Conformational Arrangements of UbcH7-Ubiquitin with OspG and 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

The E2-ubiquitin conjugate is a key regulator of ubiquitination and is therefore an important component of cellular homeostasis. Disruptions to proper E2-ubiquitin functioning have implications in diseases such as shigellosis and Parkinson’s disease discussed here. E2-ubiquitin conjugates like UbcH7-ubiquitin are extremely dynamic and can adopt multiple conformations in solution or bound to target proteins. However, the conformational arrangements that UbcH7-ubiquitin adopts while free in solution, bound to the shigellosis-associated kinase OspG or to the Parkinson’s disease-related E3 ligase parkin are unknown. Also unknown, is a mechanistic explanation for how UbcH7-ubiquitin interactions with OspG and parkin are associated with disease. Here, we determined the crystal structure of OspG bound to UbcH7-ubiquitin, the crystal structure of autoinhibited full-length human parkin with and without a phosphorylation-mimetic, the crystal and NMR structures of activated full-length human parkin bound to a phosphorylated-ubiquitin molecule, and an NMR structure of activated human parkin bound to both phosphorylated-ubiquitin and UbcH7-ubiquitin. This work determined that UbcH7-ubiquitin predominantly occupies closed states in solution but binds to OspG and parkin in open conformations. Further key findings include showing that UbcH7-ubiquitin is a biological target of OspG and that OspG involvement in shigellosis is to halt host ubiquitination by competitively binding to UbcH7-ubiquitin in a way that mimics host HECT E3 binding. We showed that parkin is autoinhibited through interdomain interactions. Phosphorylation of autoinhibited parkin primes phosphorylated-ubiquitin binding and this binding relieves autoinhibition by inducing allosteric rearrangements in parkin to allow subsequent UbcH7-ubiquitin engagement. Finally, we showed that certain hereditary variants in parkin are likely associated with autosomal recessive juvenile parkinsonism due to a loss in the ability to interact with UbcH7-ubiquitin. Research here has significant implications for understanding the basis of shigellosis and hereditary forms of Parkinson’s disease, and has contributed significant molecular understandings for the use in developing therapeutics.

Keywords: Parkinson’s disease, shigellosis, ubiquitination, nuclear factor-kappaB, parkin, PINK1, OspG, UbcH7-ubiquitin, structural biology, NMR, crystallography
Co-Authorship

Chapter 2

This chapter contains sections from the following published manuscript written by Dr. Andrey Grishin, Dr. Miroslaw Cygler and Dr. Gary Shaw:

Grishin, A.M.¹, Condos, T.E.C.², Barber, K.R.², Campbell-Valois, F.-X.³,⁴, Parsot, C.³,⁴, Shaw, G.S.², and Cygler, M.¹,⁵ (2014). Structural Basis for the Inhibition of Host Protein Ubiquitination by Shigella Effector Kinase OspG. Structure 22, 878–888.

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Dr. Andrey Grishin performed the crystallography experiments to solve the structure of OspG bound to UbcH7-ubiquitin (PDB ID code 4Q5E) from UbcH7-ubiquitin protein samples prepared by Tara Condos and Kathy Barber. Kathy Barber performed the ubiquitination assay experiments and prepared Figure 2.11. All other experiments and data analyses presented here were performed by Tara Condos.
Chapter 3

This chapter contains sections from the following published manuscript written by Dr. Atul Kumar, Dr. Jacob Aguirre, Tara Condos, Dr. Gary Shaw and Dr. Helen Walden:

Kumar, A.1, †, Aguirre, J.D.2, †, Condos, T.E.C.2, †, Martinez-Torres, R.J.1, †, Chaugule, V.K.1, Toth, R.1, Sundaramoorthy, R.3, Mercier, P.2, Knebel, A.1, Spratt, D.E.2, Barber, K.R.2, Shaw, G.S.2, and Walden, H.1 (2015). Disruption of the autoinhibited state primes the E3 ligase parkin for activation and catalysis. EMBO 34, 2506–2521.

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Dr. Atul Kumar performed the crystallography experiments to solve the structure of parkin UblR0RBR (PDB ID code 5C1Z) and parkin UblR0RBR$^{S65D}$ (PDB ID code 5C23) presented in Figure 3.8. Dr. Gary Shaw prepared Figure 3.8AB and Figure 3.19 while Dr. Helen Walden prepared Figure 3.8CD. Dr. Julio Martinez-Torres performed several ITC experiments presented in Table 3.4. Dr. Julio Martinez-Torres also performed mutagenesis experiments that provided an unambiguous restraint between residue H302 in parkin R0RBR and pS65 in pUb for HADDOCK docking protocols. All other experiments and data analyses presented here were performed by Tara Condos.
Chapter 4

This chapter contains sections from the following published manuscript written by Dr. Atul Kumar, Dr. Gary Shaw and Dr. Helen Walden:

Kumar, A.¹, Chaugule, V.K.¹, Condos, T.E.C.², Barber, K.R.², Johnson, C.¹, Toth, R.¹, Sundaramoorthy, R.³, Knebel, A.¹, Shaw, G.S.², and Walden, H.¹ (2017). Parkin-phosphoubiquitin complex reveals cryptic ubiquitin-binding site required for RBR ligase activity. Nature Structural and Molecular Biology 24(5), 475-483.

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Dr. Atul Kumar performed the crystallography experiments to solve the structure of parkin pUb-UblR0RBR (PDB ID code 5N2W) presented in Figure 4.6. Dr. Viduth Chaugule performed mutagenesis and ubiquitination assays to determine several ambiguous restraints used for docking UbcH7-ubiquitin to pUb-R0RBR in Table 4.3. Dr. Viduth Chaugule performed the ITC experiments presented in Figure 4.8BC. All other experiments and data analyses presented here were performed by Tara Condos.
Acknowledgments

I would first like to thank my supervisor Dr. Gary Shaw for having given his guidance, expertise and support during the entirety of this work. Thank-you for leading by example as a successful and equitable mentor. Thank-you to Dr. Helen Walden for supervising me during a research fellowship to her lab. I am grateful for having gotten the opportunity to experience work in a new environment and for having learnt crystallography approaches. Thank-you to everyone who has collaborated on publishing a paper. It has been exceptionally rewarding seeing our hard work acknowledged with such high-calibre journal publications. Thank-you to my committee members Dr. David Litchfield and Dr. Brian Shilton for having written countless reference letters and for your perspectives on my research. Thank-you to funding agencies at Western, the OGS program, NSERC and Boehringer Ingelheim for making this research possible. Thanks to Kathy Barber for being a wonderfully organized and approachable lab manager as you have helped a great deal. Thanks to all lab members both past and present for having made this research that much more enjoyable day-in and day-out. Finally, thanks to my family for their ever-lasting support.
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<th>Description</th>
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<tbody>
<tr>
<td>ΔG</td>
<td>gibb’s free energy</td>
</tr>
<tr>
<td>ΔH</td>
<td>enthalpy change</td>
</tr>
<tr>
<td>ΔS</td>
<td>entropy change</td>
</tr>
<tr>
<td>ARJP</td>
<td>autosomal recessive juvenile parkinsonism</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CSP</td>
<td>chemical shift perturbation</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>copper ion</td>
</tr>
<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>deuterium Oxide</td>
</tr>
<tr>
<td>DSS</td>
<td>4,4-dimethyl-4-silapentane-1-sulfonic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E2-Ub</td>
<td>E2-ubiquitin conjugate</td>
</tr>
<tr>
<td>FF</td>
<td>fast flow</td>
</tr>
<tr>
<td>g</td>
<td>gravity force</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HADDOCK</td>
<td>high ambiguity driven protein-protein docking</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to the E6AP carboxyl terminus</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HHARI</td>
<td>human homologue of Ariadne</td>
</tr>
<tr>
<td>HMQC</td>
<td>heteronuclear multiple quantum coherence</td>
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<tr>
<td>HNCA</td>
<td>hydrogen nitrogen carbon α correlation</td>
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<tr>
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<td>HOIL-1-interacting protein</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
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<tr>
<td>K&lt;sub&gt;a&lt;/sub&gt;</td>
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$K_d$  dissociation constant

tilde{kDa}  kilodalton

$KH_2PO_4$  potassium dihydrogen phosphate

LB  luria broth

mAU  milli-absorbance unit

$MgCl_2$  magnesium chloride

MOPS  3-$(N$-morpholino)-propane-1-sulfonic acid

MW  molecular weight

N  stoichiometry constant

$NaCl$  sodium chloride

$Na_2HPO_4$  disodium phosphate

$NaH_2PO_4$  monosodium phosphate

$NaOAc$  sodium acetate

NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells

NMR  nuclear magnetic resonance spectroscopy

NOESY  nuclear overhauser effect spectroscopy

OD$_{600}$  optical density at wavelength 600 nm

PCR  polymerase chain reaction

PD  Parkinson’s disease

PDB  protein databank

pI  isoelectric point

PINK1  PTEN-induced putative kinase 1

R  gas constant

RBR  RING1-between-RING2

R0RBR  RING0-RING1-between-RING2

RING  really interesting new gene

rmsd  room mean squared difference

SEC  size-exclusion chromatography

SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis

SUMO  small ubiquitin-like modifiers

T  temperature constant
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>T3SS</td>
<td>type 3 secretion system</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris (2-carboxyethyl) phosphine hydrochloride</td>
</tr>
<tr>
<td>TEV</td>
<td>tobacco etch virus</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TROSY</td>
<td>transverse relaxation-optimised spectroscopy</td>
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<tr>
<td>Ub</td>
<td>ubiquitin</td>
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<tr>
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<td>Asn (N)</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>aspartate</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>cysteine</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>glutamate</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>glycine</td>
</tr>
<tr>
<td>His (H)</td>
<td>histidine</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>isoleucine</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>leucine</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>lysine</td>
</tr>
<tr>
<td>Met (M)</td>
<td>methionine</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>proline</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>serine</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>threonine</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Val (V)</td>
<td>valine</td>
</tr>
</tbody>
</table>
Preface

This thesis closely examines an element common to both shigellosis and Parkinson’s disease: the E2-ubiquitin conjugate. The E2-ubiquitin conjugate is a key regulator of ubiquitination with implications in both diseases. Because ubiquitination aids in managing cellular homeostasis to a great extent, it’s no surprise that disease occurs when disruptions to this pathway develop. This thesis focuses on the underlying structural and atomic-level mechanisms that lead to these two very different diseases by specifically examining two sets of interactions with UbcH7-ubiquitin. The first part of Chapter 1 is an introduction to shigellosis and the bacterial effector kinase OspG, while the second part of Chapter 1 is an introduction to Parkinson’s disease and the E3-ligase parkin. Chapter 2 focuses on the shigellosis-inducing bacterial pathogen OspG interaction with UbcH7-ubiquitin while Chapters 3 and 4 focus on the Parkinson’s disease-associated E3 ligase parkin interaction with UbcH7-ubiquitin. Chapter 5 will summarize results while bringing to-light the significance of findings and discussing future directions.
Chapter 1
INTRODUCTION

1.1 Shigellosis

1.1.1 Shigellosis

Shigellosis is characterised by inflammation and destruction of the intestinal epithelium caused by mucosal penetration of the gram-negative bacteria *Shigella* (Labrec et al., 1964). Infection is often accompanied by chronic diarrhea, dehydration and abdominal discomfort (Speelman et al., 1984). This contagious disease is transmitted by the faecal-oral route or by contaminated food and water (Wharton et al., 1990). Shigellosis continues to be a problem in developing countries, as it contributes to 164,000 deaths each year (Kotloff et al., 2017). *Shigella* are highly infectious (DuPont et al., 1989) and are showing emerging signs of antibiotic resistance (Nüesch-Inderbinen et al., 2016). There is currently no cure for shigellosis and the only preventative measures include improved sanitation (Kotloff et al., 1999).

1.1.2 Shigella mode of infection

Once ingested, the bacterial pathogen *Shigella* travels through the gastrointestinal tract to the colon and rectum. *Shigella* bypass the protective intestinal epithelium by targeting M cells using a type 3 secretion system (T3SS) that acts as a needle to inject approximately 30 virulent effector proteins into the host gut epithelial cell cytoplasm (Galán and Wolf-Watz, 2006; Parsot, 2009). The proteins that comprise the T3SS and a majority of the injected effector proteins are encoded on a large virulence plasmid (Buchrieser et al., 2000). T3SSs are relatively well-conserved across bacterial pathogens and are composed of a base that scaffolds to the bacterial membrane, a needle that extends away from the bacterial surface and a cap that regulates effector secretion (Galán et al., 2014). When the cap contacts a host plasma membrane, T3SSs form a translocon pore for effector protein delivery to the host cell cytoplasm (Veenendaal et al., 2007).

*Shigella* effector proteins (Table 1.1) are distinct from other bacterial pathogens and are used at early, middle or late stages of invasion to evade the host defence system and manipulate cellular functions (Ogawa et al., 2008; Phalipon and Sansonetti, 2007). At the earliest stage, *Shigella* contact host cells and secrete effectors like IpaA-D and
IpgD involved in T3SS translocon pore formation and the protrusion of membrane ruffles around the bacteria that result in bacterial entry into host cells by macropinocytosis (Ménard et al., 1996). Upon invasion, Toll-like and Nod-like receptors recognise common bacterial lipopolysaccharides and peptidoglycans released by the cytoplasm-localised *Shigella* which leads to activation of a host immune and inflammatory defense response (Fritz et al., 2006). Host immune polymorphonuclear leukocytes then migrate to the invaded epithelium and paradoxically disrupt the cell lining allowing rapid *Shigella* invasion of epithelial cells while leading to severe inflammatory destruction characteristic of shigellosis (Sansonetti, 2001). Once bacteria invade host cells, the middle and late stages of *Shigella* invasion involve proteins that circumvent the host innate immune response in order to colonize and replicate. Effectors OspF, OspG and OspI as well as the IpaH family of proteins dampen this initially useful host inflammatory response by inhibiting the NF-κB and MAPK signaling pathways to allow bacterial intracellular survival, replication and cell-to-cell movement (Sansonetti and Di Santo, 2007).

### 1.1.3 Host immune inflammatory response NF-κB pathway

To counter *Shigella* pathogenic infection, host intestinal epithelial cells induce an innate immune response by undergoing activation of pro-inflammatory pathways such as the nuclear factor-κappaB (NF-κB) pathway (Figure 1.1A) (Sansonetti, 2004). Under non-infected conditions, transcription factor NF-κB is retained in the cytoplasm, bound to inhibitor 1κBs like 1κBα. Upon bacterial infection and consequent stimulation by pro-inflammatory cytokines, 1κBα is phosphorylated by 1κB kinases (1κK), leading to phosphorylated-1κBα dissociation from NF-κB. The ubiquitination pathway (section 1.2.4) and NF-κB pathway now intersect as phosphorylated-1κBα is targeted for ubiquitination by the E3 SCFβ-TrCP ligase complex for consequent degradation by the 26S proteasome. Nuclear dissociation signals locate dissociated NF-κB to the nucleus for the activation of target genes and an inflammatory response reflected in destruction of the intestinal epithelium seen in shigellosis (Liang et al., 2004; Karin and Ben-Neriah, 2000). Eventually, polymorphonuclear leukocytes such as neutrophils from the host adaptive immune response eliminate *Shigella* and the infection within 5-7 days (Mandic-Mulec et al., 1997).
Table 1.1 Selected *Shigella* effector proteins with known functions.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Onset</th>
<th>Targets</th>
<th>Biochemical Activity</th>
<th>Role in infection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IpaB</td>
<td>Early</td>
<td>Caspase, α5β1, CD44 and Mad2L2</td>
<td>T3SS translocon</td>
<td>Translocon pore formation, macrophage apoptosis and host cell-cycle arrest</td>
<td>(Page et al., 1999) (Skoudy et al., 2000) (Lafont et al., 2002)</td>
</tr>
<tr>
<td>IpaC</td>
<td>Early</td>
<td>Vimentin and keratin intermediate filaments</td>
<td>T3SS translocon</td>
<td>Translocon pore formation, actin polymerization, docking and effector induction</td>
<td>(Terry et al., 2008) (Mounier et al., 2009) (Du et al., 2016) (Russo et al., 2016)</td>
</tr>
<tr>
<td>IpgD</td>
<td>Early</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
<td>Phosphatase of phosphatidylinositol 4,5-bisphosphate</td>
<td>Bacterial invasion and host-cell survival</td>
<td>(Niebuhr et al., 2000) (Mayo and Donner, 2001) (Mellouk et al., 2014) (Garza-Mayers et al., 2015)</td>
</tr>
<tr>
<td>VirA</td>
<td>Middle</td>
<td>Rab1</td>
<td>GTPase-activating protein of Rab1</td>
<td>Bacterial escape from host autophagy</td>
<td>(Germane et al., 2008) (Dong et al., 2012) (Campbell-Valois et al., 2015)</td>
</tr>
<tr>
<td>OspF</td>
<td>Middle</td>
<td>MAP kinases</td>
<td>Phosphothreonine lyase of MAPK</td>
<td>Innate host immune response suppressor</td>
<td>(Arbibe et al., 2007) (Li et al., 2007) (Zhu et al., 2007)</td>
</tr>
<tr>
<td>OspI</td>
<td>Middle</td>
<td>Ubc13</td>
<td>Deaminase of the E2 Ubc13</td>
<td>Innate host immune response suppressor</td>
<td>(Sanada et al., 2012) (Nishide et al., 2013)</td>
</tr>
<tr>
<td>OspG</td>
<td>Late</td>
<td>E2-ubiquitin conjugates</td>
<td>Kinase (unknown target) and competitively binds to E2-ubiquitin</td>
<td>Innate host immune response suppressor</td>
<td>(Kim et al., 2005) (Zhou et al., 2013) (Grishin et al., 2014) (Pruneda et al., 2014)</td>
</tr>
<tr>
<td>IpaHβ8</td>
<td>Late</td>
<td>Glomulin</td>
<td>E3 ubiquitin ligase of glomulin</td>
<td>Activates host immune response inflammasomes for macrophage cell death</td>
<td>(Suzuki et al., 2014)</td>
</tr>
<tr>
<td>Ipaα8</td>
<td>Late</td>
<td>U2AF35 and NEMO/IKKγ</td>
<td>E3 ubiquitin ligase of U2AF35 and NEMO/IKKγ</td>
<td>Innate immune response suppressor</td>
<td>(Okuda et al., 2005) (Ashida et al., 2010)</td>
</tr>
</tbody>
</table>
1.1.4 OspG and E2-ubiquitin

In the year 2000, six new Shigella virulence plasmid genes were discovered and identified as the osp (outer Shigella protein) genes (Buchrieser et al., 2000). OspG is one of the approximately 30 virulent effector proteins secreted by Shigella and is encoded by the ospG gene. OspG is a serine/threonine kinase that lacks a typical kinase activation loop suggesting that it may require the binding of a host factor for activation (Zhou et al., 2013). OspG has been shown to dampen host inflammatory responses in order to facilitate colonization and invasion. This dampening is done by preventing the NF-κB pathway degradation of inhibitor IκBα (Kim et al., 2005; Zhou et al., 2013), however the mechanism used by OspG to inhibit NF-κB-directed ubiquitination of IκBα is currently unknown. The ubiquitination system is frequently hijacked by bacterial pathogens (Jiang and Chen, 2011; Rytkönen and Holden, 2007) and studies on OspG suggest that it plays a role in this manipulation. Studies have shown that the presence of OspG still results in the accumulation of phosphorylated- IκBα and the formation of E2-ubiquitin (E2-Ub) conjugates (Kim et al., 2005) suggesting that OspG acts to prevent IκBα degradation during the ubiquitination steps involving E2-Ub conjugate binding to the E3, or ubiquitin (Ub) transfer to substrate. OspG has been shown to bind to free Ub and polyubiquitin chains (Zhou et al., 2013), as well as to E2s and E2-Ub conjugates (Kim et al., 2005), however the preferred biological target has not yet been confirmed. The ability of OspG to bind to different E2-Ub conjugates suggests that it may interfere with the functioning of a large number of host E3s.
1.2 Parkinson’s Disease

1.2.1 Parkinson’s disease

The first cases of Parkinson’s disease (PD) were documented in 1817 by Dr. James Parkinson who termed the disease as a type of “shaking palsy” accompanied with extremely slow-progressing symptoms of involuntary tremor and a propensity to “bend the trunk forwards” (Parkinson, 1817). Dr. Parkinson noted the belief that it was a disease of the nervous system and an injury of the medulla spinalis, extending to the medulla oblongata. The study of Parkinson’s disease clinical features, pathophysiology, genetics and biochemical pathways involved has come a long way since then. Today, we know Parkinson’s disease to be the second most common neurodegenerative disease estimated to affect 1% of the population over 60 years of age (Tysnes and Storstein, 2017). It is diagnosed by having tremor at rest, rigidity, bradykinesia and postural instability which are symptoms that collectively describe parkinsonism (Jankovic, 2008). Parkinson’s disease is slow-progressing having a late onset of 65-70 years of age and long-term effects on emotional and mental well-being of sufferers (Huber et al., 1988), (Schrag et al., 2000). The pathophysiology of Parkinson’s disease in patients is characterised by the neurodegeneration of dopaminergic neurons in the substantia nigra of the midbrain (Hornykiewicz, 1966; Riederer and Wuketich, 1976) often accompanied by the presence of Lewy bodies composed of protein aggregates such as α-synuclein in the brainstem (Lewy, 1912; Lewy, 1923).

1.2.2 Familial PD and autosomal recessive juvenile Parkinsonism

Familial Parkinson’s disease has a hereditary component attributable to mutations in over 10 genes discovered to-date (Table 1.2). The first gene discovered with mutations predisposing familial Parkinson’s disease was PARK1 which encodes for the functionally unknown protein α-synuclein (Polymeropoulos et al., 1997), which is a hallmark protein found aggregated in Lewy bodies (Spillantini et al., 1997). Interestingly, mutations in the genes encoding for α-synuclein and another familial Parkinson’s disease -associated gene that encodes the serine/threonine kinase LRRK2 (Funayama et al., 2002) are showing emerging links to the more common sporadic form of Parkinson’s disease (Gilks et al., 2005; Mueller et al., 2005; Lesage and Brice, 2012) suggesting that there may be a more
prevalent genetic component to sporadic cases than previously assumed. Autosomal recessive juvenile Parkinsonism (ARJP) is a specific hereditary form of Parkinson’s disease and accounts for approximately 10% of all Parkinson’s disease cases. ARJP differs from common idiopathic forms by having an earlier onset, an even slower progression, occasional dystonia and a long-lasting response to levodopa medicines. 50% of all ARJP cases are attributable to mutations in the \( \textit{PARK2} \) gene, encoding for the E3 ubiquitin-ligase parkin (Kitada et al., 1998), which will be a main focus of this thesis.

1.2.3 \textit{Molecular pathology underlying PD}

Parkinson’s disease is believed to be caused by a combination of genetic predispositions described above as well as environmental factors (Corrigan et al., 1998) that lead to oxidative damage of mitochondrial proteins (Alam et al., 1997), consequent mitochondrial dysfunction (Schapira et al., 1990) and neuronal cell death in the substantia nigra of the midbrain. Damaged mitochondrial turnover is typically regulated by mitophagy (mitochondrial autophagy) and in part by the ubiquitin-proteasome system, however these pathway activities are found to malfunction in Parkinson’s disease cases (McNaught et al., 2003). \textit{PINK1} and \textit{parkin} are mitochondrial proteins that play a vital role in regulating mitophagy and the ubiquitination-proteasome system. However, mutations found in the genes encoding for them lead to a loss of function of these systems and consequent associations with ARJP (Kitada et al., 1998; Valente et al., 2004). A decrease in proteasome activity often leads to an increase in cytotoxic misfolded proteins and cell death. For instance, large components of Lewy bodies present in Parkinson’s disease patients are misfolded \( \alpha \)-synuclein aggregates (Spillantini et al., 1997), further suggesting evidence of impaired protein degradation associated with Parkinson’s disease.
Table 1.2 Genes implicated in familial Parkinson’s disease. Mutations in just over 10 genes have been discovered for association with familial forms of Parkinson’s disease. The most commonly mutated and well-studied genes with their respective encoded proteins are listed below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Protein Function</th>
<th>Inheritance</th>
<th>Pathophysiology</th>
<th>Clinical symptoms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1 and PARK4</td>
<td>α-synuclein</td>
<td>Unknown, flexible and unstructured synaptic protein</td>
<td>Autosomal dominant and sporadic</td>
<td>Loss of dopaminergic neurons, Lewy bodies (contain α-synuclein aggregates)</td>
<td>Fast progression, parkinsonism, initially levodopa responsive, cognitive decline, dementia, age of onset &lt;50</td>
<td>(Polymeropoulos et al., 1997) (Spillantini et al., 1997) (Ohtake et al., 2004) (Nemani et al., 2010)</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin</td>
<td>E3 ubiquitin ligase</td>
<td>Autosomal recessive</td>
<td>Loss of dopaminergic neurons, absence of Lewy bodies</td>
<td>Slow progression, parkinsonism, dystonia, levodopa responsive, age of onset &lt;40 years</td>
<td>(Kitada et al., 1998) (Khan et al., 2003) (Mori et al., 2003)</td>
</tr>
<tr>
<td>PARK5</td>
<td>UCHL1</td>
<td>Ubiquitin hydrolase</td>
<td>Autosomal dominant</td>
<td>Broad neurodegeneration, Lewy bodies (contain UCHL1 aggregates)</td>
<td>Slow progression, parkinsonism, levodopa responsive, age of onset &gt;50 years</td>
<td>(Leroy et al., 1998) (Bilguvar et al., 2013)</td>
</tr>
<tr>
<td>PARK6</td>
<td>PINK1</td>
<td>Ser/Thr kinase</td>
<td>Autosomal recessive</td>
<td>Loss of dopaminergic neurons, Lewy bodies</td>
<td>Slow progression, parkinsonism, dystonia, levodopa responsive, age of onset &lt;40 years</td>
<td>(Valente et al., 2004) (Ibáñez et al., 2006) (Samaranch et al., 2010)</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>Molecular chaperone</td>
<td>Autosomal recessive</td>
<td>Unknown</td>
<td>Slow progression, dystonia, levodopa responsive, age of onset &lt;40 years</td>
<td>(Bonifati et al., 2003) (Shendelman et al., 2004) (Zhou et al., 2006)</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>Ser/Thr kinase</td>
<td>Autosomal dominant and sporadic</td>
<td>Loss of dopaminergic neurons, Lewy bodies</td>
<td>Slow progression, parkinsonism, levodopa responsive, age of onset &gt;50 years</td>
<td>(Funayama et al., 2002) (Haugarvoll and Wszolek, 2009) (Greggio et al., 2009)</td>
</tr>
</tbody>
</table>
1.2.4 Ubiquitination pathway

The ubiquitin-proteasome pathway is a major protein degradation system in eukaryotic cells. Many regulatory protein levels involved in cellular pathways are controlled by ubiquitin-proteasome-mediated degradation such as the NF-κB pathway transcriptional regulator NF-κB and its inhibitor IκBα (Palombella et al., 1994), (Chen et al., 1995), as well as mitochondrial proteins like α-synuclein (Bennett et al., 1999). Ubiquitination is a post-translational modification involving a series of sequential steps that requires a ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzyme (Hershko et al., 1983). The C-terminal of ubiquitin (Ub) is first ATP-dependently activated by an E1 and is subsequently transferred to an active site cysteine on an E2 to form a thioester-bonded E2-Ub conjugate. This conjugate then binds to an E3 ligase, whereby Ub is either transferred directly to a substrate lysine ε-amino group for formation of an amide isopeptide linkage, or alternatively to the E3 catalytic cysteine for formation of another thioester-bonded complex prior to the final covalent transfer to substrate (Figure 1.1B). The nature of the Ub linkage to substrate confers the substrate cellular fate. For instance, the linkage of a single Ub molecule to a substrate in a mono-ubiquitinated manner is a signal for that substrate to undergo specific protein interactions or to confer cellular localisation. Moreover, Ub molecules can be attached to any one of the seven lysine residues on the first Ub molecule in a sequential manner to create a poly-ubiquitin chain. Substrates are targeted for proteasomal degradation by the 26S proteasome with K48- and K11-linked chains, while K63-linked chains signal the substrate for either NF-κB pathway activation, DNA repair or lysosomal targeting. Other possible Ub chain linkage types exist with little known function (Ciechanover and Brundin, 2003).
Figure 1.1 NF-κB and ubiquitination pathways. (A) An excerpt from the NF-κB pathway shows phosphorylation of inhibitor IκBα by IκB kinase (IκK) leading to IκBα dissociation from NF-κB. The dissociated NF-κB is now targeted for nuclear localisation while phosphorylated-IκBα is targeted for ubiquitination and consequent degradation by the 26S proteasome. (B) The ubiquitination pathway showing Ub first ATP-dependently activated by an E1-activating enzyme. Ub is then transferred to an E2-conjugating enzyme, forming an E2-Ub conjugate that adopts either open, backbent or closed conformations. The E2-Ub conjugate binds to an E3-ligase and Ub is then transferred to substrate (using RING E3-ligases) or is first transferred to the E3 catalytic cysteine (using HECT and RBR E3 ligases) and then to substrate. K48- and K11-linked poly-ubiquitinated substrates are targeted for degradation by the 26S proteasome.
1.2.5 E3 Ub-ligases

Eukaryotic cells have evolved hundreds of E3 Ub-ligases (E3s) equipped with a variety of regulatory elements, catalytic functions and substrate-recruiting modules in order to facilitate efficient ubiquitination of a substrate protein. E3s from eukaryotic cells are grouped into three classes based on structure and the mechanism employed to facilitate Ub transfer to a substrate. These classes include: RING (really interesting new gene), HECT (homologous to the E6AP carboxyl terminus) and the recently discovered RBR (RING-IBR-RING, where IBR stands for in-between-RING) (Figure 1.2).

RING E3 ligases comprise the largest family of E3s with more than 600 predicted members in the human genome. All RING E3 ligases contain a RING domain that acts as a scaffold to properly bind and orient the E2-Ub conjugate (Zheng et al., 2000) in closed conformations (Soss et al., 2013; Pruneda et al., 2012) for enhanced reactivity of Ub and direct transfer from E2 to substrate lysine residues. RING E3s use a canonical surface for interaction with the E2 that includes the RING loop Zn$^{2+}$-loop L1, RING helix H1 and RING Zn$^{2+}$-loop L2 (Budhidarmo et al., 2012), while the RING Zn$^{2+}$-loop L1 makes contacts with Ub (Figure 1.3A). RING E3s contain a highly conserved basic "linchpin" residue located in the RING Zn$^{2+}$-loop L2 that acts to stabilize E2-Ub conjugates in a closed conformation through a hydrogen bond network to the E2 loop 7 and Ub C-terminal tail (Pruneda et al., 2012) likely to optimise positioning of the E2-Ub thioester for nucleophilic attack by the substrate lysine and for a consequent thioester aminolysis reaction. This binding mechanism and closed E2-Ub orientation has been observed in many RING E3:E2-Ub crystal structures such as the RNF4 RING E3:E2-Ub structure (PDB ID 4AP4) (Plechanovova et al., 2012) structure, the BIRC7 RING E3:E2-Ub structure (PDB ID 4AUQ) (Dou et al., 2012) and the RNF165 RING E3:E2-Ub structure (PDB ID 5D0M) (Wright et al., 2016), suggesting that the mechanism is universal to many RING E3:E2-Ub pairings (Figure 1.6). The E2s play an important role in determining the final substrate polyubiquitination linkage type, however it is the coordination between both E2 and the RING E3 that confers final specificity (Stewart et al., 2016).

HECT E3 ligases make up a smaller group of E3s with approximately 30 members in the human genome. HECT E3s contain a bilobal HECT domain composed
of an N-lobe and C-lobe that are connected by a flexible short hinge. Distinct from RING E3s, HECT E3s recruit the E2 within the E2-Ub conjugate to the HECT N-lobe while the C-lobe makes contacts with Ub so that the conjugate takes on an open conformation as seen in a NEDD4L HECT E3:E2-Ub structure (PDB ID 3JW0) (Kamadurai et al., 2009). Also distinct from RING E3s, HECT E3s undergo a transthiolation reaction to form an E3-Ub intermediate prior to engaging in a thioester aminolysis reaction to attach Ub to a substrate (Rotin and Kumar, 2009). Upon binding of the E2-Ub conjugate, crystal structures reveal the presence of a large distance, upwards of 50 Å, between the E3 and E2 catalytic cysteines such as in the E6AP HECT E3:E2 structure (PDB ID 1C4Z) (Huang et al., 1999). However, structures have also shown that HECT domains have the ability to undergo conformational rearrangement in which the C-lobe is brought into closer proximity to the E2 catalytic site through flexibility of the HECT bilobal hinge as seen in the WWP1 HECT E3 structure (PDB ID 1ND7) (Verdecia et al., 2003). Upon loading of Ub to the HECT E3 catalytic cysteine, the Ub tail takes on an extended conformation for substrate lysine nucleophilic attack (Maspero et al., 2013). Unlike RING E3s, substrate polyubiquitination linkage specificity is enforced primarily by the HECT E3 rather than the E2 (Kim and Huibregtse, 2009).

RBR E3 ligases are the newest discovered class of E3 ligases with over 10 members in the human genome. RBR E3 ligases contain a conserved RING1-IBR-RING2 motif. RBR E3 ligases use a RING/HECT hybrid mechanism for conferring ubiquitination (Wenzel et al., 2011) whereby the E2 within the E2-Ub conjugate is recruited to the RING1 domain (similar to RING E3 mechanisms), and Ub is transferred to a conserved catalytic cysteine in RING2 by a transthiolation reaction prior to thioester aminolysis transfer to a substrate lysine (similar to HECT E3 mechanisms). RBR E3 ligases are uniquely regulated through autoinhibition and activation. For instance, the RBR E3 ligase HOIP autoinhibition is mediated by its ubiquitin-associated (UBA) domain and requires binding to other regulatory proteins for activation (Stieglitz et al., 2013; Lechtenberg et al., 2016). As well, the RBR E3 ligase HHARI autoinhibition is regulated through its Ariadne domain as it is seen occluding the catalytic cysteine on RING2 and requires binding to another regulatory protein for activation (Duda et al.,...
Structures of autoinhibited HHARI also show that the RING2 and a bound E2 would require large conformational rearrangements in order to bring the two catalytic sites into close proximity much like the mechanism used by HECT E3s. RBR E3 RING1 domains are structurally similar to RING E3 domains, but RBR E3 RING1 have an additional elongated RING1 Zn\(^{2+}\)-loop L2 (Spratt et al., 2014) and they lack the typical basic “linchpin” residue that RING E3 RING domains use to position the E2-Ub conjugate in a closed conformation (Pruneda et al., 2012) (Figure 1.3B). Despite these differences, RBRs are expected to use a similar RING1 Zn\(^{2+}\)-Loop L1, RING1 helix H1 and RING1 Zn\(^{2+}\)-loop L2 for interaction with the E2 within the E2-Ub conjugate (Figure 1.3A), however there is still uncertainty for what conformational arrangement the whole E2-Ub conjugate uses for recruitment. The unique RING1 domain features of RBR E3 ligases mentioned above are proposed to direct this E2-Ub conformational arrangement (Dove et al., 2017) and disfavour closed E2-Ub binding (Dove et al., 2016) suggesting that more open E2-Ub conformations are favoured upon RBR E3 ligase binding. Three crystal structures of RBR E3 ligases bound to E2-Ub conjugates have recently been published with various open orientations of Ub within the E2-Ub conjugate. A HOIP:E2-Ub structure (PDB ID 5EDV) shows Ub in an open conformation oriented towards the E2 backside while making secure interactions with the RING1 and IBR (Lechtenberg et al., 2016). In this structure, an allosteric Ub molecule is found activating HOIP so that RING1 helix H3 is straightened and a neighbouring HOIP molecule shows the RING2 poised for transthiolation with the backbent E2-Ub conjugate. Two HHARI:E2-Ub structures also show Ub in open conformations, however Ub is oriented towards the closed E2 surface while making contact to the UBA-like domain in one structure (PDB ID 5TTE) (Yuan et al., 2017) or not making any contacts in the other (PDB ID 5UDH) (Dove et al., 2017). In both of these structures, the RING1 helix H3 is bent which is an emerging feature of autoinhibition (Wauer et al., 2015; Kumar et al., 2017) and no regulatory proteins are bound to HHARI to activate it, suggesting that the open conformation with Ub oriented towards the E2 backside seen in the activated HOIP structure is the optimal primed binding state. Finally, and similar to HECTS E3s, polyubiquitin chain type specificity is directed by the catalytic domain of the RBR E3s rather than the E2 (Stieglitz et al., 2013).
Figure 1.2 Domain architectures of RING, RBR and HECT E3 ligases. Domain architectures are presented for (A) RING, (B) RBR and (C) HECT E3 ligases. All E3 ligases were aligned to the E2 binding site which is the RING1 for RING E3 ligases and RBR E3 ligases, or the HECT N-lobe domain for HECT E3 ligases. Ub from the E2-Ub conjugate must attach to the catalytic site located on RING2 for RBR E3 ligases or to the HECT C-lobe for HECT E3 ligases, but the distance from the E2 binding site is too far in these inactive E3 states. The E2 binding site and catalytic site domains for both RBR and HECT E3 ligases are linked together by a flexible linker that acts as a hinge to bring the two domains together in HECT E3 ligases. RING1: RING1 domain, BIR: Baculovirus IAP (inhibitor of apoptosis) repeat, SIM: SUMO-interacting motifs, Ubl: ubiquitin-like domain, RING0: RING0 domain, IBR: in-between-RING domain, RING2: RING2 domain, UBA-like: ubiquitin-associated-like domain, Ariadne: ariadne domain, PUB: PNGase/ubiquitin-associated domain, NZF: NPL4 zinc-fingers, LDD: linear ubiquitin chain determining domain, AZUL: AZUL/ N-terminal zinc-binding domain, C2: C2 domain, WW: WW domain, HECT N-lobe and C-lobe: bilobal HECT domain.
Figure 1.3 Comparison of E2 binding to RING E3 and RBR E3 RING domains. (A) Ribbon diagram comparison of RING E3 and RBR E3 RING domains (grey) bound to E2s (green). E2s share a well-conserved surface used for interacting with RING domains that includes the E2 helix H1, loop L4 and loop L7. RING domains use Zn$^{2+}$-loop L1, helix H1 and Zn$^{2+}$-loop L2 to bind to E2s. Structures of RING E3 ligases RNF4 (PDB ID 4AP4), BIRC7 (PDB ID 4AUQ) and RNF165 (PDB ID 5D0M) as well as RBR E3s HOIP (PDB ID 5EDV), HHARI (PDB ID 5UDH) and parkin (PDB ID 5C1Z) were superimposed to all atoms in the RING domain using the super command in PyMOL. All structures contained bound E2s except parkin. (B) RING E3 and RBR E3 RING domain Zn$^{2+}$-loop L2 sequence alignment comparison. RING E3 RING domains contain a basic linchpin residue (red highlight) in Zn$^{2+}$-loop L2 responsible for orienting E2-Ub conjugates in closed conformations. RBR E3 RING1 domains do not contain a basic linchpin residue and have typically elongated Zn$^{2+}$-loop L2s. These two differences are proposed to facilitate E2-Ub orientations in open conformations.
1.2.6 Parkin cellular function and activation

Parkin is an RBR E3 ligase that mediates mitochondrial quality control by regulating damaged mitochondrial turnover (mitophagy) and ubiquitination of mitochondrial proteins. Although parkin is widely expressed throughout the body, the effects of its malfunctioning are inexplicably most influential in dopaminergic neurons. Parkin was thought to be constitutively active until 2011, when it was discovered that parkin required regulatory elements to relieve its autoinhibition (Chaugule et al., 2011). PINK1 kinase works upstream of parkin to relieve this autoinhibition and together they mediate mitophagy as follows (Figure 1.4). PINK1 kinase localises to the outer mitochondrial membrane upon mitochondrial damage (Narendra et al., 2010) where it phosphorylates low levels of parkin and Ub already present at the mitochondria, acting as a positive feed-forward signal to recruit cytosolic parkin to the mitochondria (Narendra et al., 2008; Kim et al., 2008; Shiba-Fukushima et al., 2012). Importantly, both Ub and the parkin Ubl domain are specifically phosphorylated by PINK1 at a conserved S65 and both phosphorylation events activate parkin E3 ligase activity (Sha et al., 2010; Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Ordureau et al., 2014). Activated parkin then poly-ubiquitinates outer mitochondrial proteins (Sarraf et al., 2013) with K6-, K11-, K48-, and K63-linkages (Ordureau et al., 2014) leading to the formation of an autophagosome around the heavily ubiquitinated mitochondria, which then signals the damaged mitochondria to be eliminated by mitophagy (Ashrafi et al., 2014; Lazarou et al., 2015; Heo et al., 2015). Although phosphorylation of both Ub and parkin are greatly validated events that lead to parkin activation, an atomic-level structural approach to determining why parkin is autoinhibited and how activation is achieved has not been determined.
Figure 1.4 The role of parkin and PINK1 in cellular mitophagy. First, damage occurs to mitochondria leading to depolarisation of the mitochondrial membrane potential. Second, PINK1 kinase is recruited to the outer mitochondrial membrane where it phosphorylates low levels of parkin and Ub. Third, cytosolic parkin is recruited to the mitochondria in response to mitochondrial phosphorylation signals. Both Ub and parkin phosphorylation are events that activate parkin E3 ligase activity to ubiquitinate mitochondrial proteins. Fourth, a phagophore forms around the heavily-ubiquitinated mitochondria inducing mitophagy of the damaged mitochondria.
1.2.7 Parkin structure

Parkin comprises Ubl-RING0-RING1-IBR-RING2 domains (Figure 1.5). Prior to starting my research, there were no full-length structures available and only little was known about the individual domains. Previously thought to be constitutively active, parkin was eventually recognised as being autoinhibited and that this autoinhibition was regulated through the ubiquitin-like (Ubl) domain (Chaugule et al., 2011). The N-terminal Ubl domain shares 32% sequence identity with Ub resulting in a similar structural fold that confers many binding interactions such as substrate recognition (Fallon et al., 2006; Trempe et al., 2009) and proteasome association (Sakata et al., 2003). Connecting the Ubl domain to RING0 is a long and poorly conserved flexible tether approximately 64 residues long. RING0 is a recently discovered domain found to coordinate two zinc ions (Hristova et al., 2009) and was not predicted from sequence alignments. However, rather than adopting a canonical RING configuration, it is now known to have a hairpin arrangement unique to parkin. RING1 is the only correctly named RING domain in parkin having a cross-brace zinc coordination topology. Because RING1 and RING2 were originally proposed to be structurally similar to other RING E3 RING domains, both were proposed to be binding site for E2s, however today only RING1 is considered an actual E2 recruitment domain. The IBR domain coordinates two zinc ions in a novel fold (Beasley et al., 2007), however its functional role is unclear. Following the IBR domain is a flexible 34 residue linker with little known function that precedes RING2. RING2 is another misnamed domain that coordinates two zinc ions and actually resembles the IBR fold (Spratt et al., 2013). RING2 harbours a catalytic cysteine C431 that is conserved amongst RBR E3s. C431 is capable of forming a thioester bond with Ub (Wenzel et al., 2011; Stieglitz et al., 2012) as a result of a transthioleation reaction with an E2-Ub conjugate prior to catalyzing transfer towards a substrate (Figure 1.1B).
Figure 1.5 Parkin domains and ARJP variants. (A) Human parkin domain architecture and ARJP variants. UblR0RBR is the nomenclature used to describe full-length parkin in this thesis and R0RBR describes a truncated form containing RING0-RING1-IBR-RING2 domains. Selected ARJP variants are shown above the domain structure and manifest throughout the entire parkin sequence. (B) Ribbon diagrams of individual parkin domains. Structures are coloured and aligned to their corresponding domains in panel A. Structures include human Ubl (PDB ID 1IYF), human IBR (PDB ID 2JMO) and Drosophila melanogaster RING2 (PDB ID 2LWR).
1.2.8 ARJP parkin variants

Parkin is encoded by the PARK2 gene in which mutations account for 50% of all ARJP cases (Kitada et al., 1998). There are over 120 reported parkin mutations to-date including exon deletions, exon multiplications, missense and nonsense mutations (Cruts et al., 2012). ARJP gene mutations that result in residue variants can be found throughout all domains of parkin (Figure 1.5A) having numerous effects on parkin solubility, protein interactions, catalytic functioning and recruitment to mitochondria. For instance, A46P and V56E are parkin ARJP variants found in the Ubl domain that make the Ubl domain highly insoluble (Safadi et al., 2011). T240R and R271S are variants located at the proposed E2 binding site, suggesting a link between ARJP and a loss of binding to the E2. The catalytic C431F ARJP variant has been shown to reduce or completely eliminate parkin activity (Chen et al., 2010; Shin et al., 2011). Finally, non-ARJP variants H302A and S65A are proposed to impair parkin activation and have shown impaired parkin recruitment to the mitochondria (Tang et al., 2017). The elucidation of complete full-length parkin structures would provide extensive breakthroughs into determining structural-based origins of parkin-associated ARJP cases.

1.2.9 E2-Ub conjugates

Approximately 40 E2 ubiquitin-conjugating enzymes (E2s) exist in the human genome and they function mainly as Ub carriers within the ubiquitination pathway (Stewart et al., 2016). E2s interact with E1 and E3 enzymes to transfer Ub to a substrate. Functional work has shown that E2s have individual reactivity profiles and they can specify poly- or mono-ubiquitin chains when paired with RING E3s (Christensen et al., 2007; Rodrigo-Brenni and Morgan, 2007; Windheim et al., 2008). For instance, UbcH5c can transfer Ub to cysteine or lysine residues, explaining its ability to pair with RING, HECT or RBR E3 ligases (Wenzel et al., 2011). However, UbcH7 is inefficient at transferring Ub to lysine residues and rather it only exhibits reactivity towards cysteines, suggesting that UbcH7 can only be paired with RBR and HECT E3s (Wenzel et al., 2011). Contrary to RING E3:E2 pairings, E2s play a smaller role in Ub chain-linkage specificity when paired with HECT and RBR E3s (Kim and Huibregtse, 2009; Stieglitz et al., 2013). These mechanistic differences can be simplified by remembering that the last
enzyme attached to Ub prior to substrate ubiquitination typically confers linkage specificity (Stewart et al., 2016). Together, the intrinsic reactivity of the E2 can be predictive of the type of E3 that it functions with as well as the final substrate Ub-linkage product.

All E2s contain a conserved catalytic domain of approximately 150 amino acids that shares a consistent canonical structure equipped with an active site cysteine for accepting and transferring Ub. This core adopts an α/β fold with typically four α-helices and four β-sheets (Stewart et al., 2016). The E2 helix H1, loop L4 and loop L7 elements make up a relatively well-conserved surface that is used for binding to RING domains (Budhidarmo et al., 2012) (Figure 1.3A). Solution studies show that E2-Ub conjugates can be extremely dynamic when Ub is conjugated to the E2 catalytic cysteine. These dynamics are the result of flexibility in the C-terminal tail of Ub and therefore conjugates can take on an array of closed, open or backbent orientations that are identified based on Ub positioning in relation to the E2 (Pruneda et al., 2011; Page et al., 2012). Closed states involve the Ub hydrophobic I44 patch making contacts with the E2 crossover helix H2. Ubc1-Ub was the first example of a conjugate to take on a closed state in solution (Hamilton et al., 2001). Open states extend Ub away from making any contact with the E2, and backbent states fold Ub back onto E2 loops L2, L4, or L5. E2s have a tendency to favour one of these orientations in solution, specific to each E2, and can bind to E3s in the same orientation. For instance, the Ubc13-Ub conjugate favours a closed conformation in solution (Pruneda et al., 2011; Page et al., 2012), and can also bind to E3s in a closed conformation (Middleton et al., 2017). Despite the tendency for an individual E2 to adopt a certain conformation in solution, these conformations can change dramatically upon binding to an E3. For instance, the UbcH5c-Ub conjugate populates extended open and backbent conformations in solution (Pruneda et al., 2011; Page et al., 2012), but has a propensity to bind in a closed state to a RING/U-Box E3 (Pruneda et al., 2012). As discussed in section 1.2.5, structural studies to-date indicate that regardless of the preferred state in solution, E2-Ub conjugates bind to RING E3 ligases in a closed conformation and to HECT or RBR E3 ligases in open conformations (Figure 1.6).
Figure 1.6 E2-Ub conjugate closed, open and backbent conformations. Ribbon diagrams are presented of E2-Ub conjugates showing a spectrum of adoptable conformations. Closed states show Ub using its I44 hydrophobic patch to interact with the E2 crossover helix H2, open states show Ub extending away from the E2 while making little to no contact, and backbent states show Ub in proximity to the E2 “backside” which includes E2 loops L2, L4 or L5. E2s share a canonical fold as seen when all atoms are aligned using the align command in PyMOL. Structures used were RING RNF4:UbcH5a-Ub (PDB ID 4AP4) (closed), HECT NEDD4L:UbcH5b-Ub (PDB ID 3JW0) (open), RBR HOIP:UbcH5b-Ub (PDB ID 5EDV) (open) and UbcH5b-Ub (PDB ID 3A33) (backbent). Full E3 cartoon structures were omitted for clarity.
1.3 Scope of Thesis

E2-ubiquitin conjugates are key regulators of ubiquitination and are extremely dynamic while being able to adopt multiple conformations. Because ubiquitination plays a large role in cellular homeostasis, diseases can arise when activity is compromised. Common to many bacterial pathogens like the shigellosis-inducing *Shigella*, the host ubiquitination pathway is often hijacked by bacterial effector proteins like OspG. As another example, patients with parkin-linked ARJP can have hindered ubiquitination activities due to hereditary variants in parkin. This thesis examines underlying molecular mechanisms contributing to shigellosis and Parkinson’s disease by examining the conformational arrangements of UbcH7-Ub in solution, bound to the shigellosis-associated kinase OspG and bound to the Parkinson’s disease-associated E3 ligase parkin. Research here has significant implications for understanding the molecular basis of shigellosis and hereditary forms of Parkinson’s disease.

The first goal was to determine how the bacterial effector OspG helps bacteria to hijack host ubiquitination by seeing how UbcH7-Ub interacts with OspG. To do-so, isothermal titration calorimetry (ITC) and nuclear magnetic resonance spectroscopy (NMR) were used to determine whether an E2-Ub conjugate like UbcH7-Ub was a biological target of OspG by looking at binding affinity. Crystallography and NMR were used to characterise the OspG:UbcH7-Ub interaction and to determine which conformation UbcH7-Ub adopts upon binding. The OspG:UbcH7-Ub crystal structure was compared to other structures of E2-Ub conjugates bound to E3s to determine whether OspG mimics a type of E3 binding. Based on results suggesting that OspG would competitively bind to E2-Ub conjugates, ubiquitination assays were performed in the presence of OspG to view whether bacterial OspG reduced host E3 activity.

The second goal was to determine how parkin autoinhibition is regulated by its Ubl domain, and how both phosphorylation of parkin S65 and Ub S65 (pUb) activate this autoinhibition. To do-so, we used crystallography to determine the structures of full-length parkin and full-length parkin\(^{S65D}\). ITC was used to determine how phosphorylation signals on parkin and Ub either allosterically or directly affect pUb binding. NMR was used to determine a docked model of pUb bound to parkin. NMR was also used to perform competitive binding experiments and illustrate how pUb
binding affects parkin inhibition regulated by the autoinhibitory Ubl domain. Results from this section have had significant impact on the molecular understandings of parkin autoinhibition and activation.

The third goal was to determine how UbcH7-Ub engages with parkin and to show how ARJP mutations affect this event. To do-so, we first determined the crystal structure of pUb bound to full-length parkin. NMR was used to determine the conformation of UbcH7-Ub in solution and while bound to parkin by determining a docked pUb-bound parkin:UbcH7-Ub model. Finally, ubiquitination assays were performed to determine how ARJP parkin variants affect activity. Together, research here impacts molecular understandings of UbcH7-Ub conformational arrangements, proper parkin functioning, and provides an explanation for how ARJP parkin variants result in disease.
1.4 References


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Chapter 2

STRUCTURAL BASIS FOR THE INHIBITION OF HOST PROTEIN UBIQUITINATION BY SHIGELLA EFFECTOR KINASE OSPG

2.1 Introduction

The shigellosis-inducing bacterial pathogen *Shigella* secretes OspG into host cells to reduce host inflammatory responses upon infection (Kim et al., 2005). Dampening of the inflammatory response is specifically done by the ability of OspG to prevent the NF-κB pathway degradation of inhibitor IκBα (Kim et al., 2005; Zhou et al., 2013). Inhibition of NF-κB-directed ubiquitination of IκBα has been suggested to occur when OspG manipulates the crucial step of host E2-Ub conjugate binding to host E3s by an unknown mechanism (Kim et al., 2005).

Structures of free and E3-bound E2-Ub conjugates show that Ub can be found in open, closed, or backbent orientations relative to the E2 that it is linked to. E2 conjugating enzymes use a conserved region for binding to RING- and HECT-type E3 enzymes that includes residues from the α-helix H1, loop L4 and loop L7 of the E2 (Budhidarmo et al., 2012).

I studied the interaction of OspG with Ub, UbcH7 and UbcH7-Ub to determine the mechanism of how OspG inhibits IκBα ubiquitination. My research determined that the biological binding target of OspG is the E2-Ub conjugate and that OspG inhibits E3 ubiquitination by competitively binding to the E2-Ub conjugate. OspG was found to mimic host HECT E3 enzymes by orienting UbcH7-Ub in an open conformation and by binding to the region on UbcH7 that is typically used for binding to E3 ligases.

*Sections of this chapter have been taken from the following published paper: Grishin, A.M., Condos, T.E., Barber, K.R., Campbell-Valois, F.X., Parsot, C., Shaw, G.S., and Cygler, M. (2014). Structural basis for the inhibition of host protein ubiquitination by Shigella effector kinase OspG. Structure 22, 878–888.*
2.2 Materials and Methods

2.2.1 Source of materials

BL21(DE3)pLysS cells containing yeast Ub^{K48R/G76C} in a pET3a plasmid and BL21(DE3)CodonPlus-RIL cells containing human His-tagged UbcH7^{C17S/C137S} in a pET28a plasmid (Serniwka and Shaw, 2008) were obtained from Kathy Barber (University of Western Ontario, Canada). BL21(DE3)Star cells containing a truncated version of *Shigella flexneri* 2a strain 2457T His-tagged OspG (residues 26-196) in a pMCSG7 plasmid were obtained from Dr. Andrey Grishin (University of Saskatchewan, Canada). Dr. Andrey Grishin also supplied OspG^{L99R/P102E}, OspG^{F154R} and OspG^{L99R/P102E/F154R} which had variants that were incorporated using QuickChange mutagenesis. All UbcH7 samples used in this chapter were UbcH7^{C17S/C137S}.

2.2.2 Expression and purification of UbcH7^{C17S/C137S}

Unlabelled UbcH7^{C17S/C137S} in BL21(DE3)CodonPlus-RIL cells was grown in LB media for ITC experiments, while ^{15}N-labelled UbcH7^{C17S/C137S} was obtained by growing the cells in M9 minimal media supplemented with ^{15}NH_{4}Cl for NMR experiments. Labelled and unlabelled growths contained kanamycin and chloramphenicol. Cells were grown at 37°C to an OD_{600} of 0.8 and expression was induced with 1 mM IPTG at 30°C for 12 hours. Cells were harvested by centrifugation at 6000 × g for 10 minutes and resuspended in 50 mM Tris, 200 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer. Cells were lysed in the presence of protease inhibitor using an Avestin EmulsiFlex-C5 homogenizer and were further centrifuged at 148,230 × g for 70 minutes. The supernatant was collected and His-tagged UbcH7^{C17S/C137S} was purified on a HisTrap FF column by batch-binding and washing with 50 mM Tris, 200 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer. His-tagged UbcH7^{C17S/C137S} was eluted with 50 mM Tris, 200 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer, TEV-cleaved at 25°C for 1 hour, and dialysed against 50 mM Tris, 200 mM NaCl, 250 μM TCEP, pH 8.0 buffer at 4°C overnight. TEV-cleaved UbcH7^{C17S/C137S} was purified on a second HisTrap FF column using 50 mM Tris, 200 mM NaCl, 250 μM TCEP, pH 8.0 wash buffer and the purified flowthrough was collected for experimental use. Purity was monitored by SDS-PAGE.
2.2.3 Expression and purification of Ub\textsuperscript{K48R/G76C}

Unlabelled Ub\textsuperscript{K48R/G76C} in BL21(DE3)pLysS cells was grown in LB media for ITC experiments, while \textsuperscript{15}N-labelled Ub\textsuperscript{K48R/G76C} was obtained by growing the cells in M9 minimal media supplemented with \textsuperscript{15}NH\textsubscript{4}Cl for NMR experiments. Labelled and unlabelled growths contained ampicillin and chloramphenicol. Cells were grown at 37°C to an OD\textsubscript{600} of 0.5 and expression was induced with 0.4 mM IPTG while remaining at 37°C. Unlabelled growths were expressed for 4 hours and labelled growths for 8 hours. Cells were harvested by centrifugation at 6000 × g for 10 minutes, resuspended in 20 mM NaOAc, 1 mM EDTA, pH 5.0 buffer and flash-frozen in liquid nitrogen. Cells were lysed in the presence of protease inhibitor by thawing and sonication. Lysate pH was then dropped to just below 5.0 with HCl to precipitate contaminating proteins, but not Ub\textsuperscript{K48R/G76C}. The lysate was further centrifuged at 148,230 × g for 70 minutes and the supernatant was run through a HiTrap Q anion exchange column. Ub\textsuperscript{K48R/G76C} is positively charged in 20 mM NaOAc, 1 mM EDTA, pH 5.0 wash buffer because its pI is 6.55, so it is collected in the flowthrough of the HiTrap Q column. Ub\textsuperscript{K48R/G76C} was purified on a final Sephadex G-75 size-exclusion column using 25 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0 buffer. Ub\textsuperscript{K48R/G76C} purity was monitored by SDS-PAGE throughout both HiTrap Q and Sephadex G-75 column purifications.

2.2.4 Expression and purification of OspG and OspG variants

Unlabelled OspG and its variants OspG\textsuperscript{L99R/P102E}, OspG\textsuperscript{F154R} and OspG\textsuperscript{L99R/P102E/F154R} in BL21(DE3)Star cells were all grown, expressed and purified similarly with the following protocol. Cells were grown in LB media containing ampicillin at 37°C to an OD\textsubscript{600} of 0.6 when 0.5g L-arabinose was added to each 1L growth and the temperature was dropped to 20°C. After 30 minutes, expression was induced with 1 mM IPTG for 16 hours. Cells were harvested by centrifugation at 6000 × g for 10 minutes and resuspended in 50 mM HEPES, 1M NaCl, 250 μM TCEP, 25 mM imidazole, pH 7.5 buffer. Cells were lysed in the presence of protease inhibitor using an Avestin EmulsiFlex-C5 homogenizer and further centrifuged at 148,230 × g for 70 minutes. The supernatant was collected and His-tagged OspG was purified on a HisTrap FF column by batch-binding and washing with 50 mM HEPES, 1M NaCl, 250 μM
TCEP, 25 mM imidazole, pH 7.5 buffer. His-tagged OspG was eluted with 50 mM HEPES, 400 mM NaCl, 250 μM TCEP, 250 mM imidazole, pH 7.5 buffer, TEV-cleaved at 25°C for 1 hour, and dialysed against 50 mM HEPES, 150 mM NaCl, 250 μM TCEP, pH 7.5 buffer at 4°C overnight. TEV-cleaved OspG was purified on a second HisTrap FF column using 50 mM HEPES, 1M NaCl, 250 μM TCEP, 25 mM imidazole, pH 7.5 wash buffer and the purified flowthrough was collected. OspG purity was monitored by SDS-PAGE throughout both HisTrap FF column purifications.

2.2.5 UbcH7-Ub disulphide-linked conjugate formation and purification

Purified Ub\(^{K48R/G76C}\) and UbcH7\(^{C17S/C137S}\) were incubated separately with 2 mM TCEP at room temperature for 30 minutes to reduce di-Ub and di-UbcH7 disulphide bonds. 200 μM Ub\(^{K48R/G76C}\) and 100 μM UbcH7\(^{C17S/C137S}\) were combined and dialyzed against 4L of 85 mM Na\(_2\)HPO\(_4\), 15 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, 10 μM CuCl\(_2\), pH 7.5 buffer. Dialysis was performed at 4°C and buffer was changed twice over the course of 48 hours. During this Cu\(^{2+}\)-induced oxidation reaction, Ub’s C-terminal G76C reacts with UbcH7’s catalytic C86 to form the UbcH7-Ub disulphide-linked conjugate (Serniwka and Shaw, 2009). Excess Ub\(^{K48R/G76C}\) was added in order to deplete UbcH7\(^{C17S/C137S}\) for aid in purification. The newly formed UbcH7-Ub disulphide-linked conjugate was then purified on a Sephadex G-75 size-exclusion column using 25 mM Tris, 1 mM EDTA, 250 mM NaCl, pH 7.5 buffer. UbcH7-Ub disulphide-linked conjugate formation and its purity after Sephadex G-75 purification was monitored by non-reducing SDS-PAGE.

2.2.6 ITC of OspG and variants binding to Ub, UbcH7 and UbcH7-Ub

ITC experiments were performed on a Microcal VP-ITC system (GE Healthcare) using freshly purified proteins. Ub\(^{K48R/G76C}\), UbcH7\(^{C17S/C137S}\) or UbcH7-Ub disulphide-linked conjugate were titrated into the calorimeter cell containing OspG. UbcH7-Ub disulphide-linked conjugate was also titrated into the calorimeter cell containing OspG\(^{L99R/P102E}\) or OspG\(^{L99R/P102E/F154R}\). Calorimeter cell and syringe protein concentrations varied between 0.01-1.22 mM for each experiment depending on the magnitude of heats observed. All experiments were performed 2-3 times each using 100
mM NaH₂PO₄, 100 mM NaCl, pH 7.5 buffer at 25°C. Protein concentrations were verified using amino acid analysis from the SPARC BioCentre Amino Acid Facility (Toronto, Canada). Data was analysed using a single-site binding model in OriginLab to determine stoichiometry (N), association (Kₐ) and enthalpy change (ΔH) values. Gibb’s free energy (ΔG) was calculated using equation 1 while entropy change (ΔS) was calculated using equation 2:

\[ ΔG = -RT ln K_a \]  
\[ ΔG = ΔH - TΔS \]

2.2.7 NMR of OspG binding to Ub, UbcH7 and UbcH7-Ub

All NMR experiments were collected on a 600 MHz Varian Inova spectrometer (Biomolecular NMR Facility, University of Western Ontario, Canada) equipped with a triple- resonance probe and x,y,z gradients. Samples were prepared in 100 mM NaH₂PO₄, 100 mM NaCl, pH 7.5 buffer with 10% D₂O (v/v) and 1% 10 mM DSS (v/v). Samples contained 100 μM of ¹⁵N-labelled Ub^K⁴⁸R/C⁷⁶G, ¹⁵N-labelled UbcH⁷⁷C¹⁷⁵S/C¹³⁷S or UbcH7-Ub disulphide-linked conjugate in which only the UbcH7 moiety was ¹⁵N-labelled. For protein interaction studies, ¹H-¹⁵N HSQC spectra were collected of each ¹⁵N-labelled sample in the absence and presence of 200 μM unlabelled OspG at 25°C using the sensitivity-enhanced method (Barbato et al., 1992). Ub^K⁴⁸R/C⁷⁶G spectral windows were 8000.0 Hz centred on 4.790 ppm for ¹H and 2005.0 Hz centred on 115.5 ppm for ¹⁵N, while UbcH⁷⁷C¹⁷⁵S/C¹³⁷S spectral windows were 7002.8 Hz centred on 4.800 for ¹H and 2005.0 Hz centred on 115.5 ppm for ¹⁵N. All samples were referenced to the DSS internal standard and all data was collected using identical spectral parameters.

Data was processed using 60°-shifted cosine bell-weighting functions in the ¹H and ¹⁵N dimensions using NMRPipe (Delaglio et al., 1995) and was analyzed using NMRView (Johnson and Blevins, 1994). ¹H-¹⁵N resonance assignments were performed using previously reported assignments (Hamilton et al., 2000), (Serniwka and Shaw, 2008). Chemical shift perturbations (CSPs) were calculated using equation 3:

\[ CSP = \sqrt{(ΔδH)^2 + ((ΔδN) × 0.2)^2} \]

CSPs were mapped to the OspG:UbcH7-Ub crystal structure (PDB ID code 4Q5E) using PyMOL (Delano, 2002).
2.3 Results

2.3.1 UbcH7\textsuperscript{C17S/C137S} was expressed and purified

Unlabelled and \textsuperscript{15}N-labelled UbcH7\textsuperscript{C17S/C137S} was successfully purified using an initial HisTrap FF column followed by TEV-cleavage and a second HisTrap FF column. Purity was monitored successfully by SDS-PAGE as seen by the expected band around 18 kDa (Figure 2.1). Unlabelled UbcH7\textsuperscript{C17S/C137S} was later used for ITC experiments involving OspG binding. \textsuperscript{15}N-labelled UbcH7\textsuperscript{C17S/C137S} was later used for NMR studies involving OspG binding.

2.3.2 Ub\textsuperscript{K48R/G76C} was expressed and purified

Unlabelled and \textsuperscript{15}N-labelled Ub\textsuperscript{K48R/G76C} was successfully purified using an initial HiTrap Q anion exchange column followed by a Sephadex G-75 size-exclusion column. Purity was monitored successfully by SDS-PAGE as seen by the expected band around 8.6 kDa (Figure 2.2). Unlabelled Ub\textsuperscript{K48R/G76C} was later used for ITC experiments involving OspG binding. \textsuperscript{15}N-labelled Ub\textsuperscript{K48R/G76C} was later used for NMR studies involving OspG binding.

2.3.3 OspG and OspG variants were expressed and purified

OspG and its variants OspG\textsuperscript{L99R/P102E} and OspG\textsuperscript{L99R/P102E/F154R} were all successfully purified using an initial HisTrap FF column followed by TEV-cleavage and a second HisTrap FF. Purity was monitored successfully by SDS-PAGE as seen by the expected band around 20.1 kDa (Figure 2.3). OspG\textsuperscript{F154R} expression and purification did not yield sufficient amounts for experimental use. Unlabelled OspG and its variants were later used for ITC experiments involving Ub, UbcH7 and UbcH7-Ub binding. Unlabelled OspG was also used for NMR studies involving \textsuperscript{15}N-labelled Ub, \textsuperscript{15}N-labelled UbcH7 and \textsuperscript{15}N-labelled UbcH7-Ub binding.
**Figure 2.1 UbcH7<sup>C17S/C137S</sup> purification.** Unlabelled UbcH7<sup>C17S/C137S</sup> was purified on a HisTrap column followed by TEV-cleavage and a second HisTrap column. Purity was verified by SDS-PAGE with the expected MW of UbcH7<sup>C17S/C137S</sup> being around 18 kDa.
Figure 2.2 Ub\textsuperscript{K48R/G76C} purification. Unlabelled Ub\textsuperscript{K48R/G76C} was purified on a (A) HiTrap Q column followed by a (B) Sephadex G-75 column. Purity was verified by SDS-PAGE with the expected MW of Ub\textsuperscript{K48R/G76C} being around 8.6 kDa. Because buffer conditions during Sephadex G-75 column purification and gel preparation contained no reducing agent, a small band around 17 kDa is observed corresponding to di-Ub\textsuperscript{K48R/G76C} because disulphide bridges are formed between two molecules.
Figure 2.3 OspG and OspG variants purifications. (A) OspG, (B) OspG\textsuperscript{L99R/P102E} and (C) OspG\textsuperscript{L99R/P102E/F154R} were purified on a HisTrap column followed by TEV-cleavage and a second HisTrap column. Purity was verified by SDS-PAGE with the expected MW of OspG being around 20 kDa.
2.3.4 UbcH7-Ub disulphide-linked conjugate was formed and purified

UbcH7-Ub disulphide-linked conjugate was successfully formed under Cu\(^{2+}\) oxidizing conditions by adding excess Ub\(^{K48R/G76C}\) to UbcH7\(^{C17S/C137S}\) to deplete UbcH7\(^{C17S/C137S}\) supplies and to facilitate its subsequent purification by Sephadex G-75 size-exclusion. Purity was monitored successfully by non-reducing SDS-PAGE as seen by the expected band around 26.7 kDa (Figure 2.4). Unlabelled UbcH7-Ub was later used for ITC experiments involving OspG binding. UbcH7-Ub in which only the UbcH7 moiety was \(^{15}\)N-labelled was used for NMR experiments involving unlabelled OspG binding.

2.3.5 The E2-Ub conjugate is the biological binding target of OspG

ITC was used to determine the affinity of interaction of OspG with Ub, UbcH7 and UbcH7-Ub (Figure 2.5). Results showed that OspG binds tighter to the UbcH7-Ub conjugate (K\(_d\) 580 ± 20 nM) than to Ub (K\(_d\) 9 ± 0.4 μM) or UbcH7 (K\(_d\) 86 ± 3 μM) (Table 2.1). All stoichiometry constants (N) were approximately equal to 1, which indicates a 1:1 binding site. The change in enthalpy (ΔH) for OspG binding to UbcH7-Ub is more negative than OspG binding to Ub or UbcH7 which suggests that UbcH7-Ub binds with a larger surface area than the individual Ub and Ubc7 components. The change in entropy (ΔS) for OspG binding to Ubch7-Ub is the most negative which suggests that that system is becoming more ordered. UbcH7 and Ub within the conjugate are flexible in relation to one another in solution and OspG binding to the conjugate may stabilize this flexibility, leading to a decrease in entropy. Gibbs free energy (ΔG) is most negative for OspG binding to UbcH7-Ub than to Ub or UbcH7 which suggests that UbcH7-Ub binding is the most favourable. Results here suggest that UbcH7-Ub binds cooperatively to OspG and that OspG preferentially recruits an E2-Ub conjugate rather than the individual Ub or E2 components.

ITC was also used to determine the affinity of interaction of UbcH7-Ub with the variants OspG\(^{L99R/P102E}\) and OspG\(^{L99R/P102E/F154R}\). Mutations were chosen because they are located at the proposed interaction site between OspG and UbcH7, and they were expected to reduce or eliminate the binding seen by ITC between OspG and UbcH7-Ub. ITC results showed that UbcH7-Ub binding to OspG\(^{L99R/P102E}\) (K\(_d\) 620 ± 30 nM) and
OspG<sup>L99R/P102E/F154R</sup> (K<sub>d</sub> 320 ± 10 nM) was not affected by the mutations and that binding was similar to UbcH7-Ub and wild-type OspG binding. OspG binds much tighter to Ub than to UbcH7, and so it is possible that Ub within the conjugate directs binding to OspG. For this reason, OspG<sup>L99R/P102E</sup> and OspG<sup>L99R/P102E/F154R</sup> may still be able to bind tightly to the UbcH7-Ub conjugate because the interaction site on OspG for Ub is still intact.

<sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra were collected of UbcH7-Ub, where only the UbcH7 moiety was <sup>15</sup>N-labelled, in the absence and presence of unlabelled OspG to assess the strength of binding. The addition of OspG to UbcH7-Ub resulted in significant broadening of a large majority of signals indicating the formation of a large 46.8 kDa complex between OspG and UbcH7-Ub (Figure 2.6A). A third spectrum was collected with the addition of 8 mM DTT to the NMR sample containing OspG bound to UbcH7-Ub in order to observe the strength of binding after the reduction and elimination of the disulphide bond between UbcH7 and Ub. The amide resonances for UbcH7 residues C86 and L87 were shifted with respect to their positions seen when UbcH7 was conjugated to Ub, indicating that the UbcH7-Ub complex is no longer intact with DTT added. The reappearance of many signals indicated the loss of the large OspG:UbcH7-Ub complex (Figure 2.6B). These findings suggest that OspG binds tightly to UbcH7-Ub, but not to free UbcH7, further suggesting that UbcH7-Ub is the biological binding target of OspG.
Figure 2.4 UbcH7-Ub disulphide-linked conjugate formation and purification. (A) UbcH7-Ub disulphide-linked conjugate was formed under Cu$^{2+}$ oxidizing conditions by adding Ub$^{K48R/G76C}$ and UbcH7$^{C175S/C137S}$ together. (B) UbcH7-Ub disulphide-linked conjugate was then purified on a Sephadex G-75 column. Purity was verified by non-reducing SDS-PAGE with the expected MW of UbcH7-Ub being around 27 kDa.
Figure 2.5 ITC of OspG variants binding to Ub, UbcH7 and UbcH7-Ub. Isotherm graphs shown are for: (A) OspG and Ub, (B) OspG and UbcH7, (C) OspG and UbcH7-Ub, (D) OspG<sup>L99R/P102E</sup> and UbcH7-Ub, (E) OspG<sup>L99R/P102E/F154R</sup> and UbcH7-Ub. The upper panels represent raw data, and the lower panels represent integrated heat changes performed assuming a single-site binding model. Data was collected at 25°C with 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, pH 7.5 buffer conditions.
Table 2.1 Thermodynamics of OspG binding to Ub, UbcH7 and UbcH7-Ub. Values are shown for stoichiometry (N), dissociation constant ($K_d$), enthalpy change ($\Delta H$), entropy change ($\Delta S$) and Gibbs free energy ($\Delta G$).

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<tr>
<th>Cell Protein</th>
<th>Titrant</th>
<th>N</th>
<th>$K_d$ ($\mu$M)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (kJ/mol)</th>
<th>$\Delta G$ (kJ/mol)</th>
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<tr>
<td>OspG</td>
<td>Ub</td>
<td>0.97±0.01</td>
<td>9.1±0.4</td>
<td>-5.5±0.1</td>
<td>23±1</td>
<td>-29±1</td>
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<tr>
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<td>UbcH7</td>
<td>0.96±0.02</td>
<td>86±3</td>
<td>-27±1</td>
<td>-4±1</td>
<td>-23±1</td>
</tr>
<tr>
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<td>0.58±0.02</td>
<td>-126±1</td>
<td>-90±2</td>
<td>-36±1</td>
</tr>
<tr>
<td>OspG L99R/P102E</td>
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<td>0.62±0.03</td>
<td>-88±1</td>
<td>-53±2</td>
<td>-35±2</td>
</tr>
<tr>
<td>OspG L99R/P102E</td>
<td>UbcH7-Ub</td>
<td>0.83±0.01</td>
<td>0.32±0.01</td>
<td>-194±3</td>
<td>-157±4</td>
<td>-37±1</td>
</tr>
</tbody>
</table>
Figure 2.6 NMR of UbcH7-Ub binding affinity to OspG. (A) Selected regions of $^1$H-$^{15}$N-HSQC spectra of UbcH7-Ub in which only UbcH7 is $^{15}$N-labelled in the absence (black contours) and presence (red contours) of 2 equivalents unlabelled OspG. Significant signal broadening indicates a large 46.8 kDa complex formed between OspG and UbcH7-Ub. (B) Selected regions of $^1$H-$^{15}$N-HSQC spectra of the same OspG:UbcH7-Ub sample from before but with 8 mM DTT added (blue contours) superimposed with the spectra collected in panel A (black and red contours). The reappearance of signals indicates the loss of the large OspG:UbcH7-Ub binding complex. UbcH7 residues C86 and L87 shift with respect to the unbound UbcH7-Ub spectrum affirming that DTT reduced and eliminated the disulphide bond between UbcH7 and Ub. All data was collected at 25°C in 100 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7.5 buffer and all peak contour levels are shown equal between spectra.
2.3.6 OspG and UbcH7-Ub surfaces of interaction determined by NMR

$^1\text{H}-^{15}\text{N}$ HSQC NMR spectra were collected of $^{15}\text{N}$-labelled UbcH7 in the absence and presence of unlabelled OspG to determine the interface on UbcH7 used for interaction (Figure 2.7A). UbcH7 amide resonances were successfully assigned using previously reported assignments. The measured chemical shift perturbations (CSPs) were minor and exhibited fast exchange indicating a weak interaction. UbcH7 resonances that either disappear or change the most upon addition of OspG include R6 (helix H1), K64 (loop L4) and K96 (loop L7) (Figure 2.7B). These residues comprise a surface on UbcH7 that is typically involved in E3 binding and were plotted onto the structure of OspG:UbcH7-Ub (PDB ID code 4Q5E) using PyMOL showing good agreement with surfaces used for interaction in the crystal structure (Figure 2.9).

$^1\text{H}-^{15}\text{N}$ HSQC NMR spectra were collected of $^{15}\text{N}$-labelled Ub in the absence and presence of unlabelled OspG to determine the interface on Ub used for interaction (Figure 2.8A). Ub amide resonances were successfully assigned using previously reported assignments. The measured CSPs were large and exhibited slow exchange indicating a moderately tight interaction. Ub resonances that change the most upon addition of OspG include A46, Q49 and H68 (Figure 2.8B). These residues are found within the surface on Ub known as the hydrophobic patch and were plotted onto the structure of OspG:UbcH7-Ub (PDB ID code 4Q5E) using PyMOL showing good agreement with surfaces used for interaction in the crystal structure (Figure 2.9).
Figure 2.7 NMR of the UbcH7 binding interface with OspG. (A) $^1$H-$^{15}$N HSQC NMR spectra of $^{15}$N-labelled UbcH7$^{C17S/C137S}$ in the absence (black contours) and presence of 2 equivalents unlabelled OspG (green contours). (B) Measured UbcH7 CSPs upon OspG binding. Grey bars indicate that the resonance was undetectable. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were minor and exhibited fast exchange indicating a weak interaction. All data was collected at 25°C in 100 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7.5 buffer.
Figure 2.8 NMR of the Ub binding interface with OspG. (A) $^1$H-$^{15}$N HSQC NMR spectra of $^{15}$N-labelled Ub in the absence (black contours) and presence of 2 equivalents unlabelled OspG (orange contours). (B) Measured Ub CSPs upon OspG binding. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were large and exhibited slow exchange indicating a moderately tight interaction. All data was collected at 25°C in 100 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7.5 buffer.
Figure 2.9 OspG:UbcH7-Ub crystal structure with NMR binding interface. Surfaces of UbcH7 (green) conjugated to Ub (orange) are shown binding to a ribbon diagram of OspG (PDB ID code 4Q5E). Residues in UbcH7 and Ub determined to be important for binding OspG by NMR are highlighted in magenta. UbcH7 residues important for binding include R6 (helix 1), K64 (loop 4) and K96 (loop 7). Ub residues important for binding include A46, Q49 and H68. Residues determined by NMR to be important for OspG binding align well with surfaces used for contact within the crystal structure.
2.3.7 *OspG mimics E3s by binding UbcH7-Ub in an open conformation*

The crystal structure of UbcH7-Ub bound to OspG (PDB ID code 4Q5E) was determined by collaborator Dr. Andrey Grishin (University of Saskatchewan, Canada), while the interpretation that revealed that UbcH7-Ub binds in an open conformation was achieved through my individual work (Figure 2.10A). This finding is similar to a separate crystal structure in which the UbcH5b-Ub conjugate is seen binding in an open conformation to HECT E3 NEDD4L (PDB ID code 3JW0) (Kamadurai et al., 2009) (Figure 2.10B). Superposition of the two E2 components shows that the two arrangements are very similar. A third crystal structure of UbcH5a-Ub bound to RING E3 RNF4 (PDB ID code 4AP4) (Plechanovová et al., 2012) shows the contrasting capability of the E2-Ub conjugate to orient itself in a closed conformation (Figure 2.10C). These observations suggested that OspG may interfere with host E3-mediated ubiquitination by recruiting an E2-Ub conjugate through HECT-like structural mimicking.
Figure 2.10 UbcH7-Ub binds to OspG in an open conformation. (A) Ribbon diagram of UbcH7-Ub bound in the open conformation to OspG (PDB ID code 4Q5E). (B) Ribbon diagram of UbcH5b-Ub bound in the open conformation to HECT E3 NEDD4L (PDB ID code 3JW0). In NEDD4L, only residues 734–796 and 872–949 are shown for clarity. (C) Ribbon diagram of UbcH5a-Ub bound in the closed conformation to RING E3 RINF4 (PDB ID code 4AP4). Only residues 131–195 from RNF4 are shown for clarity. Residues from each E2 found in the helix αH1, loops L4 and loops L7 conservatively make contacts with E3s and are highlighted in red. Residues from Ub’s hydrophobic patch that make important contacts with OspG are also highlighted in red. Panels were prepared by aligning all atoms in the E2 structures of UbcH7, UbcH5b, and UbcH5a using the align command in PyMOL.
2.3.8 OspG inhibits E3 parkin ubiquitination activity

To investigate whether or not OspG can inhibit E3 ubiquitination activity, Western blots following E3 parkin autoubiquitination activity were performed in the absence and presence of OspG. Results showed a strong decrease in the appearance of parkin autoubiquitination bands when increasing amounts of OspG were present, and parkin ubiquitination activity was almost completely lost at an UbcH7:OspG molar ratio of 1:1 (Figure 2.11A). OspG<sup>L99R/P102E</sup> and OspG<sup>F154R</sup> contain residue variants at the binding interface with UbcH7 and were made to abolish OspG binding to the UbcH7-Ub conjugate. Western blot analysis showed that parkin ubiquitination activity was not affected in the presence of these variants in comparison to wild type OspG as expected because OspG can no longer competitively bind to UbcH7-Ub (Figure 2.11B). In conclusion, results from Western blot analyses show that OspG can inhibit the ubiquitination activity of an E3 and that the binding site of UbcH7 onto OspG is necessary for this inhibition. Since OspG has previously been shown to bind to several E2-Ub conjugates, and since the E2-Ub conjugate functions upstream of E3s, then it is logical to suggest that OspG can inhibit the activity of multiple E3s.
Figure 2.11 OspG inhibits E3 parkin ubiquitination activity. (A) Western blot analysis following ΔUbID-parkin autoubiquitination using anti-parkin (top) and anti-Ub (bottom) antibodies from 4%-15% SDS-PAGE. The first two lanes of each blot contain either Ub or ΔUbID-parkin alone. Remaining lanes contain E1, ATP and reactants indicated above the lanes. OspG amounts are equivalents to UbcH7. Autoubiquitination products are represented as [Ub]n. (B) Coomassie-stained 8% Bis-Tris SDS-PAGE as described in (A).
2.4 Discussion

2.4.1 Model for OspG inhibition of host ubiquitination

*Shigella* infect host cells by secreting effector proteins to manipulate host cellular pathways as a way to survive and replicate. Previous studies have shown that the role of OspG during cellular invasion is to prevent the NF-κB pathway degradation of phosphorylated-IκBα. This manipulation involves hijacking host ubiquitination pathways at the step involving E2-Ub conjugate binding to an E3 (Kim et al., 2005; Zhou et al., 2013), however a structural explanation for how this event occurs has been unknown until now. We determined the crystal structure of OspG bound to UbcH7-Ub and this structure shows a similar mode of binding as seen in another structure of OspG bound to UbcH5c-Ub (PDB ID code 4BVU) (Pruneda et al., 2014), suggesting that OspG binds to E2-Ub conjugates in a universal manner. The manner by which OspG orients the E2-Ub conjugate in an open conformation is reminiscent of the HECT E3 NEDD4L:UbcH5b-Ub structure (PDB ID code 3JW0) (Kamadurai et al., 2009). Since conformations of E2-Ub conjugates are dictated by the E3:E2 pairing (Page et al., 2012), these results suggest that OspG mimics HECT E3 binding to the E2-Ub conjugate. We used NMR and crystallography to show that key residues in UbcH7 used for interaction with OspG include R6 (α-helix H1), K64 (loop L4) and K96 (loop L7). These residues are located in the conserved regions on E2s used for binding to RING and HECT E3s (Budhidarmo et al., 2012) further suggesting that OspG mimics E3 binding. This similar binding mode may also account for the observed ability of OspG to bind a number of different E2s (Kim et al., 2005). Together, comparisons here suggest that OspG mimics HECT E3s by orienting the E2-Ub conjugate in a similar open conformation and by binding to the same surface on E2s that is typically used to recruit the E3.

OspG has been shown to bind to free Ub (Zhou et al., 2013), as well as to E2s and their E2-Ub conjugate forms (Kim et al., 2005). We used NMR and ITC to show that OspG preferentially binds to the UbcH7-Ub conjugate with tight affinity ($K_d$ 580 ± 20 nM) in comparison to the individual UbcH7 ($K_d$ 86 ± 3 μM) and Ub ($K_d$ 9 ± 0.4 μM) moieties, suggesting that the conjugate is the biological target of OspG. Since OspG lacks a typical kinase activation loop (Zhou et al., 2013), the binding to a host factor such as UbcH7-Ub may serve to activate OspG kinase activity, for a currently unknown
function. Studies have already shown that both Ub and E2-Ub conjugates can activate OspG autophosphorylation or phosphorylation of artificial histone substrates (Zhou et al., 2013; Grishin et al., 2014; Pruneda et al., 2014). The affinity that OspG has for UbcH7-Ub is much tighter than when compared to typical E3 affinities for E2-Ub conjugates. For instance, the E3 E6AP binds to UbcH7-Ub with only a moderate $K_d$ 6.5 μM affinity (Purbeck et al., 2010). This finding suggests that OspG would compete with E3s for binding to the E2-Ub conjugate. We confirmed this hypothesis by following ubiquitination assays of an E3 in the absence and presence of OspG, and showed that OspG can inhibit E3 ubiquitination.

Here, we propose a mechanism for how OspG inhibits typical host NF-κB pathway degradation of phosphorylated-IκBα. OspG is first secreted into the host cell cytoplasm by the *Shigella* T3SS. OspG competitively binds to host E2-Ub conjugates with high affinity leading to its own activation while preventing typical host E3 binding to the E2-Ub conjugate. OspG binds to E2-Ub conjugates in a manner that mimics host HECT E3 binding. The curtailment of host E3 binding to E2-Ub conjugates results in a reduction in host ubiquitination of substrates such as phosphorylated-IκBα, explaining how OspG inhibits the NF-κB pathway degradation of phosphorylated-IκBα.
2.5 References


Chapter 3
DISRUPTION OF THE AUTOINHIBITED STATE PRIMES THE E3 LIGASE PARKIN FOR ACTIVATION AND CATALYSIS

3.1 Introduction
Autosomal recessive juvenile Parkinsonism (ARJP) is a hereditary form of Parkinson’s disease in which 50% of cases are directly linked to mutations in the E3 ubiquitin-ligase parkin (Kitada et al., 1998). Parkin’s protective features mediate mitochondrial control in dopaminergic neurons by attaching a small ubiquitin molecule to damaged proteins in a process called ubiquitination (Grenier et al., 2013).

Parkin autoinhibition is known to be regulated by the autoinhibitory parkin Ubl domain (Chaugule et al., 2011). The discovery of parkin autoinhibition has driven efforts to uncover activators such as the upstream kinase PINK1 (Clark et al., 2006; Park et al., 2006). PINK1 can phosphorylate both Ub and the parkin Ubl domain at S65 leading to activation of parkin ubiquitination activity (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). PINK1 can phosphorylate preformed mitochondrial Ub chains allowing parkin to bind tightly for retention at the mitochondria and for enhancement of ubiquitination activity suggesting a feed-forward mechanism (Ordureau et al., 2014).

Research here was performed to determine a structural model for how parkin autoinhibition and activation occurs. We determined the crystal structure of autoinhibited parkin and a docked model of pUb bound to parkin. We present a molecular mechanism showing that parkin is autoinhibited through interdomain interactions, that parkin S65 phosphorylation optimises pUb binding and that pUb binding regulates activation through release of the Ubl domain. The model presented provides important insights into parkin regulation and can help explain how ARJP variants affect parkin activity.

*Sections of this chapter have been taken from the following published paper:
3.2 Materials and Methods

3.2.1 Source of materials

BL21(DE3) *E. coli* cells containing human His-SUMO-tagged parkin R0RBR (residues 141-465), human His-SUMO-tagged parkin R0RBR<sup>380-384G</sup> (residues 141-465) and human His-SUMO-tagged parkin Ubl<sup>S65E</sup> (residues 1-76) in pET-SUMO plasmids as well as BL21(DE3)CodonPlus-RIL cells containing non-cleavable *Pediculus* GST-tagged PINK1 (residues 126-575) in a pGEX6P-1 plasmid were obtained from Dr. Helen Walden (University of Dundee, Scotland). BL21(DE3)CodonPlus-RIL *E. coli* cells containing yeast His-tagged Ub in a pMCG7 plasmid were obtained from Dr. Hong Ling (University of Western Ontario, Canada). Ubl and R0RBR will be the nomenclature used to describe the truncated forms of parkin described above while UblR0RBR will be used to describe full-length parkin used in this chapter.

3.2.2 Mutagenesis of Ubl<sup>S65E</sup>

A S65E substitution was incorporated into Ub using the following protocol. Plasmids were isolated from harvested JM109 *E. coli* cells using the Bio Basic Inc. EZ-10 spin column miniprep kit. Forward and reverse primers were designed containing the desired mutations (Table 3.1). Base pair mutations were incorporated into DNA using the QuikChange Site-Directed Mutagenesis protocol (Agilent) and Polymerase chain reaction (PCR). Methylated parental strands that remained in the PCR reaction were digested by DpnI for 16 hours at 37°C. All PCR products were transformed into JM109 and BL21(DE3)CodonPlus-RIL *E. coli* cells. JM109 *E. coli* cells with the transformed plasmids were plated on antibiotic-resistant plates, colony-picked and grown in LB. Plasmids were isolated from harvested cells using the Bio Basic Inc. EZ-10 spin column miniprep kit and were sequenced (London Regional Genomics Centre, Canada) to ensure that the correct mutations were incorporated.
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<td>Forward: 5’- caacatccaaaggaagaaactctagaata - 3’</td>
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<td>Reverse: 5’ - gaccaagttaagttaagtttttgtggtg - 3’</td>
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3.2.3 Expression and purification of R0RBR

Unlabelled R0RBR in BL21(DE3) cells was grown in LB media. 1 L growths were supplemented with kanamycin for antibiotic resistance and 1 mL 0.5 mM ZnCl$_2$ because R0RBR coordinates Zn$^{2+}$ ions. Cells were grown at 37°C to an OD$_{600}$ of 0.8 and expression was induced with 0.1 mM IPTG at 16°C for 16 hours.

$^{13}$C$^{15}$N$_2$H- and $^{12}$C$^{14}$N$_2$H-labelled R0RBR in BL21(DE3) cells were grown in D$_2$O MOPS media supplemented with desired isotopes. pET-SUMO plasmids containing R0RBR were first freshly transformed into BL21(DE3) cells and plated on kanamycin plates. Colonies were picked and inoculated into a 25 mL MOPS media starter containing kanamycin that was incubated at 37°C for 24 hours. The 25 mL starter was spun down at 3,000 × g for 20 minutes and 10°C. The spun-down cells were resuspended in a 150 mL sterilized 70%- D$_2$O MOPS media starter with kanamycin and were incubated for approximately 8 hours at 37°C until the OD$_{600}$ reached 0.8. The 150 mL 70%- D$_2$O starter was spun down at 3,000 × g for 20 minutes and 10°C. The spun-down cells were resuspended in two 1 L flasks containing sterilized 100%- D$_2$O pH 7.5 MOPS media with kanamycin and either 2 g $^{12}$C$^2$H-D-glucose, 2 g $^{13}$C$^2$H-D-glucose, 1 g $^{15}$NH$_4$Cl or 1 g $^{14}$NH$_4$Cl isotopes. Cells were grown at 37°C for approximately 12 hours until the OD$_{600}$ reached 0.8. Temperature was turned down to 16°C for 1 hour and 1 mL 0.5 mM ZnCl$_2$ was added to each 1 L flask. Expression was induced with 0.1 mM IPTG for 8 hours.

Both unlabelled and labelled R0RBR cell cultures were harvested and purified using the following protocol. Cells were harvested by centrifugation at 6000 × g for 10 minutes and resuspended in 50 mM Tris, 350 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer. Cells were lysed in the presence of protease inhibitor using an Avestin EmulsiFlex-C5 homogenizer and were further centrifuged at 148,230 × g for 70 minutes. The supernatant was collected and His-SUMO-tagged R0RBR was purified on a HisTrap FF column by batch-binding and washing with 50 mM Tris, 200 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer. His-tagged R0RBR was eluted with 50 mM Tris, 200 mM NaCl, 250 μM TCEP, 25 mM imidazole, pH 8.0 buffer, Ulp1-cleaved at 25°C for 1 hour, and dialysed against 50 mM Tris, 200 mM NaCl, 250 μM TCEP, pH 8.0 buffer at 4°C overnight. Ulp1-cleaved R0RBR was purified on a second
HisTrap FF column using 50 mM Tris, 200 mM NaCl, 250 μM TCEP, pH 8.0 wash buffer and the purified flowthrough was collected. R0RBR was run on a final Superdex 75 size-exclusion column using a flow rate of 0.3 mL/min and collecting 0.5 mL fractions. Fractions containing the desired R0RBR protein were collected for experimental use. Purity was monitored by SDS-PAGE.

3.2.4 Expression and purification of UbS65E

12C14N2H-labelled parkin UbS65E was expressed, harvested and purified using the same protocol for deuterated-labelled R0RBR growths (section 3.2.3) except 0.5 mM IPTG was used for induction and no ZnCl2 was added to growths because the Ubl domain does not coordinate Zn2+ ions.

3.2.5 Expression and purification of Ub and UbS65E

Unlabelled Ub and UbS65E in BL21(DE3)CodonPlus-RIL cells were grown in LB media. 13C14N1H-labelled Ub as well as 13C14N1H- and 13C15N1H-labelled UbS65E growths were performed in M9 minimal media supplemented with 2 g 13C1H-D-glucose, 1 g 15NH4Cl or 1 g 14NH4Cl isotopes. The unlabelled and labelled growths described above were supplemented with ampicillin and chloramphenicol at 37°C until an OD600 of 0.8 was reached and expression was induced with 0.5 mM IPTG at 37°C for 8 hours.

12C14N2H-labelled Ub and 12C15N2H-labelled UbS65E in BL21(DE3)CodonPlus-RIL cells were grown in D2O M9 minimal media supplemented with desired isotopes for NMR experiments. pMCG7 plasmids containing His-tagged Ub and UbS65E were first freshly transformed into BL21(DE3)CodonPlus-RIL cells and plated on ampicillin/chloramphenicol plates. Colonies were picked and inoculated into a 25 mL LB media starter containing ampicillin/chloramphenicol that was incubated at 37°C for 24 hours. The 25 mL starter was spun down at 3,000 × g for 20 minutes and 10°C. The spun-down cells were resuspended in a 150 mL sterilized 70%-D2O M9 minimal media starter with ampicillin/chloramphenicol and were incubated for approximately 8 hours at 37°C until the OD600 reached 0.8. The 150 mL 70%- D2O starter was spun down at 3,000 × g for 20 minutes and 10°C. The spun-down cells were resuspended in two 1 L flasks containing sterilized 100%- D2O pH 7.5 M9 minimal media with
ampicillin/chloramphenicol and either 2 g $^{12}\text{C}_2\text{H}_2\text{D}_2\text{-glucose}$, 2 g $^{13}\text{C}_2\text{H}_2\text{D}_2\text{-glucose}$, 1 g $^{15}\text{NH}_4\text{Cl}$ or 1 g $^{14}\text{NH}_4\text{Cl}$ isotopes. Cells were grown at 37°C for approximately 12 hours until the OD$_{600}$ reached 0.8. Expression was induced with 0.5 mM IPTG at 37°C for 8 hours. All labelled and unlabelled growths were harvested and protein was purified using the same protocol for UbcH7$^{C17S/C137S}$ (section 2.2.2). Purity was monitored by SDS-PAGE.

3.2.6 Expression and purification of PINK1

Unlabelled GST-tagged PINK1 in BL21(DE3)CodonPlus-RIL cells was grown and expressed in LB media containing ampicillin/chloramphenicol at 37°C to an OD$_{600}$ of 0.8. Expression was induced with 50 µM IPTG at 26°C for 16 hours. Unlabelled PINK1 cells were harvested by centrifugation at 6000 x g for 10 minutes and resuspended in 50 mM Tris, 250 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, pH 7.5 lysis buffer. Cells were lysed in the presence of protease inhibitor using an Avestin EmulsiFlex-C5 homogenizer and were further centrifuged at 148,230 x g for 70 minutes. The supernatant was collected and non-cleavable GST-tagged PINK1 was purified on a GSTrap FF column by batch-binding and washing with 50 mM Tris, 250 mM NaCl, 1 mM DTT, pH 7.5 wash buffer. GST-tagged PINK1 was eluted with 50 mM Tris, 150 mM NaCl, 1 mM DTT, 10 mM GSH, pH 7.5 elution buffer and was dialysed against 50 mM Tris, 250 mM NaCl, 1 mM DTT, pH 7.5 buffer at 4°C overnight to remove all traces of GSH. Purity was monitored by SDS-PAGE.
3.2.7 Optimisation of pUb formation and purification

Assays to optimise pUb formation were performed as follows. All sample reactions were performed with 10 µM PINK1 and 100 µM Ub in 50 mM Tris, 0.5 mM DTT, 10 mM MgCl2, 100 µM ATP, pH 7.5 buffer at 24°C while dialysing against the same fresh ATP buffer unless stated otherwise. Three conditions were assayed: (1) dialysis or no dialysis to fresh ATP buffer, (2) ATP concentrations of 100 µM, 500 µM, 1 mM, 5 mM or 10 mM, and (3) Ub concentrations of 100 µM or 500 µM.

Once optimal conditions were identified, unlabelled and $^{12}$C$^{14}$N$^2$H-labelled pUb was synthesised using unlabelled or labelled Ub that was purified as in section 3.2.5 and the optimised protocol for pUb synthesis in section 3.3.6. Unlabelled or labelled pUb was then purified from PINK1 and any unphosphorylated-Ub as follows. The finished reaction mixture was first dialysed against 20 mM Bis-Tris propane, pH 8.7 buffer for 12 hours. The mixture was purified on a HiTrap Q anion exchange column. Using 20 mM Bis-Tris propane, pH 8.7 wash buffer, unphosphorylated Ub flows through while pUb and PINK1 bind to the column because they have sufficient negative charge. 2 mL fractions were then collected using a 0-100% 20 mM Bis-Tris propane, 0.5 mM NaCl, pH 8.7 elution buffer gradient and 0.8 mL/min flow rate. pUb elutes around 40% of the elution gradient while PINK1 elutes around 100% because it is more negatively charged than pUb. The pH of eluted fractions was neutralized by adding 1 M Tris, pH 7.5 buffer. Fractions containing purified pUb were collected for experimental use. Integrity and purity of pUb was assessed by mass spectrometry and Wako Phos-tag-gel analysis.

3.2.8 Production of a 1:1 complex of R0RBR bound to pUb

$^{12}$C$^{14}$N$^2$H-labelled pUb was added 1.5 times in excess to $^{13}$C$^{15}$N$^2$H-labelled R0RBR so that pUb would saturate R0RBR. The 1:1 complex mixture was purified from unbound pUb on a Superdex 75 size-exclusion column in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP pH 7.0 buffer using a flow rate of 0.3 mL/min and collecting 1 mL fractions. Fractions containing the desired pUb-R0RBR complex were collected for experimental use. Purity was monitored by SDS-PAGE.
3.2.9 ITC of R0RBR binding to Ub, Ub^{S65E} and pUb

ITC experiments were performed on either a Microcal VP-ITC system (GE Healthcare) or a Nano ITC system (TA Instruments) using freshly purified proteins. Ub, Ub^{S65E} or pUb were titrated into the calorimeter cell containing R0RBR or a construct of parkin containing a stretch of glycine substitutions from residues 380-384 termed R0RBR^{380-384G}. Calorimeter cell and syringe protein concentrations varied between 0.01-1.5 mM for each experiment depending on the magnitude of heats observed. Each experiment was performed 2-3 times each using 50 mM HEPES, 50 mM NaCl, 250 µM TCEP, pH 7.5 buffer at 25°C. Protein concentrations were verified using amino acid analysis from the SPARC BioCentre Amino Acid Facility (Toronto, Canada). Data from the Microcal VP-ITC system was analysed with OriginLab while data from the Nano ITC system was analysed with NanoAnalyze using a single-site binding model to determine stoichiometry (N), association (K_a) and enthalpy change (ΔH) values. Gibb’s free energy (ΔG) and entropy change (ΔS) from the Microcal VP-ITC system experiments were calculated using the same equations in section 2.2.6, while ΔG and ΔS from the Nano ITC system experiments were calculated using equation 2 from section 2.2.6 and NanoAnalyze software respectively.

3.2.10 Circular dichroism of Ub, Ub^{S65E} and pUb

Spectra were collected on a Jasco J-810 instrument using 30 µM of each sample in 10 mM KH_2PO_4, 1 mM EDTA, pH 7.0 buffer at 25°C. Structure curves were recorded and averaged using five scans from 260-190 nm. Ellipticity signals were converted to mean residue ellipticity units using the following equations:

\[ \text{MRW} = \frac{\text{MW}}{n} \] \hspace{1cm} (1)

\[ [\theta] = \frac{\theta \times \text{MRW}}{10 \times C \times l} \] \hspace{1cm} (2)

MRW is the mean residue weight of the protein in g/mol, MW is the molecular weight of the protein and n is the number of residues. \([\theta]\) is the mean residue ellipticity in deg·cm^2/dmol, \(\theta\) is the degree of ellipticity in mdeg, C is the concentration in mg/mL and \(l\) is the pathlength of the cuvette in cm. Thermal denaturing spectra recorded at 218 nm from 5-105°C were used to make melting curves. Ellipticity signals were processed
using GraphPad Prism software and melting temperatures were calculated using a nonlinear fit to the data.

3.2.11 NMR spectroscopy setup and processing

All NMR data was collected at 25°C on a Varian Inova 600 MHz NMR spectrometer equipped with a triple resonance cryogenic probe and z-field gradients. Samples were prepared in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP pH 7.0 buffer with 10% D2O (v/v) and DSS as an internal reference. All $^1$H-$^{15}$N HSQC spectra (Kay et al., 1992) were collected in TROSY mode (Pervushin et al., 1997) to follow amide backbone chemical shift perturbations (CSPs). R0RBR spectral windows were 8000.0 Hz centred on 4.780 ppm for $^1$H and 1700.0 Hz centred on 118.0 ppm for $^{15}$N, while Ub spectral windows were 7002.8 Hz centred on 4.790 for $^1$H and 2000.0 Hz centred on 120.2 ppm for $^{15}$N. All $^1$H-$^{13}$C HMQC spectra (Tugarinov et al., 2004) were collected to monitor chemical shifts of Ub side chain methyl groups using spectral windows of 7000.0 Hz centred on 4.790 ppm for $^1$H and 10,000 Hz centred on 35.00 ppm for $^{13}$C. CSPs for amide backbone resonances were calculated using equation 3, while CSPs for side chain methyl groups were calculated using equation 4:

$$\text{CSP}_{\text{amide}} = \sqrt{(\Delta \delta H)^2 + ((\Delta \delta N) \times 0.2)^2}$$  \hspace{1cm} (3)

$$\text{CSP}_{\text{methyl}} = \sqrt{(\Delta \delta H)^2 + ((\Delta \delta C) \times 0.3)^2}$$  \hspace{1cm} (4)

All data was processed using 60°-shifted cosine bell-weighting functions using NMRPipe and NMRDraw (Delaglio et al., 1995), and was analysed using NMRViewJ (Johnson and Blevins, 1994).
3.2.12 NMR assigning of Ub and Ub\textsuperscript{S65E} backbone and methyl groups

Backbone amide \textsuperscript{1}H, \textsuperscript{15}N assignment for unbound Ub\textsuperscript{S65E} was made by collecting \textsuperscript{1}H-\textsuperscript{15}N HSQC and HNCA (Kay et al., 1990) spectra of \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{1}H-labelled Ub\textsuperscript{S65E} in non-TROSY mode and by using them in conjunction with previous assignments of Ub\textsuperscript{K48R} (Hamilton et al., 2000). The \textsuperscript{1}H, \textsuperscript{15}N backbone chemical shift assignments for Ub\textsuperscript{S65E} have been deposited to the Biological Magnetic Resonance Bank under the accession number 25708.

\textsuperscript{1}H, \textsuperscript{13}C side-chain methyl identification of yeast Ub and Ub\textsuperscript{S65E} in the free state and bound to R0RBR was performed by collecting \textsuperscript{1}H-\textsuperscript{13}C HMQC spectra (Tugarinov et al., 2004) of \textsuperscript{13}C\textsuperscript{14}N\textsuperscript{1}H-labelled Ub alone and bound to \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2}H-labelled R0RBR. The same experiments were collected of \textsuperscript{13}C\textsuperscript{14}N\textsuperscript{1}H-labelled Ub\textsuperscript{S65E} alone and bound to \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2}H-labelled R0RBR. Resonance identification was performed using well-aligned and previously assigned yeast Ub\textsuperscript{K48R} resonances (Hamilton et al., 2000). The \textsuperscript{1}H, \textsuperscript{13}C side-chain methyl chemical shift assignments for Ub and Ub\textsuperscript{S65E} both bound to R0RBR have been deposited to the Biological Magnetic Resonance Bank under accession numbers 25707 and 25709 respectively. A list of all NMR experiments performed can be found in Table 3.2.

3.2.13 NMR of R0RBR interactions with Ub, Ub\textsuperscript{S65E}, pUb and PO\textsubscript{4}

TROSY \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra were collected in variations of selectively-labelled R0RBR with selectively-labelled Ub, Ub\textsuperscript{S65E}, pUb and PO\textsubscript{4} titrations. \textsuperscript{1}H-\textsuperscript{13}C HMQC spectra were collected in variations of selectively-labelled Ub or Ub\textsuperscript{S65E} with selectively-labelled R0RBR titrations. CSP analysis was performed on amide backbone and side chain methyl groups to determine ambiguous restraints used for docking pUb to R0RBR. CSPs used for docking were mapped to the docked pUb-R0RBR complex computed in section 3.2.14 using PyMOL (Delano, 2002). TROSY \textsuperscript{1}H-\textsuperscript{15}N NOESY experiments were used with TROSY \textsuperscript{1}H-\textsuperscript{15}N HSQC and \textsuperscript{1}H-\textsuperscript{13}C HMQC spectra to determine distance restraints between amide backbone protons on \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2}H-labelled R0RBR and side chain methyl protons on \textsuperscript{13}C\textsuperscript{14}N\textsuperscript{1}H-labelled Ub or Ub\textsuperscript{S65E}. A list of all NMR experiments performed can be found in Table 3.2.
Table 3.2 NMR experiments of R0RBR with Ub, Ub\textsuperscript{S65E}, pUb, PO\textsubscript{4} and Ubl\textsuperscript{S65E}. All experiments were performed at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer. All \textsuperscript{1}H -\textsuperscript{15}N HSQC spectra were collected in TROSY mode unless otherwise noted.

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<th>\textsuperscript{13}C\textsuperscript{15}N-H R0RBR</th>
<th>\textsuperscript{12}C\textsuperscript{15}N-H R0RBR</th>
<th>\textsuperscript{12}C\textsuperscript{14}N-H Ub</th>
<th>\textsuperscript{13}C\textsuperscript{14}N-H Ub</th>
<th>\textsuperscript{12}C\textsuperscript{14}N-H Ub\textsuperscript{S65E}</th>
<th>\textsuperscript{13}C\textsuperscript{14}N-H Ub\textsuperscript{S65E}</th>
<th>\textsuperscript{13}C\textsuperscript{14}N-H pUb</th>
<th>\textsuperscript{12}C\textsuperscript{14}N-H PO\textsubscript{4}</th>
<th>\textsuperscript{12}C\textsuperscript{14}N-H Ubl\textsuperscript{S65E}</th>
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* Collected in non-TROSY mode
** A 1:1 complex was purified using a Superdex 75 size-exclusion column as in section 3.2.8
*** Performed in duplicate
3.2.14 HADDOCK of R0RBR and pUb binding

Interacting residues determined while following both R0RBR and Ub\textsuperscript{S65E} amide backbone resonance CSPs during NMR experiments were defined as those that shifted greater than the average +1 standard deviation and had greater than 20% side chain accessible surface area. Interacting residues determined while following both Ub and Ub\textsuperscript{S65E} side chain methyl group resonance CSPs during NMR experiments were defined as having shifted and were used regardless of accessible surface area due to having a limited number of methyl-containing residues. Unambiguous restraints determined from TROSY \textsuperscript{1}H\textsuperscript{15}N NOESY NMR data and 1 unambiguous restraint determined through mutagenesis experiments were used for docking. The full list of ambiguous and unambiguous restraints used for docking are shown in Table 3.3.

pUb was docked to R0RBR using HADDOCK (Dominguez et al., 2003). The coordinates from the crystal structure of UblR0RBR\textsuperscript{S65D} (PDB ID code 5C23) were used for docking after removal of the Ubl domain and adjoining linker coordinates. A phosphate group was added at S65 to Ub coordinates (PDB ID code 1UBQ) (Vijay-Kumar et al., 1987) and missing regions in the R0RBR coordinates were modelled-in using the Modeller (Eswar et al., 2006) plug-in for UCSF Chimera (Pettersen et al., 2004). An upper distance limit of 5.0 Å was set for ambiguous distance restraints while unambiguous distance restraints were set to 3.0 Å. Standard parameters were used except inter_rigid (0.1) which was set to allow tight packing of the two proteins, and the unambiguous force constants were increased by five-fold compared to the ambiguous constants. A total of 1,000 initial complexes were calculated and the best 100 structures were water-refined.

3.2.15 NMR heteronuclear NOEs of R0RBR binding to pUb

\textsuperscript{1}H-\textsuperscript{15}N heteronuclear NOE NMR experiments (Farrow et al., 1994) were collected using a sample of \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2}H-labelled R0RBR and \textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled pUb. Proton saturation was achieved through a 5 second irradiation time following an 11 second relaxation delay. The equivalent non-saturated experiment contained a 16 second relaxation delay. Both saturated and non-saturated experiments were conducted in duplicate and NOEs were averaged.
Table 3.3 HADDOCK restraints for R0RBR and pUb binding. Restraints were determined using NMR CSP analysis, $^1$H-$^{15}$N NOESY experiments, mutagenesis and ITC data.

<table>
<thead>
<tr>
<th>Restraint Type</th>
<th>Experiment</th>
<th>R0RBR</th>
<th>pUb</th>
</tr>
</thead>
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<tr>
<td>Unambiguous</td>
<td>Mutagenesis</td>
<td>H302 atom N</td>
<td>pS65 atom P</td>
</tr>
<tr>
<td></td>
<td>NMR $^1$H-$^{15}$N NOESY</td>
<td>E353 atom N</td>
<td>L8 atom HD21</td>
</tr>
<tr>
<td></td>
<td>NMR $^1$H-$^{15}$N NOESY</td>
<td>L342 atom N</td>
<td>L73 atom HD11</td>
</tr>
</tbody>
</table>
3.2.16 NMR of Ub$^{S65E}$ and Ubl$^{S65E}$ competitive binding to R0RBR

Competitive binding experiments that assessed the binding of Ub$^{S65E}$ and Ubl$^{S65E}$ to R0RBR utilised $^1$H-$^{13}$C HMQC and TROSY $^1$H-$^{15}$N HSQC spectra collected to follow side chain methyl CSPs of $^{13}$C-$^{14}$N-$^1$H-labelled Ub$^{S65E}$ alone, with 1 equivalent of $^{13}$C-$^{15}$N-$^2$H-labelled R0RBR and with 1 equivalent of $^{12}$C-$^{14}$N-$^2$H-labelled Ubl$^{S65E}$ added to the Ub$^{S65E}$ and R0RBR sample.

3.3 Results

3.3.1 Ub$^{S65E}$ was synthesised

Prior to determining a protocol to synthesise pUb, Ub$^{S65E}$ was used as a functional mimetic of S65 phosphorylation. For this reason, a S65E substitution was incorporated into Ub. Sequencing results verified that QuikChange Site-Directed Mutagenesis and PCR successfully incorporated the substitution with the previously described primers (Table 3.1).

3.3.2 R0RBR was expressed and purified

Unlabelled and labelled R0RBR (parkin residues 141-465) were successfully expressed and purified using an initial HisTrap FF column followed by Ulp1-cleavage, a second HisTrap FF column and a final Superdex 75 size-exclusion column. Purity was monitored by SDS-PAGE as seen by the expected band around 36 kDa (Figure 3.1). Mass spectrometry was previously used and correctly verified the mass of R0RBR. Unlabelled R0RBR was later used for ITC experiments, while labelled R0RBR was used for NMR experiments.
Figure 3.1 R0RBR purification. $^{13}$C$^{15}$N$_2$H-labelled R0RBR was purified on (A) a HisTrap column followed by Ulp1-cleavage and a second HisTrap column, and (B) a final Superdex 75 size-exclusion column. Purity was verified by SDS-PAGE with the expected MW of R0RBR being around 36 kDa.
3.3.3 Ubl<sup>S65E</sup> was expressed and purified

Prior to determining a protocol for sufficient production of pUbl, Ubl<sup>S65E</sup> (parkin residues 1-76) was used as a mimetic. ¹²C¹⁴N²H-labelled Ubl<sup>S65E</sup> was successfully expressed and purified using an initial HisTrap FF column followed by Ulp1-cleavage, a second HisTrap FF column and a final Superdex 75 size-exclusion column. Purity was monitored by SDS-PAGE as seen by the expected band around 8.9 kDa (Figure 3.2). Mass spectrometry was previously used and correctly verified the mass of Ubl<sup>S65E</sup>. ¹²C¹⁴N²H-labelled Ubl<sup>S65E</sup> was later used for NMR competition interaction studies with Ub<sup>S65E</sup> and R0RBR.

3.3.4 Ub and Ub<sup>S65E</sup> were expressed and purified

Unlabelled Ub and Ub<sup>S65E</sup>, ¹³C¹⁴N¹H- and ¹²C¹⁴N²H-labelled Ub, as well as ¹³C¹⁴N¹H-, ¹³C¹⁵N¹H- and ¹²C¹⁵N²H-labelled Ub<sup>S65E</sup> were successfully purified using an initial HisTrap FF column followed by TEV-cleavage and a second HisTrap FF column. Purity was monitored by SDS-PAGE as seen by the expected band around 8.6 kDa (Figure 3.3). Mass spectrometry was previously used and correctly verified the mass of Ub. Unlabelled Ub and Ub<sup>S65E</sup> were later used for ITC and circular dichroism experiments, while labelled Ub and Ub<sup>S65E</sup> were used for NMR experiments.

3.3.5 PINK1 was expressed and purified

Unlabelled GST-tagged PINK1 kinase was successfully purified using a GSTrap FF column. Purity was monitored by SDS-PAGE as seen by the expected band around 64 kDa (Figure 3.4). PINK1 purity was sufficient for later enzymatic use in phosphorylating Ub samples.
Figure 3.2 Ubl$^{S65E}$ purification. $^{12}$C-$^{14}$N$_2$H-labelled Ubl$^{S65E}$ was purified on (A) a HisTrap column followed by Ulp1-cleavage and a second HisTrap column, and (B) a final Superdex 75 size-exclusion column. Purity was verified by SDS-PAGE with the expected MW of Ubl$^{S65E}$ being around 8.9 kDa.
Figure 3.3 Ub and Ub$^{S65E}$ purification. (A) WT Ub and (B) $^{12}$C$^{15}$N$_2$H-labelled Ub$^{S65E}$ were purified on a HisTrap column followed by TEV-cleavage and a second HisTrap column. Purity was verified by SDS-PAGE with the expected MWs of Ub and Ub$^{S65E}$ being around 8.6 kDa.
**Figure 3.4 PINK1 purification.** GST-tagged PINK1 was purified on a GSTrap column. Purity was verified by SDS-PAGE with the expected MW being around 64 kDa.
3.3.6 pUb formation and purification was optimised

pUb is formed when Ub is phosphorylated by PINK1 under optimal conditions, therefore a protocol for pUb synthesis was optimised in order to minimize reagent requirements and the time required for formation. Conditions that were assayed included comparing dialysis against fresh ATP buffer with no dialysis (Figure 3.5A), ATP concentrations (Figure 3.5B) and Ub concentrations (Figure 3.5C). Assays showed that optimal pUb formation required 10 µM PINK1 with 100 µM Ub in 50 mM Tris, 0.5 mM DTT, 10 mM MgCl₂, 5 mM ATP, pH 7.5 buffer while dialysing against the same fresh ATP buffer at 24°C for 1 hour. Higher concentrations of 500 µM Ub can be sufficiently phosphorylated if the reaction time is extended to 4 hours with 10 mM ATP concentrations. Assays were visualised by Phos-tag gel.

Unlabelled and ¹²C¹⁴N²H-labelled pUb was successfully purified using a HiTrap Q anion exchange column. Purity was monitored by Phos-tag gel (Figure 3.6AB). Integrity of pUb was confirmed by mass spectrometry as seen by a 8,908 Da MW which corresponds to the addition of 1 phosphate group to Ub (Figure 3.6C). Unlabelled pUb was later used for ITC and circular dichroism experiments while ¹²C¹⁴N²H-labelled pUb was used for NMR experiments.

3.3.7 A 1:1 complex of R0RBR to pUb was purified

In order to obtain a 1:1 complex of ¹³C¹⁵N²H-labelled R0RBR bound to ¹²C¹⁴N²H-labelled pUb, the two proteins were co-purified on a Superdex 75 size-exclusion column because pUb binds tight enough to R0RBR to allow the high MW 1:1 complex to be separated from unbound pUb. The elution profile was monitored by absorbance and denaturing SDS-PAGE as seen by the expected bands around 36 kDa for R0RBR and 8.9 kDa for pUb that co-elute in early fractions. Later fractions contain unbound pUb and were discarded (Figure 3.7). The purified complex was used in NMR experiments.
Figure 3.5 pUb formation optimisation. Phos-tag gels show pUb formation was optimised by assaying (A) dialysis or no dialysis against fresh ATP buffer, (B) ATP concentrations and (C) Ub concentrations. Optimal conditions for pUb formation include using 10 µM PINK1 with 100 µM Ub in 50 mM Tris, 0.5 mM DTT, 10 mM MgCl₂, 5 mM ATP, pH 7.5 buffer while dialysing against the same fresh ATP buffer at 24°C for 1 hour, as denoted by * in panel B. Higher concentrations of 500 µM Ub can be sufficiently phosphorylated if the reaction time is extended to 4 hours with higher 10 mM ATP concentrations as denoted by * in panel C.
Figure 3.6 pUb purification. Phostag-gels showing (A) Ub was successfully phosphorylated by PINK1 to form pUb and (B) pUb purification on a HiTrap Q column. (C) Integrity was verified by mass spectrometry as seen by the observed 8,908 Da MW peak corresponding to pUb.
Figure 3.7 pUb-R0RBR complex purification. (A) Absorbance elution profile of pUb-R0RBR purified on a Superdex 75 10/300 size-exclusion column from unbound pUb. pUb-R0RBR co-elutes as seen by the earlier shift in absorbance in comparison to the unbound R0RBR profile. (B) Superdex 75 10/300 size-exclusion elution profile of pUb-R0RBR purification visualised by SDS-PAGE with pUb-R0RBR eluting in fractions 21-24 and unbound pUb eluting in fractions 26-29.
3.3.8 *Parkin autoinhibition is regulated through interdomain interactions*

To determine a structural understanding for parkin autoinhibition, a crystal structure of UblR0RBR was solved to 1.8 Å and comprises the five domains of parkin: Ubl, RING0, RING1, IBR and RING2 (Figure 3.8A). Residues 383–390 and 406–413 within a large linker connecting IBR and RING2 domains are missing in the density due to high flexibility. Parkin takes on a globular structure with many notable interdomain interactions that illustrate an autoinhibited state. The largest buried interface is 2,150 Å² comprising interactions between the autoinhibitory Ubl domain to RING1 and IBR domains with predominant contacts between the Ubl sheets β3 and β5 to RING1 helix H1. The extent and nature of the Ubl/RING1 interface suggests it is important for autoinhibition of parkin which is consistent with several activating ARJP mutations that are found at this interface such as R42P, A46P and R33Q within the Ubl domain (Chaugule et al., 2011). Further contacts from N8 in the Ubl β1-β2 sheet loop sits between E310 and Q311 of a bent RING1 helix H3, as well as H11 within the Ubl β1-β2 sheet loop is seen interacting with P333 and K369 of the IBR. RING1 takes on a canonical RING fold and is the suggested E2 binding site. However, two more justifications for parkin autoinhibition exist when considering E2 binding. First, residues 390-400 comprise a repressor (REP) helix that interacts with RING1 and blocks accessibility to the E2 (Figure 3.8A). Second, when an E2 is modelled onto the RING1 binding site, transthiolation cannot occur due to a large 50 Å distance between the E2 catalytic cysteine and the RING2 catalytic C431. Furthermore, RING0/RING2 interactions bury the catalytic C431 making it inaccessible for transthiolation. Together, the full-length crystal structure of parkin shows that large conformational changes are required to relieve autoinhibition mediated through the presence of (1) Ubl interactions, (2) REP/RING1 interactions blocking the E2 binding site, (3) a large 50 Å distance between the E2 and RING2 C431 catalytic sites and (4) RING0/RING2 interactions that occlude the catalytic C431.
3.3.9 *Parkin phosphorylation leads to remodelling of a hinge interface*

Superposition of the UblR0RBR and R0RBR structures (PDB ID code 4I1H) reveal good alignment with an rmsd of 1.8 Å. The observed difference is largely due to a change in positioning of the IBR domain which experiences a 12 Å swing from a hinge at the RING0/RING1 interface (Figure 3.8B). The RING0/RING1 hinge interface comprises a basic triad that includes residues K151, H302 and R305 that are thought to coordinate pUb binding. To observe the effects of phosphorylation at parkin S65, a crystal structure of phosphorylation-mimetic UblR0RBR<sup>S65D</sup> (PDB ID code 5C23) was solved to 2.4 Å. Superposition of UblR0RBR and UblR0RBR<sup>S65D</sup> reveal an excellent 0.58 Å rmsd indicating that no global conformational changes occur. However, close observation and comparison of the hinge interface between the structures reveals that H227, E300 and H302 residues are remodelled to produce a continuous basic patch when UblR0RBR has a S65D substitution, much like the R0RBR structure (Figure 3.8CD). This data suggests that the addition of a negative charge or phosphate group at S65 of parkin is enough to allosterically alter the distal RING0/RING1 hinge interface and prime parkin for optimal pUb binding.
Figure 3.8 Crystal structure of UbIR0RBR. (A) Ribbon diagram of UbIR0RBR parkin (PDB ID code 5C1Z) showing the Ubl domain (green), RING0 (blue), RING1 (purple), IBR (black), repressor element (REP) (yellow) and RING2 (brown). Loops outside domains are modelled in cyan. Zinc atoms are represented as grey spheres. (B) Overlay of UbIR0RBR (coloured as in panel A) with R0RBR (PDB ID code 4I1H) (grey). Absence of the Ubl domain causes the IBR domain to swing 12 Å from a hinge opening at the RING0/RING1 interface. (C) Ribbon and stick comparison of the UbIR0RBR, UbIR0RBR<sup>S65D</sup> (PDB ID code 5C23) and R0RBR structures. H227, E300 and H302 at the RING0/RING1 hinge interface are remodelled in the UbIR0RBR<sup>S65D</sup> structure to resemble the R0RBR hinge interface. (D) Surface representation of the continuous basic patch formation at the RING0/RING1 hinge interface comprising K151, H302 and R305 when the phosphorylation-mimetic S65D substitution is present. Panels were prepared by aligning all RING0/RING2 atoms with the align command in PyMol.
3.3.10 Phosphorylation causes pUb to bind parkin with high affinity

ITC was used to determine the affinity of interaction of R0RBR, UblR0RBR and pUblR0RBR with Ub, Ub\textsuperscript{S65E} and pUb (Figure 3.9). Results showed that R0RBR and pUb bind with high affinity (K\textsubscript{d} 16 ± 2 nM), phosphorylation-mimetic Ub\textsuperscript{S65E} binds with an intermediate affinity (K\textsubscript{d} 6.6 ± 0.1 μM) and unmodified Ub binds with weak affinity (K\textsubscript{d} 40 ± 1 μM) (Table 3.4). All stoichiometry constants (N) are approximately equal to 1, which indicates a 1:1 binding site. The change in entropy (ΔS) is identically positive for R0RBR binding to pUb and Ub\textsuperscript{S65E} (+78 kJ/mol) suggesting that pUb and Ub\textsuperscript{S65E} increase disorder in the system to the same extent. The ΔS for pUb and Ub\textsuperscript{S65E} binding to R0RBR is more positive than the ΔS of Ub binding, suggesting that pUb and Ub\textsuperscript{S65E} increase disorder more than Ub. Gibbs free energy (ΔG) is most negative for R0RBR binding to pUb and Ub\textsuperscript{S65E} than to Ub which suggests that pUb and Ub\textsuperscript{S65E} binding are more favourable. The affinity of pUb for UblR0RBR (K\textsubscript{d} 160 ± 20 nM) and pUblR0RBR (K\textsubscript{d} 17 ± 5 nM) (Ordureau et al., 2014) shows that phosphorylation of the Ubl domain causes more than a 10-fold increase in affinity of pUb for parkin. pUb binding to UblR0RBR is also highly entropically driven (+86 kJ/mol). Results here suggest that phosphorylation of Ub at S65 is a modification that results in parkin being able to recruit pUb with high affinity and that this recruitment is enhanced when parkin is also phosphorylated at S65. These binding events are highly entropic indicating that binding leads to increased movement or disorder in the system, likely due to parkin undergoing a structural change.
Figure 3.9 ITC of R0RBR variants binding to Ub, Ub\textsuperscript{S65E} and pUb. Isotherm graphs shown are for: (A) R0RBR and Ub, (B) R0RBR and Ub\textsuperscript{S65E}, (C) R0RBR and pUb, (D) R0RBR\textsuperscript{380-384G} and Ub, (E) R0RBR\textsuperscript{380-384G} and Ub\textsuperscript{S65E}. The upper panels represent raw data, and the lower panels represent integrated heat changes performed assuming a single-site binding model. Data was collected at 25°C with 50 mM HEPES, 50 mM NaCl, 250 µM TCEP, pH 7.5 buffer conditions.
Table 3.4 Thermodynamics of R0RBR constructs binding to Ub, UbS65E and pUb. ITC experiments were collected at 25°C with 50 mM HEPES, 50 mM NaCl, 250 µM TCEP, pH 7.5 buffer conditions and were performed in duplicate. Values are shown for stoichiometry (N), dissociation constant (Kd), enthalpy change (ΔH), entropy change (ΔS) and Gibbs free energy (ΔG). Relative exchange rates determined by NMR are presented for comparison. Dashed lines indicate that data was not collected.

<table>
<thead>
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<th>Cell Protein</th>
<th>Titrant</th>
<th>N</th>
<th>Kd (µM)</th>
<th>ΔH (kJ/mol)</th>
<th>TAS (kJ/mol)</th>
<th>ΔG (kJ/mol)</th>
<th>NMR Exchange Rate</th>
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<tbody>
<tr>
<td>R0RBR Ub</td>
<td>1.1 ± 0.0</td>
<td>40 ± 1</td>
<td>30 ± 1</td>
<td>55 ± 1</td>
<td>-25 ± 1</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>R0RBR UbS65E</td>
<td>1.1 ± 0.0</td>
<td>6.6 ± 0.1</td>
<td>48 ± 0</td>
<td>78 ± 1</td>
<td>-30 ± 1</td>
<td>Slow-intermediate</td>
<td></td>
</tr>
<tr>
<td>R0RBR pUb</td>
<td>0.93</td>
<td>0.016 ± 0.002*</td>
<td>32</td>
<td>78</td>
<td>-46</td>
<td>Slow</td>
<td></td>
</tr>
<tr>
<td>R0RBR380-384G Ub</td>
<td>0.9 ± 0.0</td>
<td>44 ± 1</td>
<td>14 ± 0</td>
<td>39 ± 0</td>
<td>-25 ± 0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>R0RBR380-384G UbS65E</td>
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<td>8.2 ± 0.6</td>
<td>30 ± 0</td>
<td>59 ± 0</td>
<td>-29 ± 0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>UblR0RBR Ub</td>
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<td>45 ± 9*</td>
<td>10</td>
<td>34</td>
<td>-24</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>UblR0RBR pUb</td>
<td>1.09</td>
<td>0.16 ± 0.02*</td>
<td>50</td>
<td>86</td>
<td>-36</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>UblR0RBR Ub</td>
<td>0.37 ± 0.04**</td>
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<td></td>
<td></td>
<td></td>
<td>-</td>
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<tr>
<td>pUbR0RBR Ub</td>
<td>0.017 ± 0.005**</td>
<td></td>
<td></td>
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</table>

* Data collected by Dr. Julio Martinez-Torres
** Data from Ordureau et al (2014)
3.3.11 Phosphorylation of Ub causes a loss of stability

Circular dichroism (CD) spectropolarimetry thermal denaturation and structural curves were produced for Ub, Ub$^{S65E}$ and pUb to assess protein stability and fold. The melting temperatures for Ub (80.5°C), Ub$^{S65E}$ (77.7°C) and pUb (74.0°C) indicate that phosphorylation causes Ub to become less stable (Figure 3.10A). CD structural curves produced for Ub, Ub$^{S65E}$ and pUb assessed protein fold (Figure 3.10B) and showed a large dip around 205 nm indicative of the presence of alpha-helices in all curves. pUb has a less negative mean residue ellipticity than Ub indicating that it is less folded than Ub.

3.3.12 Ub$^{S65E}$ backbone amide assignments

Backbone $^1$H, $^{15}$N assignment for Ub$^{S65E}$ was performed for use in later NMR interaction studies with R0RBR because chemical shifts for residue resonances around S65 deviate from previous Ub$^{K48R}$ assignments. Assignments were made by collecting $^1$H-$^{15}$N HSQC and HNCA spectra of $^{13}$C$^{15}$N$^1$H-labelled Ub$^{S65E}$ (Figure 3.11AB) and using them in conjunction with previous backbone amide $^1$H, $^{15}$N assignments of Ub$^{K48R}$. The largest changes in backbone amide chemical shifts in Ub$^{S65E}$ in comparison to wild type Ub occur at residues adjacent to S65E such as Q62, K63, E64, E65, T66 and L67.
Figure 3.10 Circular dichroism of Ub, Ub\textsuperscript{S65E} and pUb. (A) CD thermal denaturation curves were recorded at 218 nm from 5-105°C and (B) CD structural-fold curves were an average of five scans from 260-190 nm for Ub, Ub\textsuperscript{S65E} and pUb. Spectra were collected on a Jasco J-810 instrument using 30 µM of each sample in 10 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM EDTA, pH 7.0 buffer at 25°C. Thermal denaturation and structural curves suggest that phosphorylation is a modification that destabilizes Ub.
Figure 3.11 Ub$^{S65E}$ backbone amide assignments. (A) HNCA strip plot of $^{13}$C$^{15}$N$^1$H-labelled Ub$^{S65E}$ indicating connectivity (dashed line) between the i and i-1 residues from K63, E64, E65, T66 and L67. Peaks corresponding to Cα are shown (black contours). Amino acid labels are listed above the strips. (B) $^1$H-$^{15}$N HSQC NMR spectra of $^{12}$C$^{15}$N$^2$H-labelled Ub (black contours) and $^{13}$C$^{15}$N$^1$H-labelled Ub$^{S65E}$ (purple contours). Resonances corresponding to Q62, K63, E64, E65, T66 and L67 experience the greatest CSP from wild type Ub as indicated by arrows. (C) Measured Ub$^{S65E}$ CSPs compared to wild type Ub. The horizontal dashed line indicates the average CSP ± 1 standard deviation. Data was collected at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
3.3.13 Identifying pUb-bound R0RBR HSQC spectrum resonances

Identifying resonances in the $^1$H-$_{15}$N HSQC spectrum of pUb-bound $^{13}$C$_{15}$N$_2^{2}$H-labelled R0RBR is difficult because binding produces very large, slow-exchanging resonance CSPs that are compounded with the presence of over 320 resonances. In order to follow the movement of R0RBR resonances and to identify the new locations when bound to pUb, a series of TROSY $^1$H-$_{15}$N HSQC spectra were collected of titration experiments between $^{13}$C$_{15}$N$_2^{2}$H-labelled R0RBR with 1 and 3 equivalents of Ub, 1 equivalent of Ub$^{S65E}$ and 1 equivalent of pUb (Table 3.2). Analysis of the spectra showed that Ub titrations cause resonances in R0RBR to experience fast exchange corresponding to a weak affinity of binding, Ub$^{S65E}$ titration causes resonances in R0RBR to experience slow-intermediate exchange corresponding to moderate binding and pUb titration causes resonances in R0RBR to experience slow exchange corresponding to tight binding. The relative affinities determined by NMR exchange rates are in good agreement with affinities determined by ITC (Table 3.4). Accordingly, the magnitude of CSPs observed in R0RBR for each form of Ub titrated corresponds with the relative affinity of binding. For instance, Ub has a weak affinity and CSPs observed in R0RBR were the smallest in magnitude even with 3 equivalents of Ub added. pUb has a tight affinity for R0RBR and showed the largest magnitude of CSPs, whereas Ub$^{S65E}$ has a moderate affinity for R0RBR and showed CSPs in R0RBR that were intermediate in magnitude between those observed for Ub and pUb. The trajectory of R0RBR resonances with the three forms of Ub followed a near-linear path and aided in the identification of R0RBR resonances that experienced large CSPs when pUb was bound (Figure 3.12).
Figure 3.12 NMR titrations of Ub, Ub\textsuperscript{S65E} and pUb into R0RBR. Selected regions shown are TROSY $^1$H-$^15$N HSQC spectra of $^{13}$C-$^15$N-$^2$H-labelled R0RBR taken alone (black contours), with 1 and 3 equivalents Ub (red and orange contours respectively), with 1 equivalent Ub\textsuperscript{S65E} (yellow contours) and with 1 equivalent of pUb (cyan contours). R0RBR residue resonances shown that are perturbed upon binding are at the binding interface and include Y312 (RING1 alpha helix), D280 (RING1 unique beta sheet), as well as G308 and G152 (RING0/RING1 hinge). The trajectory of R0RBR resonances with Ub (fast exchange), Ub\textsuperscript{S65E} (slow-intermediate exchange) and pUb (slow exchange) follow a near-linear path.
3.3.14 pUb binds to the RING0/RING1 hinge with the pS65 interface

In order to determine the surfaces used for interaction between R0RBR and pUb, NMR titration experiments of selectively labelled R0RBR with Ub, Ub\(^{S65E}\), pUb and PO\(_4\) were conducted as well as TROSY NOESY experiments between R0RBR and Ub or Ub\(^{S65E}\) (Table 3.2). CSP analysis of TROSY \(^1\)H-\(^{15}\)N HSQC NMR data shows that the addition of pUb to R0RBR results in a cluster of residues with significant chemical shift changes in R0RBR including Y149, C150, K151 and G152 in the RING0 domain, R275, F277, V278, D280, Q282, G284, Y285 and S286 in β1–β2 of RING1, and I306, G308, E309, Y312 and N313 in helices H2 and H3 of RING1 (Figures 3.12 and 3.13). These residues surround the hinge region at the RING0/RING1 interface that includes the basic triad composed of K151, H302 and R305 proposed to be responsible for interaction with pS65 of pUb. There are no significant chemical shift changes in the RING2 domain or at the RING0/RING2 interface, indicating that pUb binding does not alter the interface between these two domains where the catalytic cysteine is located. Data here reinforces the notion that the hinge region at the RING0/RING1 interface is the pUb-binding site.

Analysis of TROSY \(^1\)H-\(^{15}\)N HSQC and \(^1\)H-\(^{13}\)C HMQC NMR data acquired of selectively labelled R0RBR with Ub\(^{S65E}\) shows that Ub\(^{S65E}\) uses residues predominantly in the loop 1 (K6, T7, L8 and T9), hydrophobic patch surrounding pS65 (R42, L43, I44, F45, A46, K48, and Q49), and C-terminal tail (V70, L71, L73, R74) for interaction with R0RBR (Figure 3.14). The experiment titrating PO\(_4\) into \(^{13}\)C\(^{15}\)N\(^2\)H-labelled R0RBR was designed to show the site of interaction between pUb pS65 and R0RBR, but it showed no observable changes in the \(^1\)H-\(^{15}\)N HSQC spectrum of R0RBR.

TROSY \(^1\)H-\(^{15}\)N NOESY experiments between selectively-labelled R0RBR and Ub did not provide any unambiguous restraints due to a weak affinity of interaction, however the same experiments conducted between Ub\(^{S65E}\) and R0RBR provided 2 unambiguous restraints due to the higher affinity of interaction (Table 3.3). Mutagenesis experiments determined 1 unambiguous restraint between pUb pS65 and R0RBR H302 located in the RING0/RING1 hinge interface.
Figure 3.13 NMR of the R0RBR binding interface with pUb. (A) TROSY $^1$H-$^15$N HSQC NMR spectra of $^{13}$C$^{15}$N-$^2$H-labelled R0RBR in the absence (black contours) and presence of 1 equivalent $^{12}$C$^{14}$N-$^2$H-labelled pUb (blue contours). (B) Measured R0RBR CSPs upon pUb binding. Grey bars indicate that the resonance was undetectable. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were very large and exhibited slow exchange indicating a tight affinity. pUb-R0RBR was purified as a 1:1 complex on a Superdex 75 size-exclusion column. Data was collected at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
Figure 3.14 NMR of the Ub$^{565E}$ binding interface with R0RBR. (A) TROSY $^1$H-$^{15}$N HSQC NMR spectra of $^{12}$C$^{15}$N$^2$H-labelled Ub$^{565E}$ in the absence (black contours) and presence of 1 equivalent $^{12}$C$^{14}$N$^2$H-labelled R0RBR (red contours). (B) Measured Ub$^{565E}$ CSPs upon R0RBR binding. Grey bars indicate that the resonance was undetectable. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were large and exhibited slow-intermediate exchange indicating a moderate affinity. Data was collected at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
3.3.15 pUb-R0RBR HADDOCK structure

In order to understand how pUb binds to parkin, a model for the pUb-R0RBR complex was determined. pUb was successfully docked to R0RBR in HADDOCK using data from NMR experiments (Table 3.3), mutagenesis experiments and ITC. The location and orientation of pUb with respect to R0RBR was similar in all 100 water-refined complexes and the best 20 complexes had a backbone rmsd of 0.36 ± 0.02.

The structure shows that pUb orientation is governed by the loop 1 in pUb that interacts with residues on the helix H3 of the RING1 domain and residues in the adjacent IBR domain. The phosphate group at S65 locks pUb in place making key contacts with the RING0/RING1 hinge composed of K151, H302 and R305. The C-terminal tail of pUb including residues V70 and L71 runs parallel to helix H3 of the RING1 domain and also makes contacts with the IBR. In addition, pUb residues I44 and A46 in the β3–β4 region intercalate between strands β1–β2 and helix H3 of the RING1 domain (Figure 3.15). Interestingly, the short β1–β2 region in RING1 appears to be absent in all other RING domain protein structures and the position of the β1–β2 is replaced by a ubiquitin-associated-like domain in a RBR E3 ligase HHARI structure (Duda et al., 2013). This finding might suggest an important role for the β1–β2 of RING1 in parkin for pUb recognition, and that other RBR E3 ligases have specialized recognition domains for pUb as well.
Figure 3.15 pUb-R0RBR HADDOCK structure. The surface of R0RBR (grey) is shown binding to a ribbon diagram of pUb (orange). Residues used for ambiguous HADDOCK restraints are highlighted in blue for parkin and red for pUb. Unambiguous restraints are labelled and were the following: pUb L8 to R0RBR E353, pUb pS65 to R0RBR H302, and pUb L73 to R0RBR L342. R0RBR residues important for binding are located in β-sheet 1 (β1), β-sheet 2 (β2), helix 2 (H2) and helix 3 (H3) of the RING1 domain. pUb residues important for binding are located in loop 1, β-sheet 3 (β3), β-sheet 4(β4) and the C-terminal tail. The location and orientation of pUb with respect to R0RBR was similar in all 100 water-refined complexes and the best 20 complexes had a backbone rmsd of 0.36 ± 0.02.
3.3.16 R0RBR backbone flexibility with pUb bound

Heteronuclear NOE experiments were conducted to determine the effect that pUb binding has on R0RBR backbone flexibility. $^1$H-$^{15}$N NOE experiments of $^{13}$C$^{15}$N$^2$H-labelled R0RBR with 1 equivalent of $^{12}$C$^{14}$N$^2$H-labelled pUb bound were acquired (Figure 3.16) and compared to unbound $^{13}$C$^{15}$N$^2$H-labelled R0RBR experiments. Upon addition of pUb, notable flexibility is seen in the linker region of parkin from residues 380-390 and from 410-412. Those two regions are separated by residues 391-403 which form the repressor (REP) helix that compacts tightly with RING1. Residues in this helix show high rigidity from NOE data indicating that this helix is likely still interacting with the RING1 domain. Residues that surround the RING0/RING1 hinge interface show high flexibility such as residues 157-160 that comprise the N-terminal region of RING0, and residues K299, F304 and N313 from RING1. A large number of dispersed residues spanning the entire IBR domain become more rigid upon pUb binding likely because the C-terminal tail of pUb binds the IBR providing supporting interactions to lock it in place.
Figure 3.16  R0RBR backbone flexibility with pUb bound. (A) $^1$H-$^{15}$N heteronuclear NOE NMR of $^{12}$C$^{15}$N$^2$H-labelled R0RBR showing relative parkin flexibility and rigidity upon 1 equivalent of $^{12}$C$^{14}$N$^2$H-labelled pUb binding. Flexibility is present in the linker region of R0RBR from residues 380-390 and 410-412. Flexibility is also noted in residues 157-160, K299, F304 and N313 which surround the RING0/RING1 hinge. Residues 391-409 that form the REP helix in the linker region of R0RBR stay rigid upon pUb binding, suggesting that it still compacts tightly with the RING1 domain. Residues spanning the entire IBR domain become more rigid upon pUb binding. (B) Ribbon and stick figure of the pUb-R0RBR HADDOCK structure showing R0RBR (grey), pUb (orange), and flexible residues (green) from panel A data. Both saturated and non-saturated experiments were conducted in duplicate and NOEs were averaged. NMR data was collected at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
3.3.17 pUb binding induces a structural change in parkin

Examination of the UblR0RBR crystal structures showed that the RING0/RING1 hinge interface is altered when the Ubl domain is either absent or phosphorylated at S65 and that this event facilitates pUb binding. We therefore proposed that a similar allosteric change in parkin structure might occur upon pUb binding. Consistent with this, pUb binding results in R0RBR exhibiting several chemical shift changes in residues at the junction of the C-terminus of the bent helix H3 and the IBR domain as well as in a stretch of residues from 379-395 (Figure 3.17) that are located in a flexible linker between IBR and RING2 domains. These residues are not directly involved in making contact with pUb and we therefore proposed that the chemical shift changes seen were the result of a pUb-induced structural change. The binding of pUb to R0RBR is an endothermic binding event marked by large positive enthalpy (32 kJ/mol) and positive entropy (78 kJ/mol) changes (Table 3.4). The large increase in entropy suggests that pUb binding drives an increase in disorder or movement of the system which can be explained by an allosteric structural change. A R0RBR\textsuperscript{380-384G} construct in which a stretch of residues from 380-384 were mutated to glycines was used to determine if the binding affinity of Ub and Ub\textsuperscript{S65E} to R0RBR\textsuperscript{380-384G} was disrupted by ITC (Figure 3.9). Results showed that R0RBR\textsuperscript{380-384G} binds to Ub (44 ± 1 μM) and Ub\textsuperscript{S65E} (8.2 ± 0.6 μM) with similar affinities as wild type R0RBR (Table 3.4). This confirmed that pUb does not use this stretch of residues in R0RBR for binding and rather, CSPs seen in R0RBR residues 379-395 are likely the result of an allosteric structural change. Structures of the isolated IBR domain show little structure in the C-terminal portion of the bent helix H3 (Beasley et al., 2007) which is in contrast to the UblR0RBR crystal structures that show a well-defined bent helix. Taken together, this data suggests that pUb binding results in an increase in movement and loss of structure near the C-terminus of RING1 helix H3 and at the beginning of the IBR domain.

Chemical shift changes seen in residues 379-395 that form the flexible linker between IBR and RING2 as well as residues at the junction of the C-terminus of helix H3 and the IBR domain were later confirmed to be the result of structural changes in parkin. Specifically, the bent RING1 helix H3 straightens when pUb binds as seen in crystal structures of the pUb-parkin complexes (Kumar et al., 2017; Wauer et al., 2015).
Figure 3.17 pUb binding causes allosteric changes in R0RBR. (A) Selected TROSY $^1$H-$^{15}$N HSQC NMR spectral regions of $^{13}$C-$^{15}$N-$^2$H-labelled R0RBR in the absence (black contours) and presence of 1 equivalent of $^{12}$C-$^{14}$N-$^2$H-labelled pUb (blue contours). Resonances for residues in the R0RBR linker located between IBR and RING2, including residues 379-395, are distal from the pUb binding site yet experience a change in chemical shift upon pUb binding. Residues A379, S384 and D394 that are located in this linker region have large CSPs and are shown here. NMR data was collected at 25°C in 25 mM HEPES, 100 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
3.3.18 pUb binding causes displacement of the Ubl domain from parkin

The UblR0RBR\textsuperscript{S65D} crystal structure shows that phosphorylation of the Ubl domain optimises the RING0/RING1 interface for pUb binding while NMR and ITC experiments show that pUb binding causes a structural change in the RING1 helix H3 near the IBR domain. These allosteric structural changes suggested that pUbl and pUb cannot be bound to parkin simultaneously since the proposed sight of structural rearrangement is adjacent to the Ubl domain. To test this hypothesis, competitive binding experiments were performed by NMR to see if the N-terminal Ubl\textsuperscript{S65E} domain could still bind to the C-terminal R0RBR portion while Ub\textsuperscript{S65E} is bound. Ubl\textsuperscript{S65E} and Ub\textsuperscript{S65E} were used as mimetics of pUbl and pUb, which are the product of PINK1 kinase phosphorylation in the actual parkin activation pathway. \textsuperscript{1}H-\textsuperscript{13}C HMQC spectra were collected to follow side chain methyl group CSPs of \textsuperscript{13}C\textsuperscript{14}N\textsuperscript{2H}-labelled Ub\textsuperscript{S65E} alone, with 1 equivalent of \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2H}-labelled R0RBR and with 1 equivalent of \textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2H}-labelled Ubl\textsuperscript{S65E} added to the Ub\textsuperscript{S65E} and R0RBR sample. Experiments showed slow-exchange chemical shift changes and considerable peak broadening in Ub\textsuperscript{S65E} resonances such as I44, A46, T6 and V70 when R0RBR was added indicating slow-intermediate exchange and moderately tight binding. When Ubl\textsuperscript{S65E} was added to the Ub\textsuperscript{S65E} and R0RBR sample, there were no visible changes in Ub\textsuperscript{S65E} chemical shifts or peak intensities suggesting that Ub\textsuperscript{S65E} remained bound to R0RBR (Figure 3.18A). TROSY \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra of the same samples were collected to follow amide resonances in \textsuperscript{13}C\textsuperscript{15}N\textsuperscript{2H}-labelled R0RBR and showed that the addition of Ubl\textsuperscript{S65E} to the Ub\textsuperscript{S65E}-R0RBR sample produced no further CSPs in R0RBR (Figure 3.18B), indicating that Ubl\textsuperscript{S65E} can no longer bind to R0RBR while Ub\textsuperscript{S65E} is bound. Taken together, these results were the first to suggest that pUb binding causes the release of pUbl from parkin. This would also indicate that activation of parkin through pUb binding would prevent re-engagement with the Ubl domain until pUb is released.
Figure 3.18 pUb binding displaces the Ubl domain from R0RBR.  (A) (Top) Selected regions of $^1$H-$^1$C HMQC spectra for $^{13}$C-$^{15}$N-$^1$H-labelled Ub$^{S65E}$ alone (black contours) and with 1 equivalent of R0RBR (orange contours). Boxes show the position of the bound Ub$^{S65E}$ signal upon R0RBR binding visible at lower contour levels.  (Bottom) The same sample following the addition of 1 equivalent of unlabelled Ubi$^{S65E}$ (magenta contours) shows no change in the intensities or position of the bound Ub$^{S65E}$ signals indicating that Ub$^{S65E}$ is still bound to R0RBR in the presence of Ubi$^{S65E}$.  (B) (Left) TROSY $^1$H-$^{15}$N HSQC spectra for $^{13}$C-$^{15}$N-$^1$H-labelled R0RBR alone (black contours) and with 1 equivalent of Ub$^{S65E}$ (orange contours). Many CSPs are seen in R0RBR indicating that Ub$^{S65E}$ is bound. (Right) The same sample following the addition of 1 equivalent of unlabelled Ubi$^{S65E}$ (magenta contours) shows no change in the intensities or position of the Ub$^{S65E}$-bound R0RBR signals indicating that the Ubi$^{S65E}$ is unable to bind to R0RBR in the presence of Ub$^{S65E}$. 
3.4 Discussion

3.4.1 A molecular model for parkin inhibition and activation

Parkin autoinhibition and activation has been well-studied and structural explanations discovered here are in good agreement with literature. The Ubl domain plays a key role in regulating parkin activity by having an autoinhibitory effect when present (Chaugule et al., 2011). The catalytic cysteine C431 is occluded by the RING0/RING2 interactions (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013) and a REP helix blocks the E2 binding site on RING1 (Trempe et al., 2013). Comparing the structures of UblR0RBR, UblR0RBR\textsuperscript{S65D} and R0RBR shows no observable change in the environment around the catalytic C431 which indicates that phosphorylation and release of the Ubl domain are events that activate parkin through means other than by altering the C431 environment. CSP experiments of R0RBR binding to pUb complement this finding by showing minimal chemical shift changes for residues in this region and indicate that parkin does not undergo any large structural change that exposes C431 upon pUb binding (Figure 3.13). Heteronuclear NOE data shows that pUb binding does not affect rigidity of the repressor helix blocking the E2 binding site on RING1 (Figure 3.16), suggesting that pUb binding does not cause release of the occluding interaction. Rather, results here suggest that pUb works to activate parkin through relief of autoinhibition caused by the Ubl domain (Figure 3.18).

The discovery of such autoinhibition has driven efforts to uncover activators of parkin such as the upstream kinase PINK1 (Clark et al., 2006; Park et al., 2006). PINK1 phosphorylates both Ub and parkin Ubl domain at residue S65 leading to the activation of parkin ubiquitination activity (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Until now, a molecular basis for parkin activation by PINK1 has been unknown. Based on our structural, biochemical and biophysical approaches, we present a mechanistic model of parkin inhibition and allosteric activation (Figure 3.19). First, parkin rests in the autoinhibited state while engaged in intramolecular interactions to the Ubl domain. PINK1 then phosphorylates both Ub and the Ubl domain to induce a remodelling of the RING0/RING1 hinge interface composed of K151, H302 and R305 responsible for interaction with the phosphate on pUb (Figure 3.8). pUb can then bind to this basic patch with high affinity (K\textsubscript{d} 17 ± 5 nM) (Ordureau...
et al., 2014), which is more than a 10-fold increase in affinity compared to when the Ubl phosphorylation signal is not present ($K_d$ 160 ± 29 nM). This pUb binding leads to an entropically-favoured (Table 3.4) allosteric structural change in the RING1 helix H3 validated by CSPs seen in distal regions from the pUb binding (Figure 3.17). Two recent crystal structures showing pUb bound to R0RBR parkin (Wauer et al, 2015) and UblR0RBR parkin (Kumar et al., 2017) confirm our pUb-R0RBR structure and show that the observed entropically-driven structural change is due to straightening of the RING1 helix H3. pUb binding leads to release of the autoinhibitory Ubl domain and exposure of the E2-Ub binding surface for enhanced recruitment of the E2-Ub conjugate. A recent crystal structure of E2-Ub bound to RBR E3 ligase pUb-HOIP strengthens the hypothesis that the E2-Ub will be oriented to bind this pocket (Lechtenberg et al., 2016). Although there is still some debate over the order of events, phosphorylation of both Ub and Ubl enhances activation of parkin and both are likely major mechanisms of parkin regulation. For example, parkin can be phosphorylated by PINK1 in the absence of pUb, but the addition of pUb enhances parkin phosphorylation, suggesting that pUb-parkin is a better substrate for PINK1 (Kazlauskaite et al., 2015). At the same time, when the only ubiquitin source is non-phosphorylatable Ub$_{S65A}$, parkin is still phosphorylatable (Ordureau et al., 2015). Finally, another study shows that parkin associates tightly with ubiquitin chains only when both ubiquitin and parkin are phosphorylated (Ordureau et al., 2014), proposing that both phosphorylation signals are a part of a feed-forward mechanism.

3.4.2 Parkin mutations and Parkinson’s disease

The mechanistic model for pUb-induced activation of parkin proposed here involves an autoinhibited structure, numerous binding sites and structural changes. The multiple steps that lead to parkin activation explain why ARJP mutations have different effects on parkin activity. The model presented here provides important insights into parkin regulation that will be key to designing therapeutics that can control the activity of parkin and may be useful in the treatment of hereditary forms of Parkinson’s disease.
Figure 3.19 Model for parkin inhibition and activation. (1) Inhibition: Parkin is autoinhibited in the absence of pUb. PINK1 activation leads to phosphorylation of S65 in both parkin and Ub. (2) Optimisation: Parkin phosphorylation stabilizes the flipped-out conformation of H302, thus optimising the pUb binding site. (3) Release: pUb binds to the optimised RING0/RING1 hinge, leading to a structural change near the RING1/IBR interface and displacement of the Ubl domain. (4) Engagement: The Ub and E2 binding surfaces uncovered by displacement of the Ubl domain engage with charged E2-Ub conjugate poised for ubiquitin transfer. The yellow box represents the REP repressor that blocks the E2 binding site on RING1.
3.5 References


Chapter 4

THE CLOSED UBC7-UBIQUITIN CONJUGATE UNDERGOES REARRANGEMENT FOR LOADING ONTO ACTIVATED PARKIN IN AN OPEN CONFORMATION

4.1 Introduction

Parkin RBR E3 ubiquitin-ligase autoinhibition and activation is highly regulated whereby the malfunctioning of these steps is attributed with autosomal recessive juvenile parkinsonism (ARJP) (Kitada et al., 1998). Parkin adopts an autoinhibited conformation mediated by the Ubl domain (Kumar et al., 2015), the presence of a repressor REP linker blocking the RING1:E2 binding site and by occlusion of the catalytic C431 through RING0/RING2 interactions (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). Previous work has shown that PINK1 phosphorylation of both parkin and Ub at S65 activate parkin (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Studies propose that activation is done through relief of Ubl association and movement of RING1 helix H1 leading to the emergence of a E2-Ub binding surface (Kumar et al., 2015). However, structural insights into relief of the catalytic C431 occlusion and RING1:E2 binding site occlusion are still unknown in addition to how the E2-Ub conjugate interacts with parkin.

The goal of this research was to determine how parkin recognises E2-Ub. We confirmed that pUb binding opens a RING1/IBR pocket to allow E2-Ub engagement with parkin by determining the crystal structure of pUb-UblR0RBR and an NMR structure of pUb-R0RBR bound to UbcH7-Ub. UbcH7-Ub was found to have a closed conformation in solution that undergoes rearrangement to bind parkin in an open conformation. This binding was able to bypass REP occlusion of the RING1:E2 binding site and cause rearrangement around the RING0/RING2 interface occluding the catalytic C431. Furthermore, we were able to associate parkin certain ARJP variants with disease by determining the effects of ARJP variants on parkin activity.

4.2 Materials and Methods

4.2.1 Source of materials

BL21(DE3) *E. coli* cells containing human His-SUMO-tagged parkin UblR0RBR (residues 1-465) and human His-SUMO-tagged parkin Ubl (residues 1-76) in pET-SUMO plasmids were obtained from Dr. Helen Walden (University of Dundee, Scotland). BL21(DE3) *E. coli* cells containing *Drosophila melanogaster* His-SUMO-tagged parkin RING2 (residues 410-482) in pET-SUMO plasmids were obtained from Emmy Sun (University of Western Ontario, Canada). BL21(DE3)CodonPlus-RIL *E. coli* cells containing non-cleavable human His-tagged Uba1 in a pET3a plasmid were obtained from Dr. Hong Ling (University of Western Ontario, Canada). pUblR0RBR protein was synthesised by Dr. Atul Kumar for use in SEC experiments. All other proteins were obtained in plasmids as in sections 2.2.1 and 3.2.1. Ubl, R0RBR and RING2 will be the nomenclature used to describe the truncated forms of parkin described above while UblR0RBR will be used to describe full-length parkin used in this chapter.

4.2.2 Mutagenesis of UbcH7<sup>C17S/C86K/C137S</sup>, Ub<sup>A0C</sup> and parkin variants

A C86K substitution was made to the catalytic cysteine of UbcH7<sup>C17S/C86K/C137S</sup>. All UbcH7 constructs used in this chapter were UbcH7<sup>C17S/C86K/C137S</sup> unless otherwise stated. As well, an A0C substitution was incorporated into Ub just before the N-terminal starting sequence, and full-length parkin UblR0RBR constructs (residues 1-465) with ARJP (T240R, R271S, N273S, R402C) and non-ARJP (R314A, P335G/C337G, W403A) variants were generated. Each mutation was incorporated using the same protocol in section 3.2.2. Forward and reverse primers were designed containing the desired mutations (Table 4.1).
Table 4.1 Mutagenesis primers for UbcH7\(^{C17S/C86K/C137S}\), Ub\(^{A0C}\) and parkin variants.

<table>
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<tr>
<th>DNA Template</th>
<th>Mutation</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>UbcH7(^{C17S/C137S})</td>
<td>C86K</td>
<td>Forward: 5’- cgaaaagggcaggtcaaatgcagtagattagtcg - 3’  &lt;br&gt; Reverse: 5’- gactaattactgctacatgctcttccttgag - 3’</td>
</tr>
<tr>
<td>Ub</td>
<td>A0C</td>
<td>Forward: 5’- gtacttcecaatcaattgcactgcaaatatgcctgc - 3’  &lt;br&gt; Reverse: 5’- gacgaaatttctgaatgctagttgacag - 3’</td>
</tr>
<tr>
<td>UblR0RBR</td>
<td>T240R*</td>
<td>Forward: 5’- cggaacactacactgctcctgtaacagagctggacag - 3’  &lt;br&gt; Reverse: 5’- cgggetctgtcgctgctcagagcagttgagcag - 3’</td>
</tr>
<tr>
<td>UblR0RBR</td>
<td>R271S*</td>
<td>Forward: 5’- atactgttctagactaatagctgctgcttgg - 3’  &lt;br&gt; Reverse: 5’- cggatcattgaggctgtcacatgtgctgctt - 3’</td>
</tr>
<tr>
<td>UblR0RBR</td>
<td>N273S*</td>
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</tr>
<tr>
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<td>P335G/C337G</td>
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<tr>
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<td>R402C*</td>
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</tr>
<tr>
<td>UblR0RBR</td>
<td>W403A</td>
<td>Forward: 5’- gcgcgacgagctgtgagactgactgactgcgagcc - 3’  &lt;br&gt; Reverse: 5’- ggttctttggaggctgcttccgcacgcactgcctg - 3’</td>
</tr>
<tr>
<td>R0RBR</td>
<td>W403A</td>
<td>Forward: 5’- gcgcgacgagctgtgagactgactgactgcgagcc - 3’  &lt;br&gt; Reverse: 5’- ggttctttggaggctgcttccgcacgcactgcctg - 3’</td>
</tr>
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*ARJP variant
4.2.3 Expression and purification of UbcH7\textsuperscript{C17S/C86K/C137S}

Unlabelled UbcH7 in BL21(DE3)CodonPlus-RIL cells were grown in LB media, while \textsuperscript{12}C\textsuperscript{15}N\textsuperscript{1}H-labelled UbcH7 growths were performed in M9 minimal media supplemented with 1 g \textsuperscript{15}NH\textsubscript{4}Cl. Unlabelled and non-deuterated-labelled expressions/purifications were performed according to the protocol in section 2.2.2.

\textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled and \textsuperscript{12}C\textsuperscript{15}N\textsuperscript{2}H-labelled UbcH7 in BL21(DE3)CodonPlus-RIL cells were grown in D\textsubscript{2}O M9 minimal media supplemented with 2 g \textsuperscript{12}C\textsuperscript{2}H-D-glucose, 1 g \textsuperscript{15}NH\textsubscript{4}Cl or 1 g \textsuperscript{14}NH\textsubscript{4}Cl isotopes. Expression and purifications were performed using the same protocol in section 3.2.5, but with the following changes: growth media contained kanamycin and chloramphenicol while expression was induced with 0.7 mM IPTG at 16°C for 12 hours.

4.2.4 Expression and purification of Ub and Ub\textsuperscript{A0C}

Unlabelled Ub and Ub\textsuperscript{A0C} in BL21(DE3)CodonPlus-RIL cells were grown in LB media. Unlabelled expressions and purifications were performed according to the protocol in section 3.2.5.

\textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled Ub was expressed and purified using the same protocol as in section 3.2.5. Selective-labelling was used to express Ub so that it was \textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled but with the side-chain terminal methyl groups of valine, isoleucine and leucine residues being \textsuperscript{13}C\textsuperscript{1}H-labelled (Goto et al., 1999). This \textsuperscript{13}C\textsuperscript{1}H-VIL \textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled Ub was expressed and purified using the protocol in section 3.2.5, but with the following exceptions: cells were grown in 100% D\textsubscript{2}O media containing 1 g \textsuperscript{14}NH\textsubscript{4}Cl and 2 g \textsuperscript{12}C\textsuperscript{2}H-D-glucose isotopes until an OD\textsubscript{600} reached 0.5 when 50 mg/L \textsuperscript{13}C\textsubscript{4} 3,3-D\textsubscript{2} α-ketobutyric acid and 85 mg/L \textsuperscript{13}C\textsubscript{5} 3-D\textsubscript{1} α -ketoisovaleric acid were added. Cells continued to grow until the OD\textsubscript{600} reached 0.8 when expression was induced with 0.5 mM IPTG at 37°C for 8 hours.
4.2.5 Expression and purification of parkin constructs and variants

Unlabelled, $^{13}$C$^{15}$N$^2$H- and $^{12}$C$^{14}$N$^2$H-labelled R0RBR in BL21(DE3) cells were expressed and purified using the protocol in section 3.2.3. All other parkin constructs and variants were expressed and purified using the same protocol but with the following exceptions. Unlabelled UblR0RBR, UblR0RBR $^{T240R}$, UblR0RBR $^{R271S}$, UblR0RBR $^{N273S}$, UblR0RBR $^{R314A}$, UblR0RBR $^{P335G/C337G}$, UblR0RBR $^{R402C}$ and UblR0RBR $^{W403A}$ expression was induced with 25 µM IPTG and the final Superdex 75 size-exclusion column was not used. Unlabelled Ubl expression was induced with 0.5 mM IPTG and no ZnCl$_2$ was added to growths because the Ubl domain does not coordinate Zn$^{2+}$ ions.

$^{12}$C$^{15}$N$^1$H-labelled RING2 and R0RBR $^{W403A}$ in BL21(DE3) cells were grown in M9 minimal media supplemented with $^{15}$NH$_4$Cl isotopes. Growths were supplemented with kanamycin at 37°C until an OD$_{600}$ of 0.8 was reached and expression was induced with 0.5 mM IPTG for RING2 or 0.1 mM IPTG for R0RBR $^{W403A}$ at 16°C for 8 hours. 1 mL 0.5 mM ZnCl$_2$ was also added to each 1 L flask of R0RBR $^{W403A}$ upon induction.

4.2.6 Expression and purification of Uba1

Unlabelled His-tagged Uba1 in BL21(DE3) CodonPlus-RIL cells was grown in LB media for use in ubiquitination assays and later synthesis of the stable UbcH7-Ub isopeptide-linked conjugate. Growths contained ampicillin and chloramphenicol. Cells were grown at 37°C to an OD$_{600}$ of 0.8. After turning the temperature down to 16°C and waiting 20 minutes, expression was induced with 0.5 mM IPTG at 16°C for 12 hours. Cells were harvested by centrifugation at 6000 × g for 10 minutes and resuspended in 50 mM Tris, 200 mM NaCl, 250 µM TCEP, 25 mM imidazole, pH 8.0 buffer. Cells were lysed in the presence of protease inhibitor using an Avestin EmulsiFlex-C5 homogenizer and were further centrifuged at 148,230 × g for 70 minutes. The supernatant was collected and His-tagged Uba1 was purified on a HisTrap FF column by batch-binding and washing with 50 mM Tris, 200 mM NaCl, 250 µM TCEP, 25 mM imidazole, pH 8.0 buffer. 5% and 17.5% of elution buffer that contained 50 mM Tris, 200 mM NaCl, 250 µM TCEP, 250 mM imidazole at pH 8.0 was used to further wash the batch-bound His-tagged Uba1. The His-tagged Uba1 was then eluted with 100% elution buffer and was collected for experimental use. Purity was monitored by SDS-PAGE.
4.2.7 Expression and purification of PINK1

Unlabelled GST-tagged PINK1 was expressed and purified using the same protocol in section 3.2.6.

4.2.8 pUb and pUbl formation and purification

Unlabelled and $^{12}$C$^{14}$N$^{2}$H-labelled pUb was synthesised using unlabelled or labelled Ub that was purified as in section 3.2.5 and the optimised protocol for pUb synthesis in section 3.3.6. Unlabelled pUbl was synthesised using unlabelled Ubl that was purified as in section 3.2.4 and the optimised protocol for pUb synthesis shown in section 3.3.6.

4.2.9 UbcH7-Ub isopeptide-linked conjugate formation and purification

Assays to optimise the formation of UbcH7-Ub isopeptide-linked conjugate were performed. Conditions that were manipulated included reaction time, buffer type, temperature, pH as well as concentrations of Mg$^{2+}$/ATP, Ub, UbcH7$^{C17S/C86K/C137S}$ and Uba1. All UbcH7-Ub conjugates used in this chapter were isopeptide-linked.

The optimised protocol for UbcH7-Ub isopeptide-linked conjugate formation is as follows. 200 µM His-tagged Ub, 400 µM UbcH7$^{C17S/C86K/C137S}$, 25 µM His-tagged Uba1 and 10 mM Mg$^{2+}$/ATP were added together in 50mM CHES, 150 mM NaCl, pH 9.0 buffer. The pH was slowly adjusted to 9.0 using 1 M NaOH and the sample mixture was then incubated at 37°C for 6-16 hours. UbcH7 is the limiting reagent and the final reaction results in 50% of it being conjugated.

UbcH7-Ub isopeptide-linked conjugate was purified from the above mixture using the following protocol. The reaction mixture was dialysed against 2 L of 50 mM Tris, 200mM NaCl, 250 µM TCEP, pH 8.0 at 4°C for 2 hours. The mixture was first purified on a HisTrap FF column using the protocol in section 2.2.2 to eliminate unconjugated UbcH7 from the mixture because it is the only protein without a His-tag. Next, the eluted His-tagged proteins were TEV-cleaved and dialysed using the protocol in section 2.2.2. Uba1 contains a non-cleavable His-tag, therefore free His-tagged Ub and conjugated His-tagged Ub are the only proteins that get cleaved. After TEV-cleavage and dialysis, the protein mixture was purified on a second HisTrap FF column.
using the protocol in section 2.2.2. His-tagged Uba1 sticks to the column while untagged UbcH7-Ub and Ub flow through. The flowthrough was collected and UbcH7-Ub was purified from the lower molecular weight of Ub on a HiLoad Superdex 16/60 size-exclusion column using 25 mM HEPES, 100 mM NaCl, 500 μM TCEP, pH 7.0 buffer, a 0.3 mL/min flow rate and collecting 2 mL fractions. Integrity and purity of isopeptide-linked UbcH7-Ub was assessed by mass spectrometry and SDS-PAGE.

4.2.10 SEC of parkin binding to pUb, Ub, UbcH7 and UbcH7-Ub

50 μl samples containing 40 μM R0RBR, UblR0RBR or pUblR0RBR with 40 μM pUb, Ub, UbcH7 or UbcH7-Ub were prepared in 20 mM Tris, 75 mM NaCl, 250 μM TCEP, pH 8 buffer and loaded onto a Superdex 75 10/300 size-exclusion column. Runs were performed with a 0.5 mL/min flow rate while collecting 1 mL fractions. Elution profiles were verified by SEC-MALS and SDS-PAGE.

4.2.11 ITC of R0RBR variants binding to Ub, UbcH7 and UbcH7-Ub

ITC experiments were performed on either a Microcal VP-ITC system (GE Healthcare) or a Nano ITC system (TA Instruments) using the same protocol as in section 3.2.9.

4.2.12 NMR spectroscopy setup and processing

All NMR data was collected, processed and analysed using the same parameters and protocols in section 3.2.11 with the following additions. 1H-15N HSQC spectra were collected in TROSY mode (Pervushin et al., 1997) or non-TROSY mode (Kay et al., 1992) to follow amide backbone chemical shift pertubations (CSPs) as noted in Table 4.2. UbcH7 spectral windows were 8000.0 Hz centred on 4.780 ppm for 1H and 1700.0 Hz centred on 118.0 ppm for 15N.
4.2.13 NMR of pUb-R0RBR interactions with Ub, UbcH7 and UbcH7-Ub

TROSY $^1$H-$^{15}$N HSQC spectra were collected in variations of selectively-labelled pUb-R0RBR or R0RBR with selectively-labelled Ub, UbcH7 and UbcH7-Ub. $^1$H-$^{13}$C HMQC spectra were collected using conjugated and unconjugated $^{13}$C$^1$H-VIL $^{12}$C$^{14}$N$^2$H Ub with selectively-labelled pUb-R0RBR. CSP analysis was performed on amide backbone and side chain methyl groups to determine ambiguous restraints used for docking UbcH7-Ub to pUb-R0RBR. CSPs used for docking were mapped to the docked pUb-R0RBR:UbcH7-Ub complex computed in section 4.2.14 using PyMOL (Delano, 2002). A list of all NMR experiments performed can be found in Table 4.2.
Table 4.2 NMR experiments for pUb-R0RBR:UbcH7-Ub structure determination. All experiments were performed at 25°C in 25 mM HEPES, 50 mM NaCl, 500 µM TCEP pH 7.0 buffer with 10% D2O (v/v) and DSS as an internal reference on a Varian Inova 600 MHz NMR spectrometer. Experiments were run in either TROSY or non-TROSY mode, as noted. All pUb-R0RBR samples here were purified as a 1:1 complex on a Superdex 75 size-exclusion column as in section 3.2.8. All UbcH7 constructs were UbcH7\textsuperscript{C17S/C86K/C137S} unless stated otherwise. All pUb moieties were \textsuperscript{12}C\textsuperscript{14}N\textsuperscript{2}H-labelled.

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* Collected in non-TROSY mode
** Performed in duplicate
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* Collected in non-TROSY mode
** Performed in duplicate
4.2.14 HADDOCK of pUb-R0RBR and UbcH7-Ub binding

Interacting residues determined while following pUb-R0RBR and UbcH7-Ub amide backbone resonance CSPs during NMR experiments were defined as those that shifted greater than the average +1 standard deviation and had greater than 20% side chain accessible surface area. Several interacting residues were determined by mutagenesis and ubiquitination assays. Passive residues that neighboured active residues were included as interacting residues and were defined as having greater than 20% side chain accessible surface area as well as having a noticeable chemical shift change. A full list of restraints used for docking is shown in Table 4.3.

UbcH7 and Ub were docked to pUb-R0RBR sequentially in HADDOCK (Dominguez et al., 2003) using 1 unambiguous restraint to position the C-terminal G76 of Ub in proximity to the catalytic C86K of UbcH7. The coordinates from the crystal structure of pUb-UblR0RBR (PDB ID code 5N2W) were used for docking after removal of the Ubl domain and adjoining linker coordinates. R0RBR REP linker residues (387-405) occluding the RING1 binding site were moved on average 5.3 Å in PyMOL (Delano, 2002) in order to facilitate UbcH7 docking. Missing regions in the R0RBR coordinates were modelled-in using the Modeller (Eswar et al., 2006) plug-in for UCSF Chimera (Pettersen et al., 2004). Ub coordinates (PDB ID code 1UBQ) (Vijay-Kumar et al., 1987) were used as well as UbcH7 coordinates isolated from the OspG:UbcH7-Ub crystal structure (PDB ID code 4Q5E) (Grishin et al., 2014). An upper distance limit of 4.0 Å was set for ambiguous distance restraints while the unambiguous distance restraint was set to 6.8 Å. Standard parameters were used except inter_rigid (0.1) which was set to allow tight packing of the proteins, and the unambiguous force constants were increased by five-fold compared to the ambiguous constants. A total of 1,000 initial complexes were calculated and the best 100 structures were water-refined.
Table 4.3 HADDOCK restraints for pUb-R0RBR:UbcH7-Ub docking. Restraints were determined using NMR CSP analysis and mutagenesis data.

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<th>Restraint Type</th>
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*Restraints determined by Dr. Viduth Chaugule
4.2.15 Ubiquitination assays of ARJP parkin variants

A maleimide-containing fluor DyLight$^{800}$ was attached to Ub$^{A0C}$ using the Thermo Fisher Scientific protocol for DyLight Sulfhydryl-Reactive Dyes to make Ub$^{A0C-800}$. Ubiquitination assays were performed in triplicate using the following reagents: 1 µM E1 Uba1, 5 µM E2 UbcH7$^{C17S/C137S}$, 5 µM E3 UblR0RBR or variants, 40 µM Ub, 10 µM Ub$^{A0C-800}$ and 5 mM Mg$^{2+}$/ATP. E3 ARJP and non-ARJP variants assayed were UblR0RBR$^{T240R}$, UblR0RBR$^{R271S}$, UblR0RBR$^{N273S}$, UblR0RBR$^{R314A}$, UblR0RBR$^{P335G/C337G}$, UblR0RBR$^{R402C}$ and UblR0RBR$^{W403A}$. Each reaction was performed either in the absence or presence of 5 µM pUb. Reactions were run for 30 minutes at 30°C in 50 mM HEPES, pH 7.4 buffer and were quenched with SDS/DTT when complete. Assays were visualized by SDS-PAGE and on a LI-COR Odyssey Classic imaging system at 800 nm. Images were processed in ImageJ (Schneider et al., 2012).
4.3 Results

4.3.1 UbcH7\textsuperscript{C17S/C86K/C137S} , Ub\textsuperscript{A0C} and parkin variants were synthesised

The study of parkin and UbcH7-Ub interactions requires reducing conditions in order to eliminate the possibility of disulphide bridges forming between cysteine-rich parkin molecules. For this reason, the disulphide-linked UbcH7-Ub conjugate used previously was replaced with a more stable isopeptide-linked UbcH7-Ub conjugate for all parkin interaction studies. A C86K substitution was made to the catalytic cysteine of UbcH7\textsuperscript{C17S/C137S} to enable the formation of an isopeptide-bond between C86K of UbcH7 and the C-terminal G76 of Ub during later conjugate synthesis. This E2 substitution has been used successfully to form other E2-Ub isopeptide-linked conjugates for interaction studies with other E3s (Plechanovova et al., 2012).

In order to track ubiquitination activity of parkin in later assays, Ub was later tagged with a fluor. DyLight\textsuperscript{800} is a maleimide-containing fluor that reacts with sulfhydryl groups of target compounds to form a stable thioether-conjugated bond. Ub requires a free cysteine for conjugation to occur and therefore a A0C substitution was incorporated into Ub just before the N-terminal starting sequence.

Several UblR0RBR constructs with ARJP and non-ARJP variants positioned at the proposed UbcH7-Ub binding interface were generated to follow their effects on ubiquitination activity: T240R, R271S, N273S, R314A, P335G/C337G, R402C and W403A. A R0RBR\textsuperscript{W403A} construct was created for NMR studies as well. Sequencing results verified that QuikChange Site-Directed Mutagenesis and PCR successfully incorporated each substitution with the previously described primers (Table 4.1).

4.3.2 UbcH7\textsuperscript{C17S/C137S} and UbcH7\textsuperscript{C17S/C86K/C137S} were expressed and purified

Unlabelled and \textsuperscript{12C\textsuperscript{15}N\textsuperscript{1}}H-labelled UbcH7\textsuperscript{C17S/C137S} as well as unlabelled, \textsuperscript{12C\textsuperscript{14}N\textsuperscript{2}}H- and \textsuperscript{12C\textsuperscript{15}N\textsuperscript{2}}H-labelled UbcH7\textsuperscript{C17S/C86K/C137S} were successfully purified as monitored in section 2.3.1. Unlabelled UbcH7\textsuperscript{C17S/C137S} was later used for ubiquitination assays and ITC while unlabelled UbcH7\textsuperscript{C17S/C86K/C137S} was used for ITC, size-exclusion chromatography (SEC) and one NMR experiment. Labelled UbcH7\textsuperscript{C17S/C137S} as well as labelled UbcH7\textsuperscript{C17S/C86K/C137S} were later used for NMR experiments.
4.3.3 *Ub and Ub$^{A0C}$ were expressed and purified*

Unlabelled Ub and Ub$^{A0C}$ were successfully purified as monitored in section 3.3.4 and were later used for SEC, ITC, ubiquitination assays and NMR experiments. $^{12}$C$^{14}$N$^2$H-labelled Ub and $^{13}$C$^1$H-VIL $^{12}$C$^{14}$N$^2$H-labelled Ub were successfully purified as monitored in section 3.3.4 and were later used for NMR experiments.

4.3.4 *Parkin constructs and variants were expressed and purified*

The following unlabelled full-length parkin constructs (residues 1-465) were successfully purified, monitored by SDS-PAGE as seen by the expected band around 51.6 kDa in Figure 4.1, and verified by mass spectrometry for later use in SEC and ubiquitination assays: UbIR0RBR 51,642.87 Da, UbIR0RBR$^{T240R}$ 51,699.72 Da, UbIR0RBR$^{R271S}$ 51,572.88 Da, UbIR0RBR$^{N273S}$ 51,616.93 Da, UbIR0RBR$^{R314A}$ 51,556.69 Da, UbIR0RBR$^{P335G/C337G}$ 51,556.42 Da, UbIR0RBR$^{R402C}$ 51,589.79 Da and UbIR0RBR$^{W403A}$ 51,528.81 Da (Figure 4.2). Unlabelled R0RBR (parkin residues 141-465) was successfully purified for later use in SEC and ITC experiments as monitored in section 3.3.2. Unlabelled Ubl (parkin residues 1-76) was successfully purified for later use in NMR experiments as monitored in section 3.3.3.

$^{12}$C$^{15}$N$^1$H-labelled RING2 (*Drosophila* parkin residues 410-482) was successfully purified for later use in NMR experiments. Purity was monitored successfully by SDS-PAGE as seen by the expected band around 8.3 kDa (Figure 4.1). $^{13}$C$^{15}$N$^2$H- and $^{12}$C$^{14}$N$^2$H-labelled R0RBR as well as $^{12}$C$^{15}$N$^1$H-labelled R0RBR$^{W403A}$ were successfully purified for later use in NMR experiments as monitored in section 3.3.2.

4.3.5 *Uba1 was expressed and purified*

Unlabelled His-tagged Uba1 was successfully purified using a HisTrap FF column. Purity was monitored successfully by SDS-PAGE as seen by the expected band around 118 kDa (Figure 4.3). Unlabelled His-tagged Uba1 was used for ubiquitination assays and later synthesis of the stable UbcH7-Ub isopeptide-linked conjugate.
Figure 4.1 UblR0RBR and RING2 purification. (A) Unlabelled UblR0RBR was purified on a HisTrap column followed by Ulp1-cleavage and a second HisTrap column. Purity was verified by SDS-PAGE with the expected MW of UblR0RBR being around 51.6 kDa. (B) 12C15N1H-labelled RING2 was purified on a HisTrap column followed by Ulp1-cleavage, a second HisTrap column and (C) a final Superdex 75 size-exclusion column. Purity was verified by SDS-PAGE with the expected MW of RING2 being around 8.3 kDa.
Figure 4.2 Mass spectrometry of UblR0RBR variants. Integrity was verified of (A) UblR0RBR, (B) UblR0RBR\(^{T240R}\), (C) UblR0RBR\(^{R271S}\), (D) UblR0RBR\(^{N273S}\), (E) UblR0RBR\(^{R314A}\), (F) UblR0RBR\(^{P335G/C337G}\), (G) UblR0RBR\(^{R402C}\) and (H) UblR0RBR\(^{W403A}\) by mass spectrometry as seen by the expected observed MW peaks. Each UblR0RBR variant showed 2 additional prominent peaks with MWs of 28 Da and 56 Da below the expected MW corresponding to the correct variant having a loss of 1 and 2 carbonyl groups respectively.
Figure 4.3 *His-tagged Uba1 purification*. Unlabelled His-tagged Uba1 was purified on a HisTrap column. Purity was verified by SDS-PAGE with the expected MW of His-tagged Uba1 being around 118 kDa.
4.3.6 PINK1 was expressed and purified

Unlabelled GST-tagged PINK1 kinase was successfully purified as monitored in section 3.3.5 and was later used for phosphorylation of Ub and Ubl.

4.3.7 pUb and pUbl were purified

Unlabelled pUbl and $^{12}$C$^{14}$N$^2$H-labelled pUb were successfully purified as monitored in section 3.3.6 for use in NMR experiments. Unlabelled pUb was successfully purified as monitored in section 3.3.6 for use in SEC, ITC and ubiquitination assays.

4.3.8 UbcH7-Ub isopeptide-linked conjugate was formed and purified

Assays were performed in order to optimise UbcH7-Ub isopeptide-linked conjugate formation (Figure 4.4) and a protocol was successfully developed as shown in section 4.2.9. Assays showed that the best conditions for enzymatically forming UbcH7-Ub included incubating 200 µM His-tagged Ub, 400 µM UbcH7$^{C17S/C86K/C137S}$, 25 µM His-tagged Uba1 and 10 mM Mg$^{2+}$/ATP in 50mM CHES, 150 mM NaCl, pH 9.0 buffer at 37°C for 6-16 hours. UbcH7-Ub was purified using an initial HisTrap FF column followed by TEV-cleavage, a second HisTrap FF column and a final HiLoad Superdex 16/60 size-exclusion column. Purity was monitored by SDS-PAGE as seen by the expected band around 26.9 kDa (Figure 4.5). Integrity of UbcH7-Ub was confirmed by mass spectrometry as seen by a 26,860.27 Da MW peak (Figure 4.5D). Unlabelled UbcH7-Ub was later used for ITC and SEC while selectively labelled UbcH7-Ub conjugates were used for NMR experiments.
Figure 4.4 UbcH7-Ub isopeptide-linked conjugate formation assays. Conditions manipulated included (A) buffer type, (B) temperature, (C) Mg\(^{2+}\)/ATP concentration, (D) pH, (E) His-Uba1 concentration, (F) UbcH7\(^{C17S/C86K/C137S}\) concentration, (G) His-Ub concentration and (H) reaction time. All reactions were performed at 37°C for 6 hours in 50mM CHES, 150 mM NaCl, pH 9 buffer with 20 µM Uba1, 50 µM UbcH7\(^{C17S/C86K/C137S}\), 200 µM Ub and 10 mM Mg\(^{2+}\)/ATP with only one of these conditions being varied as noted on each panel. Reactions were monitored by SDS-PAGE as seen by the appearance of His-tagged UbcH7-Ub being formed at a MW of 29.9 kDa.
Figure 4.5 UbcH7-Ub isopeptide-linked conjugate purification. (A) His-tagged UbcH7-Ub was enzymatically conjugated as seen by the appearance of a band being formed at a MW of 29.9 kDa. UbcH7-Ub was then purified on (B) a HisTrap column followed by TEV-cleavage and a second HisTrap column, and (C) a final HiLoad Superdex 16/60 size-exclusion column. Purity was verified by SDS-PAGE with the expected MW of UbcH7-Ub being around 26.9 kDa. (D) Integrity was verified by mass spectrometry as seen by the observed 26,860.27 Da MW peak corresponding to isopeptide-linked UbcH7-Ub.
4.3.9 pUb binding leads to allosteric opening of a Ub-binding pocket

The 2.7 Å crystal structure of human UblR0RBR^{A84–143} in complex with pUb reveals the activated state of parkin comprising the five domains Ubl, RING0, RING1, IBR and RING2 (Figure 4.6A). Comparison of activated pUb-UblR0RBR (PDB ID code 5N2W) to inactive UblR0RBR (PDB ID code 5C1Z) shows that while there are some small rearrangements, the autoinhibitory Ubl domain remains packed against helix H3 of RING1. The largest structural rearrangement resulting from pUb binding is the straightening of the previously bent helix H3 in RING1 which leads to global movement of the IBR domain by 22 Å (Figure 4.6B). Previous NMR and thermodynamic data of pUb binding to parkin suggested that RING1 helix H3 would undergo rearrangement due to observed CSPs and an increase in entropy of the system. These observations are confirmed as seen in our crystal structure, as well as in the structure of Pediculus humanus pUb-R0RBR (Wauer et al., 2015). Importantly, movement of the IBR domain results in loosening of the Ubl/IBR interface, further affirming that pUb binding causes release of the Ubl domain by inducing weakened Ubl interactions. Interestingly, movement of the IBR domain induced by pUb binding also creates of a pocket in between RING1 and IBR. Analysis of the pUb-R0RBR crystal packing showed that this void is occupied by the Ubl domain of a neighbouring parkin molecule. Since the Ubl domain and Ub share homology and sequence similarity, we proposed that the pocket created by pUb-induced structural rearrangements would be the Ub binding site for the UbcH7-Ub conjugate.
Figure 4.6 Crystal structure of pUb-UblR0RBR. (A) Ribbon diagram of pUb-UblR0RBR$^{\Delta84-143}$ (PDB ID code 5N2W) showing pUb (orange), the Ubl domain (green), RING0 (blue), RING1 (white), IBR (cyan), repressor element (REP) (yellow) and RING2 (purple). The phosphate group on pUb is shown as red sticks. (B) Overlay of activated pUb-UblR0RBR (coloured as in panel A) with inactive UblR0RBR (PDB ID code 5C1Z) (grey). pUb binding causes straightening of RING1 helix H3 and a global 22 Å movement of the IBR domain. This movement of the IBR domain leads to a loss of Ubl/IBR contacts and an open RING1/IBR pocket for Ub binding. Panels were created using PyMol and alignment was performed to all atoms in UblR0RBR using the align command.
4.3.10 pUb must activate R0RBR to allow UbcH7-Ub engagement

In order to determine under what conditions UbcH7-Ub binds to parkin optimally, SEC, ITC and NMR experiments were performed. Three constructs of parkin (R0RBR, UblR0RBR and pUblR0RBR) were assayed in the presence and absence of pUb in order to determine relative binding affinities to UbcH7-Ub by SEC. Results showed that UbcH7-Ub would only co-elute with the pUblR0RBR construct and only when pUb was present (Figure 4.7). This indicates that parkin is required to be phosphorylated at S65 and have pUb bound in order to bind to UbcH7-Ub optimally. pUb binding is necessary because it allosterically opens a RING1/IBR pocket for Ub within the conjugate to bind parkin however, the necessity for a phosphorylation signal at S65 on parkin was not known, so we proposed that pUbl may be stabilizing UbcH7-Ub binding by making contacts with UbcH7. To test this, we collected $^1$H-$^{15}$N HSQC spectra of $^{12}$C$^{15}$N$^1$H-labelled UbcH7$^{C17S/C137S}$ with unlabelled pUbl (Table 4.2). Results indicated that the isolated pUbl domain does not interact with UbcH7, suggesting that parkin phosphorylation at S65 functions to stabilize UbcH7-Ub binding by a different means.

To further characterise the importance of creating a pocket for Ub within the UbcH7-Ub conjugate to bind, ITC experiments were performed to compare both pUb-pUblR0RBR and pUb-pUblR0RBR$^{E321A}$ binding to UbcH7-Ub (Figure 4.8BC). E321A is located on the RING1 helix H3 proposed to be a part of the binding site for Ub within the UbcH7-Ub conjugate. Importantly, this residue is occluded by RING1/IBR interactions in the absence of pUb binding, and is only accessible for Ub binding when pUb activates parkin to open the RING1/IBR pocket. UbcH7-Ub bound to activated pUb-pUblR0RBR ($K_d$ 0.46 ± 0.03 µM) much tighter than to activated pUb-pUblR0RBR$^{E321A}$ ($K_d$ 9.42 ± 0.63 µM), indicating an important role for E321 binding to Ub within the UbcH7-Ub conjugate. To ensure that this reduction in affinity was the result of perturbing Ub binding rather than UbcH7 within the conjugate, further ITC experiments were performed and showed that UbcH7 alone bound to both pUb-pUblR0RBR ($K_d$ 19 ± 1.3 µM) and pUb-pUblR0RBR$^{E321A}$ ($K_d$ 23.5 ± 2.1 µM) with similar affinities (Table 4.4). Together, this data suggests that Ub within the UbcH7-Ub conjugate interacts with the RING1 helix H3 of parkin and analysis of crystal structures.
show that this site is only accessible when pUb is bound to allosterically open the
RING1/IBR pocket.

Further ITC experiments were performed to determine the affinities of inactive
R0RBR (no pUb bound) for Ub (K_d 40 ± 1 µM), UbcH7^{C17S/C137S} (K_d 5.5 ± 0.2 µM) and
UbcH7-Ub (K_d 30 ± 3 µM) (Table 4.4). UbcH7-Ub was expected to bind tighter to
inactive R0RBR than the free UbcH7^{C17S/C137S} and free Ub only if both moieties within
the conjugate were binding using one continuous patch with synergistic binding
contributions. However it was found that UbcH7-Ub only bound with an apparent K_d
that is an averaged intermediate of the UbcH7^{C17S/C137S} and Ub affinities, indicating that
UbcH7-Ub is likely not binding to R0RBR using one continuous binding surface. This
result affirms the importance of having pUb bound in order to allow opening of the
RING1/IBR pocket and full engagement of the UbcH7-Ub conjugate since UbcH7-Ub
binds to pUb-pUblR0RBR (K_d 0.46 ± 0.03 µM) with a much higher affinity than to
R0RBR alone.
Figure 4.7 Parkin and UbcH7-Ub SEC elution profiles. Combinations of pUb, Ub, UbcH7 and UbcH7-Ub were run on a Superdex 75 10/300 size-exclusion column with (A) R0RBR, (B) UblR0RBR and (C) pUblR0RBR. The addition of pUb to all three parkin constructs results in a shift in absorbance to the left (cyan) indicating that pUb binds tight enough to co-elute with all three constructs. UbcH7-Ub only binds tight enough to co-elute with pUb-pUblR0RBR as shown by the additional second shift in absorbance to the left in panel C (orange) suggesting an importance for pUb and a phosphorylation signal on the Ubl domain. The standard reference peak is the black elution profile of the unbound parkin constructs in each panel. V₀ represents the void volume. (D) SDS-PAGE gel of pUblR0RBR elution profiles showing composition of samples loaded onto the column (Load) and in the 9-11 mL (9-11) fractions. UbcH7-Ub is only present in the 9-11 mL fractions for pUb-pUblR0RBR as denoted by *. Results were verified by SEC-MALS as measured by a large pUb-pUblR0RBR:UbcH7-Ub complex of MW 87.4 kDa co-eluting.
Figure 4.8 ITC of R0RBR constructs binding to Ub, UbcH7 and UbcH7-Ub. (A) Isotherm graphs of R0RBR with Ub (left), R0RBR with UbcH7\textsuperscript{C17S/C137S} (middle) and R0RBR with UbcH7-Ub (right). Results indicate that UbcH7-Ub binds to R0RBR with an apparent $K_\text{d}$ that is an intermediate between Ub and UbcH7 affinities. (B) Isotherm graphs of UbcH7 with pUb-pUblR0RBR (left) and UbcH7 with pUb-pUblR0RBR\textsuperscript{E321A} (right), and (C) UbcH7-Ub with pUb-pUblR0RBR (left) and UbcH7-Ub with pUb-pUblR0RBR\textsuperscript{E321A} (right). Results indicate that E321 is important for conjugated-Ub binding and that this residue does not perturb UbcH7 binding. The upper panels represent raw data, and the lower panels represent integrated heat changes performed assuming a single-site binding model. Data was collected at 25°C with 50 mM HEPES, 50 mM NaCl, 250 µM TCEP, pH 7.5 buffer conditions.
Table 4.4 Thermodynamics of parkin binding to Ub, UbcH7 and UbcH7-Ub. ITC experiments were collected at 25°C with 50 mM HEPES, 50 mM NaCl, 250 μM TCEP, pH 7.5 buffer conditions and were performed in duplicate. Values are shown for stoichiometry (N), dissociation constant (K_d), enthalpy change (ΔH), entropy change (ΔS) and Gibbs free energy (ΔG). Relative exchange rates determined by NMR are presented for affinity comparison. Dashed lines indicate that data was not collected.

<table>
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<th>N</th>
<th>K_d</th>
<th>ΔH</th>
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<th>NMR Exchange Rate</th>
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<td>40 ± 1</td>
<td>30 ± 1</td>
<td>55 ± 1</td>
<td>-25 ± 1</td>
<td>Fast</td>
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<tr>
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<td>UbcH7C17S/C137S</td>
<td>1.1 ± 0.0</td>
<td>5.5 ± 0.2</td>
<td>26 ± 0</td>
<td>56 ± 1</td>
<td>-30 ± 1</td>
<td>Slow</td>
</tr>
<tr>
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<td>30 ± 3</td>
<td>7.6 ± 1.4</td>
<td>33 ± 1</td>
<td>-25 ± 2</td>
<td>Fast (Ub) and slow (UbcH7)</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
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* Data collected by Dr. Viduth Chaugule
4.3.11 UbcH7-Ub takes on a closed conformation in solution

CSP analysis comparing $^1$H-$^{15}$N HSQC spectra of free $^{12}$C$^{15}$N$^2$H-labelled UbcH7 and free $^{12}$C$^{15}$N$^2$H-labelled Ub to spectra collected of their conjugated form (Figure 4.9A) can reveal important insights into the closed, open or backbent states of UbcH7-Ub in solution. The comparison revealed that upon conjugation, UbcH7 and Ub exhibit CSPs that localise to a surface of interaction between the two molecules. For instance, CSPs seen in Ub were hallmarks of the closed state localising to the hydrophobic patch (G47-L50), loop L1 (T9), and C-terminal tail (V70 and R72-G76) (Figure 4.9B) while the UbcH7 cross-over helix spanning residues K100-N113 exhibited CSPs also indicative of the closed state (Figure 4.9C). UbcH7 also presents CSPs from residues in neighbouring space to the catalytic C86K in loop L7 as well as H76, D80, Q116, E118, H119 and L121, but these are due to a significant change in chemical environment from C86K being conjugated G76 of Ub rather than from a binding interaction. Taken together, results suggest that UbcH7-Ub takes on a closed conformation in solution.
Figure 4.9 NMR shows that UbcH7-Ub takes on a closed conformation. (A) $^1$H-$^{15}$N HSQC NMR spectra of free $^{12}$C$^{15}$N$^{2}$H-labelled UbcH7 (red contours), free $^{12}$C$^{15}$N$^{2}$H-labelled Ub (yellow contours) and $^{12}$C$^{15}$N$^{2}$H-labelled UbcH7-Ub conjugate (black contours). (B) Measured Ub CSPs upon UbcH7-Ub conjugation. CSPs were of moderate magnitude and localised to loop L1 (T9), the hydrophobic patch (G47-L50) and C-terminal tail (V70 and R72-G76). (C) Measured UbcH7 CSPs upon UbcH7-Ub conjugation. CSPs were of moderate magnitude and localised to the crossover helix spanning residues K100-N113 as well as to regions surrounding the catalytic C86K such as loop L7. Significant CSPs were mapped to the surfaces of both Ub and UbcH7 (PDB ID 4Q5E) in PyMOL. CSPs seen in both UbcH7-Ub are hallmarks of the closed state. Horizontal dashed lines indicate the average CSP ± 1 standard deviation. Data was collected at 25°C in 25 mM HEPES, 50 mM NaCl, 500 μM TCEP, pH 7.0 buffer.
4.3.12 UbcH7 binds to RING1 and Ub binds to the RING1/IBR pocket

In order to determine the surfaces used for interaction between pUb-R0RBR and UbcH7-Ub, NMR titration experiments were conducted using selectively labelled pUb-R0RBR with UbcH7-Ub as well as constructs of R0RBR with the individual UbcH7 or Ub moieties in order to obtain well-defined surfaces (Table 4.2). In all NMR experiments involving pUb-R0RBR, pUb was $^{12}$C$^{14}$N$^2$H-labelled and the complex was co-eluted on a Superdex 75 size-exclusion column. CSP analysis of TROSY $^1$H- $^{15}$N HSQC NMR data showed that the addition of deuterated UbcH7-Ub to $^{13}$C$^{15}$N$^2$H-labelled pUb-R0RBR resulted in significant line broadening and that resonance CSPs were of moderate magnitude exhibiting slow-exchange indicative of a large complex being formed. Resonance CSPs spanned regions of the RING1, IBR and REP linker (Figure 4.10). To specify the individual binding surface on R0RBR for UbcH7, unlabelled UbcH7 was titrated into $^{13}$C$^{15}$N$^2$H-labelled R0RBR. Results showed that UbcH7 caused resonance signals to become undetectable or show small CSPs around the canonical E2-E3 binding site on RING1 loop L1 (C241-T242), RING1 helix H1 (L266, V269, R271-L272) and RING1 Zn$^{2+}$-loop L2 (A291-G292) as well as to a large portion of the REP linker (Y391-R392, D394-R396 and A398-K408). The pUb-UblR0RBR crystal structure and $^1$H- $^{15}$N heteronuclear NOE data (Figure 3.16) indicate that pUb binding does not affect placement of the highly-electrostatic autoinhibitory REP linker and rather, it indicates that the REP still blocks the UbcH7 binding site on RING1. However, the most pronounced CSPs upon UbcH7 binding are found in this REP region. Since CSPs are seen in the RING1 as well, this indicates that UbcH7 is capable of moving the REP linker in order to bind to the canonical RING1 surface. This also indicates that UbcH7 is either making contacts directly with the REP, or causing it to be displaced completely. To specify the individual binding surface on R0RBR for Ub, $^{13}$C$^1$H-VIL $^{12}$C$^{14}$N$^2$H-labelled Ub was titrated into $^{13}$C$^{15}$N$^2$H-labelled pUb-R0RBR. Overall, results showed CSPs in the RING1/IBR pocket including RING1 helix H1 (V269, R271-L272), the straightened RING1 helix H3 (R314, E322), IBR β1 (V330-C332 and R334) and loop L4 (F364, R366, C368), and in the linker (A379-F381 and A383-T386). However, several of the RING1 helix H3 resonances were broadened by prior pUb binding and were therefore difficult to visualize in the NMR spectra. $^1$H- $^{15}$N cross-saturation data was collected for
all experiments using $^{13}$C$^1$H-VIL $^{12}$C$^{14}$N$^2$H-labelled Ub with $^{13}$C$^{15}$N$^2$H-labelled pUb-R0RBR in order to specify a surface on R0RBR for Ub binding, however data did not reveal a definitive surface.

Determining surfaces on UbcH7-Ub used for binding to pUb-R0RBR was straightforward. CSP analysis of TROSY $^1$H-$^{15}$N HSQC NMR followed the titration of unlabelled pUb-R0RBR into $^{12}$C$^{15}$N$^2$H-labelled UbcH7-Ub, where both UbcH7 and Ub moieties were labelled (Figure 4.11). Significant line broadening was observed indicative of tight binding, and resonance CSPs were of moderate magnitude. Importantly, both UbcH7 and Ub resonances experienced slow exchange indicating that both molecules were making tight contact with pUb-R0RBR rather than having one molecule bind and the other “float”. CSPs observed in UbcH7 localised to the canonical E2 surface typically used for E3 interactions including residues in the helix H1 (R6, M8-K9, L11 and I14), loop L4 (A59-Y61 and F63-K64) and loop 7 (E93-N94, K96, A98 and T101-D102). CSPs seen in UbcH7 helix H1 extended over a larger portion of the helix than is typically seen for binding to E3s, suggesting that there may be additional pUb-R0RBR contacts being made to this portion of the helix. CSPs observed in Ub localised to a large portion of the I44 hydrophobic patch including residues in the β1-L1-β2 region (F4-V5, T7-G10 and I13-T14), β3 (R42-L43) and β4 (Q49). CSPs also localised to a large part of the C-terminal tail including V70-L71, L73 and G75-C76. A last notable cluster of CSPs were noticed at the C-terminal end of helix H1 (E34 and I36). Several $^1$H-$^{13}$C HMQC experiments were collected of experiments using $^{13}$C$^1$H-VIL $^{12}$C$^{14}$N$^2$H-labelled Ub with $^{13}$C$^{15}$N$^2$H-labelled pUb-R0RBR and CSP analysis from those spectra were used to complete the Ub surface interaction dataset.
Figure 4.10 NMR of the pUb-activated R0RBR binding interface with UbcH7-Ub. (A) TROSY $^1$H-$^{15}$N HSQC NMR spectra of $^{13}$C-$^{15}$N-$^2$H-labelled R0RBR bound to unlabelled pUb in the absence (black contours) and presence of 1 equivalent UbcH7-Ub where UbcH7 is $^{12}$C-$^{14}$N-$^2$H-labelled and Ub is $^{13}$C-$^1$H-VIL $^{12}$C-$^{14}$N-$^2$H-labelled (purple contours). (B) Measured pUb-R0RBR CSPs upon UbcH7-Ub binding. Grey bars indicate that the resonance was undetectable. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were moderate in magnitude and exhibited slow exchange indicating a tight affinity. pUb-R0RBR was purified as a 1:1 complex on a Superdex 75 size-exclusion column. Data was collected at 25°C in 25 mM HEPES, 50 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
Figure 4.11 NMR of the UbcH7-Ub binding interface with pUb-R0RBR. (A) TROSY $^1$H-$^{15}$N HSQC NMR spectra of $^{12}$C$^{15}$N$^2$H-labelled UbcH7-Ub in the absence (black contours) and presence of 1 equivalent $^{12}$C$^{14}$N$^2$H-labelled pUb-R0RBR (red contours). UbcH7 residues are indicated in green lettering and Ub residues are blue. (B) Measured UbcH7-Ub CSPs upon pUb-R0RBR binding. Grey bars indicate that the resonance was undetectable. The horizontal dashed line indicates the average CSP ± 1 standard deviation. CSPs were large and exhibited slow exchange for both UbcH7 and Ub resonances indicating a tight affinity. pUb-R0RBR was purified as a 1:1 complex on a Superdex 75 size-exclusion column. Data was collected at 25°C in 25 mM HEPES, 50 mM NaCl, 500 μM TCEP, pH 7.0 buffer.
4.3.13 The closed UbcH7-Ub state is released upon binding pUb-R0RBR

Close analysis of TROSY \(^1\)H-\(^{15}\)N HSQC NMR data collected while titrating deuterated pUb-R0RBR into \(^{12}\)C\(^{15}\)N\(^2\)H-labelled UbcH7-Ub (Figure 4.11) revealed important features about UbcH7-Ub conformations when this data was compared to \(^1\)H-\(^{15}\)N HSQC spectra collected of free \(^{12}\)C\(^{15}\)N\(^2\)H-labelled UbcH7, free \(^{12}\)C\(^{15}\)N\(^2\)H-labelled Ub and \(^{12}\)C\(^{15}\)N\(^2\)H-labelled UbcH7-Ub (Figure 4.9). Initially, resonances for UbcH7 F22, V40, N43, N56 and Q106 as well as Ub resonances for G47 and Q49 show CSPs when UbcH7 becomes conjugated to Ub that are consistent with the closed conformation of UbcH7-Ub. When deuterated pUb-R0RBR is titrated into this UbcH7-Ub conjugate however, these residue resonances revert back to similar positions seen in the spectra collected of free UbcH7 and free Ub (Figure 4.12). This finding suggests that the surfaces that these residues comprise are no longer interacting with one-another in a closed state, nor are they interacting with pUb-R0RBR. Rather it suggests that these residues are experiencing chemical environments similar to those seen in free UbcH7 and free Ub states, which is only possible if UbcH7-Ub is binding to pUb-R0RBR in an open conformation. Figure 4.12 also shows that both UbcH7 and Ub resonances within the conjugate experience slow exchange when binding to pUb-R0RBR indicative that both moieties are binding to pUb-R0RBR with tight affinity. Apart from G47 and Q49, the majority of Ub residues that interact with UbcH7 in a closed conformation also interact with pUb-R0RBR. When bound to pUb-R0RBR, the majority of residues do not revert back to similar chemical shifts as the free Ub state and rather they experience a new chemical environment suggesting that these residues are interacting with pUb-R0RBR. Overall, these results show that the closed UbcH7-Ub conjugate conformation is released upon interaction with parkin.
Figure 4.12 The closed UbcH7-Ub state is released upon binding to pUb-R0RBR. (A) Ribbon diagram representations of the free and conjugated UbcH7-Ub states. (B) TROSY $^1$H,$^{15}$N HSQC NMR spectra were taken of free $^{12}$C,$^{15}$N,$^2$H-labelled UbcH7 (green contours) and free $^{12}$C,$^{15}$N,$^2$H-labelled Ub (cyan contours). UbcH7 residues are labelled green and Ub residues are labelled cyan. These two labelled moieties were then conjugated together (black contours). UbcH7 resonances for F22, V40, N43, N56 and Q106 and Ub resonances for G47 and Q49 are perturbed upon conjugate formation and form a surface that interacts in the closed state. UbcH7-Ub was then titrated with 0.5 equivalents (yellow contours) and 1 equivalent (purple contours) of $^{12}$C,$^{14}$N,$^2$H-labelled pUb-R0RBR. The same UbcH7 and Ub resonances revert back to the free state chemical shifts suggesting that UbcH7 and Ub release interaction with one-another and bind to pUb-R0RBR in a more open conformation. Both UbcH7 and Ub residue resonances exhibit slow exchange indicating that both molecules interact tightly with pUb-R0RBR.
4.3.14 pUb-R0RBR:UbcH7-Ub HADDOCK structure

In order to understand how UbcH7-Ub binds to parkin, a model for the pUb-R0RBR:UbcH7-Ub complex was determined. UbcH7-Ub was successfully docked to pUb-R0RBR in two steps by sequentially docking UbcH7 and Ub in HADDOCK using data from NMR experiments, mutagenesis experiments and 1 unambiguous restraint from Ub G76 to the catalytic C86K in UbcH7 (Table 4.3). The location and orientation of UbcH7 with respect to pUb-R0RBR was similar in all 100 water-refined complexes and the best 20 complexes had a backbone rmsd of 0.47 ± 0.07. Likewise, the location and orientation of Ub with respect to pUb-R0RBR:UbcH7 was similar in all 100 water-refined complexes and the best 20 complexes had a backbone rmsd of 0.71 ± 0.10.

The pUb-R0RBR:UbcH7-Ub structure shows that UbcH7-Ub takes on an open conformation, poised for transthiolation (Figure 4.13). UbcH7 makes canonical E2-E3 contacts and Ub uses an altered version of its hydrophobic patch to position itself in a large pocket formed by RING1, IBR and linker residues 379-386. The Ub surface used for interaction is considered an altered form of the hydrophobic patch because loop L4 (residues F45, A46 and G47), comprising a part of the typical hydrophobic patch, makes no contacts with R0RBR. UbcH7 contacts are seen between helix H1 to R0RBR RING1 loop L1, UbcH7 loop L4 to R0RBR RING1 helix H1, and UbcH7 loop L7 to R0RBR RING1 Zn$^{2+}$-loop L2 with additional contacts being made to the REP linker. Ub binding is governed predominantly by contacts from β1-L1-β2 in Ub to the IBR. Interestingly, Ub loop L1 residues T7, L8, T9 and G10 are a distinguishing feature from the Ubl domain in parkin where the corresponding residues in Ubl are F7, N8, S9, and S10. This variation may suggest an important guiding role for loop L1 in both Ubl and Ub binding to parkin and account for the fact that the two proteins have overall similar tertiary structures, yet different modes of interaction. Further contacts stabilizing Ub binding to the R0RBR pocket are also seen between R0RBR linker residues 379-386 to the loop L1 and C-term of Ub, and between Ub loop L3 to R0RBR RING1 helices H1 and H3. An important feature for Ub binding is the fact that the RING1/IBR pocket is only formed when pUb is also bound to R0RBR. This confirms that parkin is activated by pUb binding to induce a conformational change that results in the opening of this Ub binding pocket (Kumar et al., 2017; Wauer et al., 2015).
**Figure 4.13 pUb-R0RBR:UbcH7-Ub HADDOCK structure.** The surface of R0RBR (grey) is shown binding to ribbon diagrams of pUb (orange) as well as UbcH7 (green) conjugated to Ub (cyan). UbcH7-Ub binds in an open conformation poised for transthiolation where UbcH7 uses canonical E2-E3 interactions and Ub binds to the RING1/IBR pocket. R0RBR residues used for ambiguous HADDOCK restraints are highlighted green and cyan while UbcH7-Ub restraints are highlighted black. 1 unambiguous restraint was used between the C-terminal G76 of Ub and C86K of UbcH7. R0RBR uses RING1 loop L1, RING1 helix H1, RING1 Zn$^{2+}$-loop L2 and REP to bind UbcH7 helix H1, loop L4 and loop L7 whereas R0RBR uses RING1 helix H1, RING1 helix H3, IBR and linker residues 379-386 to bind Ub β-sheet β1, loop L1, β-sheet β2, loop L3 and the C-terminal tail. The location and orientation of UbcH7 and Ub docks with respect to R0RBR were similar in all 100 water-refined complexes and the best 20 complexes had a backbone rmsd of 0.47 ± 0.07 for UbcH7 and 0.71 ± 0.10 for Ub.
4.3.15 UbcH7-Ub binding alters the RING0/RING2 interface

CSP analysis of the TROSY \(^{1}\text{H}\)\(^{15}\text{N}\) HSQC NMR data of \(^{13}\text{C}\)^{15}\text{N}\(^2\text{H}\)-labelled pUb-R0RBR with deuterated UbcH7-Ub also revealed significant chemical shift changes around W403 and the RING0/RING2 interface in addition to the CSPs attributable to being at the UbcH7-Ub binding interface (Figure 4.14). W403 comprises a portion of the REP linker and is a pillar residue for packing the REP linker against RING1. It is also located adjacent to the C-terminal V465 at the RING0/RING2 interface in crystal structures. The W403A variant has been shown to increase parkin activity (Trempe et al., 2013) proposed to be a result of the REP linker releasing interaction with RING1 to allow access of the E2 to bind RING1 (Riley et al., 2013). However, the W403A variant may also result in the disruption of the RING0/RING2 interface. To test this, we collected a \(^{1}\text{H}\)\(^{15}\text{N}\) HSQC NMR spectrum of \(^{12}\text{C}\)^{15}\text{N}\(^1\text{H}\)-labelled R0RBR\(^{W403A}\) and performed CSP analysis using TROSY \(^{1}\text{H}\)\(^{15}\text{N}\) HSQC NMR data of \(^{13}\text{C}\)^{15}\text{N}\(^2\text{H}\)-labelled R0RBR as a comparison. Many resonances were undetectable around W403A and the rest of the REP linker as expected, and moderate-sized CSPs extended to the RING2/RING0 interface much like the CSPs that were seen when UbcH7-Ub bound to pUb-R0RBR. Since all structures of parkin to-date show that C431 is occluded by RING0 interactions, then it is possible that the CSPs seen around the RING0/RING2 interface upon UbcH7-Ub binding were reporting a conformational change to allow exposure of the catalytic C431.

Since UbcH7-Ub binding to pUb-R0RBR showed large CSPs in the parkin REP linker that connects to the RING2, we wondered whether this was due to direct binding of UbcH7 to the REP linker or due to complete displacement of the REP linker from RING1. To test this, we collected \(^{1}\text{H}\)\(^{15}\text{N}\) HSQC NMR spectra of an isolated \(^{12}\text{C}\)^{15}\text{N}\(^3\text{H}\)-labelled \textit{Drosophila melanogaster} RING2 (residues 410-482) construct that includes the REP linker. Six equivalents of unlabelled UbcH7 were titrated into this RING2 construct, but no interaction was observed, suggesting the possibility that the CSPs seen in the REP linker are due to displacement from the RING1 rather than from binding to UbcH7. A similar \(^{1}\text{H}\)\(^{15}\text{N}\) HSQC experiment was collected titrating six equivalents of unlabelled Ub into \(^{12}\text{C}\)^{15}\text{N}\(^1\text{H}\)-labelled RING2. Since Ub ultimately attaches to C431 in RING2 through a transthiolation reaction, we wondered if there might be an observable interaction between the two molecules, however no interaction was detectable.
Figure 4.14 UbcH7-Ub binding alters the RING0/RING2 interface. (A) CSPs were measured for TROSY \(^1\)H,\(^{15}\)N HSQC NMR spectra collected of \(^{13}\)C,\(^{15}\)N\(^2\)H-labelled R0RBR bound to unlabelled pUb in the presence of 1 equivalent \(^{12}\)C,\(^{14}\)N\(^2\)H-labelled UbcH7-Ub and were modelled onto the surface diagram of R0RBR (PDB ID code 5N2W). (B) Significant CSPs from panel A that were at the UbcH7-Ub binding interface. (C) Significant CSPs from panel A that resulted in a change in environment around W403A and the RING0/RING2 interface potentially result in exposure of C431. Grey bars indicate that the resonance was undetectable. Horizontal dashed lines indicate the average CSP ± 1 standard deviation. Data was collected at 25°C in 25 mM HEPES, 50 mM NaCl, 500 µM TCEP, pH 7.0 buffer.
4.3.16 ARJP variants impair parkin ubiquitination activity

Ubiquitination assays were performed with ARJP and non-ARJP UblR0RBR variants in order to assess effects on parkin activity (Figure 4.15) and to obtain ambiguous restraints for HADDOCK pUb-R0RBR:UbcH7-Ub structure calculations (Table 4.3). Results showed that UblR0RBR\textsuperscript{T240R}, UblR0RBR\textsuperscript{R271S}, UblR0RBR\textsuperscript{R314A} and UblR0RBR\textsuperscript{P335G/C337G} all showed significant decreases in activity while UblR0RBR\textsuperscript{W403A} showed increased activity. UblR0RBR\textsuperscript{N273S} and UblR0RBR\textsuperscript{R402C} showed no significant change in activity. T240R and R271S variants are located at UbcH7 binding site while R314A and P335G/C337G variants are located at the proposed Ub binding site. Since these variants resulted in a reduction in parkin activity, it suggests that these residues are important for making interactions with the UbcH7-Ub conjugate. W403A is a proposed activating variant that is suggested to increase parkin activity by freeing inhibitory intramolecular interactions of the REP linker with RING1 (Trempe et al., 2013; Riley et al., 2013) or by altering the RING0/RING2 interface as shown by CSP data. This variant indeed showed a marked increase in ubiquitination activity even in the absence of pUb suggesting that W403A is affecting parkin structure in a manner to become active.

N273S was proposed to be a variant located at the Ub binding site but did it not have an effect on parkin activity, therefore this residue must not be important for interaction with Ub. The R402C variant was used to test the conformation of UbcH7-Ub used for binding to parkin. If UbcH7-Ub was to bind to parkin in a closed conformation, then this residue would be important for making interactions with Ub. However, this residue variant did not result in a reduction in parkin activity and rather it increased activity to a small extent, suggesting that R402 does not interact with Ub and that UbcH7-Ub does not bind to parkin in a closed conformation.

T240R, R271S, N273S and R402C are all ARJP mutations. Since T240R and R271S variants are located at the UbcH7 binding interface and resulted in reduced parkin activity, it is likely that these variants are associated with ARJP due to their ability to abolish UbcH7 binding to parkin. N273S and R402C variants resulted in no significant change in parkin activity, suggesting that they work to disrupt the parkin pathway by some other means.
Figure 4.15 Ubiquitination activity of ARJP and non-ARJP parkin variants. (A) Assays were performed in triplicate using E1 Uba1, E2 UbcH7C17S/C137S, E3 UbIR0RBR variants, Ub, UbcH7C17S/C137S and Mg²⁺/ATP in the presence or absence of pUb at 30°C for 30 minutes. Assays were imaged by SDS-PAGE and a LI-COR Odyssey Classic imaging system at 800 nM. (B) Gel images were processed with ImageJ and data was analysed using a two-tailed student t-test where * represents $P < 0.05$. Mean activity and ± standard error of the mean (s.e.m.) for n=3 replicates are presented. All time zero measurements, 30 minute time measurements and 30 minute time measurements with pUb were compared to their corresponding wild type parkin control measurements. T240R and R271S are at the proposed UbcH7 binding site, R314A and P335G/C337G are at the proposed Ub binding site and W403A is a proposed activating mutation that relieves catalytic C431 occlusion.
4.4 Discussion

4.4.1 A molecular model for E2-ubiquitin engagement with parkin

Extensive research on the RBR E3 ligase parkin has shown that its autoinhibition is regulated by the Ubl domain (Kumar et al., 2015), a repressor REP linker blocking the RING1:E2 binding site and by occlusion of the catalytic C431 through RING0/RING2 interactions (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). Previous studies have shown that pUb binding and parkin S65 phosphorylation events together are sufficient to relieve autoinhibition (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Similar to parkin, the RBR E3 ligases HOIP and HHARI are autoinhibited and are activated by binding to other proteins suggesting a common requirement for distinct modes of regulation. HOIP autoinhibition is mediated by its ubiquitin-associated (UBA) domain (Stieglitz et al., 2013; Lechtenberg et al., 2016) and HHARI autoinhibition is regulated through its Ariadne domain (Dove et al., 2016; Duda et al., 2013). The binding of pUb to parkin causes release of the autoinhibitory Ubl domain (Kumar et al., 2015) and the opening of a RING1/IBR pocket for E2-Ub to bind (Figure 4.6) as confirmed in a Pediculus humanus pUb-R0RBR crystal structure (Wauer et al., 2015). However, pUb binding does not explain relief of autoinhibition caused by the REP blocking RING1 from E2 binding or occlusion of the catalytic C431 through RING0/RING2 interactions because both pUb-bound parkin crystal structures still show these inhibitory characteristics and similar findings are shown by heteronuclear NOE data (Figure 3.16). NMR experiments performed here indicate that UbcH7-Ub binding to pUb-R0RBR causes large CSPs in the REP linker around W403 located adjacent to the RING0/RING2 interface (Figure 4.14). Ubiquitination assays performed with UblR0RBR\textsuperscript{W403A} also indicate an important role in this region for activating parkin activity. This data together suggests that UbcH7-Ub binding is sufficient to move the autoinhibitory REP linker and likely cause relief of catalytic C431 occlusion.

RBR E3 ligases and RING E3 ligases have RING domains that are structurally similar and bind to E2s in a similar fashion (Budhidarmo et al., 2012) using the E3 RING loop L1, RING1 helix H1 and RING1 Zn\textsuperscript{2+}-loop L2 with the E2 helix H1, loop L4 and loop L7, much like what was observed for UbcH7 binding to R0RBR (Figure 4.10 and...
However, RBR (and HECT) E3s bind to extended conformations of the E2-Ub conjugate in order to promote Ub transfer to the catalytic E3 cysteine, while RING E3s bind to closed conformations in order to promote direct Ub transfer to substrates. RBR RING1 domains differ from canonical RING E3 domains by having an elongated RING1 Zn$^{2+}$-loop L2 (Spratt et al., 2014) and the absence of a basic “linchpin” residue that typically makes contact with the E2-Ub conjugate in a closed conformation (Pruneda et al., 2012), and these differences are proposed to direct E2-Ub conformational arrangements upon binding to E3s. The absence of a “linchpin” and the elongation of RING1 Zn$^{2+}$-loop L2 are features of parkin that help explain the ability to disfavour binding to the closed E2-Ub state much like HHARI inhibits closed E2-Ub binding conformations (Dove et al., 2016; Dove et al., 2017). In agreement with another study, we found that UbcH7-Ub predominantly occupies closed states in solution (Figure 4.9) (Dove et al., 2016), however we also showed that UbcH7-Ub undergoes rearrangement to bind parkin in an open conformation (Figure 4.12). The pUb-R0RBR:UbcH7-Ub structure (Figure 4.13) shows UbcH7-Ub binding in a very similar open conformation as shown in the crystal structure of the RBR E3 ligase HOIP:E2-Ub (Figure 4.16) with only slight differences in E2 angling and a small difference in the position of β1-L1-β2 in Ub (Lechtenberg et al., 2016). Specifically, both structures show Ub oriented in an open conformation positioned closer towards the “backside” surface of UbcH7. Two crystal structures of RBR E3 ligase HHARI bound to UbcH7-Ub show similar placement of UbcH7, however the orientation of Ub is open with a specific positioning closer towards the “closed” surface of UbcH7. In these structures, Ub is making contacts to the UBA-like domain in one structure (Yuan et al., 2017) and not making any contacts in the other (Dove et al., 2017). The full HOIP:E2-Ub structure shows the catalytic site on the RING2 domain of a neighbouring HOIP molecule extended and poised for transthiolation with the E2-Ub conjugate, suggesting that the open conformation we see in our HADDOCK pUb-R0RBR:UbcH7-Ub structure is the correctly primed binding state for the E2-Ub conjugate.

Together, research here shows that binding of pUb to parkin opens a RING1/IBR pocket for E2-Ub to bind (Figure 4.6). UbcH7-Ub is closed in solution (Figure 4.9), and undergoes conformational rearrangement (Figure 4.12) to bind parkin in an open state
poised for transthiolation (Figure 4.13). UbcH7-Ub binding is sufficient to move the REP linker and bind to the RING1 (Figure 4.10). Movement of the REP linker alters the environment around W403A and the RING0/RING2 interface (Figure 4.14), likely to expose the catalytic C431 for transthiolation. In the proposed model, Ub G76 within the conjugate remains 50 Å away from the RING2 catalytic site suggesting that parkin will eventually undergo a conformational change to bring both sites together for transthiolation to occur.

4.4.2 Parkin mutations and Parkinson’s disease

Based on work to determine the structure of pUb-R0RBR:UbcH7-Ub, ubiquitination assays showed that several parkin residues had a critical role for binding to UbcH7-Ub. UblR0RBR$^{T240R}$ and UblR0RBR$^{R271S}$ ARJP variants showed a decrease in ubiquitination activity which was the result of a loss of ability for parkin to bind to the conjugate. This finding provides a direct association between the structure and function of parkin to the diseased-state. Together, research performed here provides an understanding of parkin regulation and a framework for the design of small molecules to modulate parkin activity in hereditary forms of Parkinson’s disease.
Figure 4.16 Conformations of E2-Ub conjugates bound to RBR E3 ligases. The HADDOCK structure of parkin R0RBR (grey surface) is shown binding to the ribbon diagrams of pUb (orange) as well as to UbcH7 (lime green) conjugated to Ub (cyan). Crystal structure ribbon diagrams of the RING1 and E2-Ub conjugates for HOIP:UbcH5b-Ub (navy Ub) (PDB ID code 5EDV), HHARI:UbcH7-Ub (magenta Ub) (PDB ID code 5UDH) and HHARI:UbcH7-Ub (purple Ub) (PDB ID code 5TTE) were superimposed to pUb-R0RBR:UbcH7-Ub using all atoms in RING1 domains and the super comand in PyMOL. Only the RING1 domain of each RBR E3 is shown for clarity. UbcH7 binding and orientation to parkin R0RBR from the HADDOCK structure (lime green) is very similar to other E2 enzymes (dark green) binding to RBR E3 ligases. The orientation of UbcH7-Ub bound to pUb-activated parkin closely resembles the open conformation of UbcH5b-Ub bound to HOIP. Both of these structures show Ub stabilized by IBR domain interactions and the E2-Ub conjugate poised for transthiolation. Not shown, the HOIP structure shows a neighbouring catalytic RING2 domain positioned for conjugate transthiolation suggesting that open conformation with Ub positioned closer towards he “backside” of the E2 is a primed binding state for the conjugate.
4.5 References


Chapter 5

SUMMARY AND FUTURE DIRECTIONS

5.1 E2-ubiquitin conformations

Since E2-Ub conjugates are extremely dynamic (Pruneda et al., 2011; Page et al., 2012) and play important roles in ubiquitination, this thesis sought to determine how the UbcH7-Ub conjugate behaves in solution, while bound to the shigellosis-associated kinase OspG and while bound to the Parkinson’s disease-associated E3 ligase parkin. NMR found that UbcH7-Ub adopts predominantly closed states while free in solution but that UbcH7-Ub undergoes rearrangement to bind to OspG and parkin in open conformations as confirmed by the OspG:UbcH7-Ub crystal structure and the docked pUb-bound parkin:UbcH7-Ub NMR model. Since these results suggest that UbcH7-Ub must undergo a conformational rearrangement, future work will likely explore what features govern E2-Ub conformations and whether it is a characteristic of the E2 or E3 that does-so. Work in this area will allow us to predict which conformations different E3:E2-Ub pairings will adopt while leading to a better understanding of ubiquitination mechanisms.

5.2 OspG and shigellosis

Common to many bacterial pathogens like the shigellosis-inducing *Shigella*, the host ubiquitination pathway is often hijacked by bacterial effector proteins like OspG. We used NMR and ITC to show that the E2-Ub conjugate is a biological target of OspG. Since OspG is a kinase lacking a typical kinase activation loop, it has been proposed that OspG may require binding to a host factor for activation. Indeed, studies have already shown that both Ub and E2-Ub conjugates can activate OspG autophosphorylation or phosphorylation of artificial histone substrates (Zhou et al., 2013; Grishin et al., 2014; Pruneda et al., 2014). With this said, future work on OspG will likely focus on determining biological substrate phosphorylation targets, and on determining how these phosphorylation signals help *Shigella* invasion. Like other *Shigella* effectors, OspG is secreted in a precisely-timed manner as a way to control effector activity. Since OspG is not directly involved in the first stages of translocon pore formation or bacterial invasion,
it is likely secreted in later stages of host cell manipulation to exert its effect on dampening host inflammatory responses (Kim et al., 2005; Zhou et al., 2013). As more and more *Shigella* effector proteins are studied, it will be interesting to find in which order OspG is secreted relative to other effectors. Since OspG is now amongst one of the more well-studied *Shigella* effector proteins, future work will likely focus on expanding our knowledge about the function and timing of other effectors. As a final note, we were able to determine the crystal structure of OspG bound to UbcH7-Ub lending to significant molecular insights for how therapeutics and competitive drug inhibitors can be designed to halt OspG activation and the prevalence of shigellosis.

### 5.3 Parkin and ARJP

Crystal structures determined here of full-length human parkin and full-length human parkin$^{S65D}$ revealed the autoinhibited state which is regulated through many interdomain interactions including the presence of the autoinhibitory Ubl domain. Structural work on other RBR E3 ligases such as HOIP and HHARI have extended the observation of autoinhibition as a common mode of regulation for RBR E3 ligases (Stieglitz et al., 2013; Lechtenberg et al., 2016; Dove et al., 2016; Duda et al., 2013). It will be interesting to see whether the inhibitory features seen in these structures like the bent RING1 helix H3 become hallmark indicators of autoinhibition in other RBR E3 ligases once more structures are deposited. Research here also showed that phosphorylation of the Ubl domain at S65 leads to rearrangement of a hinge composed of basic residues between RING0/RING1 and that this hinge rearrangement optimises pUb binding to parkin as seen by ITC and the docked pUb-R0RBR NMR model. It will be interesting to determine how PINK1 accesses the Ubl at S65 in future work. PINK1 requires the Ubl I44 surface for interaction (Rasool et al., 2018), however this same surface is seen interacting with parkin RING1 in the autoinhibited state.

The crystal structure of full-length human parkin bound to pUb as well as the NMR model of pUb-R0RBR:UbcH7-Ub determined here both show the activated state of parkin. The structures show pUb bound to the RING0/RING1 hinge which results in straightening of RING1 helix H3 and global movement of the IBR domain. Important competitive binding studies performed by NMR showed the first instance of pUb binding
to parkin that causes release of the autoinhibitory Ubl domain to create a pocket for E2-Ub engagement. Another structure of activated RBR E3 HOIP shows an allosteric Ub molecule bound to the same position on RING1 as our parkin structures, and this structure also shows that the allosteric Ub molecule causes straightening of the HOIP RING1 helix H3 (Lechtenberg et al., 2016). This may suggest a universal role for an allosteric pUb molecule to activate other RBR E3 ligases and this is likely to be an area of study in the future. Moreover, in order to predict future pUb-binding partners, work will likely be done to try to find common structural elements in other proteins such as the parkin RING0/RING1 hinge basic triad composed of K151, H302 and R305 residues.

Another unresolved question revolves around determining what happens to the pUbl domain once it is displaced from parkin. Unphosphorylated Ubl has been found to aid parkin in substrate recognition (Fallon et al., 2006; Trempe et al., 2009) and proteasome association (Sakata et al., 2003). Future work will likely examine whether pUbl is still involved in these tethering events, whether it interacts with a different domain on parkin, or whether it is simply displaced with a loss of interaction capabilities.

NMR work performed here showed the first occurrence of a change in environment around the catalytic C431 in RING2 driven by E2-Ub binding. Next steps will be to determine whether E2-Ub binding causes release of the RING2 and to determine how the RING2 comes into close proximity to the E2-Ub conjugate for transthiolation to occur. It is well known that HECT E3s have a flexible linker between the E2-Ub-recruiting domain and the catalytic domain which is able to bring both domains into close proximity for transthiolation. It will be interesting to determine whether the RBR E3 stretch of residues that connects to the RING2 uses a similar mechanism of flexibility.

Finally, ubiquitination assays performed here suggest a link between disease and a loss of parkin activity due to the inability for E2-Ub to bind to parkin. The continuation of research on parkin structure and function using parkin ARJP variants will further our understandings of how disease is caused and how we can develop therapeutics for managing the progression.
5.4 Significance of work

Prior to research here, knowledge about UbcH7-Ub conformations in solution, while bound to the shigellosis-associated kinase OspG and bound to the Parkinson’s disease-associated E3 ligase parkin was non-existent. The tertiary structures of both OspG and full-length human parkin were also not known. Parkin was thought to be constitutively active and no knowledge existed that indicated that Ub could be phosphorylated by PINK1, let alone that pUb binding could lead to parkin activation. Furthermore, knowledge for how OspG:E2-Ub and parkin:E2-Ub interactions contribute to disease was also not known. That being said, our group has made significant contributions to the discovery of both OspG and parkin structure-function relationships. Knowledge about OspG functioning, as well as the structure, autoinhibition and activation of parkin has made extremely large steps in the past few years, and our research has contributed extensive structural and mechanistic insights into these understandings. In all, structural and mechanistic insights researched here will have a significant impact on the understanding of disease and the development of drug therapeutics in the future.
5.5 References


CURRICULUM VITAE

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University Educational Background

Western University Doctor of Philosophy in Science, Biochemistry 2012-18
University of Dundee Research Fellowship, Biochemistry Jun-Aug 2015
University of Ottawa Honours Major Biochemistry, Minor Business 2008-12

Selected Scholarships and Awards

NSERC Alexander Graham Bell CGS – Doctoral $70,000 2016-18
   Top-ranked doctoral applicant according to academics, research potential and leadership

Western Doctoral Excellence Award $10,000 2016-18
   Outstanding academic achievement of Western’s best and brightest scholars

Ontario Graduate Scholarship $15,000 (declined second offer) 2015-17
   Merit-based according to academics, research and extra-curriculars

Western Graduate Research Scholarship $22,500 2012-17
   Student making good progress in research with minimum 78% average

Young Investigator’s Award $450 Aug 2016
   For substantial contributions to the field of biological magnetic resonance, ICMRBS Kyoto, Japan

Drs. Madge and Charles Macklin Fellowship for Publication $4,500 Jun 2016
   Most significant doctoral first-author publication in a peer-reviewed journal

Boehringer-Ingelheim Travel Grant $4,500 Jun-Aug 2015
   Travel and accommodation for University of Dundee fellowship

Schulich Scholarship for Medical Research $4,000 2015
   Ontario Graduate Scholarship holder, based on academic achievement and research merit

CIS Academic All-Canadian 2012-13
   Western varsity athlete with 80% average

Women’s Soccer Scholarship $3,250 2008-12
   uOttawa varsity soccer student-athlete with good academic standing
**Research Experience**

PhD Research, Western University 2012-18  
Supervisor: Dr. Gary Shaw  
Project Title: Conformational Arrangements of UbcH7-Ubiquitin with OspG and Parkin

PhD Research Fellowship, University of Dundee Jun-Aug 2015  
Supervisor: Dr. Helen Walden  
Project Title: Using X-ray Crystallography to Understand Parkin Function

Undergraduate Summer Research, Western University May-Aug 2011 and 2012  
Supervisor: Dr. Gary Shaw  
Project Title: Using NMR to Characterise OspG and E2-Ubiquitin Binding

**Teaching Experience**

Western Biochemistry Research Mentor for Undergraduate Projects 2014-2018  
Western University Biochemistry Lab BCH3380 Teaching Assistant 2013-2017  
Western University Molecular Biology BCH4420 Teaching Assistant 2014-2015

**Publications**


*Authors contributed equally and work was featured on the cover of EMBO journal

Selected Presentations and Abstracts

SDCI Biology Guest Lecturer Oct 2017
“Research in Biochemistry: Parkin and Parkinson’s Disease”
Oral Presentation
Strathroy District Collegiate Institute, Strathroy, Ontario, Canada

MOOT NMR Sep 2017
“Conformational Arrangement of the E2-Ubiquitin Conjugate for Parkin Loading”
Poster Presentation
Western University, London, Ontario, Canada

Collaboration of Practitioners and Researchers Seminar Feb 2017
“Linking Parkin Function to Parkinson’s Disease”
Oral Presentation
Western University, London, Ontario, Canada

Western Biochemistry Graduate Seminar Jan 2013-16
"Conformational Arrangements of UbcH7-Ubiquitin with OspG and Parkin"
1 oral presentation, 2 poster presentations
Western University, London, Ontario, Canada

“Conformational Arrangement of the E2-Ubiquitin Conjugate for Parkin Loading”
Poster Presentation
Kyoto International Conference Center, Kyoto, Japan

Inspiring Young Women in STEM Conference Mar 2016
“A Day in the Life of a Graduate Student”
Poster Presentation
Western University, London, Ontario, Canada

MOOT NMR Oct 2015
“Parkin is Activated by Displacement of its Ubiquitin-Like Domain”
Poster Presentation
McMaster University, Hamilton, Ontario, Canada

London Health Research Day Apr 2015
"Parkin Activation by Phosphorylated-Ubiquitin”
Poster Presentation
London Convention Centre, London, Ontario, Canada

International Union of Crystallography Congress Aug 2014
"Bacterial Effector Kinases"
Acknowledgment and presentation given by co-author Andrey Grishin
Montreal, Quebec, Canada