51,52). Therefore, we wondered whether an I44A substitution, a conservative substitution commonly employed to disrupt UIM binding (53,54), could still be phosphorylated by PINK1. I44 is distant in structure (>12 Å) and primary sequence from S65, and should not sterically impede the phosphoryl-transfer reaction catalyzed by PINK1. However, I44A-UBL was severely impaired in its ability to be phosphorylated, as observed by Phos-tag SDS–PAGE. While native UBL was 50% phosphorylated within approximately 2 minutes *in-vitro*, nearly no phosphorylated I44A-UBL was observed after the same time (Figure 4.1 B). This provides evidence that the hydrophobic patch of UBL is involved in the recruitment of PINK1 or the phosphoryl-transfer reaction.
Figure 4.1. Detection of parkin Serine 65 phosphorylation by PINK1.

(A) Structure-based sequence alignment of parkin’s UBL domain from *Homo sapiens* (human), *Rattus norvegicus* (rat), *Drosophila melanogaster* (fly) and *Pediculus humanus* (louse). The sequence of human ubiquitin is shown at bottom. Areas of high sequence conservation are highlighted in green and the S65 phosphorylation site is highlighted in blue. A linear domain map showing UBL secondary structure elements is shown above for reference.

(B) PINK1-phosphorylation time-course of UBL and I44A-UBL monitored by Phos-Tag SDS–PAGE. The retarded band represents the phosphorylated species.

(C) Detection of phosphorylated parkin using a pSer65-specific antibody. Purified parkin and Ser65-phosphorylated parkin (pParkin) of known concentrations were probed by Western Blot, detected by a fluorescent secondary antibody at 700 nm.

(D) PINK1-phosphorylation time-course of full-length parkin and UBL under identical conditions monitored by a pSer65-specific antibody. Fluorescence was measured and plotted against time to determine relative rate constants (k) of phosphorylation.
Similar attempts were made to characterize phosphorylation of full-length forms of parkin by Phos-Tag SDS–PAGE. However, poor resolution and a “laddering” of gel bands corresponding to parkin precluded a conclusive analysis of phosphorylation rates in the context of the full-length protein. This is likely a by-product of the zinc-binding nature of parkin, which could competitively chelate variable numbers of Zn$^{2+}$ ions from the Phos-Tag gel matrix. Therefore, a pSer65-parkin phospho-specific antibody was instead used to characterize *in-vitro* phosphorylation of parkin by fluorescence. The specificity of the antibody for the phosphorylated form of parkin was verified using purified native parkin and Ser65-phosphorylated parkin (*Figure 4.1 C*). Negligible background fluorescence was observed for non-phosphorylated parkin at concentrations below 1 µM, therefore 1 µM protein was used in subsequent phosphorylation reactions. Time-course reactions of parkin showed the isolated UBL domain was phosphorylated approximately 16x faster than the full-length protein under identical conditions (*Figure 4.1 D*). This result is consistent with the hypothesis of the hydrophobic patch recruiting PINK1, which is occupied by autoinhibitory associations in full-length parkin and consequently slows the rate of phosphorylation relative to the isolated UBL.

To further examine the molecular determinants of parkin phosphorylation, especially in the context of disease-causing mutations, relative rates of phosphorylation were determined for parkin and disease-state parkin mutants. Five PD-causing parkin mutants were chosen; G12R, R33Q, R42P, P37L and T55I, sampling the structure of the UBL domain without affecting the antibody epitope (residues 60-72) (*Figure 4.2 A*). Time-course phosphorylation experiments were performed with both full-length parkin (1:2
Figure 4.2. Disease-state mutations impair S65 phosphorylation by PINK1. 
(A) Structure of parkin UBL domain (PDB: 2ZEQ), showing the positions of disease-state mutants examined in this study. The S65 phosphorylation site is also shown in red. 
(B) Purified parkin and UBL mutants. 10X concentrated loading controls used for time course experiments. 
(C) Representative phosphorylation time-course experiments showing Ser65-phosphorylation of purified wild-type and disease state mutants of parkin. The R42P mutant could not be purified in the context of the isolated UBL. 
(D) Relative rates of parkin phosphorylation determined from time-course experiments as in B. The presented rates are a global fit of 3 independent experiments ± SE (represented by error bars). * and ** represent statistically significant deviation (P ≤ 0.05 and P ≤ 0.01, respectively) from wild-type as determined by a two-tailed T-test.
ratio PINK1:parkin) and the isolated UBL (1:20 ratio PINK1:UBL) variants (Figure 4.2 B–C). Whether full-length parkin or isolated UBL were used, the G12R and R33Q mutants were significantly impaired in PINK1-phosphorylation relative to the wild-type protein (Figure 4.2 D). Conversely, the P37L and T55I mutants were phosphorylated at a rate comparable to their wild-type counterparts. In all replicates, however, the slowest mutant to be phosphorylated was R42P in the context of full-length parkin. This mutation is known to unfold the UBL domain in-vitro and consequently could not be purified in the context of the isolated UBL (23). Unexpectedly, none of the tested disease-state parkin variants were impaired in phosphorylation to the extent of the I44A substitution studied earlier. The data show that certain disease-state mutations in parkin’s UBL domain are compromised in their ability to be phosphorylated at serine 65.

4.3.2 Phosphorylation of the UBL decreases its thermodynamic stability

Compared to UBL, phosphorylated UBL (pUBL) was more difficult to handle, with decreased solubility and a greater propensity to precipitate. It was hypothesized that phosphorylation negatively impacted the stability of the UBL fold. Circular dichroism (CD) spectra for native UBL and pUBL showed both proteins had a similar spectral signature with minima observed near 205 nm and 222 nm (Figure 4.3 A), characteristic of a β-grasp ubiquitin fold. However, thermally-treated pUBL showed minimal change in ellipticity near 218 nm, in contrast with UBL which underwent a large and obvious unfolding transition. The lack of change to this region of the CD spectrum of pUBL suggested heating promoted β-aggregation rather than thermal unfolding.
Figure 4.3. S65 phosphorylation destabilizes the UBL domain of parkin.

(A) Circular dichroism (CD) spectra showing thermal denaturation of UBL and pUBL. Spectra were collected from 5 °C (blue curves) to 75 °C (red curves) in 10 °C increments. At right, a temperature-course experiment is shown monitoring change in CD ellipticity in UBL and pUBL at 205 nm. (B) Urea denaturation of UBL and pUBL monitored by CD spectropolarimetry from 0 M urea (blue curves) to 6.5 M urea (red curves) in 0.5 M increments. At right, the unfolding profile of UBL and pUBL as a function of urea is shown by CD ellipticity at 222 nm. Observed denaturation midpoint ± SE is indicated beside each curve. (C) Bis-ANS fluorescence in the presence of UBL and pUBL. An increased fluorescence for pUBL indicates a greater exposed hydrophobic surface than for UBL.
To eliminate interferences from aggregated species in the experiment, the stability of pUBL was tested against chemical denaturation by urea. In this experiment, urea solubilizes an unfolded, random coil form of the protein rather than forming aggregates. Chemical denaturation confirmed pUBL was significantly less stable than UBL, undergoing a much earlier unfolding transition in urea (50% unfolded at 3.3 M vs 4.4 M urea, respectively) (Figure 4.3 B). Thermodynamically, this indicates pUBL is approximately 4.3 kJ/mol less stable than its unphosphorylated form and would be approximately 2% unfolded in its native state. Since pUBL was less stable and more susceptible to aggregation than UBL, the fluorescent probe, 4,4’-bis-1-anilinonaphthalene-8-sulfonate (bis-ANS) was used to probe for changes in hydrophobicity upon phosphorylation. Interestingly, bis-ANS showed markedly greater fluorescence in the presence of pUBL compared to UBL (Figure 4.3 C), suggesting the hydrophobic surface of pUBL is altered. Taken together, the results show that phosphorylation of UBL is associated with structural changes that perturb its folding pathway and thermodynamic stability by modifying the hydrophobicity of pUBL.

4.3.3 Solution NMR structure of phosphorylated Parkin UBL

To identify the structural effects of phosphorylation and establish how this modifies the UBL association with parkin, the solution structure of pUBL was determined using NMR spectroscopy (Figure 4.4 A, Table 4.1). The 25 lowest-energy structures of pUBL form a canonical β-grasp ubiquitin-like fold: a four-strand β-sheet (β1, M1-R6; β2, F13-V17; β3, L41-I44; β4, V67-Q71) and two α-helices (α1, I23-Q34; α2, V56-C59). However, obvious changes occur near the S65 site of phosphorylation, where ligation of the
Figure 4.4. Solution structure of Ser65-phosphorylated parkin UBL.

(A) pUBL structure (closest to average) represented as a cartoon with secondary structure as described in the text. (B) Expanded view of pUBL structure around pSer65. (C) UBL structure (extracted from PDB 5C1Z) is shown for comparison. (D) Expanded view of pUBL structure showing hydrophobic residues affected by phosphorylation. (E) Chemical shift perturbations in methyl resonances upon Ser65 phosphorylation. Resonance assignments for methyl groups in pUBL are indicated (red spectrum), reporting changes to the hydrophobic core upon phosphorylation. (F) Chemical shifts observed during the pH titration of UBL (black curves) and pUBL (red curves). ¹H chemical shifts were measured at each pH and fit by nonlinear regression to determine pKa ± SE (indicated beside the curve).
Table 4.1. Structural statistics for 25 lowest energy structures of pUBL

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<th>Completeness of resonance assignments</th>
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<td>Sidechain (C,H)</td>
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<tr>
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<td>Long-range (</td>
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<td>Max. distance constraint violation, Å(^c)</td>
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<td>Bond angles, º</td>
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<td>Additionally favored</td>
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<tr>
<td>Generously favored</td>
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<td>Disallowed</td>
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<tr>
<th>RMSD to Mean Structure (Å)(^e)</th>
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<tr>
<td>Heavy</td>
<td>1.104 ± 0.123</td>
<td>Backbone</td>
</tr>
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</table>

\(^a\) Talos-derived phi/psi angles  
\(^b\) As reported by Xplor-NIH, using all residues  
\(^c\) No distance violation < 0.1Å  
\(^d\) As reported by Procheck, using all residues  
\(^e\) RMSD to averaged coordinates using residues 1-71
phosphate group rotates the protein backbone and exposes the sidechain of phosphoserine 65 (pSer65) to a solvent accessible position. This conformational change eliminates a hydrogen bond between the S65 hydroxyl and backbone amide of Q62. The reorganization is translated to the upstream residues, L61-I66 (Figure 4.4 B–C), forming a new α-helix-like structural element (α2-L, L61-Q64) that appears as a one-turn helix in water refined structures. Secondary structure propensity scores (40) of pUBL Cα/Cβ chemical shifts are in agreement with calculated structures, as were \(^3\)J\(^{HNHA}\) coupling constants (44) and chemical shift indices (55), including the observed helical propensity in α2-L (Figure 4.5). As a result of these structural changes in pUBL, sidechain atoms of D62, Q63 and Q64 are oriented away from the backbone into solvent, while the aliphatic sidechain of L61 is buried into the protein core. \(^1\)H\(^{15}\)N HSQC data also report the change in backbone structure upon phosphorylation, with large chemical shift changes observed for residues near pSer65 (Figure 4.6), consistent with observations in a previous study (20). Backbone amides of residues D60 through I66 all undergo chemical shifts larger than 0.5 ppm. Significant chemical shift changes were also propagated to more distant residues comprising the β-sheet face and hydrophobic patch of pUBL including residues R6, I44, H68 and V70.

To investigate changes in hydrogen bonding in the pUBL fold, hydrogen-deuterium exchange experiments were collected by NMR. After 20 minutes in D\(_2\)O solvent, only ~15% of pUBL amide resonances were detected in \(^1\)H\(^{15}\)N HSQC spectra (Figure 4.7). Conversely, ~26% of amides in unphosphorylated UBL were observable after the same time. For instance, amide protons of V67 and I69 were protected from solvent exchange
Figure 4.5. Secondary structure propensity in pUBL.
Secondary structure map from the solved pUBL structure is displayed above for reference. (A) Secondary structure propensity (SSP) for pUBL. Positive values are indicative of α-helical propensity and negative values indicate a propensity to β structure. SSP was determined from CA and CB chemical shift assignments, using the software “SSP”. pSer65 was omitted from analysis. (B) Residue plot of $^3J_{HNHA}$ coupling constants obtained from quantitative $^3J$ correlation experiments. Secondary structure thresholds are indicated by dashed lines. (C) Chemical shift index analysis using HA, CA, and CO chemical shifts. A red symbol indicates an α-helical chemical shift propensity. A blue symbol indicates β-strand propensity.
Figure 4.6. Amide chemical shift perturbations in UBL upon phosphorylation. 
(A) $^{1}H$-$^{15}N$ HSQC spectra showing backbone amide resonances in parkin UBL (UBL, black spectrum) and following phosphorylation (pUBL, red spectrum). Resonance assignments are labeled in red for pUBL. Where large chemical shift changes occur, an arrow is indicated to show the chemical shift from UBL upon phosphorylation.
(B) Weighted chemical shift perturbations upon phosphorylation. CSP was calculated as $\text{CSP} = [\Delta \delta_{HN}^2 + 0.2\Delta \delta_{N}^2]^{0.5}$.
Figure 4.7. Backbone amide protection in UBL and pUBL.

$^1$H-$^15$N HSQC spectra of parkin UBL and pUBL in 10% $D_2O$ (top panels) and following exchange into 100% $D_2O$ (bottom panels). NMR spectra in $D_2O$ were collected approximately 20 minutes following first contact with 100% $D_2O$ (total 9.5 minutes experiment acquisition time). Resonances that are detectable after exchange into 100% $D_2O$ are indicated.
in UBL but showed faster exchange in pUBL and were not observable after 20 minutes. The loss of signal is indicative of overall weaker structural bonding in the pUBL structure, and supports an overall decrease in protein stability. Since deuterium exchange for most amides was faster than could be detected by NMR, amide temperature coefficients were also determined to obtain a comprehensive analysis of hydrogen bonding throughout the entire protein backbone in UBL and pUBL. Backbone amide protons that exchange rapidly with water exhibit a strong linear change with temperature, whereas protons involved in hydrogen bonds are more protected from this effect (43,56). Therefore the slope of a plot of $^1$H chemical shift vs temperature represents the temperature coefficient ($\Delta\delta_{\text{NH}}/\Delta T$), where a less-negative slope is indicative of hydrogen bonding or rigidity of the amide (57). Comparison of measured temperature coefficients in pUBL vs UBL agrees remarkably with the initial amide chemical shift analysis. In particular, amide temperature coefficients ($\Delta\delta_{\text{NH}}/\Delta T$) for pUBL showed large decreases near the S65 phosphorylation site, including Q63 and Q64 in the $\alpha_2$-L structural element (Figure 4.8), indicative of amide hydrogen bonding. In contrast, the amide of pSer65 showed increased temperature dependency, reflecting the loss of structural hydrogen bonds with F4 in $\beta_1$, consequently shortening the terminal $\beta_4$ strand. These findings were also corroborated by $^1$H-$^{15}$N heteronuclear NOE measurements that showed phosphorylation increases flexibility in the backbone loop of D62-Q64 and releases pSer65 and I66 from the $\beta_4$ strand of the $\beta$-sheet (Figure 4.9). While S65 and I66 showed heteronuclear NOE values near the average for structured regions of the UBL, the intensity of these NOEs were markedly reduced in pUBL, indicative of a loss of structure in this region.
Figure 4.8. Amide temperature coefficients in UBL and pUBL.

(A) Residue plots demonstrating the effect of temperature on chemical shift in UBL (black) and pUBL (red) backbone amides (note: several residues are near-perfectly overlapped). Amide proton chemical shifts were plotted against temperature and fitted by linear regression. The x-axis of each plot spans 279–313 K and the y-axis spans 0.5 ppm ($^1$H). Residues with slope ($\Delta\delta_{NH}/\Delta T$, the temperature coefficient) less negative than $-4.5$ ppb/K are indicated by an asterisk in each plot. (B) Plot of temperature coefficient vs. chemical shift deviation for amide protons in pUBL. A cutoff line identified in Andersen et al., $\Delta\delta_{NH}/\Delta T = -2.11 - (\text{CSD} \times 2.41)$, to include hydrogen-bonding amides is indicated by a dashed line. Residues that fall on or below the cutoff are listed.

pUBL residues ≤ cutoff

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<tr>
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<td>K32</td>
<td>Q63</td>
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<td>E28</td>
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Figure 4.9. Backbone flexibility increases around the phosphorylation site in pUBL. 
$^1$H-$^{15}$N Heteronuclear NOE measurements for UBL (black squares) and pUBL (red circles) 
backbone amides. The values plotted are an average of two independent experiments at 
600 MHz, indicated by error bars. The average NOE for data within 1 SD of the mean is 
indicated by a dashed line for each species. The S65/pSer65 data point is indicated for 
reference.
A hydrophobic core comprising residues M1, V3, V5, I23, F45, A46, L50, V56, V67 and centered around L61 exists in pUBL as supported by extensive backbone and sidechain NOEs between these aliphatic residues. This network also exists in unphosphorylated UBL, where the S65 hydroxyl group is partially buried, forming a structural hydrogen bond across this core to the backbone amide of D62. The movement of pSer65 into the bulk solvent uncovers a portion of this hydrophobic core in pUBL (Figure 4.4 D). While backbone $^1$H-$^{15}$N HSQC data do not report this change, $^1$H-$^{13}$C HSQC spectra demonstrate extensive changes in methyl resonance positions of M1, V3, I23, A46, L50 and L61 upon phosphorylation (Figure 4.4 E), despite being distant in space from the phosphate group in our structure. In addition, the structure shows pSer65 resides adjacent to I66 that results in large chemical shift changes to its sidechain methyl atoms. These changes to the hydrophobic core reaffirm the bis-ANS experiments that show modified hydrophobicity in pUBL upon phosphorylation.

4.3.4 Phosphoserine 65 modifies the ionization network in pUBL

In pUBL, the pSer65 phosphate is directed towards R6 and H68 in most of the calculated structures (avg distance: 4.8 Å), owing to multiple NOEs observed between I66, R6 and H68 (Figure 4.4 B). Therefore, it was hypothesized that a transient ionic interaction may exist between the negative phosphate group on pSer65 and basic sidechains of R6 or H68. Consistent with this idea, chemical shifts obtained from $^1$H-$^{15}$N HMBC and $^1$H-$^{13}$C HSQC spectra confirmed the $\text{N}_\varepsilon$2-protonated tautomer of H68, with $\text{N}_\varepsilon$2 oriented towards pSer65 in all calculated structures [(35,36)]. To further investigate this, pH titrations were performed to measure the pKa of histidines in UBL and pUBL by tracking the sidechain imidazole $\text{H}_\varepsilon$ chemical shift in $^1$H-$^{13}$C HSQC spectra. Owing to the central position of R6
in β1 (Figure 4.4 B), pKa’s for both H11 and H68 in UBL (6.01 and 5.50, respectively) were suppressed from values expected for an average histidine (~6.6) (58). However, while phosphorylation had no effect on the pKa of H11, the pKa of H68 increased by a half unit from 5.50 to 5.97 in pUBL (Figure 4.4 F). Therefore, this change in pKa can be attributed to the proximity of the negatively charged pSer65 sidechain, which could engage the ionized form of H68.

It was noted that in addition to H68, sidechain methyl groups of L61 in pUBL (but not UBL) titrated with pH, suggesting their chemical environment is also affected by ionization. Fitting of the L61 titration data results in a pKa of 6.41 (Figure 4.4 D). Since this value is distinct from the histidine pKa and L61 is distant from H68 in our pUBL structures, it was suspected this might be reporting ionization of the pSer65 phosphate from the −1 to −2 charge state. This was confirmed by measuring the titration of the pSer65 amide proton, calculating a similar pKa of 6.47, consistent with observations for phosphoproteins (59,60). This indicates the ionization network in pUBL is altered upon S65 phosphorylation, and provide evidence that pSer65 partially neutralizes the H68 positive charge.

4.3.5 Phosphorylation alters the autoinhibitory association in parkin

Given that parkin autoinhibition is maintained through intramolecular interactions with the β-sheet face and I44 patch of UBL, we sought to examine how phosphorylation might directly perturb this binding interface. The decreased affinity of pUBL for RORBR was reported in Chapter 2 (see Figure 2.13), and other studies have also reported a loss of UBL–RORBR interaction upon phosphorylation (14,20). The results presented in Chapter
2 in particular showed ~10-fold weaker binding of pUBL vs UBL measured by isothermal titration calorimetry in *trans* (13). To investigate the structural basis of the weakened affinity, the surface electrostatic potential of the UBL–RORBR binding interface was calculated (61). On its parkin-interacting surface, UBL has an overall positive surface that interacts with an overall negative surface on RORBR (Figure 4.10 A). Upon phosphorylation, the predominantly −2 charge of the O-phosphate group changes the surface to a more negative state by partially neutralizing charges on the β-sheet face of pUBL (Figure 4.10 A). The additional negative potential on the pUBL surface is unfavorably brought into close proximity to the negative binding pocket on RORBR. A model of pUBL-bound parkin was generated maintaining the I44-centered binding interface observed in crystal structures (13,14) (Figure 4.10 B–C). In this model, I44 and V70 maintain contact with L266, as their aliphatic sidechains show minimal change in structure and chemical environment upon phosphorylation (Figure 4.4 E). However, pSer65 is brought into close proximity of the RING1 domain, specifically the carboxylate group of D274, resulting in electrostatic repulsion and disruption of the polar interactions of D274 with H68 and R6 in UBL (Figure 4.10 C). These changes to electrostatic interactions in the binding pocket provide a rationale for the poorer autoinhibition observed in phosphorylated parkin.

To further examine how phosphorylation of the UBL domain modulates its interaction with parkin, the pUBL-RORBR interaction was probed by TROSY NMR spectroscopy from the perspective of RORBR. Consistent with the calorimetric data, NMR experiments showed pUBL is still able to bind parkin in *trans*, despite the weakened affinity. Many of the perturbed residues in RING1 and BRcat are affected in a similar fashion for binding
Figure 4.10. Changes to surface electrostatic potential upon phosphorylation of UBL disrupt autoinhibitory interactions with RORBR.

(A) Surface potential is indicated by color in red (negative), white (neutral), and blue (positive), as calculated by PDB2PQR and APBS. Surface potential was calculated separately for RORBR, UBL (left and middle), and pUBL (right). At left, UBL-bound parkin is shown for reference. In middle and right panels, UBL and pUBL, respectively, are shown dissociated from RORBR to reveal potential on the interacting surface.

(B) Structure of parkin RORBR (PDB: 5C1Z, residues 142–465) with pUBL aligned in the UBL binding region. Unresolved tether residues (A383–A390) were modelled in UCSF Chimera.

(C) Expanded view of B, showing proposed pUBL-binding interface. Residue numberings are coloured according to their respective domains. pSer65 is brought into close proximity of D274 (<5 Å), a possible source of charge repulsion.
of pUBL and UBL, indicating the RING1/BRcat interface is still used for pUBL recruitment (Figure 4.11). Further, no chemical shift changes were observed in the RINGO or Rcat domains upon binding of pUBL, indicating the arrangement of these domains is not modified nor does pUBL interact specifically with either domain. The most significant changes in chemical shift upon phosphorylation occur in the tether region (residues F381-T415) connecting the BRcat and Rcat domains (Figure 4.11). Much of this region is flexible based on heteronuclear NOE experiments (see Figure 2.7) and is unresolved in crystal structures (13,62). Chemical shift perturbations also showed that a portion of this tether (residues F381-G385) lies near the UBL domain in the autoinhibited state. However, phosphorylation of the UBL leads to more pronounced changes in the magnitude and direction of chemical shifts for several residues in the tether (G385-E395) when pUBL is titrated into RORBR parkin (Figure 4.11). Notably, no chemical shift changes were observed to residues beyond R396, a region that includes W403, and is proposed to suppress ubiquitin ligase activity by blocking the E2 binding site (62,63). These differences when pUBL is bound, especially in residues G385-E395, suggest these are specifically reporting the phosphate moiety and indicate that pSer65 is likely near this portion of the tether.

4.3.6 pUb activation leads to an extended parkin structure

Most biophysical experiments to evaluate the impact of S65 phosphorylation have been conducted in trans, using pUBL and RORBR constructs of parkin. To examine how phosphorylation might alter the structure of full-length parkin in cis and to identify how pUb activation further modulates this, analytical ultracentrifugation was used to probe the hydrodynamic shape of parkin in solution. We purified native parkin, fully S65-
Figure 4.11. Interaction of UBL and pUBL with RORBR parkin.

(A) Selected 600 MHz $^1$H-$^{15}$N TROSY HSQC spectra of $^2$H/$^{15}$N-labelled parkin RORBR highlighting differences in UBL (purple, top panels) and pUBL (red, bottom panels) interactions with the tether domain. UBL and pUBL interactions were ~96% and ~92% saturated, respectively, according to affinities in Table 2.2.

(B–C) Full chemical shift perturbation (CSP) plots for RORBR domain interactions with UBL (purple) and pUBL (red). Grey bars indicate resonances that broaden or shift beyond assignment in the bound form. CSP was calculated as $\text{CSP} = [\Delta \delta_{HN}^2 + 0.2\Delta \delta_N^2]^{0.5}$.
phosphorylated parkin (pParkin) and homogenous pParkin/pUb complexes by size exclusion chromatography (13,19). Both parkin and pParkin displayed similar sedimentation profiles and rates ($S_{20,w} = 3.86$ S and 3.88 S, respectively), indicating both forms of parkin are monomeric and retain the same compact shape under the conditions tested, where the UBL (pUBL) domain is associated with the RORBR domains (Figure 4.12 A, black and red). To complement these studies, hydrodynamic properties of autoinhibited parkin were calculated using available three-dimensional structures (13,20,50). Results from this computational approach (3.86 S, $R_G = 27$ Å) match nearly perfectly with the experimental data for parkin and pParkin. In contrast, pParkin/pUb was reproducibly found to sediment faster by ultracentrifugation, at 4.02 S (Figure 4.12 A, blue). Whereas an increase in sedimentation coefficient is expected due to the addition of pUb (~9 kDa), the modest increase in the sedimentation rate must be offset by a change in hydrodynamic shape, reflected in the frictional coefficient ($f/f_0$). Indeed, $f/f_0$ increased from 1.30 to 1.37 with the addition of pUb, indicating pParkin/pUb adopts a more extended conformation in solution. Hydrodynamic calculations using the autoinhibited structure (13) with pUb bound to the observed pUb-binding site (20) yielded a sedimentation coefficient of 4.56 S, much larger than observed experimentally (4.02 S). Together, the data show pParkin/pUb does not maintain a compact structure.

In order to determine the position of pUBL in the pParkin/pUb complex, 200 models were generated where pUBL is randomly displaced from RORBR by its disordered 65-residue linker (residues G77-I142) (49). Visual inspection shows that pUBL samples the available conformational space surrounding RORBR in different models (Figure 4.12 B). As expected, the generated structures occupied a Gaussian distribution of sedimentation
Figure 4.12. Hydrodynamic shape analysis of PINK1-activated parkin.

(A) Sedimentation velocity experiments show little change in hydrodynamic shape upon UBL phosphorylation. A more extended conformation is observed only in the presence of pUb (higher f/f₀). Example surface representations for the given hydrodynamic shapes are shown. (B) Ten representative pParkin/pUb models with a “displaced” pUBL domain. 200 models were generated, allowing pUBL linker to sample random conformations relative to the RORBR/pUb complex (shown dark/light grey). Modelled structures are shown according to their sedimentation coefficients as in C. (C) Distribution of sedimentation coefficients of activated pParkin/pUb models and autoinhibited pParkin/pUb. Histogram x-axis shows calculated S_{20,w} values grouped by ± 0.05 S.
coefficients centered on an average of 3.89 S (Figure 4.12 C). The most extended model had a sedimentation coefficient of 3.36 S, reflecting pUBL displaced by a near-linear linker (R_G = 54 Å). In contrast, the most compact model observed was autoinhibited pParkin/pUb (4.56 S, R_G = 27 Å). Taking all calculated structures into account, the observed sedimentation coefficient (4.02 S) for pParkin/pUb is most consistent with an average structure whereby pUBL is displaced from RORBR with R_G ≈ 32 Å, occupying a range of positions dictated by the configuration of the disordered linker.

To support a model that both phosphorylation of parkin and engagement by pUb are needed to release the pUBL domain, a series of T2-filtered ^1H-^15N HSQC experiments were collected for parkin, pParkin, and pParkin/pUb. Because backbone ^15N atoms in parkin have a short bulk T2 (20 ms) consistent with its 52-kDa size, a short T2 relaxation period was used to effectively suppress most signals arising from the structured domains of parkin. As expected, the resulting ^1H-^15N HSQC spectrum of parkin showed only a series of sharp signals arising from the flexible linker (residues K76–R140), the tether region (E382–T414), and multiple loops in the protein (Figure 4.13 A). Remarkably, the addition of pUb to pParkin resulted in the complete appearance of amide resonances assigned to pUBL (Figure 4.13 C) due to a much longer T2 of these atoms after displacement from RORBR. Notably, in pParkin (without pUb engagement) resonances corresponding to pUBL showed only marginal increases in intensities (Figure 4.13 B), consistent with pParkin maintaining a compact shape, albeit sampling a displaced structure more often due to the weakened affinity of pUBL for RORBR. This is exemplified by the progressive increase in intensity of resonances such as A31, which is not directly involved in the autoinhibitory interaction, but reports the overall T2 of the...
Figure 4.13. pUb displaces pUBL in full-length, phosphorylated parkin. 

$^{1}{H}$-$^{15}$N CPMG, T2-filtered HSQC spectra (scaled identically) of (A) $^{15}$N-labelled full-length parkin, (B) $^{15}$N-labelled pParkin, and (C) $^{15}$N-labelled pParkin after addition of excess, unlabelled pUb. Spectra were collected with a 30-ms spin-echo period to attenuate signals from the fastest-relaxing component. The much slower decay of free UBL signal compared to parkin can therefore serve as a reporter for release of autoinhibition. In the autoinhibited state, only signals from flexible loops are visible (A). After PINK1 phosphorylation (B) and to a greater extent after pUb binding (C), pUBL is released from RORBR and relaxes as a smaller molecular mass entity, enabling its detection beyond the applied spin-echo period. (D) Superposition of $^{1}{H}$ traces through the resonance for Ala 31, showing the progressive release of the UBL with each phosphorylation event.
UBL domain with each phosphorylation event (Figure 4.13 D). These results support the sedimentation velocity experiments that show the full extrusion of the UBL domain occurs only upon PINK1 phosphorylation and recruitment of pUb.

4.4 Discussion

Phosphorylation of both parkin and ubiquitin at serine 65 are, at present, the most convincing activators of parkin’s ubiquitin ligase activity. However, a recent crystal structure of pUb showed the ubiquitin structure is largely unchanged upon phosphorylation (18). The UBL domain of parkin shares high sequence and structural similarity with ubiquitin, but is significantly less thermodynamically stable (23). Consequently, phosphorylated parkin has evaded crystallographic analysis. In this study, the structure of pUBL was solved in solution, revealing significant changes to the secondary structure and hydrophobicity of the protein upon phosphorylation. Phosphorylation of S65 results in a further loss of UBL thermodynamic stability, likely a result of structural rearrangements in the hydrophobic core that largely maintains the fold of ubiquitin-like proteins (64,65). Importantly, the pUBL structure stands in stark contrast to the pUb structure where the phosphate group is accommodated in the loop preceding S65 and maintains a hydrogen bond with the backbone amide of Q62 (18). Conversely, in pUBL this hydrogen bond is abolished and the phosphoserine group rotates the backbone forming a new structural element, α2-L, and instead engages in an ionic interaction with H68. The orientation of pSer65 in pUBL is therefore opposite to that observed in pUb and
provides an explanation for the key differences in physical and functional properties between pUb and pUBL.

In all calculated structures, the sidechain of pSer65 in pUBL faces towards Ne2 of H68, engaging in an ionic interaction that was supported by a decrease in the pKa of H68 upon phosphorylation. This association was also evidenced by the neutralization of surface electrostatic potential across the β-sheet face of UBL upon phosphorylation. Since this region of UBL forms the autoinhibitory interface with RORBR in parkin, this provides a rationale for the weakened affinity between pUBL and RORBR that has been observed in trans (13,14,20). It is conceivable this occurs by pSer65 disrupting the critical H68–D274 ionic interaction at the autoinhibitory interface. It is also noteworthy that this disruption does not fully abolish pUBL binding to RORBR, but rather shifts the equilibrium of the pUBL–RORBR association. Therefore, it is conceivable that UBL phosphorylation serves a dual purpose: it optimizes the pUb-binding site and primes the upstream region of the tether for remodeling to accommodate the incoming E2~Ub conjugate, as supported by previous affinity measurements (14).

Unquestionably, the analytical ultracentrifugation data and T2 relaxation experiments show the full displacement of the UBL domain occurs only upon both phosphorylation of parkin and recruitment of phosphoubiquitin. Significant changes to both the frictional ratio (which reports the shape of a molecule) and T2 relaxation time of UBL (which reports molecular tumbling and indirectly, molecular size) occurred only upon engagement by pUb, providing strong evidence that pUb indispensible to parkin activation. Notably, the hydrodynamic properties determined here experimentally for
autoinhibited and activated parkin were in excellent agreement with previous molecular dynamics simulations (66). The allosteric pUBL/pUb relationship highlights the importance of both PINK1 phosphorylation signals in remodeling parkin to an extended structure where the E2–Ub binding site is presumed to be more accessible.

Interestingly, certain Parkinson’s disease mutations in the UBL domain were found to impede S65 phosphorylation by PINK1. In particular, the G12R, R33Q and R42P variants were phosphorylated significantly more slowly than the wild-type protein. This suggests these mutants may be impaired in E3 ligase activity and consequently in activating mitophagy, a possible cause of their deleterious effects. In contrast, two mutations, P37L and T55I, did not affect the ability of the UBL to be phosphorylated by PINK1. This was unexpected given T55I was the closest tested mutation to the S65 phosphorylation site in both sequence and structure. Rather, the efficiency of phosphorylation seemed to be more closely correlated with the stability of the UBL fold. The R33Q and R42P substitutions have markedly decreased thermodynamic stabilities relative to the wild-type protein, with the R42P substitution resulting in complete unfolding of the UBL domain (23,24). Likewise, we observed the I44A substitution could not be efficiently phosphorylated by PINK1. These results suggest the hydrophobic patch and overall fold of the UBL may govern PINK1 recruitment, rather than a consensus sequence around S65, which is not immediately obvious.

Beyond activation of parkin’s E3 ligase potential, it will also be interesting to see how UBL phosphorylation affects its function with other UBL-interacting motifs, including the S5a proteasomal subunit and deubiquitinating enzymes, including ataxin-3 (67,68).
The pUBL structure solved here and model of phosphorylation-induced UBL release will be vital to further understanding the full cellular significance of parkin function and increase our knowledge of regulation in the ubiquitin-proteasome system.

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Chapter 5

Probing the catalytic mechanism of parkin ubiquitination

5.1 Introduction

Parkin belongs to the family of E3 ubiquitin ligases known as RING1–BRcat–Rcat (RBR) ubiquitin ligases (1-3). This family of E3 ligases catalyze ubiquitination using a unique hybrid mechanism, employing both an E2-recruiting RING domain and a covalent catalytic intermediate with ubiquitin (4). This covalent intermediate is absolutely dependent on a conserved catalytic cysteine in the C-terminal Rcat domain (4-6). Experiments showed this intermediate is readily hydrolyzed by 2-mercaptoethanol, suggesting the cysteine linkage is a thiolester bond with the C-terminus of ubiquitin (4-6). The Rcat~Ub thiolester intermediate prepares the Ub moiety for transfer to a substrate protein lysine, presumably the ultimate target of the ubiquitination cascade.

Recent structures of RBR proteins including parkin, HHARI and HOIP have revealed a conserved fold for the catalytic Rcat domain (7-13). The structures show Rcat domains adopt a unique bilobal fold governed by two structural Zn$^{2+}$ ions that are coordinated in a linear fashion (see Figure 5.1 A, C). The proposed catalytic cysteine is surface exposed and lies adjacent to zinc site II and the C-terminus of the Rcat domain. Interestingly, these structures are completely distinct from canonical zinc-binding RING domains commonly found in ubiquitin ligases. Instead, the Rcat fold closely resembles BRcat (Benign Rcat) domains found upstream in sequence of Rcat domains in all RBR E3 ligases. (14). An
important distinction is that BRcat domains show no intrinsic catalytic activity due to lack of the catalytic cysteine that is conserved in Rcat domains (4,14).

The precise chemical mechanism of ubiquitin transfer is unknown for RBR ubiquitin ligases, although it is possible that these enzymes function in a similar manner to HECT-type ubiquitin ligases and deubiquitinating (DUB) enzymes that catalyze ubiquitination and deubiquitination, respectively, through thiolester intermediates. The active site structures of many cysteine-dependent DUBs are well studied and function similar to papain-like cysteine proteinase enzymes (15-18). In these enzymes, a conserved histidine residue is polarized by a nearby acidic residue, consequently raising the pKa of the histidine. This histidine serves to deprotonate a catalytic cysteine that acts as a nucleophile on the protein substrate, forming a thiolester intermediate with the active site cysteine. This cascade repeats with a water molecule acting as the nucleophile to hydrolyze the protein substrate. It is conceivable this process proceeds analogously in RBR ubiquitin ligases, with a nucleophilic substrate lysine substituting for water in the second half of the reaction, forming an isopeptide bond between ubiquitin and substrate.

The catalytic triads of these enzymes are well suited to analysis by Nuclear Magnetic Resonance (NMR) spectroscopy, owing to the high sensitivity of a nucleus’ chemical shift to atomic structure, ionization, and hydrogen bonding (19). The chemical shifts of histidine nuclei in particular are remarkably sensitive to ionization, allowing identification of protonation, tautomeric state and pKa determination (20-22). Similarly, deprotonation of the thiol group in cysteine can be studied by NMR spectroscopy,
specifically by analysis of β proton and carbon chemical shifts, although these phenomena are notoriously less sensitive than for histidine (23).

This study investigates the catalytic mechanism employed by the Rcat domain in parkin, using NMR spectroscopy to elucidate ionization properties in the Rcat active site. The presence of a conserved, functional catalytic triad was established that likely exists across the entire family of RBR ligases. Quantitation of sidechain pKa’s for the active site residues in Rcat was conducted, revealing that a central histidine acts as a base catalyst throughout the catalytic mechanism. Further, the application of irreversible electrophilic ubiquitin probes was comprehensively studied to develop a method to generate stable Rcat~Ub conjugates, that mimic the covalent intermediate formed during catalysis. Together, the work provides new insight into the chemical mechanism of parkin ubiquitination and the thiolate-imidazolium ion pair that enhances the reactivity of a catalytic cysteine in Rcat-containing ubiquitin ligases.

5.2 Materials and Methods

5.2.1 Cloning, Protein Expression and Purification

*Drosophila melanogaster* parkin Rcat (residues 410-482) was overexpressed as a His-SUMO fusion protein in *E. coli* BL21(DE3). Single point mutants were generated using a modified site-directed mutagenesis protocol (24). Cells were grown in LB or minimal media (supplemented with 500 µM ZnCl₂) at 37 °C until an OD₆₀₀ of 0.6 was reached at which point cells were cooled to 16 °C and induced with 1 mM IPTG for 16 hours. Cells
were harvested and resuspended in lysis buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 25 mM imidazole, pH 8.0). Cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin), ultracentrifuged at 41,000 rpm (124,000 x g) and proteins purified by Ni\textsuperscript{2+} affinity using a HisTrap FF column on an AKTA FPLC (GE Healthcare). Affinity captured proteins were eluted from the column with elution buffer (50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 500 mM imidazole, pH 8.0). Fractions containing the eluted protein of interest were pooled, Ulp1 protease was added to cleave the His-SUMO tag (~1:50 ratio protease:protein) and dialyzed overnight at 4 °C against lysis buffer. After cleavage was complete, proteins were passed over the HisTrap FF column again in lysis buffer and the flow through was collected, containing proteins of interest. A final purification step by gel filtration was performed on a HiLoad Superdex75 pre-equilibrated in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP pH 7.0. If Rcat protein was being used to generate Rcat~Ub conjugate, Ulp1 digestion was omitted and SUMO–Rcat was instead purified by gel filtration chromatography performed at pH 8.3 without any reducing agent present.

The ubiquitin plasmid used to generate the Rcat~Ub conjugate was obtained as a kind gift from Brian Mark (University of Manitoba) in a pTXB1 vector (as a Ub/intein/CBD fusion protein). The plasmid was transformed into BL21(DE3) cells, and a single colony picked to inoculate starter cultures in LB media that were grown to saturation overnight. The next day, one litre cultures were inoculated and grown at 37 °C until an OD\textsubscript{600} of 0.6 then dropped to 25 °C and induced 15 minutes later with 1 mM IPTG. Cells were harvested 4 hours after induction and resuspended on ice in lysis buffer (50 mM HEPES, 100 mM NaOAc pH 6.5). Cells were lysed and immediately centrifuged at 41,000 rpm (124,000 x g)
g) for at least 1 hour. Clean chitin beads (3 x 15 mL slurries) were extensively pre-equilibrated with lysis buffer to remove ethanol or azide storage solution. Cleared cell lysates were added to chitin beads and allowed batch bind for 1 hour. Beads were applied to a gravity column, allowed to settle, and unbound lysate slowly passed over the beads. Chitin beads were then washed well with lysis buffer (3 manual washes with ~20 mL buffer). Beads were quickly rinsed with 10 mL cleavage buffer (lysis buffer + 100 mM MESNA), then another 10 mL fresh cleavage buffer was added and the column incubated at room temperature (or optimally, 37 °C) for 24-48 hours. Following cleavage, the column was opened and cleaved Ub~MESNA proteins were collected. Another 5 batches of 5 mL lysis buffer was added to push through remaining Ub~MESNA proteins, until all Ub~MESNA was eluted, as judged by SDS-PAGE. Ub~MESNA was immediately concentrated down to 2 mL (at 4 °C) and applied to a HiLoad Superdex75 column in 50 mM NaOAc, 100 mM NaCl, pH 5.0 (the column must be absolutely free of reducing agents and at a mildly acidic pH to stop Ub~MESNA hydrolysis). Fractions containing Ub~MESNA were pooled and used immediately or flash frozen in liquid nitrogen and stored at -80 °C.

5.2.2 Synthesis of electrophilic Ub probes

UbBr was synthesized as described in (25). Briefly, 0.9 g of bromopropylamine hydrobromide was dissolved in 10 mL of 400 µM Ub~MESNA in 50 mM NaOAc, 100 mM NaCl, pH 5.0. The solution was basified with 1.5 mL of 2 M NaOH and rapidly mixed to avoid prolonged protein exposure to NaOH. The final pH of the solution was 9.8-10.3 and was agitated/rocked for 15 minutes at room temperature. After this time, the reaction was quenched with 1.5 mL of 2 M HCl to restore the pH to near 5.0. This
solution was then dialyzed in a 10 K MWCO dialysis bag against 50 mM NaOAc, 300 mM NaCl, pH 5.0 for 8-12 hours, followed by a final dialysis for 4 hours against 25 mM HEPES, 100 mM NaCl, pH 8.3. UbBr probes were spin-concentrated and used immediately at this stage for conjugation to Rcat.

UbVS was synthesized as described in (25). Briefly, 50 mg glycine vinyl sulfone was dissolved in 600 µL of 2 mM Ub-MESNA in 50 mM NaOAc, 100 mM NaCl pH 5.0. N-hydroxysuccinimide was dissolved separately in buffer to make a 2 M stock, which was brought to pH 6.5 with NaOH. 90 µL of this 2 M stock of N-hydroxysuccinimide was added to the protein solution. To initiate the reaction, 50 µL of 2 M NaOH was added to the solution to increase the pH of the reaction to 7.6. The solution was incubated at 37 °C for 90 minutes with light shaking, and then quenched with 50 µL of 2 M HCl. The protein was subjected to gel filtration to remove excess salts and reagents and then used immediately.

5.2.3 Generation of Rcat~Ub conjugate

The optimized conditions to generate Rcat~Ub conjugate were as follows. SUMO-Rcat was purified as described to homogeneity by Ni²⁺ affinity and size exclusion chromatography leaving the SUMO fusion protein intact. SUMO-Rcat and UbBr were exchanged into 25 mM HEPES, 100 mM NaCl, pH 8.3. The two proteins were mixed together to obtain a final solution (~10 mL volume) of 100 µM SUMO-Rcat and 800 µM-1000 µM UbBr. The reaction progress was monitored by SDS-PAGE and allowed to proceed overnight. The reaction was quenched by applying the solution to a Ni²⁺ column, where unreacted Ub products were extensively washed away from SUMO-Rcat. Affinity
captured proteins were eluted stepwise with 5, 10 and 50% gradients of Ni$^{2+}$ elution buffer, 50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, 500 mM imidazole, pH 8.0. The eluted proteins were pooled, Ulp1 protease was added (1:50 ratio protease:proteins) and the solution dialyzed overnight against 50 mM Tris, 200 mM NaCl, 0.5 mM TCEP, pH 8.0. The following day, proteins were reapplied to the Ni$^{2+}$ affinity column and the affinity purification repeated, collecting flow through fractions that contained predominantly Rcat–Ub conjugate with some unreacted Rcat species. This solution was spin concentrated, and applied to a Superdex75 Increase (24mL) column in 300 µL injection batches running at 0.5 mL/min in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.0. Fractions containing Rcat–Ub as judged by SDS-PAGE were pooled and concentrated again for NMR experiments.

5.2.4 NMR Spectroscopy

All NMR data were collected on a Varian Inova 600 MHz NMR spectrometer equipped with a triple resonance cryogenic probe. All experiments were performed in NMR buffer (25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.0, 10% (v/v) D$_2$O) at 25 °C unless otherwise specified. 200 µM DSS was used as an internal reference and 300 µM imidazole was used as an internal pH indicator (26). All data were processed using NMRPipe (27) and analyzed using NMRViewJ (28).

Histidine tautomers and protonation states were determined by Cδ chemical shifts in $^1$H-$^{13}$C HSQC experiments (21). For pH titrations, $^1$H-$^{15}$N HSQC and $^1$H-$^{13}$C HSQC experiments of $^{13}$C/$^{15}$N-labelled Rcat (free or Ub-conjugated) at 200-500 µM were collected over a range of pH 5.9-10.5. To adjust the pH, approximately 1 mL of protein in
NMR buffer was rapidly mixed with 0.2-1.0 µL increments of 1 M HCl or NaOH and monitored by a micro pH meter until the desired pH was reached. The chemical shift of imidazole was also used to verify the final pH in the spectrometer (26). To calculate pKa of titrating residues, $^1$H chemical shifts of residues over the pH range were plotted and fit to a modified Henderson-Hasselbalch equation 1:

$$\delta_{\text{obs}} = \frac{(10^{pH-pKa})\delta_A + \delta_{HA}}{1 + 10^{pH-pKa}}$$

Where $\delta$ represents the chemical shift of the deprotonated ($\delta_A$) or protonated ($\delta_{HA}$) state.

### 5.2.5 Mass Spectrometry

Whole protein ESI-MS was performed on a QTof Ultima mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode with an Agilent 1100 HPLC used for flow injection. The survey range was set as m/z 600-1800, the source temperature was 80 °C and cone voltage was 50 V. Raw data were deconvoluted by MaxEnt algorithm.

Tandem ESI-MS/MS for Rcat~Ub conjugate was performed on a Thermo Orbitrap Elite mass spectrometer equipped with a nanoflex spray ESI source and run in positive ion mode. The instrument was run in FT/IT/CID Top15 mode. Trypsin digested peptides were introduced into the spectrometer from a C18 HPLC column in H$_2$O/acetonitrile. The survey range was set as 350-1800 with +1 charged ions rejected from the collision cell.
5.3 Results

5.3.1 Evidence for a catalytic triad in parkin’s Rcat domain

Recent three-dimensional structures of parkin show the active site geometry in the Rcat domain is well conserved across organisms from humans to *Drosophila melanogaster* (8-11). Additionally, NMR structures of the isolated Rcat domain show little difference when compared to structures in the context of RORBR, suggesting the presence of other domains in parkin do not modify the overall fold of Rcat (9). Due to solubility issues with human Rcat, this study describes the Rcat domain from *Drosophila melanogaster*, employing a construct spanning amino acids 410-482, according to the fly residue numbering.

To confirm the catalytic nature of the Rcat domain, the reactivity of Rcat was tested towards the irreversible ubiquitin probe, ubiquitin vinylsulfone (UbVS), an electrophilic probe that conjugates non-reversibly onto the catalytic cysteines of ubiquitin ligating and deubiquitinating enzymes (29,30). Formation of a non-reducible Rcat~Ub conjugate was dependent on the presence of the catalytic cysteine, C449 (**Figure 5.1 B**). Examination of Rcat sequences from parkin and other related RBR ubiquitin ligases revealed several conserved features in the active site around this catalytic cysteine (**Figure 5.1 C**). Firstly, the catalytic cysteine is contained within a GGCxH motif, where two tandem glycines (G447, G448 in fly parkin) form a tight turn preceding the catalytic cysteine, and a histidine residue follows at the +2 position (H451 in fly parkin). The imidazole sidechain of the histidine is therefore oriented in a similar direction as the catalytic cysteine relative to the protein backbone. Secondly, an acidic or polar residue is found further in sequence...
Figure 5.1. A catalytic triad mediates ubiquitin transfer in parkin’s Rcat domain. 

(A) Structure of parkin RORBR (PDB: 4I1H). A dashed cyan line represents missing electron density in the crystal structure. Grey spheres represent the 8 Zn\(^{2+}\) ions in the protein. The Rcat domain is colored in black with the proposed catalytic triad shown in yellow. An expanded view of the Rcat domain around the catalytic site is shown beneath. 

(B) Conjugation of an irreversible ubiquitin probe, ubiquitin vinyl sulfone (UbVS) onto the Rcat is dependent on C449. The reaction was performed at pH 7.5 where thiols, but not hydroxyls, are nucleophilic towards UbVS. 

(C) Structure-based sequence alignment of Rcat domains from *Drosophila melanogaster* (Dm) parkin, human (Hs) parkin, and four other human RBR ubiquitin ligases. Active site residues (as described in the text) are highlighted in blue. Zinc coordinating residues are highlighted in yellow.
but adjacent to the histidine sidechain in three-dimensional space (E462 in fly parkin). Finally, an aromatic residue follows distant in sequence but again adjacent in space to the histidine (W471 in fly parkin).

The structure and geometry of active sites in RBR ligases therefore resemble catalytic triads found among a broad range of ligase and hydrolase enzymes. In these enzymes, a central histidine residue serves as a general base to deprotonate a nucleophilic cysteine or serine residue, which becomes covalently bonded to the substrate of interest through a thiolester or ester linkage, respectively. To test whether catalytic residues in parkin harbor this characteristic of canonical catalytic triads, we examined the parkin Rcat domain by NMR spectroscopy. Parkin Rcat contains two histidine residues: the proposed catalytic histidine (H451) and a second histidine (H479) involved in coordination of a structural Zn$^{2+}$ ion. While the resonances corresponding to aromatic protons of H479 appear in the expected regions of a $^1$H-$^1$C HSQC spectrum, the $\delta$2 proton of H451 does not have the expected chemical shift for a histidine aromatic proton. (HB)CB(CGCD)HD correlation experiments instead assigned the H451 $\delta$2 proton at ~5.7 ppm (Figure 5.2 A), approximately 1.5 ppm ($^1$H) upfield of the average chemical shift (avg 7.19 ppm, BioMagResBank: www.bmrbr.wisc.edu/ref_info/statful). This chemical shift indicates extensive shielding occurs on the H451 $\delta$2 proton, a result of significant nearby electron density. Based on the parkin Rcat structure, a glutamate residue (E462) resides adjacent to the histidine sidechain. Therefore, we hypothesized this glutamate polarizes the histidine residue adjacent to the catalytic cysteine. While substitution of the catalytic cysteine did not affect the chemical shift of the H451 $\delta$2 proton, substitution of E462 to
Figure 5.2. Polarization of H451 is dependent on E462.

(A) Aromatic $^1$H-$^{13}$C HSQC spectrum of parkin Rcat domain superimposed with selected active site mutants, C449F (blue) and E462A (red). Substitution of E462 reverts the chemical shift of H451 $\delta_2$ to that of an average histidine resonance (shift shown by red arrow). A dashed box represents the expanded region shown in B. (B) Upfield region of the aromatic $^1$H-$^{13}$C HSQC spectrum of parkin Rcat (wild-type). The pH-dependant titration of the resonance corresponding to H451 $\delta_2$ is shown from low pH (light contours) to high pH (dark contours). Histidine atom nomenclature is shown.
alanine reverts the chemical shift of the δ2 proton to ~7.2 ppm, as expected for an average histidine (Figure 5.2 A). The assignment of histidine protons in Rcat E462A was verified using constant-time (CT) $^1$H-$^{13}$C one-bond correlation experiments, where $^{13}$C nuclei coupled to 0 or 2 carbon nuclei are in opposite phase to $^{13}$C nuclei coupled to 1 carbon nucleus, identifying the δ2 protons in histidine.

In addition to $^1$H chemical shifts, $^{13}$C chemical shifts also provide valuable information about tautomeric states of histidine. Specifically, the $^{13}$C chemical shift of the δ2 histidine carbon can distinguish between Nδ1 and Nε2-protonated tautomers (21). Analysis of $^{13}$C chemical shifts in Rcat show H451 adopts an Nε2-protonated tautomer when in the uncharged state, owing to a δ2 chemical shift well below the threshold of ~122 ppm (Figure 5.2 A). Conversely, the zinc-coordinating histidine, H479, shows a δ2 chemical shift of 126 ppm, suggesting this histidine is Nδ1-protonated, consistent with its zinc-coordinating role in the Rcat structure. Taken together, the data show H451 is polarized through an association with E462.

5.3.2 pKa determination for residues in the parkin catalytic triad

In canonical catalytic triads, the active site histidine is elevated in pKa, conferring its role as a general base (18). To determine whether the pKa of H451 is elevated in this manner, we measured the sidechain pKa by NMR spectroscopy in a pH titration. The chemical shifts of δ2 and ε1 histidine protons are highly sensitive to protonation state of the aromatic ring, reporting the average distribution of charged and neutral states in solution (20,31). Therefore, measurement of these chemical shifts over a range of pHs covering
the ionization event allows the precise determination of a histidine pKa. $^1$H-$^{13}$C HSQC spectra covering the aromatic proton region were collected for Rcat from pH 6–9. The upfield $\delta$ proton of H451 showed a pH dependent shift of 0.34 ppm ($^1$H) during the titration, confirming ionization of H451 occurs over the measured pH range (Figure 5.2 B). An even larger titration shift of $\sim$1.0 ppm ($^1$H) is observed for the $\varepsilon$1 proton of H451, reporting the same ionization event (Figure 5.3 A). The increased sensitivity of the $\varepsilon$2 proton to ionization provides the more accurate measurement of pKa (31), therefore we performed non-linear regression to fit the $\varepsilon$2 proton chemical shift to a Henderson-Hasselbalch curve, solving for a single pKa of 7.40 ± 0.01 (Figure 5.3 A). Importantly, the lack of change to resonances of F461 or the other histidine H479, which coordinates a structural Zn$^{2+}$ ion and is thus “locked” in the neutral state, confirm the protein fold was maintained over the titration. Consequently, spectra at conditions below pH 6 were not possible due to protein unfolding and precipitation, likely resulting from protonation of zinc coordinating histidine and cysteine ligands.

The observed pKa of 7.40 for H451 is significantly higher than an average histidine residue in proteins, frequently cited between 6.0-6.6 (31-33). To test whether this pKa is influenced by neighbouring residues in the active site, the pKa of H451 in the context of C449F and E462A active site mutants was also measured. C449F was used to due to this being a pathogenic ARJP mutation by elimination of the catalytic cysteine, while E462A was chosen as a simple alanine mutant to eliminate the charged nature of glutamate. While substitution of the catalytic cysteine did not change the pKa of H451, substitution of the adjacent glutamate, E462, reverted the pKa of H451 to a value of 6.71 ± 0.07
Figure 5.3. pKa determination of parkin’s catalytic histidine.

(A) Aromatic $^1$H-$^{13}$C HSQC spectrum of parkin Rcat showing the pH-dependent titration of H451 from low pH (light contours) to high pH (dark contours). Titrations were repeated with C449F and E462A substitutions and observed chemical shift plotted as a function of pH (graph at right). Plots were fit by non-linear regression to a modified Henderson-Hasselbalch equation to determine pKa ± SE. (B–C) Selected regions of $^1$H-$^{15}$N HSQC spectra during pH titrations show several resonances specifically respond to ionization of H451. Chemical shift vs pH plots for the indicated residues are shown at right.
(Figure 5.3 A). The results show that E462 chemically shields the aromatic protons of H451 and subsequently increases its pKa, supporting its proposed role as a base catalyst.

While most resonances in Rcat are unaffected over the course of the pH titrations, several other atoms in the active site do experience chemical shift changes upon ionization of the active site histidine. For example, a tryptophan sidechain itself is not ionizable, however the W471 indole proton resonance titrates simultaneously with H451 as observed in $^1$H-$^{15}$N HSQC spectrum (Figure 5.3 B). The titration curve is shifted in the E462A mutant, suggesting this tryptophan sidechain is responding specifically to ionization of H451. In another Rcat mutant lacking the catalytic histidine (H451F) the resonance for the W471 indole amine proton was unaffected by pH and undergoes no measurable titration, confirming its dependence on H451. In addition, another tryptophan in the active site, W463, also shows similar chemical shift changes in both its backbone amide and, to a lesser extent, sidechain amine protons during the pH titrations (Figure 5.3 B–C). The position of this tryptophan in the Rcat structure explains this effect, with the backbone nitrogen of W463 only 4.7 Å from the histidine aromatic ring, and the indole nitrogen separated by a larger distance of 7.9 Å. The data show that ionization of the catalytic histidine in Rcat results in chemical changes that propagate to other residues composing the active site.

Next, the pKa of the catalytic cysteine itself was investigated. Cysteine sulfhydryl groups are deprotonated, on average, near a pH of 8.6 (34). This ionization event can be observed in chemical shift changes in aliphatic beta atoms of the cysteine sidechain by NMR spectroscopy, although the magnitude of these chemical shift changes are markedly
smaller than those observed for histidine residues (23). Indeed, observation of an inflection point for the C449 beta group over the pH titration was complicated by the clear influence of multiple chemical events. As observed in $^1\text{H}$-$^{13}\text{C}$ HSQC spectra, an initial upfield shift occurs in the two C449 beta proton chemical shifts, followed by a larger downfield change in $^{13}\text{C}$ chemical shift (Figure 5.4 A). The initial $^1\text{H}$ shift could be fit to an inflection at $7.20 \pm 0.05$, before the $^1\text{H}$ shift reverses downfield again. Conversely, the $^{13}\text{C}$ chemical shift could not be fit to a pKa, as the rate of this chemical shift change continues to increase as a function of pH well beyond the pH range that could be studied (>10.5). Importantly, over the data points used for curve fitting, the chemical shifts of other Rcat cysteine residues that coordinate Zn$^{2+}$ were unchanged, again confirming the integrity of the protein fold during the experiment (Figure 5.4 A). The measured pKa is similar to that calculated for H451, although outside of the margin of error. It is worth mentioning that both pKa’s were determined from spectra collected back to back on identical samples. Therefore, the difference in fitted pKa cannot be due to differences in sample conditions or error in pH measurement. The data suggest the formation of a thiolate–imidazolium ion pair between C449 and H451 interconverting in solution near neutral pH.

Since the initial nucleophilic attack by the catalytic cysteine relies on its deprotonation, the pH dependence of this nucleophilic attack was studied using UbVS. The reactivity of Rcat towards UbVS was conducted from pH 5.8 to 9.0 and monitored by reducing SDS–PAGE. Conjugation of UbVS occurred in a pH-dependent manner, following a sigmoidal shape consistent with the pH titrations conducted by NMR. Fitting of the data results in an inflection at pH $7.10 \pm 0.07$, within error of that observed for the C449 beta proton
Figure 5.4. pKa determination of parkin's catalytic cysteine.

(A) Aliphatic $^{1}$H-$^{13}$C HSQC spectrum of parkin Rcat showing the pH-dependent titration of C449 Cβ from low pH (light contours) to high pH (dark contours). Zinc-coordinating residue, C459, is shown as a reference. Observed chemical shifts were plotted as a function of pH (graph at right). Data were fit by non-linear regression to a modified Henderson-Hasselbalch equation to determine pKa ± SE. The fit pKa excludes data points where the chemical shift reverts and begins to increase again.

(B) pH-dependent reactivity of Rcat for ubiquitin vinyl sulfone probe. Proteins were mixed at various pHs and allowed to react for 1 hour. The resulting complex migrates at ~16 kDa by reducing SDS-PAGE. At right, band intensity at ~16 kDa was measured using an Odyssey Imager (Licor), plotted against pH and fit to a modified Henderson-Hasselbalch equation to determine an “apparent” pKa ± SE.
(Figure 5.4 B). The experiment shows the catalytic cysteine in Rcat is enhanced in chemical reactivity relative to an average cysteine residue.

5.3.3 Generation of a non-hydrolyzable Rcat~Ub conjugate

After determination of the ionization properties in the free Rcat active site, it was desired to obtain similar parameters for the enzyme in the catalytic intermediate. This state is represented by a thiolester-bound Rcat~Ub complex, where the catalytic cysteine of Rcat is covalently linked to the carboxy C-terminus of ubiquitin. Due to the extremely transient nature of this intermediate, a more stable structural mimic must be employed for solution studies. The irreversible Ub probe, UbVS, satisfies this requirement by generating an thioether-bound Ub intermediate, which is non-hydrolyzable. The quantities of UbVS required for a preparative scale synthesis necessitated that UbVS be synthesized in-house. This is accomplished using a self-cleaving intein expression method to generate a C-terminally-reactive Ub moiety (Figure 5.5) (35). A “warhead” with a thiol-reactive group is then ligated to Ub to create an irreversible Ub probe. Glycine vinylsulfone, glycine vinylmethylester and glycine vinylcyanide are commonly used warheads, whereby the vinyl group reacts with an active site cysteine by a Michael addition (25,29). Various alkyl halides are also employed due to their thiol-reactive nature, with bromopropylamine and chloroethylamine being the best characterized (25).

To synthesize UbVS, a purified Ub~MESNA adduct was reacted with glycine vinylsulfone in the presence of N-hydroxysuccinimide to catalyze formation of an activated ester, as described (25). After 1.5 hours, the reaction was quenched and products assessed by LC-ESI-MS. Negligible signal corresponding to the Ub~MESNA
Figure 5.5. Reaction scheme to generate a stable Rcat–Ub conjugate. Detailed descriptions are found in Materials and Methods and Results sections of text.
reactant was observed, however multiple Ub-derivative products were detected. The dominant product was the desired product, UbVS (MW: 8623 Da), however multiple other Ub derivatives were also detected (Figure 5.6 A). Base-hydrolyzed Ub, harbouring a native C-terminal carboxylic acid, was observed (MW: 8507 Da). Moreover, adducts of +135 Da from the desired product were readily observed in the mass spectra, consistent with addition of more than one glycine vinylsulfone molecule onto the parent ubiquitin protein. The structures of these products are polymers whereby the primary amine of one glycine vinylsulfone group reacts with the vinyl group of another (Figure 5.6 A). Unfortunately, each of these adducts are still reactive Ub probes, as the terminal vinylsulfone group contains a reactive alkene. Subsequent attempts to conjugate Rcat and UbVS products therefore resulted in a mixture of Rcat~Ub products with varying separating linker lengths (Figure 5.6 B). The NMR spectra of these conjugates are highly complicated by the differing chemical environments around each of these linkers, precluding their characterization by NMR. Unfortunately, attempts to minimize polymerization by optimizing reaction conditions were unsuccessful. Subsequent investigation showed the glycine vinylsulfone reagent and UbVS showed similar polymerization patterns as tested by ESI-MS. This shows glycine vinylsulfone spontaneously undergoes a polymerization reaction in solution, generating a heterogeneous mixture of adducts.

To circumvent the issue of polymerization, synthesis of another electrophilic probe, ubiquitin bromopropylamine (UbBr) was attempted. This alkyl halide probe reacts with a catalytic cysteine through an $S_{N}2$ substitution mechanism, generating a thioether (see Figure 5.5). To synthesize UbBr, Ub~MESNA was rapidly mixed with bromoproylamine
Figure 5.6. Electrophilic vinyl sulfone generates heterogeneous Ub probes.  

(A) Deconvoluted ESI-MS spectrum of ubiquitin species following conjugation with glycine vinyl sulfone. The desired product is observed at 8623 Da (UbVS), however Ub species with multiple vinyl sulfone groups are significant contaminants (observed as +135 Da species). The proposed chemical structures of the adducts are shown, where “Ub” represents residues 1-75 in ubiquitin.  

(B) Deconvoluted ESI-MS spectrum of Rcat-Ub species generated with UbVS probes. Each species shows evidence of polymerization with multiple vinyl sulfone groups, manifesting as +135 adducts, labelled on the spectrum.
hydrobromide at high pH, allowed to react for 15 minutes with shaking and then quenched with acid. Products were assessed by LC-MS, revealing 3 main Ub species (Figure 5.7 A). UbBr was the dominant product, with the expected molecular weight (MW: 8626 Da). Base-hydrolyzed Ub from the MESNA conjugate was again observed, although to a lesser extent as before, likely due to a shorter reaction time. Finally, base-hydrolyzed UbBr was also observed (MW: 8564 Da), consistent with hydrolysis of the halide group by water to form a primary alcohol. Unlike with UbVS, the contaminant products from synthesis of UbBr are unreactive towards cysteine and therefore pose no problem in subsequent reactions. Conjugation of Rcat and UbBr subsequently generated an Rcat–Ub conjugate, as monitored by SDS-PAGE (Figure 5.7 B). Following conjugation, an additional affinity chromatography step was performed to remove unreacted Ub products, followed by gel filtration as a final sample clean-up and to remove any unreacted Rcat (Figure 5.7 C).

The final Rcat–Ub conjugate appeared pure by SDS-PAGE (Figure 5.7 C) and was next assessed by mass spectrometry. ESI-MS of the protein complex confirmed one main species of the expected molecular weight of one Rcat and one Ub molecule (Figure 5.8 A). To confirm that Rcat and Ub were linked through the catalytic cysteine, a trypsin digest of the complex was performed and tryptic peptides analyzed by tandem MS/MS. Both the expected +2 and +3 charged peptides were readily observed for a $^{13}$C/$^{15}$N-labelled Rcat peptide harbouring the catalytic cysteine (peptide MW: 1440.71), containing an additional 115.15 Da for the propyl linker and C-terminal glycine of ubiquitin. MS/MS fragmentation of these two peptides confirmed the catalytic cysteine was specifically modified, with all y and b fragment ions assigned between the two
Figure 5.7. Electrophilic alkyl halides generate “homogenous” Ub probes.

(A) Deconvoluted ESI-MS spectrum of ubiquitin species following reaction with 3-bromopropylamine. The desired Ub probe is observed at 8626 Da. Base-hydrolyzed by-products are observed but are unreactive towards thiols. The proposed chemical structures of observed species are shown, where “Ub” represents residues 1-75 in ubiquitin. (B) Reaction of Ub* with Rcat shows formation of an Rcat~Ub conjugate. Following the reaction, the protein was re-purified by Ni²⁺ affinity, cleaved with Ulp1 and another Ni²⁺ affinity step performed before concentration for size-exclusion chromatography. (C) Size-exclusion chromatography for final sample cleanup and removal of unreacted Rcat species. An SDS-PAGE gel is shown beneath the chromatogram, showing the contents of the fractions. ** denotes Rcat~Ub complex that was further characterized by mass spectrometry and NMR (lanes 14-17 were pooled).
Figure 5.8. Generation of a stable, ether-linked Rcat~Ub conjugate.

(A) Deconvoluted ESI-MS spectrum of Rcat~Ub conjugate generated using an alkyl halide Ub probe. The Ub species is at natural isotope abundance and Rcat is $^{13}$C/$^{15}$N-labeled, leading to a broad deconvoluted peak. Theoretical mass assuming complete $^{13}$C/$^{15}$N incorporation is 17262 Da. (B) Theoretical y/b ion table for a $^{13}$C/$^{15}$N-labelled Rcat tryptic peptide harboring the catalytic cysteine and ether modification. Masses correspond to those expected using the employed isotopic labelling scheme. (C) MS/MS spectrum showing fragmentation of +3 ion of theoretical peptide (m/z = 480.88). Observed y/b ions by manual fragment assignment are indicated. (D) MS/MS spectrum as in C showing fragmentation of +2 ion of theoretical peptide (m/z = 720.83).
spectra (Figure 5.8 B–D). The other cysteine residue contained within the Rcat peptide was modified with iodoacetamide during reduction and alkylation steps, confirming the UbBr probe does not modify other cysteine residues in Rcat that are zinc-coordinated.

5.3.4 Characterization of the Ub-bound catalytic intermediate

After confirming the integrity of the Rcat~Ub conjugate by mass spectrometry, the protein conjugate was characterized by NMR spectroscopy. In conjugates generated for NMR studies, the Rcat moiety was $^{13}$C/$^{15}$N isotopically-labelled while the ubiquitin moiety was at natural isotopic abundance. Optimized reaction conditions produced an NMR sample containing approximately 400 µM Rcat~Ub. $^1$H-$^{15}$N HSQC spectra of this Rcat~Ub conjugate revealed many resonances had been perturbed from their chemical shifts in the spectrum of free Rcat (Figure 5.9 A). All amide resonances for residues composing the active site loop, including G447, G448, C449, M450 and H451 were shifted from their position in free Rcat, consistent with a change in chemical environment from conjugation to Ub. Other amide resonances in the active site also showed significant perturbations, including E462, W463 and W471, which were all shown to be responsive to histidine ionization in the earlier pH titration experiments. Beyond chemical shift changes in the active site, we also observed several unexpected chemical shift changes in the N and C terminal regions of the Rcat domain in the context of the Rcat~Ub intermediate. These included E418, A424, N426 in the N-terminal region preceding Rcat, which are unstructured and unassociated with the active site in all previous NMR and X-ray crystal structures (8-11,36). Additionally, resonances at the C-terminus of parkin showed significant chemical shift changes upon Ub conjugation, despite not being directly in the active site. These C-terminal residues included amides of A478, H479 and
Figure 5.9. Chemical shift perturbations in parkin Rcat–Ub intermediate. 
(A) $^1$H-$^{15}$N HSQC spectrum of Rcat–Ub intermediate (black spectrum) superimposed with free Rcat (red spectrum). The spectra were collected under identical conditions. Amide resonances that show noticeable perturbations in the Ub-bound state are labelled on the spectrum. (B) Surface representation of parkin Rcat structure (PDB: 2LWR). Residues labelled in A are coloured blue on the surface. The catalytic cysteine, conjugated to the C-terminus of ubiquitin, is shown in red.
the terminal residue G482. Mapping of the perturbed amide resonances onto the structure of free Rcat reveals a broad interacting surface localized to one face of the molecule, where Ub presumably sits in the catalytic intermediate (Figure 5.9 B). However, the changes in chemical environment remote from the catalytic cysteine suggest there may be a minor conformational change in Rcat that occurs transiently upon conjugation of Ub. Such a change would not be unexpected given structural rearrangements that would occur to accommodate a ubiquitin molecule. Nonetheless, the majority of amide resonances are unchanged in chemical environment between Rcat and Rcat~Ub, including most zinc-coordinating residues, confirming the overall protein fold is maintained in this catalytic intermediate. To assign the amide resonances of Rcat in the Ub-bound state, three-dimensional experiments were collected in the context of Rcat~Ub. Unfortunately, signal intensities in these experiments were extremely weak for most atoms in the structured region of Rcat, especially surrounding the catalytic cysteine. This prevented a conclusive re-assignment of these peaks in the context of the Rcat~Ub intermediate.

Sufficient signal in two-dimensional spectra, however, allowed for analysis of the catalytic histidine ionization through $^1$H-$^{13}$C HSQC spectra as was described for the isolated Rcat. A constant-time experiment was first collected to verify the assignment of aromatic H451 atoms in the context of the Rcat~Ub conjugate. A resonance at ~5.9 ppm was observed in phase corresponding to one $^{13}$C nucleus coupling, most likely the H451 δ2 proton (Figure 5.10 A). Thus, the shielding of this nucleus that was described earlier in free Rcat persists in the Rcat~Ub conjugate. This would suggest that in the Rcat~Ub intermediate, E462 remains in close contact to the δ2 proton of H451, consistent with a
Figure 5.10. pKa determination of catalytic histidine in a Ub-bound intermediate.  
(A) Aromatic $^1$H-$^{13}$C HSQC spectrum of Rcat–Ub intermediate. The spectrum was collected as a constant-time experiment, where $^{13}$C nuclei coupled to zero or two carbon atoms (black) appear in opposite phase to those coupled to one carbon atom (red). Atom assignments for histidine residues are labelled. (B) Selected region of the $^1$H-$^{13}$C HSQC spectrum showing the pH-dependent titration of H451 from low pH (light contours) to high pH (dark contours). (C) Observed chemical shifts from pH titrations were plotted as a function of pH and fit by non-linear regression to determine pKa $\pm$ SE.
base-catalyst role for H451 in the second (Ub-discharging) reaction. To assess this in a quantitative manner, a pH titration was performed with Rcat−Ub and monitored through aromatic $^{1}$H−$^{13}$C HSQC spectra. As was observed in free Rcat, a pH dependent shift was readily observed for the ε1 proton resonance of H451 (Figure 5.10 B). The corresponding resonance for H479 was largely unaffected by this change in pH, consistent with a zinc coordinating role for H479 in Rcat−Ub. Fitting of the titration data for H451 revealed a single inflection corresponding to a pKa of 7.02 ± 0.03 for this histidine in the context of Rcat−Ub. While this pKa value remains increased relative to an average histidine, it is not increased to the extent observed in the free Rcat state (pKa: 7.40). However, the influence of E462 on H451 persists, as this pKa is still higher than observed in the context of the E462A mutant. This suggests this glutamate may have less of an effect on H451 in the context of the Rcat−Ub intermediate, likely dictated by the Ub adduct. The results show the Ub-bound intermediate of parkin arranges the active site in a manner that prepares the catalytic histidine to act as a base catalyst for ubiquitin discharge to a substrate lysine.

5.4 Discussion

The release of multiple RBR ubiquitin ligase structures in recent years uncovered a new mechanism of ubiquitin transfer employing Rcat domains. Solution and crystal structures showed a conserved catalytic cysteine is positioned in a manner remarkably similar to catalytic triads in other cysteine protease enzymes (7-13). Here, chemical properties of the parkin active site were studied by NMR spectroscopy, which is uniquely suited to investigating these catalytic mechanisms (19). A histidine (H451) central to the active site
structure is polarized by an association with E462, raising its pKa for its role as a base catalyst. Interestingly, the orientation of this histidine in RBRs is distinct from other catalytic triads in enzymes such as subtilisin and alpha-lytic protease, where an Nδ1-protonated histidine is hydrogen bonded to an adjacent acidic residue (19,37). Instead we show Rcat domains adopt an Ne2-protonated histidine in close contact to a negative carboxylate anion, leading to a highly shielded δ2 proton that may exist across all members of the RBR family. This histidine orientation is shared by some DUB enzymes, including UCHL1, that use a cysteine protease mechanism to perform the reverse reaction of ubiquitin ligases (38). Interestingly, DUB enzymes also harbor a highly-conserved glutamine residue in the active, proposed to stabilize the tetrahedral transition state (39). An equivalently positioned electropositive sidechain is missing in Rcat structures. However, the conserved “GG” motif preceding the catalytic cysteine is highly reminiscent of chymotrypsin-like hydrolase enzymes, where the catalytic cysteine is often preceded by GG or GS in a tight turn. In these proteases, backbone amides of these small amino acids provide an electropositive contribution to form an “oxyanion hole” that stabilizes formation of the tetrahedral transition state (40-42). Finally, the structures of Rcat domains show a conserved tryptophan sidechain positioned parallel to the histidine imidazole ring, separated by only 3.5 Å. This distance is within the ideal contact distance for π-stacking (43), suggesting the arrangement of these aromatic residues may be important over the catalytic cycle. Although the precise chemical role of this tryptophan is unknown, this rationalizes why an aromatic residue at this position is found across the family of RBR ubiquitin ligases.
It has been hypothesized that rearrangements of the Rcat active site occur during catalysis, as the distance between the histidine and cysteine sidechains (~5 Å in all Rcat structures) is too large in the resting state to favor a thiol–imidazolium ion pair (8,9). To investigate a potential active site reorganization, a structure of the covalent Rcat~Ub catalytic intermediate is highly desirable. The investigation of electrophilic ubiquitin probes for this purpose revealed several insights into the use of these probes for structural studies. Importantly, the vinyl-containing probes were prone to polymerization, and thus the synthesis of fresh probes and their chemical precursors is essential. Vinyl-containing probes, including UbVS, are commonly used in biochemical assays, where a gel mobility shift serves as a reporter of ubiquitin ligase or deubiquitinase activity. Although not resolvable by gel, it is possible (and highly probable) that these modified products are heterogeneous in nature and therefore care should be taken when interpreting these results. This explains why, despite the multitude of crystal structures of protein complexes with electrophilic ubiquitin probes, none have successfully employed UbVS, instead preferring the alkyl halide probes (17,44-47). A notable exception to this is a crystal structure of a ubiquitin C-terminal hydrolase (UCH) in complex with ubiquitin vinylmethylester (38). It is worth mentioning that the precursor for generation of the alkyl halide UbBr probe, 3-bromopropylamine, is readily available and significantly less expensive compared to precursors like glycine vinylsulfone used to synthesize UbVS.

The integrity of the covalent Rcat~Ub intermediate generated in this study was validated extensively by both mass spectrometry and NMR spectroscopy. Extensive amide chemical shifts in Rcat upon Ub conjugation support the idea of a structural rearrangement, and could support previous hypotheses of a Ub-associating region in the
unstructured N-terminus of Rcat (48,49). Importantly, the reaction scheme employed here to generate the Rcat~Ub conjugate allows for segmented isotopic labeling of either Rcat or Ub moieties, enabling its future use in a multitude of NMR studies. The obstacle of low signal intensity observed in the triple-resonance experiments could be a combined consequence of sample concentration, conformational exchange, or the size of the molecule (over 17 kDa). Nonetheless, this is promising for ultimately obtaining a three-dimensional structure of this intermediate in solution, which would be the first RBR E3~Ub catalytic intermediate. In summary, this study represents the first description of active site ionization equilibria in parkin and will be important for unraveling the full catalytic mechanism of RBR ubiquitin ligases. Further, these data will be valuable for designing therapeutics that directly perturb the catalytic machinery.

### 5.5 References


Chapter 6

Summary and Perspectives

6.1 Autoinhibition in parkin

Autoinhibition of parkin activity by its UBL domain is a fascinating evolutionary result to suppress substrate binding in a ubiquitin ligase enzyme. Parkin’s UBL domain shares high sequence and structural similarity with its own substrate, Ub, however the UBL is significantly less thermodynamically stable (1,2). It was shown in Chapter 2 the UBL associates with the C-terminal RBR region of parkin with micromolar affinity in trans, maintaining a compact tertiary structure (see Figure 2.2, Figure 2.13, Table 2.2). Surprisingly, the affinity of Ub for the same C-terminal parkin fragment was found to be an order of magnitude weaker, despite both proteins primarily employing their highly conserved I44-patch to bind to parkin. Apparently, UBL binding is preferred over Ub, possibly owing to a secondary interface near the β1-β2 connecting loop—the most divergent region in sequence and structure between UBL and Ub (see Figure 1.6). It would be interesting to see if a Ub-UBL chimera, harbouring the primary sequence of Ub but substituting the β1-β2 connecting loop from UBL, interacts with parkin more favourably than native Ub to support this hypothesis. The fact that parkin autoinhibition occurs naturally in cis is also a major factor discriminating UBL binding from Ub, as the local concentration of UBL is drastically increased by its physical tethering to the C-terminus of parkin. In this regard, it would also be interesting to test whether another
parkin chimera, with the whole N-terminal UBL substituted for Ub, is even suppressed in ubiquitin ligase activity compared to ΔUBL parkin.

There is evidence that during the ubiquitination cascade, an E2–Ub conjugate is recruited to parkin and the Ub moiety partly occupies the UBL binding site (3). This would suggest that, at some stage, Ub binding becomes preferred over UBL binding. It is possible this preference is governed by the E2 enzyme in the context of an E2–Ub conjugate, as it has been shown that E2–Ub conjugates can bind cooperatively to their respective E3 ligase partners (4). Although the E2 enzyme UbcH7 binds with comparable affinity as the UBL domain to parkin (5), it is possible that in the context of an E2–Ub conjugate this affinity is enhanced such that E2–Ub can outcompete the autoinhibitory UBL association.

In addition to UBL-mediated autoinhibition, there may be other endogenous factors that regulate parkin’s ubiquitin ligase activity. The available crystal structures of parkin show the proposed E2 binding site, the RING1 domain, is obstructed not only by the UBL domain but also by a so-called “tether” spanning residues 377-415 (6-9). In particular, a region of this tether from residues 395-404 is rigidly associated with RING1 (see Figure 2.7–2.8). Interestingly, neither UBL nor its phosphorylated form (pUBL) change the chemical environment of backbone atoms in this region (see Figure 4.11). Without further changes, this tether would block a canonical E2–Ub interaction (10). It is worth noting, however, that parkin actually lacks several conserved features of RINGs that normally engage an E2–Ub conjugate. This could suggest that parkin utilizes a non-canonical E2-binding mechanism, perhaps actually utilizing this tether to recruit E2 enzymes, rather than obstructing them. Using the amide chemical shift assignments for
this region, it should be possible to test whether these residues gain flexibility or lose flexibility upon E2 binding, representing a displacement or contribution from E2 binding, respectively. Elucidating this mechanism of E2-binding will be the next step in understanding the full catalytic cycle of parkin.

The multiple crystal structures of autoinhibited (UBL-bound) parkin and ∆UBL parkin represented a breakthrough in our understanding of parkin’s tertiary structure. The most remarkable feature of these structures was the sheer lack of difference between the UBL-bound and ∆UBL structures of parkin. Specifically, the orientation and accessibility of the catalytic cysteine was essentially identical across all structures. Indeed, the NMR chemical shift perturbation experiments of RORBR ± UBL confirmed the chemical environment of the catalytic site is not altered by UBL dissociation (see Figure 2.9). However, the NMR data allowed us to discern more subtle changes not apparent by simple superposition of the RORBR ± UBL crystal structures. Specifically, in addition to the UBL-binding interface on RING1, binding of the UBL propagates a structural change from the BRcat through helix H3 to the RINGO/RING1 interface. This revealed a hinge at the RINGO/RING1 interface that is responsive to UBL binding, causing a subtle closing/opening of parkin with UBL association/dissociation, respectively (see Figure 2.12). This conformational change is allosteric by definition, as binding of UBL induces a structural change at a secondary site. This allosteric switch could potentially be targeted by therapeutics to perturb the balance of these conformational states that govern parkin autoinhibition.
It should not have been surprising then, that nature already exploits this allostery through an endogenous effector molecule that favours the “open” state to displace the UBL. What was shocking is that this allosteric effector is in fact parkin’s substrate, ubiquitin! The discovery that ubiquitin phosphorylated at serine 65 (pUb) by PINK1 binds to this allosteric site rationalized the genetic links originally identified between PINK1 and parkin (11-13). Additionally, parkin itself is phosphorylated at the same serine 65 site in its UBL domain to further activate its ubiquitin ligase activity (14,15). Suddenly, the focus of this thesis became unraveling how these two phosphorylation events cooperate to modify the autoinhibited structure of parkin to an activated state.

6.2 Cooperation of pParkin and pUb to induce mitophagy

Remarkably, phosphorylation of parkin’s UBL decreases its affinity for RORBR (weakening the inhibitory association), while phosphorylation of Ub confers the opposite effect, drastically increasing its affinity for RORBR (see Figure 2.13, Table 2.2). The question of how phosphorylation of serine 65 in Ub and parkin’s UBL activate parkin was initially seemingly contradictory: How could a phosphate group at the identical position in two homologous molecules (Ub and UBL) confer opposite biochemical effects, yet cooperate to stimulate parkin activity? Somehow, these phosphorylation events weakened the inhibitory properties of UBL, yet enhanced binding of parkin’s substrate, Ub. The finding that pUb is primarily an allosteric activator, binding at a site distinct from the UBL, began to unravel this mystery (see Figure 2.14). The next major advancements
came from elucidating the structures of these two phosphorylated molecules. While the structure of pUb is basically unchanged upon phosphorylation, pUBL undergoes a larger structural change to accommodate the phosphate moiety (Figure 6.1). The significant structural differences in pUBL show why it cannot compete for the pUb binding site, yet can independently increase parkin activity, if only partially. The latter arises by the phosphate group in pUBL interfering with the autoinhibitory association with RING1 and the tether domain (see Figure 4.10–4.11). However, an important finding from this work is that engagement of parkin by pUb is absolutely necessary to form an extended parkin structure where pUBL is displaced, uncovering the E2~Ub binding site (see Figure 4.12–4.13).

Figure 6.1. Comparison of phosphorylated Ub and parkin UBL.
Superposition of pUBL structure (red, PDB: 5TR5) and pUb structure (gray, PDB: 4WZP). An expanded view around the phosphorylation site is shown to demonstrate the significant conformational changes observed in UBL vs. Ub upon phosphorylation. pSer65 in ubiquitin maintains a hydrogen to the backbone amide of Q62, whereas pSer65 in UBL is oriented into solvent and facing H68.
While the specific order of phosphorylation events in cells remains controversial, parkin has evolved such that each phosphorylation signal induces a positive effect on its activity while the receptors for each signal are physically distinct. This cooperativity is evidenced in two possible scenarios below:

If parkin were initially phosphorylated (Figure 6.2 A), the autoinhibitory interaction is weakened and this modestly increases its ubiquitin ligase activity, beginning to ubiquitinate substrate proteins on the mitochondrial membrane. These ubiquitin chains are subsequently phosphorylated by PINK1, retaining parkin at the mitochondrial membrane and maximizing its ubiquination activity by the full extrusion of the UBL domain. Polyubiquitin chains accumulate on the mitochondria and become phosphorylated by PINK1, and the pathway feeds forward. An unanswered question about this scenario is how PINK1 is able to phosphorylate the UBL in autoinhibited parkin if both I44 and S65 in parkin, which are needed for the phosphorylation event, are occluded in the autoinhibitory association.

If Ub were initially phosphorylated (Figure 6.2 B), this pUb signal recruits parkin and stimulates its activity by displacing the UBL domain. This allows parkin to begin synthesizing polyubiquitin chains on the mitochondria. Further, the displaced nature of the UBL domain facilitates its phosphorylation by PINK1, maintaining parkin in its activated state. The polyubiquitin chains synthesized by parkin are subsequently phosphorylated by PINK1, and the pathway feeds forward. A lingering question of this model is how the initial ubiquitin is added to a substrate if parkin is still autoinhibited. It is possible that the latent activity of autoinhibited parkin may be sufficient to catalyze this
Figure 6.2. Mechanisms of induction of a feed-forward PINK1/parkin pathway. Two potential scenarios for PINK1/parkin cooperativity. See text for a detailed description of each scenario. Blue diamonds on the OMM represent various putative parkin substrates described previously in Chapter 1. Yellow pac-man shape represents putative autophagy/mitophagy receptors. (A) Parkin is initially phosphorylated by PINK1. (B) Ub is initially phosphorylated by PINK1.
initial ubiquitination event, or perhaps this occurs by another unidentified E3. This would not be surprising, as multiple E2/E3 pairs have been previously shown to cooperate in catalyzing polyubiquitin chains (16,17). In these examples, one E2/E3 pair serves to initiate the cascade via a monoubiquitination event, while a second E2/E3 pair subsequently elongates the polyubiquitin chain. It is conceivable that such a mechanism could also occur with parkin, providing an initial ubiquitin substrate before any phosphorylation event.

Conveniently, either of the aforementioned scenarios converge by a rapid accumulation of ubiquitin chains coated over the OMM, which are subject to further phosphorylation of PINK1. Therefore, the proposed mechanisms of activation are consistent with a feed-forward amplification model to rapidly induce parkin activity at any stage in the mitophagy cascade (18). Recent work has identified that polyubiquitin chains and, more specifically, phosphorylated polyubiquitin chains, are the signal that recruits autophagy machinery to initiate mitophagy (19-21). Two autophagy receptors in particular, NDP52 and OPTN, were shown to be specifically recruited to polyubiquitin chains (and phosphorylated polyubiquitin chains) on the OMM (20,21). Interestingly, another kinase, Tank-binding kinase 1 (TBK1), was shown to facilitate this binding to polyubiquitin by phosphorylating these autophagy receptors (22). It will be interesting to further uncover potential crosstalk between these autophagy receptors and PINK1/parkin. For example, if parkin maintains a strong affinity for pUb throughout mitophagy, is parkin engulfed into lysosomes with damaged mitochondria? Presumably a recycling mechanism for parkin would be beneficial for other mitochondria that may be under similar stressors.
6.3 Mechanistic insights into RBR ubiquitin ligases

Although parkin was largely the focus of this thesis due to its direct implication with PD, it is likely that many of the findings here extend to other members of the RBR ubiquitin ligase family. All other members of this family contain the RBR domains in the same sequential order, suggesting they share at least some common catalytic features. Despite this presumption, recent structures of other RBR members HHARI and HOIP show completely different spatial orientations of the RBR domains. In parkin and HOIP structures, the RING1 domain is found central to the RBR module (7,23). Conversely, in HHARI, the BRcat domain is more central with Rcat and RING1 domains on opposite sides of BRcat and not in contact with each other (24). It is important to note that these observations have been largely made in the context of the autoinhibited enzymes, and it is likely that upon engagement by an E2~Ub conjugate and during Ub transfer these RBR enzymes undergo large conformational changes to occupy similar active catalytic orientations. The structures released to date therefore exemplify the extraordinarily diverse mechanisms by which RBR ubiquitin ligases have evolved to be structurally autoinhibited in their native states.

Additional insight into mechanisms of RBR ubiquitin transfer will certainly come by understanding how E2~Ub conjugates engage this family of ubiquitin ligases. Recent discoveries in HOIP and HHARI have shown that RING1 of these RBRs stabilize “open” conformations of E2~Ub conjugates (23,25), in contrast to canonical RING domains that stabilize predominantly “closed” E2~Ub conformations (26). Consistent with this idea, the so-called “linchpin” residue conserved throughout canonical RING domains, a
conserved basic residue (Arg/Lys) that donates a hydrogen bond to promote the closed conformation, is absent in all RBR ubiquitin ligases (25,26). Further, RING1 domains in RBR ligases contain an insertion of varying length in the L2 loop that is known to govern E2–Ub binding (10,27). This is further evidence that RING1 domains of RBR ubiquitin ligases utilize a novel method of E2–Ub recruitment.

It is now known that Rcat structures in RBR ubiquitin ligases are well-conserved, suggesting the chemistry of ubiquitin transfer is shared across RBR members. The active site chemistry in parkin was examined in this thesis to determine the roles of several conserved residues in increasing the reactivity of the catalytic cysteine. It was shown here that a conserved glutamate residue in parkin polarizes a histidine to enable deprotonation of the catalytic cysteine, analogous to papain-like catalytic triads (see Figure 5.1–5.2). It was further proposed that a “GG” motif preceding the catalytic cysteine might play a role in stabilizing the tetrahedral transition state during the Ub transfer reaction. This has direct pathological implications, as many of these residues, including G429, G430, C431 and E444 in human parkin (G447, G448, C449, E462 in Drosophila parkin) are targeted by missense mutations that cause ARJP. Interestingly, the pathogenic mutations G429E and G430D are both substitutions that insert negative charges in the loop preceding the catalytic cysteine, and would disfavour formation of the tetrahedral transition state by acting as oxyanion destabilizers. The E444Q substitution would presumably be impaired in raising the pKa of the adjacent histidine (although notably, one RBR member–HOIP–actually contains a glutamine residue at this position). Finally, the lack of an active site nucleophile altogether is a clear rationale for the deleterious effects of the C431F substitution. Future studies on the catalytic Rcat–Ub intermediate using the protocol
developed in this thesis will certainly provide additional insight into the second half of this mechanism, the discharge of Ub from Rcat onto substrate. It remains to be discovered whether Rcat domains are able to recruit substrates independently or whether other regions in parkin act as substrate scaffolds for this final Ub transfer reaction.

### 6.4 Significance of the work

It is truly remarkable how much knowledge of parkin and other RBR ubiquitin ligases has come about throughout the duration of this thesis. It is worth re-mentioning that at the commencement of this project, no structures of intact RBR domains were available and the first report of autoinhibition in parkin had only recently surfaced. Unraveling the details of this autoinhibitory mechanism in solution therefore represents a significant advancement of our understanding of parkin regulation, especially with regards to its role in activating mitophagy. This work showed that conformations of autoinhibited parkin captured in multiple crystal structures are indeed representative of the structure in solution. Importantly, this work revealed many details not discernable from these static structures. For example, many flexible tethers and loops in parkin are unresolved in crystal structures, and this thesis shows at least some of these flexible regions are involved in transient interactions with the UBL to maintain autoinhibition. Additionally, changes in NMR chemical shifts propagated through the parkin backbone helped identify a structural hinge that regulates autoinhibition in an allosteric manner. The NMR assignments for parkin determined here allowed identification of the pUb binding site on
parkin, generating a model of pUb-bound parkin. These NMR assignments will also be extremely valuable in the future to identify binding sites of potential substrates proteins, as well as small molecules that may activate or inhibit parkin activity with therapeutic applications.

The structure of the phosphorylated UBL domain solved here also provides a significant advancement in our understanding of how PINK1 relieves parkin autoinhibition. The phosphorylated form of the UBL has not been amenable to crystallization and we showed this likely results from a substantial destabilizing effect of the pSer65 group on the pUBL structure. However, the results in this dissertation add to a growing consensus that it is primarily the pUb signal that is responsible for the conformational change that relieves UBL-mediated autoinhibition (3,28-30). In this sense, it is curious the lack of significance that phosphorylation of the UBL may actually play in this displacement. If UBL phosphorylation is dispensable, it is possible that its phosphorylation is actually just a byproduct of Ub phosphorylation—the true signal for mitophagy machinery—and occurs simply due to homology between UBL and Ub. Perhaps the sequence and structure of pUBL has evolved over time to assist in amplifying this signal, rather than directly mimicking pUb. In the future, it will be interesting to see how PINK1 actually recognizes these two substrates and if PINK1 can distinguish between Ub and UBL. The findings here would suggest that PINK1 might recognize the overall Ub/UBL fold rather than a consensus sequence around the phosphorylation site, which is not immediately obvious. This could explain why positional scanning efforts to determine the optimal PINK1 substrate yielded a peptide sequence with no similarity to Ub or UBL (31) and why peptides encompassing Ser65 cannot be phosphorylated by PINK1 (15). These studies
will also shed further insight into the molecular basis of disease-state mutations, not only in parkin’s UBL, but also in PINK1 that are mutated in ARJP.

The intricate orchestration of phosphorylation and ubiquitination signals by PINK1 and parkin are a remarkable example of crosstalk between multiple post-translational modifications to regulate cellular processes. The new roles of ubiquitin as both an activator of an E3 ligase and a receptor for mitophagy machinery are remarkable examples of the non-degradative roles attainable by ubiquitination. Although commonly referred to as the Ubiquitin-Proteasome system, it is clear today the signaling role of ubiquitin in cells extends far beyond the proteolytic role for which it was originally described.

6.5 References


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POSITIONS HELD

2017-present  **Postdoctoral Fellowship**, Friedrich Miescher Institute for Biomedical Research
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• Research Project: Structures of ubiquitination assemblies in DNA-damage response
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EDUCATION

2012-2017  **PhD in Biochemistry**, Department of Biochemistry, Schulich School of Medicine and Dentistry
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• Doctoral Thesis: Autoinhibition and activation of Parkin
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2008-2012  **BMSc (Honours) in Clinical Biochemistry**, Schulich School of Medicine and Dentistry
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RESEARCH INTEREST

Proteins and enzymes account for over half of the total composition of human cells, and the magnificent diversity in their structures provides countless opportunities to regulate cellular architecture, signalling, and biological chemistry. My research interests are focused on observing and characterizing these biomolecular structures at the atomic level using state-of-the-art techniques. By understanding the composition of these cellular machines and their relationships with other macromolecules, the hope is to construct literal “pictures” of the cell and use these structures to uncover the molecular basis of human diseases and design potential pharmacological interventions.

SCHOLARSHIPS AND AWARDS

2017  Lucille & Norton Wolf London Health Research Day Trainee Publication Award - Neuroscience
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2016  Queen Elizabeth II Graduate Scholarship in Science and Technology
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2008  Western Scholarship of Distinction

PUBLICATIONS

*equal author contribution


This article was highlighted in "News & Views" and selected for the cover of EMBO J. This article has been cited 42 times as of April 25, 2017.


This article was highlighted in “News & Views" in EMBO J and Current Biology. This article was highlighted on the Michael J. Fox Foundation website for Parkinson's Research. This article has been cited 66 times as of April 25, 2017.


CONFERENCES AND SCHOLARLY PRESENTATIONS


TEACHING AND SUPERVISING EXPERIENCE

Enzyme Mechanisms and Kinetics (Biochemistry 3381A – The University of Western Ontario)

- Conducted 1 hour tutorial lectures for 200+ 3rd year undergraduate students. Reviewed challenging topics on thermodynamics and enzyme kinetics and emphasized their applications to solving scientific problems.
- Prepared and evaluated course assignment and final exam.
- Received a graduate teaching award in 2014 and nominated again in 2016.

Clinical Biochemistry Laboratory (Biochemistry 3387G – The University of Western Ontario)

- Conducted laboratory sessions on topics of molecular biology and biochemistry of clinical diagnostic tests.
- Introduced students to formal scientific writing. Evaluated and critiqued lab reports.

Mentor for undergraduate student projects (Biochemistry 4483E – The University of Western Ontario)

- Co-supervised 4 undergraduate research projects and taught students how to conduct experiments, process data, critically evaluate results and design appropriate follow-up experiments.