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Modulating Parkin E3 Ubiquitin Ligase Activity Using Phospho-Ubiquitin Variants

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Abstract

Parkin is a Parkinson’s disease-linked E3 ubiquitin (Ub) ligase that promotes mitophagy by ubiquitination of mitochondrial outer membrane proteins. Phosphorylation of Ub at Ser65 by the PTEN-induced putative kinase 1 activates parkin. The role of other Ub phosphorylation sites and the associated kinases remain unknown. We optimized genetic code expansion to produce pure site-specifically phosphorylated Ub (pUb) variants (pUb$^{S7}$, pUb$^{S12}$, pUb$^{S20}$, pUb$^{S65}$) and investigated their activity in a key neurodegenerative pathway. Purification of pUb$^{S7}$ revealed a +3 frameshifted protein (Ub $\Delta7$) that was successfully purified away from the pUb. Parkin was significantly activated when pUb$^{S12}$ or pUb$^{S65}$ was the sole parkin substrate. pUb$^{S20}$ showed dose-dependent inhibition of stimulated parkin. Producing pure phosphoprotein was essential, as we showed parkin activity is sensitive to the stoichiometry and location of phosphorylation on its substrate Ub. Further mechanistic investigations of Ub phosphorylation will identify new pathways and potential drug targets in neurodegenerative signalling networks.

Keywords

Cellular signalling, genetic code expansion, parkin, Parkinson’s disease, phosphorylation, phosphoserine, protein degradation, synthetic biology, tRNA, ubiquitin
Co-Authorship Statement

Chapter 2 contains work from a co-first authored published manuscript: Susanna George*, Jacob D. Aguirre*, Donald E. Spratt, Yumin Bi, Madeline Jeffery, Gary S. Shaw and Patrick O’Donoghue. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett.* 509: 1530-1542. Susanna George, Jacob D. Aguirre, Gary S. Shaw and Patrick O’Donoghue wrote the manuscript. Susanna George performed the experiments for Figures 2.2, 2.4, 2.5 and 2.7, and Supplemental Figures S2.9C-E, S2.11 and S2.12. Jacob D. Aguirre performed the experiments for Figure 2.3, 2.6 and 2.7, and Supplemental Figures S2.8, S2.10 and S2.12. Madeline Jeffery performed the experiment in Figure 2.1. Yumin Bi performed the experiment in Figure S2.9A,B. Susanna George, Jacob D. Aguirre, Donald E. Spratt and Yumin Bi contributed to producing and purifying the different pUb variants and reporting the yields in Table S2.1.
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AR-JP</td>
<td>autosomal recessive juvenile parkinsonism</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>elongation factor</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6-AP C terminus</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>IBR</td>
<td>in-between-ring</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>Levodopa or L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>MIRO</td>
<td>mitochondrial Rho GTPase</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>ncAA</td>
<td>non-canonical amino acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>pSer</td>
<td>O-phospho-L-serine</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>pUb</td>
<td>phosphorylated ubiquitin</td>
</tr>
<tr>
<td>pUb&lt;sup&gt;S65&lt;/sup&gt;</td>
<td>phosphorylation of ubiquitin at Ser65</td>
</tr>
<tr>
<td>RBR</td>
<td>RING-between-RING</td>
</tr>
<tr>
<td>REP</td>
<td>repressor element</td>
</tr>
<tr>
<td>RF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>release factor 1</td>
</tr>
<tr>
<td>ΔRF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>release factor 1-deficient</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SepRS</td>
<td>phosphoseryl-tRNA synthetase</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Sep&lt;/sup&gt;</td>
<td>pSer-accepting transfer RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>$\text{Ub}^{\text{IR}}$</td>
<td>$\text{Ub}_{\text{infrared}}$</td>
</tr>
<tr>
<td>Ubl</td>
<td>ubiquitin-like</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Neurodegenerative disorders

1.1.1 Prevalence of neurodegenerative disorders

Neurodegenerative disorders are debilitating conditions typified by progressive degeneration of certain regions in the brain and deposition of abnormal proteins in cells. Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS or Lou-Gehrig’s disease) and Parkinson’s disease (PD) are three of the most common neurological disorders. Reactive oxygen species (ROS) are commonly observed at increased levels in brain tissue samples of individuals with AD, ALS and PD. Increased ROS is related to the relatively high metabolic and low cellular regeneration rates in the brain. Protein nitration, often associated with oxidation of proteins, is present in greater abundance in the hippocampus and neocortex of AD patients, motor neurons of ALS patients and Lewy bodies of PD patients. The increased incidence of ROS and lack of cellular regeneration in the brain renders these diseases almost incurable. Although there are several lines of cellular defense to prevent and repair oxidative damage from ROS, neurodegenerative disorders also have reduced activity of antioxidants, such as a glutathione reduction in PD patients.

The Canadian Institute for Health Information published a report on 11 of the most common neurological conditions in Canada: AD, ALS, brain tumours, cerebral palsy, epilepsy, head injury, headaches, multiple sclerosis, PD, spinal injuries, and stroke. In the 21st century, the Public Health Agency of Canada estimated the direct costs (i.e., treatments and medication) associated with these 11 conditions to be $2.3 billion and their indirect costs (i.e., morbidity and mortality) to be $6.5 billion. Out of the total value for indirect costs associated with these diseases, the costs resulting from morbidity made up 60% to 80% for AD, cerebral palsy, epilepsy, multiple sclerosis and PD. A more recent report published by the Ontario Brain Institute focused on 13 brain disorders similar to the 11 most common neurological conditions, but including motor neuron disease, schizophrenia and spina bifida, and excluding ALS. These brain disorders
increased on average by 1.4 per 1000 persons from 2004 to 2010\(^7\). This increase is attributed to longer survival times due to improved clinical care and earlier diagnosis from advances in detection methods\(^7\). Despite the increased prevalence, effective treatments and cures for many of these neurodegenerative diseases are still far from reality. Effective therapeutic approaches are essential immediately since the costs associated with these neurodegenerative diseases represent a significant burden on the Canadian health care system.

1.1.2 Parkinson’s disease

PD is the second most common neurodegenerative disorder and predominantly affects the older population\(^8\). This chronic, progressive disorder is characterized by the loss of dopaminergic neurons resulting in tremors and postural instability\(^8\). Neurodegeneration occurs in the substantia nigra and locus coeruleus of the brain; the presence of intracytoplasmic protein aggregates termed Lewy bodies in these regions of the brain stem are elevated in patients with idiopathic PD\(^9\).

Autosomal dominant and autosomal recessive inheritance of PD have been identified\(^9\). A plethora of psychological symptoms can also be observed, such as depression, anxiety, psychosis and fatigue\(^8\). The dopamine replacement therapy L-DOPA (i.e., Levodopa or L-3,4-dihydroxyphenylalanine) was the first drug approved for PD\(^10\). L-DOPA is known to improve individual motor symptoms, but there is a large variation in the drug’s effect on the cognitive functions of different PD patients\(^10\). Presently, there is no cure for PD and treatment options, such as L-DOPA, are only used to control the intensity of the symptoms\(^8\).

Several charities, such as Parkinson Society Canada, provide education and advocacy to help alleviate the burden for individuals living with PD\(^11\). However, this does not resolve the persistent need to find a cure for PD. Recent data estimate 0.2% of adults in private households and 4.9% of adults in residential care facilities have PD\(^8\). From 2011 to 2031, the number of Canadians over 65 living with PD was predicted to double due to an increase in the aging population\(^12\). PD patients also had the highest use of prescription
medication\textsuperscript{12}. These facts highlight the importance of determining the etiology of PD in hopes of finding a cure that eventually leads to elimination of PD prevalence.

1.2 Protein quality control

1.2.1 The ubiquitination cascade

Proteins involved in the ubiquitin proteasome system (UPS) have been shown to play a significant role in PD pathogenesis\textsuperscript{13-16}. The UPS is responsible for majority of protein turnover within the cell through covalent ligation of the target substrate with ubiquitin (Ub), a highly conserved 8.5 kilodalton (kDa) protein with 76 amino acid residues (Figure 1.1)\textsuperscript{17}. The highly conserved nature of Ub is evident as human and yeast Ub have 96% amino acid sequence identity\textsuperscript{18}. There are only 3 amino acid residues that are substituted between human and yeast Ub: P19S, E24D and A28S\textsuperscript{18}.

![Figure 1.1: Generalized schematic of the UPS.](image)

E1 Ub-activating enzymes activate Ub in the presence of ATP. Ub is then conjugated to the E2 Ub-conjugating enzymes and the E3 Ub ligases catalyze the transfer of Ub onto the target substrate. Further reactions allow polyubiquitination of the substrate, which lead to subsequent substrate degradation.

Protein ubiquitination via the UPS requires the sequential action of three enzymes\textsuperscript{19}. The carboxy-terminal (C-terminal) glycine residue (G76) of Ub is activated in an ATP-
dependent step by the Ub-activating enzyme named E1. During this step, an intermediate Ub adenylate is formed with the release of inorganic pyrophosphate. A thioester linkage is subsequently formed between the glycine’s carboxyl group of Ub and the sulfhydryl group of a cysteine residue of E1 along with the release of AMP. The E2 Ub-conjugating enzyme then catalyzes the transfer of Ub through a transthioesterification reaction from E1 to the active site cysteine of E2. The final step of this cascade, transfer of Ub chains to substrate proteins, is catalyzed by E3 Ub ligases.

Bioinformatic identifications, based on protein homology, found 2 E1s, 40 E2s and over 600 E3 Ub ligases in the human proteome. The E3 family of enzymes confer the greatest substrate specificity in the UPS and they facilitate the covalent isopeptide linkage between the ε-amino group of a lysine residue of the substrate and the C-terminal carboxylate group of G76 of Ub. Further reactions allow formation of polyUb chains on the substrate through isopeptide bonds between the terminal G76 residue of each Ub unit and a specific lysine residue (most commonly K48) of the previous Ub.

The UPS is involved in several cellular processes, including development, apoptosis, and antigen processing. The involvement of the UPS in development is evident through a defect in the E3 enzyme E6-AP in patients with Angelman syndrome, a brain development disorder. Likewise, inactivation of the human E2 enzyme HR6B homolog in mice causes infertility in male mouse models. The UPS also plays a role in apoptosis induced by γ-irradiation in human lymphocytes as seen by increased Ub gene expression and increasingly ubiquitinated nuclear proteins. Similarly, apoptosis induced by the deprivation of nerve growth factor in sympathetic neurons does not occur when the UPS is inhibited. The UPS also processes many major histocompatibility complex class I antigens.

1.2.2 Parkin, an E3 Ub ligase

The E3 Ub ligase parkin and the deubiquitinating enzyme (Ub C-terminal hydrolase L1) are components in the UPS that are implicated in PD. A substrate for the UPS, α-synuclein, was also found to associated with PD through autosomal dominant inheritance. During oxidative stress, the E3 Ub ligase parkin directs damaged
mitochondria for mitophagy by tagging mitochondrial outer membrane proteins (e.g., the mitochondrial Rho GTPase, MIRO1) with polyUb chains\textsuperscript{26,27}. Although endogenous parkin is predominantly found in the cytosol, mitochondrial accumulation of parkin was observed following treatment of human embryonic kidney (HEK)-293 cells with carbonyl cyanide \textit{m}-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler that induces oxidative damage\textsuperscript{26}.

Parkin localization to the mitochondrial outer membrane was also observed in HeLa cells lines overexpressing parkin\textsuperscript{26}. Following translocation to the mitochondrial outer membrane, parkin is capable of forming K48-linked polyUb chains as well as K63-linked polyUb chains on outer membrane proteins\textsuperscript{28}. The cellular outcome of polyubiquitination is dependent on the topology of the polyUb chain linkage. Mitophagy predominantly occurs in the presence of polyUb chain linkage by K63 and protein degradation by the UPS occurs via K48-linked polyUb chains\textsuperscript{28}. These observations highlight a dual molecular function for parkin E3 Ub ligase activity: polyubiquitination of mitochondrial proteins recruits cellular components needed for mitophagy and acts as a signal for protein degradation\textsuperscript{28}.

The role of parkin E3 Ub ligase activity in mitophagy suggests the importance of parkin in neuronal tissue maintenance\textsuperscript{29}. Not surprisingly, PD-causing mutations in parkin could perturb the maintenance of neuronal tissues and could be key drug targets in PD\textsuperscript{29}. Initial evidence implicating the role of parkin in PD came from genetic mutation studies of \textit{park2} (the gene encoding parkin) showing association with autosomal recessive juvenile parkinsonism (AR-JP). AR-JP is a familial form of PD typified by early age onset\textsuperscript{13}. Loss-of-function mutations in parkin are the primary cause of AR-JP since mutations in \textit{park2} were identified in 50\% of AR-JP patients\textsuperscript{13,30}. Parkin-deficient mice also showed clear evidence of mitochondrial dysfunction and oxidative damage, two key factors leading to PD\textsuperscript{31}. Heterozygous mutations of parkin have also been implicated in the more common, late-onset form of the disease\textsuperscript{32,33}. Furthermore, parkin was shown to function as an E3 Ub ligase involved in neurotoxicity protection during stress by promoting protein degradation\textsuperscript{34-36}.
Human parkin is a 52 kDa protein with 465 amino acid residues. It consists of five domains: Ub-like (Ubl) (1-76), really interesting new gene (RING) 0 (144-216), RING1 (228-327), in-between-ring (IBR) (328-377) and RING2 (415-465). The four RING domains (including the IBR) of parkin bind two Zn$^{2+}$ ions each. To date, there has been only one high-resolution structure reported of parkin with all five of its domains and the repressor element (REP) (Figure 1.2B). However, even this structure does not include the entire sequence of full-length parkin. In order to crystallize parkin along with its amino-terminal (N-terminal) Ubl domain, the amino acid residues following the Ubl domain were truncated and fused to the RING0 domain. The lack of a full crystal structure for parkin was attributed to the highly flexible region between the Ubl and RING0 domain of parkin resulting in decreased stability. Human parkin and rat parkin have 85% amino acid sequence identity. The crystal structure of the truncated human parkin is consistent with the full-length 6.5 Å low-resolution crystal structure of rat parkin.

**Figure 1.2: Domain organization and crystal structure of parkin.** A) Linear domain organization of parkin showing structural boundaries. B) The adapted 1.8 Å resolution crystal structure of human parkin (PDB: 5C1Z) illustrates the five domains coloured as in A. The repressor element (REP) is represented in grey and the eight zinc atoms are represented as yellow spheres.
Initially, parkin was reported to be an E3 Ub ligase since it could ubiquitinate several substrates, including itself\(^{35,36}\). Unmodified parkin, however, exists in an autoinhibited state resulting in weak basal ubiquitination activity for parkin compared to other Ub ligases\(^{40-42}\). The autoinhibition is a result of the close proximity of the Ubl domain to the E2~Ub conjugate binding site that is blocked by the REP linker\(^{40,43}\). Likewise, the interaction between the RING0 and RING2 domain blocks access to the catalytic cysteine residue (C431), which also results in parkin autoinhibition\(^{40,42}\). The disease-linked C431F substitution disrupts parkin E3 Ub ligase activity in both auto-ubiquitination and substrate assays\(^{27,44-46}\). In addition, the recessive nature of AR-JP is linked to parkin auto-ubiquitination occurring in cis as opposed to trans\(^{41}\). The IBR-RING2 of parkin responsible for auto-ubiquitination was inactivated using the disease-linked T415N substitution in full-length parkin and supplemented with an IBR-RING2 candidate substrate in auto-ubiquitination assays\(^{41}\). These assays prevented auto-ubiquitination of full-length parkin, and thus confirmed cis auto-ubiquitination\(^{41}\).

Parkin belongs to the RING-between-RING (RBR) family of E3 Ub ligases\(^{47}\). Traditionally, E3s were classified into two distinct classes based on their structure and mechanism: Homologous to E6-AP C terminus (HECT)-type ligases and RING/UBOX-type ligases\(^{47}\). HECT domains are bilobal and found only at the C-terminal of proteins\(^{47}\). The N-terminal lobe binds the E2 and C-terminal contains the catalytic cysteine residue\(^{47}\). RING domains are globular and can be readily identified by a canonical spacing of conserved cysteine and histidine residues, which bind two Zn\(^{2+}\) ions to stabilize the overall domain structure and allow interaction with E2~Ub conjugates\(^{48}\).

HECT-type ligases and RING/UBOX-type ligases have different mechanisms of action and differ in the enzyme responsible for the aminolysis reaction, which involves the transfer of Ub onto the lysine residue of a substrate\(^{47}\). For reactions catalyzed by HECT-type ligases, the E3 is responsible for the aminolysis, whereas the E2 is responsible in reactions catalyzed by RING/UBOX-type ligases\(^{47}\). The RBR family that parkin belongs to is a hybrid between the HECT- and RING/UBOX-type ligases. RBR family E3 ligases have a N-terminal with a RING-type E2 binding domain and a C-terminal active site\(^{47}\). Unlike RING domains, RBR domains can be found at any location.
on their respective proteins\textsuperscript{47}. The RBR family of E3 ligases consists of 15 proteins from which parkin is functionally the best characterized\textsuperscript{49}. Post-translational regulation and autoinhibition are key characteristics of this family of E3 ligases, and parkin is no exception\textsuperscript{47}. Pathogenic parkin mutations disrupt the autoinhibition resulting from the interaction of the Ubl domain with the RBR region in parkin\textsuperscript{43}. Disruption in parkin autoinhibition leads to constitutively active parkin\textsuperscript{43}. We speculate that constitutively active parkin could lead to premature degradation of parkin. Constitutively active parkin may auto-ubiquitinate itself, and thus signal for its own degradation by the UPS before parkin reaches the mitochondria. Premature parkin degradation is expected to inhibit mitophagy.

1.3 Post-translational modifications

1.3.1 Parkin

Parkin was initially found to be S-nitrosylated \textit{in vivo} in PD mouse models and PD patient brain tissue samples\textsuperscript{50,51}. S-nitrosylation is a post-translational modification (PTM) that inhibits parkin E3 Ub ligase activity, and diminishes subsequent substrate ubiquitination and the neuroprotective function of parkin\textsuperscript{50,51}. Three cysteine residues (C59, C95 and C182) on parkin were also observed to undergo S-sulfhydration, leading to an increase in parkin E3 Ub ligase activity\textsuperscript{52}. Decreased S-sulfhydration and increased S-nitrosylation have been observed in PD patient brain samples, which is consistent with the respective impact of these modifications on parkin activity\textsuperscript{50-52}. A less recognized PTM called neddylation occurs on parkin and increases its E3 Ub ligase activity by enhancing its interaction with E2 enzymes and substrates\textsuperscript{53,54}. Parkin can also be phosphorylated to increase or decrease its activity\textsuperscript{55-57}.

1.3.2 Ubiquitin

The parkin substrate Ub can itself be modified\textsuperscript{58}. Proteomic and chromatographic studies have identified Ub acetylation, deamidation and phosphorylation sites in human cells\textsuperscript{58}. Acetylation of Ub at either of two specific lysine residues (K6 or K48) disrupts polyUb chain elongation linked by K11, K48 (predominantly seen in the UPS) and K63\textsuperscript{59}. Acetylated Ub can be transferred onto the E1 and E2 enzymes in the UPS, but blocks the
final step of the pathway where Ub chains are transferred to substrate proteins to target them for degradation\textsuperscript{59}. Mass spectrometric evidence identified Ub acetylation \textit{in vivo} where 0.03\% and 0.01\% of total Ub in cells was acetylated on K6 and K48, respectively\textsuperscript{59}. Deamidation is the irreversible conversion of an amide to an acid and this is another PTM that can occur on Ub\textsuperscript{60}. Ub is deamidated at a glutamine residue (Q40) to generate a glutamic acid residue (E40) in its place by the cycle inhibiting factor homolog in \textit{Burkholderia pseudomallei} also known as CHBP\textsuperscript{60}. This single amino acid substitution blocks discharge of Ub from the E2 to the E3 in the UPS, but does not affect the formation of E1~Ub or E2~Ub conjugates\textsuperscript{60}.

Phosphorylation of Ub is known to occur at several conserved amino acid residues: T7, T12, T14, S20, S57, Y59 and S65\textsuperscript{58,61} (Figure 1.3). In addition to proteomics, Ub phosphorylation has been detected by phosho-specific antibodies in the case of Ub phosphorylated at S65 (pUb\textsuperscript{S65})\textsuperscript{61}. All of the experimentally identified phosphorylation sites on Ub are strictly conserved from human to yeast\textsuperscript{58}. In addition, phosphorylation of Ub was observed at T7, S20, S57 and S65 in mouse as well as S20 and S57 in rat\textsuperscript{61}. For all of these sites, except S65, the respective kinases and phosphatases remain unknown\textsuperscript{58}. In addition, the molecular function and abundance in cells of phosphorylated Ub (pUb) species have yet to be defined in all cases except for pUb\textsuperscript{S20} and pUb\textsuperscript{S65} \textsuperscript{58,62}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Ub_structure.png}
\caption{Crystal structure of Ub with locations of observed phosphorylation sites.} The serine and threonine phosphorylation sites of human Ub (PDB: 1UBQ) are indicated: T7, T12, T14, S20, S57 and S65. The lysine residue (K48) most responsible for polyUb chain linkage in the UPS is also indicated.
\end{figure}
1.4 Phosphorylation

1.4.1 Activating parkin with phosphorylation

It has been recently discovered that upon mitochondrial membrane potential depolarization, phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1) is responsible for phosphorylation of parkin at a particular serine residue (S65) located in the Ubl domain of parkin\textsuperscript{57}. Phosphorylation at S65 of parkin leads to a release of autoinhibition and subsequent activation of the E3 Ub ligase activity\textsuperscript{57}. PINK1 also phosphorylates the homologous site, S65, in the substrate Ub to stimulate parkin activity\textsuperscript{63-66}.

Analysis of phosphopeptides using mass spectrometry enabled identification of pUb\textsuperscript{S65} in mitochondria isolated from wild-type cells, but not from PINK1 knockout cells following oxidative stress induced by CCCP treatment\textsuperscript{63}. A 14-fold enrichment of pUb\textsuperscript{S65} was observed in HEK-293 cells overexpressing active PINK1 as opposed to HEK-293 cells with kinase-inactive PINK1\textsuperscript{64}. In addition to the phosphorylation of single Ub molecules, PINK1 can also phosphorylate Ub in polyUb chains\textsuperscript{66}. In addition, optimal activation of parkin is only evident in the presence of PINK1-mediated phosphorylation of S65 in both parkin and Ub\textsuperscript{63-65,67}. Although the detailed mechanism of parkin activation has yet to be completely defined, several groups have presented insightful yet, in some cases, contradictory studies on the mechanism of parkin activation and polyUb chain building.

One proposed mechanism is that PINK1 phosphorylates parkin and Ub, which then proceeds to alleviate autoinhibition to stimulate parkin leading to a feed-forward model of parkin activation\textsuperscript{63,65}. On the contrary, other studies suggested phosphorylation of parkin primes the enzyme for activation by pUb\textsuperscript{S65,64,67}. It has also been suggested that phosphorylation of parkin by PINK1 occurs following parkin interaction with pUb\textsuperscript{S65} that is present in the mixture of polyUb chains on mitochondrial outer membrane proteins\textsuperscript{67}. This secondary recruitment of parkin is thought to directly activate parkin and enhance ubiquitination of mitochondrial outer membrane proteins with less specificity than phosphorylated parkin\textsuperscript{67}. The phosphorylation of parkin following interaction with
pUb$^{S65}$ could also enhance phosphorylation of parkin since parkin and PINK1 would be in close proximity$^{67}$.

Accelerated discharge of E2 was also observed in the presence of pUb$^{S65}$ as well as in the presence of parkin phosphorylated at Ser65$^{27,65,66}$. Isothermal titration calorimetry experiments have revealed that pUb$^{S65}$ binds to full-length parkin with greater than a 100-fold increase in binding affinity than the interaction between Ub and parkin$^{37,68}$. In addition, the binding affinity between parkin phosphorylated at Ser65 and pUb$^{S65}$ is about 10 times stronger than that between unmodified parkin and pUb$^{S65}$ $^{37,68}$.

Phosphorylation of parkin has also been found to be important in substrate ubiquitination$^{27}$. Parkin tags MIRO1 with polyUb chains at highly conserved lysine residues (K153, K230, K235, K330 and K572) following phosphorylation at Ser65$^{27}$. PolyUb chain formation on MIRO1 targets the protein for degradation, which is an important route to mitophagy$^{26,27}$. Mitophagy reduces mitochondrial oxidative stress and promotes cell survival. Without the clearance of damaged mitochondria in the brain, ROS will accumulate as seen in PD patients. It is interesting to note that a cohort of studies regarding pUb$^{S65}$ $^{63-66}$ only emerged following the discovery of its upstream kinase, PINK1$^{69}$.

The use of phosphomimetics in Ub and parkin (S65D/E) to study the mechanism of parkin activation was ultimately misleading$^{67}$. In vitro biochemical studies showed phosphomimetic mutations at S65 in Ub led to a loss of function, while ample evidence demonstrate pUb$^{S65}$ stimulates parkin activity$^{62-67}$. Proteomics is highly valuable and indispensable at identifying phosphorylation sites, but these methods do not measure enzymatic activity and insight into signalling mechanisms is limited to correlative studies. Biochemical and biophysical approaches with pure pUb variants, when combined with data from correlative and cell-based studies will provide a complete description of the function and impact of specific Ub phosphorylation events.

1.4.2 Site-directed phosphorylation of Ub

In addition to pUb$^{S65}$, there are 6 other experimentally verified phosphorylation sites on
Ub (T7, T12, T14, S20, S57 and Y59) for all of which the upstream kinases remain unknown. The function of Ub phosphorylated at these sites and their effect of UPS activity has yet to be determined. A major impediment to the elucidation of the function of Ub phosphorylation sites is the inability to produce site-specifically phosphorylated Ub variants. Due to the lack of knowledge on their respective kinases, there is a pressing need for alternate techniques to produce site-specifically phosphorylated Ub at all identified phosphorylation sites.

Genetic code expansion strategies enable production of site-specifically phosphorylated proteins in *Escherichia coli* (*E. coli*) and is an effective method to produce phosphoproteins. Phosphoprotein production using genetic code expansion strategies is especially valuable when the upstream kinase is unknown or difficult to obtain. There are more than 100,000 experimentally observed phosphorylation sites; for more than 90% of these sites, the upstream kinase remains unknown. The site-specific phosphorylation method optimized here reassigns the UAG stop codon to O-phospho-L-serine (pSer). A system for site-specific incorporation of phosphothreonine has yet to be developed. Since threonine and serine residues only differ by one methyl group, pSer incorporation at these positions will provide more accurate mechanistic insight than phosphomimetic mutants.

The pSer system consists of a pSer-accepting transfer RNA (tRNA<sub>Sep</sub>), its cognate phosphoseryl-tRNA synthetase (SepRS) and engineered elongation factor (EF)-Tu (EFSep). Mutation of the bacterial EF-Tu to EFSep to bind the tRNA<sub>Sep</sub> charged with pSer was required to enable translation with pSer. EFSep was engineered by identifying 6 mutations in EF-Tu that removed two negative charges in EF-Tu and introduced one positively charged amino acid to the binding pocket of EF-Tu: H67R, E216N, D217G, F219Y, T229S and N274W. This changed the charge and shape of the aminoacyl-tRNA recognition pocket so that the negatively charged pSer-tRNA<sub>Sep</sub> can be accommodated in protein synthesis.

Further mutations to enhance the pSer system and potentially overcome previously inefficient pSer incorporation led to the development of SepRS9 and EFSep21 (Figure...
1.4) SepRS9 was constructed by introducing 4 mutations in the anticodon-binding domain (E412S, E414I, P495R and I496R) and 3 additional spontaneous mutations (K347E, N352D and L512I) in SepRS. EFSep21 was constructed by introducing the substitution mutation N216V in EFSep. This improved system was expressed in BL21(DE3) and produced 3 mg·L⁻¹ E. coli culture of recombinant pSer-containing histone H3.

1.4.3 Competition and mistranslation at UAG

Another important feature of the pSer system is the use of orthogonal aminoacyl-tRNA synthetase:tRNA pairs (i.e., SepRS:tRNA-Sep). As with most genetic code expansion strategies, an amber suppressor (UAG-decoding) tRNA is used and this enables reassignment of the UAG stop codon to pSer. A limitation to orthogonal translation is low protein yield due to competition between peptide chain elongation with the aminoacylated suppressor tRNA and termination by release factor 1 (RF1).

Figure 1.4: The incorporation of pSer using genetic code expansion. The UAG codon in Ub is reassigned to pSer in E. coli in the presence of SepRS9, EFSep21 and tRNA-Sep.

This competition was overcome by the use of a RF1 deficient E. coli strain where all genomic TAG terminating coding sequences were replaced with synonymous TAA using a genome editing technique. Although the newly engineered E. coli strain had an increased pSer incorporation efficiency per cell, it was found to be deleterious to cell fitness and viability as seen by increased doubling time and decreased final cell density.
A common misconception associated with genetic code expansion strategies (i.e., the pSer system) is that amber codon translation has the same fidelity as the canonical protein synthesis\(^{80}\). Using a novel approach that facilitated characterization of organisms with expanded genetic codes, the incorporation of noncanonical amino acids (ncAAs) and natural amino acids at UAG in a recombinant green fluorescent protein (GFP) was detected in the pSer system\(^{80}\). This method also identified mistranslation as a common by-product of the pSer system in RF1 deficient \textit{E. coli}\(^{80}\). Near-cognate mis-incorporation of glutamine was most commonly observed with evidence of lysine, tyrosine and glutamic acid mis-incorporation in lower abundance than pSer\(^{80}\). The absence of RF1 is thought to be a major contributor to increased levels of mistranslation\(^{62,80}\). In a newly designed plasmid containing 5 copies of tRNA\(^{\text{Sep}}\), this mistranslation was observed to a lesser degree and pSer incorporation was found to be the most abundant\(^{80}\). Using the most efficient pSer system (SepRS\(^9\)/tRNA\(^{\text{Sep}}\) and EFSep\(^{21}\)) in RF1 deficient \textit{E. coli} did not eliminate this mistranslation, but greater yields of pSer per litre of \textit{E. coli} culture were produced compared to previously tested systems using SepRS/tRNA\(^{\text{Sep}}\) and EFSep with recombinant GFP\(^{81}\). Although a higher yield of pSer was obtained using SepRS\(^9\)/tRNA\(^{\text{Sep}}\) and EFSep\(^{21}\), Phos-tag gels show evidence of non-phosphorylated protein present in the mixture\(^{81}\). In addition to mass spectrometry, we found that Phos-tag gels are an efficient and effective tool to determine the presence and purity of phosphorylated proteins. The gel system relies on the binding of a divalent metal ion (Mn\(^{2+}\) or Zn\(^{2+}\)) to Phos-tag acrylamide, a proprietary reagent\(^{82,83}\). The divalent metal ion can bind to phosphate groups on proteins and retard their migration rate on gels\(^{82,83}\). An important factor that is often overlooked in proteins encoding ncAAs is the purity of the sample. Due to significant levels of mistranslation, careful consideration should be placed on yields reported as this could reflect total protein yield (including mistranslated products) as opposed to pure ncAA-containing protein yield.

1.5 Scope of thesis

1.5.1 Rationale

There are many traditional methods that have been used to investigate the effects and function of phosphorylation \textit{in vivo}, in cell culture models and \textit{in vitro}\(^{84}\). Genetic
methods are often used to either disable or mimic phosphorylation. Proteins with alanine mutations at known phosphorylation sites are unable to be phosphorylated at the site of the alanine mutation. Aspartic acid and glutamic acid residues are commonly used as phosphomimetic substitutions due to the single negative charge and somewhat similar structure to pSer. Despite their chemical structure similarity, it is not clear that phosphomimetics accurately mimic phosphorylation. In one example, the glutamic acid mutant of the mitogen-activated ERK activating kinase 1 reveals >70-fold lower activity when compared to a true pSer residue. Though alanine mutant and phosphomimetic substitution experiments provided important data and are useful probes of signalling pathways, they are often unable to isolate and characterize the molecular role of a specific phosphorylation or of multiple phosphorylations in specific combinations or stoichiometry.

The ability to produce designer phosphoproteins in vitro allows precise control over the stoichiometry of phosphorylation and provides biochemical, structural and mechanistic insights into the activity of these phosphoproteins. Using in vivo studies to determine the mechanism of action is complicated by interacting cellular proteins and other PTMs in cells. Cell-based studies are often correlative, and complementary biochemical studies are needed to provide a detailed mechanistic description of phosphoprotein function. The many confounding variables in vivo make it complicated to identify interactions between individual proteins, a crucial aspect in exploring the biological functions of proteins.

Since most of the phosphorylation sites on Ub are associated with unidentified kinases, genetic code expansion is an ideal strategy to produce otherwise inaccessible phosphoproteins. The efficiency of producing pSer in great abundance using the pSer system in vitro with SepRS9/tRNA<sup>Sep</sup> and EF-Sep21 makes this system an effective tool. Here, we use this method to study the effect of pUb variants on parkin E3 Ub ligase activity by site-specifically incorporating pSer at experimentally verified phosphorylation sites on Ub<sup>58,61</sup>.
1.5.2 Hypothesis

In addition to the previously identified pUb\textsuperscript{S65}, I hypothesize that parkin can be stimulated by other pUbs (i.e., pUb\textsuperscript{S12} and pUb\textsuperscript{S20}). My hypothesis will be tested by producing pure site-specifically phosphorylated Ub using the pSer system and measuring the effect of the pUb variants on parkin activity in auto-ubiquitination assays.

1.5.3 Findings and significance

In this thesis, phosphorylated Ub was produced for the first time in high yield and high purity. Production of pUb variants using the pSer system in release-factor deficient \textit{E. coli} cells revealed a new by-product of mistranslation where the in-frame UAG codon was skipped resulting in a novel phenomenon called codon skipping. This is the first published report of +3 frameshift.

The precise stoichiometric measurements of pUbs needed to stimulate parkin E3 Ub ligase activity were also investigated. Using molecular biology and biochemical techniques, the work presented in this thesis has advanced the molecular understanding of the effect of pUb on parkin activation and identified new roles for different pUbs in the activation of parkin E3 Ub ligase activity. The ability to precisely modulate the activity of parkin, as we have demonstrated here, will have implications for novel strategies to treat PD.

1.6 References


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Chapter 2

2 Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping

The activity of the Parkinson’s disease (PD)-linked E3 ubiquitin (Ub) ligase parkin is stimulated by phosphorylation of ubiquitin at Ser65 (pUb$^{S65}$). The role of other Ub phospho-sites and their kinases are unknown. We produced phosphorylated Ub (pUb) variants (pUb$^{S7}$, pUb$^{S12}$, pUb$^{S20}$, pUb$^{S57}$, pUb$^{S65}$) by genetically encoding O-phospho-L-serine with the UAG codon. In release factor 1-deficient (ΔRF1) *Escherichia coli* (*E. coli*), intended to enhance UAG read-through, we discovered Ub variants lacking the UAG-encoded residue, demonstrating previously undocumented +3 frameshifting. We successfully purified each pUb variant from mistranslated products. While pUb$^{S20}$ failed to stimulate parkin, parkin was partially active with pUb$^{S12}$. We observed significant ubiquitination when pUb$^{S65}$ was the only Ub variant in the reaction.

2.1 Introduction

In oxidative stress, the E3 Ub ligase parkin is recruited to mitochondria to tag several outer membrane proteins (i.e., Miro-1, Miro-2) with Ub chains that signal protein degradation and initiate mitophagy$^1$. The role of parkin in clearing defective mitochondria is disrupted in PD patients, and the inability for PD-linked mutants to achieve normal levels of mitochondrial degradation is thought to contribute to PD$^2$. The parkin substrate Ub was recently found to have at least seven experimentally observed phosphorylation sites in human cells$^3$-$^6$. As a result of the discovery of an upstream kinase, phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1)$^7$, specific for Ub Ser65, a series of studies followed that show phosphorylation at Ser65 in Ub stimulates parkin auto-ubiquitination activity$^8$-$^{12}$. In genetic studies, expression of the mutant Ub Ser65Ala reduced poly-Ub chain synthesis and mitophagy in osteosarcoma cells$^{10}$. The role of other Ub phosphorylation sites and the associated kinases are unknown$^2$. 
Genetic code expansion with pSer in *E. coli* enables biosynthesis of site-specifically phosphorylated recombinant protein by reassigning the UAG codon to pSer\textsuperscript{13}. The system requires a phosphoseryl-tRNA synthetase (SepRS), derived from an archaeal pathway for Cys-tRNA\textsubscript{Cys} synthesis, a mutant pSer-accepting transfer RNA (tRNA\textsuperscript{Sep})\textsuperscript{14}, and a mutant elongation factor (EF-Sep)\textsuperscript{13}. The pSer system is toxic to normal *E. coli* strains and initial experiments showed phosphoprotein production was possible but inefficient (~1 µg·L\textsuperscript{-1} culture)\textsuperscript{15}. When the pSer system is expressed in a ΔRF1 *E. coli* strain that lacks all genomic TAG codons\textsuperscript{16}, improved phosphoprotein production was observed\textsuperscript{15,17}. The phosphoprotein was, however, contaminated by incorporation of natural amino acids as a result of near-cognate UAG decoding\textsuperscript{17}, limiting the use of this approach where purified phosphoproteins are required, especially for preparative biochemical experiments. We optimized phosphoprotein expression and purification to efficiently generate and purify multiple pUb variants. We conducted an initial examination of parkin E3 Ub ligase activity *in vitro* with purified pUb variants that allowed us to precisely control the stoichiometry of phosphorylation in the Ub substrate pool.

### 2.2 Materials and Methods

#### 2.2.1 Bacterial strains and plasmids

Recombinant proteins were expressed in BL21(DE3) (Invitrogen, Carlsbad, CA, USA), or in *E. coli* ΔRF1 (C321.DA.exp, Addgene 49018) as specified. The 1\textsuperscript{st} generation pSer system is on the plasmid pKD-pSer1 (Addgene 52054) and the 2\textsuperscript{nd} generation system is on the plasmid pDS-pSer2 (see Section 2.6.1). The Ub constructs were expressed from pDS0-Ub (see Section 2.6.1).

#### 2.2.2 Phosphoprotein production and purification

Either pKD-pSer1 or pDS-pSer2 and a pDS0-Ub expression plasmid were freshly cotransformed into *E. coli* ΔRF1 (i.e., C321.DA.exp\textsuperscript{16}) or BL21(DE3) cells on selective media and grown as specified in Section 2.6.1. Cells were harvested and resuspended in ~10 mL wash buffer (50 mM Tris-HCl, 200 mM NaCl, 0.25 mM TCEP, 50 mM NaF, 2 mM NaVO\textsubscript{3}, 25 mM imidazole, pH 8.0) per litre culture. 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 tablet of ethylenediaminetetraacetic acid (EDTA)-free mini
protease inhibitor cocktail (Roche, Mississauga, ON, USA) were added to the cells. Cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, ON, Canada), centrifuged at 41,000 xg to remove cell debris, and filtered through a 0.45-µm filter for use on a gravity column or an AKTA Pure L1 fast protein liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, UK) (see Section 2.6.1). To eliminate contaminating high molecular weight proteins, fractions were subjected to a Superdex75 gel filtration column (GE Healthcare) pre-equilibrated in 25 mM HEPES, 100 mM NaCl, 0.25 mM TCEP pH 7.0. The pUb was then purified from unphosphorylated Ub using ion-exchange chromatography (see Section 2.6.1).

2.2.3 In vitro auto-ubiquitination assays

All reactions contained 0.1 µM E1 (Uba1 – see Section 2.6.1), 1 µM E2 (UbcH7), 1 µM E3 (parkin), 5 mM ATP, and a 20-µM final concentration of Ub and/or pUb in a 20-µL final reaction volume incubated at 30°C for 1 h. The reaction buffer contained 5 mM MgCl₂ and 50 mM HEPES pH 7.4. Reactions were quenched with sodium dodecyl sulfate (SDS)-loading buffer and subjected to immunoblotting using anti-parkin primary antibody (kind gift from Dr. Michael Schlossmacher, University of Ottawa) and fluorescent-labelled secondary antibody (Mandel Scientific, Guelph, ON, Canada). Blots were visualized using the Li-COR® Odyssey Infrared Imaging System.

2.3 Results

2.3.1 Efficiency of phosphoprotein biosynthesis

Genetic code expansion has relied on using stop codons (normally UAG) to insert noncanonical amino acids (ncAAs) into proteins. Competition with release factor 1 (RF1) is a limiting factor that depresses ncAA-containing protein yield. Several groups have engineered ΔRF1 E. coli strains to enhance UAG translation efficiency. We quantified pSer incorporation in two E. coli strains. A plasmid (pKD-pSer1) bearing the 1st generation pSer system (SepRS, EF-Sep and five tRNA^Sep expression cassettes) was coexpressed with a wild-type green fluorescent protein (GFP) reporter or GFP with a UAG codon at position 17. In BL21(DE3), we achieved 13% translational read-through of the UAG codon with pSer compared to normal sense codon translation (100%) (Figure
2.1) Background translation of the UAG codon in the absence of the pSer system in this strain is minimal (5%).

The same constructs were expressed in the ΔRF1 *E. coli* strain C321.ΔA.exp, in which all 321 genomic TAGs were mutated to TAA. This strain shows sense codon-like readthrough of the UAG codon when the pSer system is expressed (Figure 2.1). In the absence of the pSer system, natural aminoacyl tRNA with near-cognate anticodons decode UAG leading to 60% read-through (Figure 2.1). Although the ΔRF1 *E. coli* produces phosphoprotein efficiently per cell, contamination with natural amino acids impedes pure phosphoprotein synthesis.

Figure 2.1: Efficiency of UAG translation with pSer in (A) *E. coli* BL21(DE3) compared to (B) *E. coli* ΔRF1 (C321.ΔA.exp). In both panels, fluorescence per cell (GFP Fluorescence/OD\_600) corresponding to wild-type GFP expression (GFP WT), coexpression of the 1st generation pSer system with GFP containing a UAG codon at position 17 (GFP UAG17+pSer), expression of the GFP UAG17 variant without the pSer system (GFP UAG17-pSer), and background fluorescence (gray dashed line) from cells not expressing GFP (-GFP) are shown.

2.3.2 Impact on growth from expanding the genetic code with pSer

Expression of the pSer system in common *E. coli* strains, such as BL21(DE3), leads to significant growth defects. The pSer system is intended to direct incorporation of pSer at a UAG codon in a recombinant protein transcript, but this is not the only UAG codon in the transcriptome. There are 321 *E. coli* genes that terminate protein synthesis with a UAG codon, and pSer is incorporated in at least some of the corresponding protein products that are then unnaturally extended to the next stop codon. This unnatural protein extension is thought to lead to the observed phenotypic growth defects.
The \textit{E. coli} ΔRF1 strain used here (Figure 2.1B) lacks all genomic \textit{TAG} codons\textsuperscript{16}. Unnatural protein extension induced by the pSer system expression should be totally eliminated in this strain, which we hypothesized would in turn eliminate the growth defect. We measured the growth of several \textit{E. coli} ΔRF1 strains transformed with either empty plasmids or with a GFP-expressing plasmid (wild-type or UAG17) and with or without coexpression of pSer system components (Figure 2.2). In all strains tested, addition of the free amino acid pSer stimulated growth and led to higher final cell densities.

![Figure 2.2: Impact of genetic code expansion with pSer on bacterial growth in \textit{E. coli} ΔRF1.](image)

\begin{itemize}
  \item \textbf{A} Growth curves are shown for \textit{E. coli} ΔRF1 strains expressing the indicated GFP constructs with the specified components of the 1\textsuperscript{st} generation pSer system.
  \item \textbf{B} Growth statistics for \textit{E. coli} ΔRF1 expressing indicated components of the pSer system include: doubling time (top), maximal OD\textsubscript{600} (middle), lag time (bottom).
\end{itemize}

Interestingly, cells expressing an incomplete pSer orthogonal translation system (i.e., lacking tRNA\textsubscript{Sep} or lacking SepRS/EFSep) showed similar doubling times, but with a sharply reduced duration of the exponential phase and a maximal cell density that was 50\% less than \textit{E. coli} ΔRF1 cells expressing a complete pSer system (Figure 2.2). In comparing \textit{E. coli} ΔRF1 to strains expressing GFP and the complete pSer system, the growth curves and doubling times are essentially identical. Thus, the growth defects
observed upon expressing the pSer system in *E. coli* BL21(DE3)\(^\text{15}\) were mostly eliminated in *E. coli* \(\Delta\text{RF1}\.\)

### 2.3.3 Characterization of purified pUb

pUb\(^{\text{S65}}\) was produced by the coexpression of the Ub-UAG65 construct with the 1\(^{\text{st}}\) generation pSer system in *E. coli* \(\Delta\text{RF1}\.\) Following His\(_6\)-tag affinity chromatography, the Ub variant was analyzed by electrospray ionization mass spectrometry (ESI-MS) (Figure 2.3A). Two major mass peaks were observed corresponding to two full-length protein products: one with pSer65 (pUb\(^{\text{S65}}\)) and one with Gln65. This protein mixture was further purified using ion-exchange chromatography, which showed the two well-defined peaks, consistent with the two mass peaks observed (Figure 2.3B). The identity of pUb\(^{\text{S65}}\) was assessed by MS/MS (Figure 2.3C) and by Phos-tag gels\(^\text{23}\), which retard phosphorylated proteins so that a band-shift separates unphosphorylated Ub from pUb (Figure 2.4). As a comparison to genetically encoded pUb\(^{\text{S65}}\), pUb was also produced starting with unmodified Ub that was then phosphorylated with the upstream kinase PINK1 (pUb\(^{\text{S65}-k}\)). The enzymatically phosphorylated Ub was separated from the reaction components and unreacted Ub by ion-exchange chromatography resulting in homogenous and pure pUb\(^{\text{S65}-k}\) (Figure 2.4A). The purified genetically encoded pUb\(^{\text{S65}}\) shows the same band-shift without evidence of unphosphorylated Ub in the final ion-exchange fraction (Figure 2.4B). Tryptic digests of the ion-exchange chromatography fractions were further analyzed by MS/MS (Figure 2.3C), confirming unambiguously the homogenous purification of pUb\(^{\text{S65}}\) at a yield of 11 \(\mu\text{g} \cdot \text{L}^{-1}\) *E. coli* culture (Table S2.1).

### 2.3.4 Optimization of pUb\(^{\text{S12}}, \text{pUb}\(^{\text{S20}}, \text{pUb}\(^{\text{S57}}, \text{and pUb}\(^{\text{S65}}\) production

Parkin is also phosphorylated by PINK1 in its amino-terminal Ub-like (Ubl) domain at Ser65\(^\text{24,25}\). Expression of the Ubl domain with pSer65 (pUbl\(^{\text{S65}}\)) leads to similar purity (Figure S2.8) and yield (Table S2.1) as observed for pUb\(^{\text{S65}}\) produced with the 1\(^{\text{st}}\) generation pSer system in *E. coli* \(\Delta\text{RF1}\.\) Other pUb variants (pUb\(^{\text{S7}}, \text{pUb}\(^{\text{S57}}\)) were also produced with the 1\(^{\text{st}}\) generation pSer system in *E. coli* \(\Delta\text{RF1}\) and purified to homogeneity (Figure S2.9A,B), yet the yield was < 1 \(\mu\text{g} \cdot \text{L}^{-1}\) *E. coli* culture (Table S2.1).
To enhance production of different pUb variants, we introduced 2nd generation pSer system mutations into the pKD-pSer1 plasmid to yield pDS-pSer2. This pSer insertion system is more efficient and able to compete against RF1, so the protein production host was switched to BL21(DE3). Because near-cognate decoding of UAG is minimal in

Figure 2.3: Purification of genetically encoded pUbS65 from ΔRF1 E. coli. (A) Full-length protein ESI-MS indicates full-length protein products with Gln or pSer at position 65. (B) Ion-exchange chromatogram shows distinct separation of phosphorylated from unphosphorylated protein products. (C) MS/MS of tryptic-digested pUb run in positive ion mode (+0.1% formic acid) confirms identity of pure Ub phosphorylated Ser65 (s).

Figure 2.4: Comparison of the purity of kinase phosphorylated (A) and genetically encoded (B-D) pUb variants. (A) Preparation of pUbS65 with the upstream kinase PINK1 is shown on a Phos-tag gel; unmodified Ub (lane 1), PINK1 phosphorylated and purified pUbS65 (lane 2). (B) Fractions from ion-exchange chromatography (e.g., Figure 2.3B) that separated the contaminant Ub Gln65 (lanes 1 and 2) and pure genetically encoded pUbS65 (lane 3). (C) Ion-exchange chromatography fractions of mistranslated (unphosphorylated) Ub (lanes 1 and 2) from His6-pUbS20 (lanes 3 and 4). (D) Ion-exchange chromatography fractions of mistranslated (unphosphorylated) Ub (lanes 1 and 2) separated from pure His6-pUbS12 (lanes 3 and 4).
BL21(DE3) (Figure 2.1A), we anticipated this strain would produce only phosphorylated Ub and ion-exchange chromatography purification might be unnecessary. The yield of all pUb variants was increased 10 to 100-fold compared to the 1st generation pSer system (Table S2.1). In addition, several pUb variants (pUbS12, pUbS20, pUbS57) were all purified successfully (Figures 2.4C,D and S2.9C,E) at the milligram scale.

Near-cognate decoding of UAG was reduced in BL21(DE3) compared to E. coli ΔRF1, but unphosphorylated Ub was still produced in BL21(DE3) (Figure 2.4C,D). For the unphosphorylated Ub produced by the UAG12 variant (Figure 2.4D, lanes 1 and 2), MS/MS identified Gln at the UAG encoded site (Figure S2.9D). All experiments showed that unphosphorylated Ub resulted from mistranslation. We found no evidence of dephosphorylation (i.e., Ser at UAG encoded loci). Maximal phosphoprotein production was observed in BL21(DE3) with the 2nd generation pSer system (300 µg·L⁻¹ E. coli culture of pure pUbS20). In contrast, expression of the 2nd generation pSer system in E. coli ΔRF1 gave at most 8 µg·L⁻¹ (Table S2.1). The data suggest that in E. coli ΔRF1, pSer incorporation efficiency is not the limiting factor for recombinant phosphoprotein synthesis.

2.3.5 UAG codon skipping is a unique by-product of orthogonal translation

Full protein ESI-MS of pUbS7 and pUblS65 revealed a third and unexpected protein species. For Ub, peaks corresponding to pUbS7 and Ub Gln7 (Figure 2.5A) were observed and confirmed by MS/MS (Figure 2.5B). Likewise, for the parkin Ubl domain, pUblS65 and Ubl Gln65 were identified (Figure 2.6). However, in both cases signals corresponding to Ub lacking residue 7 (Figure 2.5A) and the Ubl domain lacking residue 65 (Figure 2.6A) were also identified. This previously unknown interpretation of the UAG codon was verified by MS/MS (Figures 2.5D and 2.6D). Glu-C digestion of the UbΔ7 protein isolated by ion-exchange chromatography identified a peptide (NLYFQSNAMQIFVK66L76TGKTITLE) that resulted from peptidyl transfer between position 6 and position 8 of the Ub variant expressed with a UAG codon at position 7. Similarly, the UblΔ65 protein product was digested with trypsin and the resulting peptide (VQNCDDLQQ64I66VHVQRPWR) confirmed deletion of the amino acid at position 65 and formation of a peptide bond between positions Gln64 and Ile66. Because we
suspected this might result from a contaminating plasmid lacking the UAG locus, the Ubl expression plasmid was freshly transformed and 10 independent clones sequence verified, showing no evidence of a deletion of the codon in the plasmid. Two of these reverified Ubl UAG65 constructs were expressed with the pSer system in ΔRF1 *E. coli*. The Ubl domain Δ65 protein product was robustly reproduced (Figure S2.10).

**Figure 2.5:** Codon skipping in a Ub variant with an in-frame UAG codon at position 7. (A) Full protein mass from ESI-MS run in positive ion mode (+0.1% formic acid) for the His6-pUbS7 variant shows peaks corresponding to translation of UAG as pSer, Gln, and as a single amino acid deletion (Δ7). Insertion of pSer (B) and Gln (C) in Ub at position 7 was confirmed by MS/MS run in positive ion mode (+0.1% formic acid). (D) MS/MS run in positive ion mode (+0.1% formic acid) of the Ub UAG7 construct also shows UAG was translated as a deletion.
To further investigate UAG codon skipping, we expressed the Ub UAG7 construct in *E. coli* ΔRF1 lacking specified components of the pSer system. MS/MS confirmed identity of the amino acids inserted at the UAG codon (Figure S2.11). Interestingly, the Ub Δ7 protein was only produced in the presence of the complete pSer system when exogenous pSer was added to the growth medium. Together, these results indicate that in *E. coli* ΔRF1, the UAG codon can be skipped or bypassed by the ribosome to yield proteins with a single residue deleted (UbΔ7 and UbΔ65).

**Figure 2.6: Codon skipping in a Ubl domain variant with an in-frame UAG codon at position 65.**
(A) Full protein mass from ESI-MS run in positive ion mode (+0.1% formic acid) for the pUbl665 variant shows peaks corresponding to translation of UAG as pSer, Gln, and as a single amino acid deletion (Δ65). Insertion of pSer (B) and Gln (C) in the Ubl domain at position 65 was confirmed by MS/MS run in positive ion mode (+0.1% formic acid). (D) MS/MS run in positive ion mode (+0.1% formic acid) of the Ubl domain UAG65 construct also shows UAG was translated as a deletion.
2.3.6 Parkin ligase activity is differentially modulated by pUb variants

PINK1 is the upstream kinase known to phosphorylate Ub specifically at Ser65. Non-covalent interaction of pUbS65 with parkin stimulates the ubiquitination activity of parkin10,11. In order to assess the integrity of genetically encoded pUbS65 to stimulate parkin ubiquitination activity, we compared its effect in auto-ubiquitination assays with PINK1 phosphorylated Ub (i.e., pUbS65-k, Figure 2.4A). Consistent with previous work10,11, we observed a significant enhancement of parkin auto-ubiquitination activity at a stoichiometry of 10% pUbS65 to 90% unmodified Ub. In addition, precise stoichiometric mixtures of 10% pUbS65 or 10% pUbS65-k to unmodified Ub (90%) led to essentially the same level of auto-ubiquitination activity (Figure 2.7A) that was, in both cases, enhanced over activity in the absence of pUb (Figure 2.7A). Similar reactions using either 10% pUbS12 or 10% pUbS20 (Figure 2.7B lane 2, C lane 4) did not stimulate parkin ubiquitination activity, indicating that the activation of parkin is specific for phosphorylation at position Ser65 in Ub.

Interestingly, we found that assays conducted using pUbS65 or pUbS65-k as the only Ub source both displayed significant activation of parkin above the background level observed with unmodified Ub (Figure 2.7A) but lower than that observed using either 10% pUbS65 or 10% pUbS65-k. This is in contrast to a previous report that indicated that 100% pUbS65 is unable to stimulate parkin12. In order to differentiate between Ub chains built with Ub versus pUb, the unmodified Ub was His6-tagged, while His6-tag was removed from pUbS65. In experiments with 100% pUbS65, the Ub chains appeared at a lower molecular weight, indicating that these chains were synthesized from only the pUb species lacking the His6-tag. In lanes with 10% pUbS65, the high molecular weight chains are dominant, indicating chains built with unmodified His6-Ub predominantly (Figure 2.7A).

In similar reactions, we monitored the abilities of pUbS12 or pUbS20 to be incorporated into Ub chains. We found that these variants do not stimulate parkin activity when included at low stoichiometry: pUbS12 (10% His6-pUbS12 + 90% Ub; Figure 2.7B, lane 2) or pUbS20 (Figure 2.7C, lane 4). At high stoichiometry, no activity is observed with 100% pUbS20 (Figure 2.7C, lane 5), but the formation of at least a single His6-pUbS12
ligated to parkin (at the expected molecular weight) suggests low parkin activity when pUb$^{S12}$ is the only source of Ub in the reaction (Figure 2.7B, lane 3).

**Figure 2.7: Stoichiometry and location of Ub phosphorylation modulates parkin activity.** (A) Parkin auto-ubiquitination assay with unmodified Ub (His$_6$-Ub), PINK1 phosphorylated Ub (pUb$^{S65-k}$) and genetically encoded pUb$^{S65}$ were visualized by an anti-parkin immunoblot (M, molecular weight marker). Reactions all contain E1, E2, full-length parkin (E3), ATP and different Ub variants: 100% unmodified His$_6$-Ub (lane 1), 10% pUb$^{S65-k} + 90\%$ His$_6$-Ub (lane 2), 100% pUb$^{S65-k}$ (lane 3), 10% genetically encoded pUb$^{S65} + 90\%$ His$_6$-Ub (lane 4) and 100% genetically encoded pUb$^{S65}$ (lane 5). (B) Auto-ubiquitination assay with Ub, pUb$^{S65-k}$, and/or genetically encoded His$_6$-pUb$^{S12}$. The stoichiometry of Ub or pUb variants included in lanes 1–9 is listed above the blot. (C) Auto-ubiquitination assay with His$_6$-Ub, pUb$^{S65-k}$, and/or genetically encoded His$_6$-pUb$^{S20}$. The stoichiometry of Ub and pUb variants included in lanes 1–11 is listed above the blot. Triangles indicate decreasing stoichiometry of phosphorylation at positions 12 (B) and 20 (C), respectively. In these experiments, the Ub His$_6$-tag was cleaved with TEV protease except where indicated by the His$_6$ prefix.
We then conducted experiments in the presence of 10% pUb\textsuperscript{S65-k} to determine if stimulated parkin could use pUb\textsuperscript{S12} or pUb\textsuperscript{S20} as substrates. In the presence of 10% pUb\textsuperscript{S65-k} with 90% pUb\textsuperscript{S12} or pUb\textsuperscript{S20}, above background auto-ubiquitination bands were observable (Figure 2.7B lane 4; Figure 2.7C lane 6). Interestingly, these reactions both showed reduced activity compared to reactions lacking phosphorylation at S12 or S20. Decreasing the occupancy of phosphorylation at position 12 (Figure 2.7B) or at position 20 (Figure 2.7C) led to concomitant increase in parkin activity.

We also observed that Ub, pUb\textsuperscript{S12}, pUb\textsuperscript{S20}, and pUb\textsuperscript{S65} are all competent substrates for the E1 and E2 enzymes as evidenced by successful E2~Ub or E2~pUb conjugate formation (Figure S2.12). Taken together, the data suggest that Ub phosphorylation at positions 12 and 20 is disruptive to poly-Ub chain formation and may function as a negative regulator for parkin activity stimulated by pUb\textsuperscript{S65}. Future experiments will show if this potential regulation is either by direct parkin inhibition or if these pUb variants are incompetent substrates for parkin.

2.4 Discussion

2.4.1 Pure designer phosphoprotein

Previous reports have shown it is possible to produce designer phosphoprotein in *E. coli\textsuperscript{13,26}*, but the efficient production of pure phosphoprotein has remained challenging. We successfully produced several pUb variants in unambiguously pure form as evidenced by MS/MS and Phos-tag gels. Strikingly, in all conditions tested, we observed no dephosphorylation of the genetically encoded pUb variants.

We found that optimization of the phosphoseryl-tRNA synthetase, and the *E. coli* host expression strain were factors that improved efficiency of phosphoprotein biosynthesis. *E. coli ΔRF1* has been described as an ideal expression strain for UAG codon reassignment\textsuperscript{16}. Ultimately, we found that BL21(DE3) is more efficient in phosphoprotein production. Despite competition between pSer insertion and RF1 in BL21(DE3), the fraction of phosphorylated protein produced was higher and BL21(DE3) grows to higher cell density compared to *E. coli ΔRF1\textsuperscript{15}*. 
We observed mistranslation in both *E. coli* BL21(DE3) and ΔRF1, and mistranslation was evident with the less efficient (1st generation) and with the more efficient (2nd generation) pSer systems. Our goal in this study was not to necessarily maximize the yield of phosphoprotein. We\textsuperscript{17} (Table S2.1) and others\textsuperscript{11,22,26} have already produced phosphoproteins in sufficient amounts for biochemical and structural studies. Rather, we were concerned with demonstrating unambiguously and in a more rigorous fashion than previous reports that we indeed obtained pure phosphoprotein. Previous work relied on MS/MS identification of pSer in the phosphoprotein\textsuperscript{11,26}, yet mistranslated protein may go undetected with this method. A modified pSer system\textsuperscript{11}, including mutations in SepRS and the anticodon stem of tRNA\textsuperscript{Sep}, recently reported similar mg·L\textsuperscript{-1} culture yields of phosphoprotein as was previously reported for the 2nd generation pSer system\textsuperscript{26}. While our yields were somewhat lower, we emphasize that we are reporting pure phosphoprotein yields, which likely reflect some loss of protein during column chromatography. We observed somewhat different efficiencies of protein production for different pUb variants. This is not unexpected and has been observed for other phosphoproteins\textsuperscript{15}, potentially resulting from codon context or differential rates of protein folding or degradation.

Our strategy enables streamlined and efficient production of phosphoproteins in *E. coli* at a scale of milligrams of pure phosphoprotein. Compared to chemical synthetic techniques (~ $5000$ mg\textsuperscript{-1} phosphoprotein), our method enables any lab to economically biosynthesize (~ $50$ mg\textsuperscript{-1} phosphoprotein reagent cost) pure phosphoproteins that are appropriate for biochemical, structural, and drug-screening applications. Importantly, this technology allows us to access any desired phosphorylation site without the need to identify and isolate active upstream kinases.

2.4.2 Codon skipping

Fascinatingly, in *E. coli* ΔRF1 pSer insertion competes not only with near-cognate translation with Gln but also with codon skipping. We have not observed codon skipping in BL21(DE3), which is RF1+ *E. coli*. However, we cannot rule out the possibility that it may occur at some level in other *E. coli* strains or naturally in living organisms. In two of the six phosphoproteins produced here, a significant amount of Ub or Ubl was produced.
lacking the UAG-encoded residue. In both cases, protein product resulting from codon skipping includes a peptide bond between the residues encoded immediately before and after the UAG codon. We hypothesize that the ribosome underwent a +3 frameshift leading to a single in-frame amino acid deletion and a novel interpretation of the UAG codon.

Aside from an obvious comparison to frameshifting\textsuperscript{27}, the only other precedent for nucleotide skipping is from recent work in mitochondria of the yeast *Magnusiomyces capitatus*. In this case, the ribosome bypasses ~ 100 nucleotide intragenic untranslated regions (UTRs) in the mRNA and forms a peptide bond between the two residues encoded on either side of the UTR\textsuperscript{28}. Together, these data indicate that genetic code expansion systems can produce unexpected translation products, and that the ribosome allows more flexible interpretations of the RNA message than previously envisioned.

2.4.3 Parkin activity is sensitive to phosphorylation site location and stoichiometry

Missense mutations in the human genes *park6* (encoding the kinase PINK1) and *park2* (encoding parkin) are found in most cases of autosomal recessive juvenile parkinsonism\textsuperscript{29}, yet the intricate interplay between these two proteins is only now being uncovered. Initial discoveries found that the PINK1 substrate is parkin itself, and phosphorylation at position Ser65 in the Ubl domain of parkin stimulated the E3 Ub ligase activity of parkin\textsuperscript{24}. The parkin substrate Ub was later found to be a substrate of PINK1 as well, and pUb\textsuperscript{S65} stimulates parkin activity to a greater extent than phosphorylation of the Ubl domain\textsuperscript{7,8,30}. In the current model for parkin activation, the Ubl domain is auto-inhibitory\textsuperscript{31}, but phosphorylation of parkin at Ser65 leads to a weaker interaction of the Ubl domain with the RING1 domain of parkin, at least partially alleviating auto-inhibition, and activating parkin\textsuperscript{32}. Similarly, PINK1 phosphorylation at Ser65 of Ub also activates parkin via pUb\textsuperscript{S65} binding with a conserved pSer-binding pocket on parkin, which induces a conformational change, causing release of the auto-inhibitory Ubl domain\textsuperscript{32-35}. Because continued PINK1 activity leads to both a release of autoinhibition and stimulation of parkin activity, this has been described as a feed-forward signalling mechanism\textsuperscript{10}. 
Previous work found parkin activation by pUb\textsuperscript{S65} is dependent on the stoichiometry of phosphorylation, with 100% pUb\textsuperscript{S65} failing to activate Ub ligation\textsuperscript{12}. Our data indicate that pUb\textsuperscript{S65} stimulates parkin activity at any stoichiometry. Some experiments presented here used what might be considered a ‘high stoichiometry’ of pUb, yet the stoichiometry and abundance of different pUb variants in the cell is not yet known. Quantitation of phosphoprotein abundance based on proteomic data suggests pUb\textsuperscript{S65} represents between 0.1% and 20% of the total cellular Ub pool\textsuperscript{10,36,37}. Recent data show that pUb\textsuperscript{S65} is not uniformly distributed in the cell. Rather, pUb\textsuperscript{S65} was shown by immunodetection to colocalize with parkin and the mitochondrial membrane\textsuperscript{38}. The abundance of pUb\textsuperscript{S65} increases with oxidative stress in cell-based studies and with age and PD status in patient tissue samples\textsuperscript{39}. These data show that under disease-relevant conditions, the concentration of pUb in the vicinity of parkin might be much higher than is apparent from bulk measurements. Antibodies to image the distribution of other pUb variants are lacking. This is an exciting direction for future work that will be facilitated by the pUb variants produced here.

Despite intense research on pUb\textsuperscript{S65}, little is yet known about how other phosphorylation sites on Ub modulate parkin activity. Both traditional and high-throughput proteomic investigations have identified variants of Ub phosphorylated at nearly all of its serine, threonine, or tyrosine residues\textsuperscript{40}. This includes observations of pUb\textsuperscript{S20} \textsuperscript{41-43}, in humans, mice, and rats\textsuperscript{3-6}. Ours is the first report of Ub phosphorylation sites (distinct from pUb\textsuperscript{S65}) that modulate the activity of an E3 Ub ligase. Our data show that phosphorylation of Ub at Ser12 or Ser20 led to pUb variants that are possibly less competent or incompetent substrates for parkin when compared with unmodified Ub or pUb\textsuperscript{S65}. Since a system for site-specific incorporation of phosphothreonine has yet to be developed, pUb\textsuperscript{S12} was used to mimic phosphothreonine at position 12 in Ub. We hypothesize that these Ub phospho-sites may negatively regulate pUb\textsuperscript{S65}-stimulated parkin in the cell. Indeed, phosphorylation sites on parkin itself (pS131\textsuperscript{44}, pY143\textsuperscript{45}) are known to negatively regulate its activity. Experiments with the other pUb variants produced here are currently underway. We anticipate that phosphorylation signalling on Ub may be a general mechanism for positively or negatively regulating the activity of the > 600 E3 Ub ligases in the human proteome.
2.5 Conclusion

There are > 100,000 experimentally documented phosphorylation sites in the human proteome\textsuperscript{46,47}. Of these, at most only 100s of sites are annotated with a biological function\textsuperscript{48}. It is estimated that human disease-causing missense mutations affect \( \sim 20,000 \) distinct phosphorylation sites\textsuperscript{47}. It is not surprising; therefore, that dysfunctional signalling is thought to drive major human diseases including cancers and neurodegeneration\textsuperscript{49,50}. The ability to synthesize site-specifically phosphorylated proteins, such as pUb or recombinant active kinases\textsuperscript{13}, enables drug-screening efforts to explore new targets and to specifically target pathological phosphorylation\textsuperscript{51}. Our insights on pUb\textsuperscript{S65} activation of parkin may be useful pharmacologically, since small molecules that mimic this phosphorylated ubiquitin variant may activate parkin and benefit PD patients. Likewise, the possible inhibition of parkin by pUb\textsuperscript{S20} could potentially regulate the levels of constitutively active parkin in patients with pathogenic parkin mutations that disrupt parkin autoinhibition. In addition, site-specific phosphorylation of the mitogen-activated ERK activating kinase 1 to activate its downstream signalling partners showcases the usefulness of genetic code expansion systems to study the molecular mechanisms of disease\textsuperscript{13}. We established an efficient route to pure recombinant phosphoprotein, and we have demonstrated that phosphoprotein purity is essential when enzyme activity depends critically on the site and occupancy of phosphorylation.

2.6 Supporting Information

2.6.1 Supporting Methods

**Bacterial strains and plasmids.** Recombinant proteins were expressed in BL21(DE3) (Invitrogen), or in *E. coli* ΔRF1 (C321.ΔA.exp, Addgene 49018) as specified. The 1st generation pSer system is on the plasmid pKD-pSer1 (Addgene 52054) that expresses SepRS, EFSep and 5 copies of the tRNA\textsuperscript{Sep} expression cassette. The Ub expression plasmid (pDS0-Ub) was constructed using a T7 promoter, T7 terminator, and a cloning site (NdeI/BamHI) into an ampicillin-resistant backbone derived from pUC19. The Ub wild type gene was PCR amplified (PfuUltra II Fusion HS DNA polymerase, Agilent) from pET3a-His\textsubscript{6}TEVyUb\textsuperscript{32} and inserted into pDS0 by restriction-free cloning. Quick-
change mutagenesis was used (as described\textsuperscript{52}) to generate Ub variants with in-frame TAG codons. DNA sequencing confirmed the correct Ub expression plasmids. pDS-pSer2 also contains 5 copies of the tRNA\textsuperscript{Sep} expression cassette and is identical to pKD-pSer1 except the SepRS and EF-Sep were mutated to their 2nd generation counterparts SepRS\textsuperscript{9} and EF-Sep\textsuperscript{21}\textsuperscript{26}.

**Protein and phosphoprotein production.** Either pKD-pSer1 or pDS-pSer2 and a pDS0-Ub expression plasmid were freshly cotransformed into *E. coli* ΔRF1 (i.e., C321ΔA.exp\textsuperscript{16}) or BL21(DE3) cells and plated on lysogeny broth (LB)-agar plates with 25 µg/mL kanamycin and 100 µg/mL ampicillin. Single colonies were picked to inoculate 10 mL starter cultures in LB (with 100 µg/mL ampicillin and 25 µg/mL kanamycin), which were grown, shaking overnight at 37°C. Once saturated, the starter culture was used to inoculate 1 L of LB media supplemented with ampicillin (100 µg/mL), kanamycin (25 µg/mL), and O-phospho-L-serine (pSer, 2.5 mM final concentration, Sigma-Aldrich). Cultures were grown at 37°C until OD\textsubscript{600} = 0.6. An additional 2.5 mM pSer was added to cultures at this point and the incubation temperature was reduced to 16°C. Protein expression was induced at OD\textsubscript{600} of 0.8 by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued for 18 hours at 16°C.

**E1, E2, and E3 protein production and purification.** Human Uba1 (E1), UbcH7 (E2), and Parkin (E3, full length) enzymes used in this study were produced and purified to homogeneity exactly as described previously\textsuperscript{32}. PINK1 phosphorylated Ub (Ub pSer\textsuperscript{65}) was prepared by incubating wild type Ub with ATP and active *Pediculus humanus* GST-PINK1 as described\textsuperscript{32}. The PINK1 phosphorylated Ub was then purified by ion-exchange chromatography as below\textsuperscript{32}.

**Affinity chromatography.** Nickel affinity purification was performed using a gravity column with 1 mL Ni-NTA resin (Thermo Fisher Scientific, Waltham, MA, USA) per litre culture or a HisTrap FF column connected to the FPLC. For nickel affinity column chromatography by gravity, cleared lysate was loaded onto the column, washed extensively with wash buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.25 mM TCEP, 50 mM NaF, 2 mM NaVO\textsubscript{3} and 25 mM imidazole) and eluted with elution buffer (250
mM imidazole in wash buffer). For nickel affinity column chromatography by FPLC, cleared lysate was loaded onto the column at 0.5 mL/min, washed with 100 column-volumes wash buffer, and eluted with 350 mM imidazole (in wash buffer). At this stage, TEV-protease was used to cleave the His<sub>6</sub>-tag from some of the Ub or pUb variants, as previously. For some Ub variants the His<sub>6</sub>-tag was retained to differentiate Ub chains by molecular weight (as noted in Figures 2.4, 2.7 and S2.12). It is well established that the presence or absence of the His<sub>6</sub>-tag has no effect on the interaction of the Ub substrate with the E1, E2 or E3 enzymes involved in the parkin ubiquitination pathway (e.g.,).

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

**Anion-Exchange chromatography.** Purification was performed on a FPLC system (GE Healthcare) using a 1 mL HiTrap Q-HP (GE Healthcare) column equilibrated with buffer C (20 mM bis-tris propane, pH 8.7). Prior to purification, samples were dialyzed overnight in buffer C. Anion-exchange chromatography was performed at a flow rate of 0.3 mL/min and 2 mL fractions were collected. Proteins were eluted using a 0-100% gradient of buffer D (20mM bis-tris propane, 500mM NaCl, pH 8.7) over 100 minutes. Cation- and anion-exchange chromatography were found to separate pUb from non-phosphorylated Ub equally well.

**Phos-tag gels.** Phos-tag acrylamide (Wako Pure Chemicals Ltd., Japan) was purchased and all Phos-tag gels were prepared according to manufacturer’s instructions with the following exceptions: ZnCl<sub>2</sub> concentration was increased to 0.77 mM and gels were run at 25 mA per gel.

**Mass spectrometry.** MS was performed both before and after ion-exchange chromatography. Samples were prepared for MS by overnight dialysis in 15 mM NH₄
acetate to remove salt adducts. Whole protein ESI-MS was performed on a Q-Tof Micro mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode (+0.1% formic acid). Samples were prepared for ESI-tandem-MS by digestion with trypsin or Glu-C protease. Briefly, 25 µg of protein was reduced with dithiothreitol and alkylated with iodoacetamide using standard protocols, followed by protease digestion using a 1:40 ratio of protease to sample. All mass spectrometry was performed at the Biological Mass Spectrometry Laboratory (The University of Western Ontario, http://www.uwo.ca/biochem/bmsl/).

**E2 loading assay.** The assay was adapted from a previous study\(^4\) with modifications as follows. The reaction contained 0.5 µM E1 (Uba1), 20 µM E2 (UbcH7), 4 µM (Figure S2.12A) or 20 µM (Figure S2.12B) total Ub or pUb, and 10 mM ATP in reaction buffer containing 50 mM HEPES and 100 mM NaCl at pH 7.0. Reactions were incubated at 37°C for 10 min. Samples were quenched with SDS non-reducing loading buffer (containing 50 mM EDTA) and incubated for 5 min at 100°C. The reaction products were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie staining.

### 2.6.2 Supporting Tables

**Table S2.1: Pure phosphoprotein yields.**

<table>
<thead>
<tr>
<th>Phosphoprotein variant</th>
<th>E. coli expression strain</th>
<th>pSer system</th>
<th>pure pUb/L E. coli culture</th>
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<tr>
<td>pUb(^565)</td>
<td>ΔRF1 (C321.ΔA.exp)</td>
<td>1(^{st}) generation</td>
<td>11 µg/L</td>
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<tr>
<td>pUb(^565)</td>
<td>ΔRF1 (C321.ΔA.exp)</td>
<td>1(^{st}) generation</td>
<td>6 µg/L</td>
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<tr>
<td>pUb(^57)</td>
<td>ΔRF1 (C321.ΔA.exp)</td>
<td>1(^{st}) generation</td>
<td>0.1 µg/L</td>
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<tr>
<td>pUb(^557)</td>
<td>ΔRF1 (C321.ΔA.exp)</td>
<td>1(^{st}) generation</td>
<td>0.4 µg/L</td>
</tr>
<tr>
<td>pUb(^512)</td>
<td>BL21(DE3)</td>
<td>2(^{nd}) generation</td>
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<tr>
<td>pUb(^520)</td>
<td>BL21(DE3)</td>
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<td>pUb(^565)</td>
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<tr>
<td>pUb(^565)</td>
<td>ΔRF1 (C321.ΔA.exp)</td>
<td>2(^{nd}) generation</td>
<td>8 µg/L</td>
</tr>
</tbody>
</table>

1\(^{st}\) generation pSer system is expressed from plasmid pKD-pSer1\(^17\); 2\(^{nd}\) generation pSer system is expressed from plasmid pDS-pSer2.
2.6.3 Supporting Figures

Figure S2.8: Production of the Ubl domain of Parkin (residues 1-76) phosphorylated at Ser65. (A) Coomassie-stained Phos-tag (top) and SDS-PAGE (bottom) gels show unmodified Ubl domain (lane 1), PINK1 phosphorylated and purified Ubl domain (lane 2), ion-exchanged purified genetically encoded pUbl\textsuperscript{S65} (lane 3), and genetically encoded pUbl\textsuperscript{S65} prior to ion-exchange chromatography showing a mixture of unphosphorylated Ubl and pUbl\textsuperscript{S65} (lane 4). Phosphorylated proteins are retarded only in the Phos-tag gel (top) and not in the SDS-PAGE gel (bottom).
Figure S2.9: Purification of His₆-pUb⁵⁷⁷ and MS/MS data for His₆-pUb⁵²⁰ as well as His₆-pUb⁵¹². His₆-pUb⁵⁷⁷ produced in E. coli ΔRF1 using the 1st generation pSer system led to pure His₆-pUb⁵⁷⁷ (lanes 4,5) as observed in Coomassie-stained (A) and immunoblotted (B, anti-His) Phos-tag gels. (C) MS/MS run in positive ion mode (+0.1% formic acid) identifies genetically encoded His₆-pUb⁵²⁰. (D) Unphosphorylated Ub produced along with His₆-pUb⁵¹² (Figure 2.4D) in BL21(DE3) from the Ub UAG12 construct was determined to contain Gln at the UAG encoded residue by MS/MS run in positive ion mode (+0.1% formic acid). (E) MS/MS run in positive ion mode (+0.1% formic acid) identifies genetically encoded His₆-pUb⁵¹².
Figure S2.10: Codon skipping resulting in the amino acid deletion at position 65 of the Ubl domain is reproducible. Full protein ESI-MS run in positive ion mode (+0.1% formic acid) for Ubl domain expressed from 3 independent clones (A-C) of the Ubl domain UAG65 consistently show protein product that corresponds to Ubl Δ65.
Figure S2.11: Specific conditions promote UAG codon skipping in *E. coli* ΔRF1. The His$_6$-Ub UAG7 construct was coexpressed with (A) pKD-SepRS/EFSep (containing the 1st generation SepRS/EFSep lacking tRNA$_{Sep}$), (B) with pKD-pSer1 (containing SepRS/EFSep and 5 x tRNA$_{Sep}$ expression cassettes), or (C) with pKD-pSer1 in media supplemented with 5 mM exogenous pSer. Each strain yielded distinct protein products from translation of the UAG codon as Gln, pSer and/or a single amino acid deletion (Δ7), confirmed by MS/MS run in positive ion mode (+0.1% formic acid) of the purified and Glu-C digested Ub. To confirm reproducibility of codon skipping at position 7, panel C is from an independent protein purification from that shown in Figure 2.5B.
Figure S2.12: E2 (UbH7) loading assay with Ub and pUb variants. E2 loading assays were performed with unmodified Ub (A, B), pUbS12 (A), pUbS65-k (B), and His6-pUbS20 (B). The reaction at t = 0 (before ATP addition) shows only the E2 and Ub reactants. Following a 10-minute reaction, the appearance of an upper band indicates successful formation of the E2–Ub or E2–pUb conjugate. Bands representing free Ub, pUb or His6-pUb are indicated along with the E2, E2–Ub, E2–pUb, or E2–His6-pUbS20 conjugates.
2.7 References


Chapter 3

3 Parkin E3 ubiquitin ligase activity is stimulated by differentially phosphorylated ubiquitin variants

Parkin is an E3 ubiquitin (Ub) ligase that is stimulated by phosphorylation of Ub at Ser65 (pUbS65). The role of other phosphorylation sites on Ub and their respective kinases remain unknown. We used fluorescently labelled phosphorylated Ub (pUb) substrates to quantitatively relate the stoichiometry of Ub phosphorylation to parkin activation. We determined labeling efficiency and optimized the concentration of labelled-Ub required to visualize a broad range of parkin activity. The sensitivity of the fluorescence-based assay was demonstrated as we unexpectedly observed significant parkin activity when unmodified Ub was the only substrate. Fluorescence measurements indicate that pUbS65-simulated parkin is 4-fold more active than autoinhibited and unstimulated parkin. We consistently observed a low level of parkin activity when pUbS12 is the only Ub variant in the reaction. Fluorescently labelled pUbS12 and anti-parkin immunoblots were used to identify two distinct parkin-pUbS12 linked chains following auto-ubiquitination of parkin.

3.1 Introduction

Autosomal recessive juvenile parkinsonism is a familial form of Parkinson’s disease (PD) where mutations in park2 account for 50% of the observed cases in patients\(^1\). Parkin is an E3 Ub ligase encoded by park2 that has been implicated in clearing defective mitochondria from cells by tagging mitochondrial outer membrane proteins with polyUb chains\(^2,3\). Upon depolarization of mitochondrial membrane potential, the phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1) phosphorylates parkin at a particular serine residue (S65) within its Ub-like (Ubl) domain\(^4\). PINK1 also phosphorylates the homologous site, S65, in Ub\(^5-8\). The combined phosphorylation of both parkin and Ub leads to maximal activation of parkin E3 Ub ligase activity\(^5-7,9\). In PD patients, the role of parkin in clearing damaged mitochondria is disrupted\(^1,2,10\).

Previously, it has been reported that pUbS65 fails to activate parkin when it is the only Ub variant present in the reaction\(^8\). Our recent findings demonstrated that pUbS65
activates parkin at any stoichiometry, including when it is the sole Ub variant (Figure 2.7). In addition, we found that parkin activity is affected by the stoichiometry and location of phosphorylation in Ub. Since the upstream kinases for several experimentally observed phosphorylation sites on Ub remain unknown, we used genetic code expansion strategies to site-specifically phosphorylate Ub at experimentally identified phosphorylation sites. Our findings showed that pUb\(^{S12}\) partially activates parkin and that increasing the stoichiometry of pUb\(^{S20}\) inhibits pUb\(^{S65}\)-stimulated parkin activity in a dose-dependent manner (Figure 2.7B,C). Activity of parkin was measured using auto-ubiquitination assays, which were visualized by immunoblotting with anti-parkin to observe Ub chains built on parkin. We found that anti-parkin immunoblots did not consistently report on parkin activity or enable quantitation of parkin activity.

Here we produced fluorescently labelled and site-specifically phosphorylated Ub variants (Ub* and pUb*) to provide quantitative reports on parkin activity with distinct phosphorylation stoichiometry in the Ub substrate pool. We introduced a single cysteine (Cys) residue at the amino (N)-terminal of our Ub and pUb constructs to enable covalent linkage with small compounds, such as Alexa Fluors or DyLight dyes. We used a sulfhydryl-reactive Dylight dye that relies on maleimide chemistry to produce Ub* and pUb*. Visualization of Ub* will allow direct measurement of Ub chains and enable more precise measurement of the stoichiometry of chain building on parkin.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids

Recombinant proteins were expressed in BL21(DE3) (Invitrogen, Carlsbad, CA, USA). The Ub expression plasmids used to make fluorescent Ub, pSG1-Ub* and pSG2-Ub*, were constructed by site-directed mutagenesis of N23C (as described) in the N-terminal His\(_6\)-tag linker sequence before the start site of Ub (i.e., -2 position) in the open-reading frame of pDS0-Ub (see Section 2.6.1) and pMCSG7-Ub, respectively. pMCSG7-Ub (a kind gift from Kathryn R. Barber, The University of Western Ontario) was constructed by ligation independent cloning of yeast Ub into pMCSG7 followed by site-directed mutagenesis of N23C in the -2 position. Site-directed mutagenesis was used (as
described\(^{16}\) to generate Ub variants with in-frame TAG codons. DNA sequencing confirmed the correct Ub\(^*\) expression plasmids. pSG1-Ub\(^*\) is the pUC19 backbone plasmid and pSG2-Ub\(^*\) is pMCSG7 backbone plasmid. pDS-pSer2 is the plasmid containing the pSer system with SepRS9, EFSep21 and 5 copies of the tRNA\(^{\text{Sep}}\) expression cassette (see Section 2.6.1).

### 3.2.2 Ub overexpression and purification

Expression plasmid pSG2-Ub\(^*\) was freshly transformed into BL21(DE3) cells and plated on lysogeny broth (LB)-agar plates with 100 µg/mL ampicillin. Single colonies were picked to inoculate 10 mL starter cultures in LB (with 100 µg/mL ampicillin), which were grown, shaking overnight at 37°C. Once saturated, the starter culture was used to inoculate 1 L of LB media supplemented with ampicillin (100 µg/mL). Cultures were grown at 37°C until OD\(_{600}\) = 0.6 and the incubation temperature was reduced to 16°C. Protein expression was induced at OD\(_{600}\) = 0.8 by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued for 20 hours at 16°C.

Cells were harvested and resuspended in ~10 mL buffer A1 (50 mM Tris-HCl, 200 mM NaCl, 0.25 mM TCEP, 25 mM imidazole, pH 7.5) per litre culture. 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 tablet of ethylenediaminetetraacetic acid (EDTA)-free mini protease inhibitor cocktail (Roche, Mississauga, ON, USA) were added to the cells. Cells were lysed using a French pressure cell press (Newport Scientific Inc, Jessup, MD, USA), centrifuged at 41,000 xg to remove cell debris, and filtered through a 1.2-µm filter for use on a gravity column. Nickel affinity purification was performed using a gravity column with 1 mL Ni-NTA resin (Thermo Fisher Scientific, Waltham, MA, USA) per litre culture. Cleared lysate was loaded onto the column, washed extensively with buffer A1 and eluted with buffer B1 (250 mM imidazole in buffer A1).

Size-exclusion chromatography was performed on an AKTA Pure L1 fast protein liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, UK) using a 24 mL Superdex 75 10/300 GL (GE Healthcare) column equilibrated with buffer C (25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP, pH 7.0) to separate Ub from higher molecular
weight bacterial proteins. Size-exclusion chromatography was performed at a flow rate of 0.1 mL/min and 0.5 mL fractions were collected. Ub eluted at approximately 0.5 column-volumes of buffer C.

3.2.3 pUb overexpression and purification

Expression plasmids pDS-pSer2 and either pSG1-Ub* or pSG2-Ub* (with TAG at positions 12 and 65 of Ub) were freshly cotransformed into BL21(DE3) cells and plated on LB-agar plates with 25 µg/mL kanamycin and 100 µg/mL ampicillin. Single colonies were picked to inoculate 10 mL starter cultures in LB (with 100 µg/mL ampicillin and 25 µg/mL kanamycin), which were grown, shaking overnight at 37°C. Once saturated, the starter culture was used to inoculate 1 L of LB media supplemented with ampicillin (100 µg/mL), kanamycin (25 µg/mL), and O-phospho-l-serine (pSer, 2.5 mM final concentration, Sigma-Aldrich). Cultures were grown at 37°C until OD<sub>600</sub> = 0.6. An additional 2.5 mM pSer was added to cultures at this point and the incubation temperature was reduced to 16°C. Protein expression was induced at OD<sub>600</sub> = 0.8 by addition of 1 mM IPTG and continued for 20 hours at 16°C.

Cells were harvested and resuspended in ~ 10 mL buffer A2 (50 mM Tris-HCl, 200 mM NaCl, 0.25 mM TCEP, 50 mM NaF, 2 mM NaVO<sub>3</sub>, 25 mM imidazole, pH 8.0) per litre culture. 1 mM PMSF and 1 tablet of EDTA-free mini protease inhibitor cocktail (Roche, Mississauga, ON, USA) were added to the cells. Cells were lysed using a French pressure cell press (Newport Scientific Inc), centrifuged at 41,000 xg to remove cell debris, and filtered through a 1.2-µm filter for use on a gravity column. Nickel affinity purification was performed using a gravity column with 1 mL Ni-NTA resin (Thermo Fisher Scientific) per litre culture. Cleared lysate was loaded onto the column, washed extensively with buffer A2 and eluted with buffer B2 (250 mM imidazole in buffer A2).

Size-exclusion chromatography was performed on a FPLC system (GE Healthcare) using a 24 mL Superdex 75 10/300 GL (GE Healthcare) column equilibrated with buffer C to separate pUb from higher molecular weight bacterial proteins. Size-exclusion chromatography was performed at a flow rate of 0.1 mL/min and 0.5 mL fractions were collects. pUb eluted at approximately 0.5 column-volumes of buffer C.
To separate pUb from non-phosphorylated Ub, anion-exchange chromatography was performed on an AKTA FPLC system (GE Healthcare) using a 1 mL HiTrap Q-HP (GE Healthcare) column equilibrated with buffer C (20 mM bis-tris propane, 250 μM TCEP, pH 8.7). Anion-exchange chromatography was performed at a flow rate of 0.1 mL/min and 1 mL fractions were collected. Proteins were eluted using a step-wise gradient of 10%, 20%, 30%, 40% and 100% buffer D (20mM bis-tris propane, 500mM NaCl, 250 μM TCEP, pH 8.7) for 10 column-volumes each.

Effective separation of pUb from non-phosphorylated Ub was confirmed using Phos-tag gels. Phos-tag acrylamide (Wako Pure Chemicals Ltd., Japan) was purchased and all Phos-tag gels were prepared according to manufacturer’s instructions with the following exceptions: ZnCl₂ concentration was increased to 0.77 mM and gels were run at 25 mA per gel.

3.2.4 Ub and pUb fluorescent labelling

Cys-Ub and Cys-pUb variants were fluorescently labelled using Dylight 800 Maleimide (Thermo Fisher Scientific). Both Cys-Ub and Cys-pUb variants were dialyzed into buffer E (50mM HEPES, 100mM NaCl, 1mM EDTA, pH 7.0). After measuring the concentration of Cys-Ub (648 μM) and Cys-pUb⁵¹² (38 μM) using the Bradford assay (Bio-Rad, Hercules, CA, USA), 100x excess of TCEP was incubated with the protein for 15 minutes to ensure reduction of all intermolecular disulfide bonds. An excess of 5x Dylight 800 Maleimide dye (3.2 mM for Cys-Ub and 0.19 mM for Cys-pUb⁵¹²) was added to the reduced protein to create irreversible thioether linkages via maleimide chemistry and produce Ub* and pUb*. The reaction was quenched with 20x excess of dithiothreitol (DTT). Ub* and pUb* variants were dialyzed into storage buffer (50 mM HEPES pH 7.4) to remove unreacted dye and then stored at 4°C.

3.2.5 Quantitative auto-ubiquitination assays

All reactions contained 0.1 µM E1 (Uba1), 1 µM E2 (UbcH7), 1 µM E3 (parkin), 5 mM ATP, and a 20 µM final concentration of Ub and/or pUb in a 10 µL final reaction volume incubated at 30°C for 1 h. Human Uba1, UbcH7, full-length parkin and PINK1 phosphorylated Ub (Ub pSer⁶⁵) were produced and purified to homogeneity as
described previously\textsuperscript{15}. The reaction buffer contained 5 mM MgCl\textsubscript{2} and 50 mM HEPES pH 7.4. Reactions were quenched with sodium dodecyl sulfate (SDS)-loading buffer, resolved by 4-12% SDS-polyacrylamide gel electrophoresis (PAGE) (Life Technologies, Carlsbad, CA, USA) and analyzed by direct fluorescence monitoring using the Li-COR\textsuperscript{®} Odyssey Infrared Imaging System at $\lambda = 800$ nm. The gels were then blotted on a PVDF membrane (Thermo Fisher Scientific) and immunodetected using anti-parkin primary antibody (Cell Signaling Technology, Danvers, MA, USA) and fluorescent-labelled secondary antibody (Thermo Fisher Scientific). Blots were prepared as previously described (see Section 2.2.3) and visualized using the Li-COR\textsuperscript{®} Odyssey Infrared Imaging System at $\lambda = 700$ nm.

3.3 Results

3.3.1 pUb production

Ub is an 8.5 kilodalton (kDa) protein comprised of 76 amino acid residues\textsuperscript{17}. Out of these 76 amino acid residues, there are no Cys residues and few aromatic amino acid residues. A reduced number of aromatic amino acid residues make it difficult to observe Ub-containing fractions on chromatograms from size-exclusion and anion-exchange chromatography since the spectrometer measures at a wavelength of 280nm\textsuperscript{18}. The lack of Cys residues prevents facile fluorescent labelling of Ub\textsuperscript{19}, a task that can be effective in quantitatively measuring chain building observed in activated parkin.

In order to enable quantitation of pUbs involved in activating parkin, a single Cys residue was introduced in the -2 position of pUb to produce Cys-pUb. Initial attempts to purify Cys-pUb variants by size-exclusion chromatography were not completely successful (Figure 3.1). The wild-type Cys-Ub (Figure 3.2) and pUb variants (Figure 2.4 B,D) were essentially pure, yet the Cys-pUb\textsuperscript{S65} and Cys-pUb\textsuperscript{S12} variants did not readily separate from higher molecular weight proteins (Figure 3.1).

The use of the pUC19 backbone plasmid (pSG1-Ub*) to produce Cys-pUb was less efficient when compared to previously obtained pUb yields (Table S2.1), even though the only variation was the introduction of a single Cys residue. We switched to the pMCSG7 backbone plasmid (pSG2-Ub*) to produce Cys-pUb since this route expressed wild-type
Cys-Ub considerably better with yields of 360 μg per litre *Escherichia coli* (*E. coli*) culture (Figure 3.2). However, purification of Cys-pUb⁶⁶ and Cys-pUb⁸¹² with the pMCSG7 backbone plasmid did not separate completely by size-exclusion chromatography (Figure 3.1C,D).

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<thead>
<tr>
<th>pSG1-Ub⁺</th>
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<td><img src="image3.png" alt="Image" /></td>
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**Figure 3.1:** Ineffective separation of Cys-pUb⁶⁶ and Cys-pUb⁸¹² by size-exclusion chromatography. Lanes show molecular weight marker (M) followed by elution fractions from size-exclusion chromatography resolved by Coomassie-stained SDS-PAGE gels of (A) Cys-pUb⁶⁶ and (B) Cys-pUb⁸¹² produced using the pUC19 backbone plasmid (pSG1-Ub⁺), and (C) Cys-pUb⁶⁶ and (D) Cys-pUb⁸¹² produced using the pMCSG7 backbone plasmid (pSG2-Ub⁺) plasmid. His₆-Cys-pUb has a molecular weight of 11.2 kDa (indicated by ⬤).

| ![Image](image5.png) |

**Figure 3.2:** High yield and purity of Cys-Ub observed in nickel-affinity column chromatography elution fractions. Lanes show molecular weight marker followed by elution fractions from nickel-affinity column chromatography of Cys-Ub. His₆-Cys-Ub has a molecular weight of 11.2 kDa (indicated by ⬤).
3.3.2 Fluorescent labelling of pUb

The Cys-pUb variants were not produced in high yield (1.15 μg per litre *E. coli* culture for Cys-pUb<sub>S65</sub> and 2.14 μg per litre *E. coli* culture for Cys-pUb<sub>12</sub>) in the pUC19 backbone plasmid. The yield of Cys-pUb<sub>S65</sub> using the pUC19 backbone plasmid was not sufficient so this Cys-pUb variant was not labelled. However, both Cys-Ub and Cys-pUb<sub>S12</sub> were purified successfully (Figures 3.2 and 3.3A) and labelled using Dylight 800 Maleimide (Figure 3.3B). By maintaining the pH between 6.5-7.5, the maleimide group present in the dye can react with sulphydryl groups present on the Cys residue in Cys-Ub or Cys-pUb to create irreversible thioether linkages and generate Ub* and pUb*<sup>19</sup>. The concentration of protein loaded on the gel was not consistent when the efficiency of labelling was measured due to underestimation of the concentration of Ub* in lane 2 (Figure 3.3B). This was accounted for when performing auto-ubiquitination assays using Ub* and pUb<sub>S12*</sub>.

![Image](image.png)

**Figure 3.3: Production of Ub* and pUb<sub>S12*</sub>.** (A) Phos-tag gel shows unmodified Ub (lane 1) and Cys-pUb<sub>S12</sub> (lane 2). (B) Ub<sub>IR</sub> scan shows labelling efficiency of and pUb<sub>S12*</sub> and Ub*.

3.3.3 Quantitation of pUb stimulation

Initially, auto-ubiquitination assays were performed using Ub* to confirm activation of parkin in the presence of 10% unlabelled pUb<sub>S65</sub> (Figure 3.4). It is evident that unstimulated parkin forms Ub* chains in the presence of 100% Ub*. Parkin is activated to a greater extent in the presence of 10% pUb<sub>S65</sub> as seen by increased Ub* chain
formation (Figure 3.4A) and the increase in densitometry (Figure 3.4B). This is consistent with our previous findings as well as finding from other groups. Increasing the concentration of parkin in the reaction from 1µM to 2µM did not show a significant increase in activity. In addition, incubation times of both 60 minutes and 90 minutes at 30ºC showed that parkin is 4-fold more active in the presence of 10% pUbS65-k than in the presence of 100% unmodified Ub* (Figure 3.4B).

Figure 3.4: Ub* is an effective tool to quantitate parkin activity. (A) Parkin auto-ubiquitination assays with fluorescently labelled unmodified Ub (Ub*) and PINK1 phosphorylated Ub (pUbS65-k) were visualized by IR scan at λ=800 nm. Reactions all contain E1, E2, full-length parkin (E3), ATP and different Ub variants. The stoichiometry of Ub* and pUbS65-k included in lanes 1-8 are listed below the gel. The reaction time and amount of parkin used in the reactions are listed above the gel. (B) The representative densitometry graph of the gel in A.

We then investigated the impact of 100% pUbS12* on parkin activity in auto-ubiquitination assays (Figure 3.5A). The Ub infrared (UbIR) scan, which observes Ub fluorescence at λ = 800 nm, together with the anti-parkin immunoblot revealed the presence of two parkin-pUbS12 bands (Figure 3.5A,B lane 3). This is consistent with our previous anti-parkin immunoblot (Figure 2.7B) since partial activation of parkin was previously observed in the presence of 100% pUbS12. In these reactions only pUbS12 was fluorescently labeled (Figure 3.5A). As a result, only chains built with pUbS12 are visible (Figure 3.5A), while all parkin-conjugated Ub chains are observable in the anti-parkin immunoblot (Figure 3.5B). Control lanes 1 and 2 lack fluorescently labeled Ub (Figure 3.5A). The corresponding anti-parkin immunoblot confirms significant activation of
parkin observed with pUb$^{S65-k}$ (Figure 3.5B, lane 1) and reduced but evident activity with 100% unmodified Ub (Figure 3.5B, lane 2).

Figure 3.5: pUb$^{S12}$ partially activates parkin in auto-ubiquitination assays. (A) Parkin auto-ubiquitination assays with unmodified Ub (Ub), PINK1 phosphorylated Ub (pUb$^{S65-k}$) and fluorescently labelled pUb$^{S12}$ (pUb$^{S12*}$) were visualized by IR scan at $\lambda=800$ nm (M, molecular weight marker). Reactions all contain E1, E2, full-length parkin (E3), ATP and different Ub variants: 10% pUb$^{S65-k}$ + 90% Ub (lane 1), 100% unmodified Ub (lane 2) and 100% pUb$^{S12*}$ (lane 3). (B) Parkin auto-ubiquitination assays with Ub (Ub), PINK1 phosphorylated Ub (pUb$^{S65-k}$) and fluorescently labelled pUb$^{S12}$ (pUb$^{S12*}$) were visualized by anti-parkin immunoblot (M, molecular weight marker). The stoichiometry of Ub and pUb variants included in lanes 1-3 are the same as in (A) and are listed below the blot. (C) Parkin auto-ubiquitination assays with unmodified Ub (Ub), PINK1 phosphorylated Ub (pUb$^{S65-k}$) and pUb$^{S12}$ were visualized by anti-parkin immunoblot (M, molecular weight marker). The stoichiometry of Ub and pUb variants included in lanes 1-5 are listed below the blot.

Further testing of pUb$^{S12}$ at different stoichiometries revealed parkin activation in the presence of 30% pUb$^{S12}$ (Figure 3.5C, lane 4) similar to patterns seen in auto-ubiquitination assays where pUb$^{S12}$ was present in mixtures with unmodified Ub and 10% pUb$^{S65}$ (Figure 2.7B, lanes 4-9). The Ub or pUb variant responsible for the chain building observed in these mixtures is inconclusive at this point. In future work, we will extend these studies to test auto-ubiquitination activity of parkin with precise stoichiometric mixtures of fluorescently labelled and unlabelled Ub, pUb$^{S65-k}$ and pUb$^{S12}$ (as in Figure 2.7B, lanes 4-9 and Figure 3.5C, lane 4). Controls used in the anti-parkin immunoblot
confirm activation of parkin by both 10% pUb\textsuperscript{S65-k} (Figure 3.5C, lane 2) and 100% pUb\textsuperscript{S65-k} (Figure 3.5C, lane 5), with reduced chain building by autoinhibited and unstimulated parkin (Figure 3.5C, lane 1).

We further optimized the fluorescent parkin assay by decreasing the amount of fluorescently labelled Ub in the reaction mixture in order to determine the optimal level of labelled Ub needed to clearly visualize and quantitate parkin activation. Auto-ubiquitination assays were performed with 100% pUb\textsuperscript{S12} where 25% of the mixture was pUb\textsuperscript{S12*} and the remaining 75% was unlabelled pUb\textsuperscript{S12} (Figure 3.6A, lane 1). The assay was also performed with 100% pUb\textsuperscript{S12} where 10% of the mixture was pUb\textsuperscript{S12*} and the remaining 90% was unlabelled pUb\textsuperscript{S12} (Figure 3.6A, lane 2). In both cases, only one band was observed unlike the intense double banding pattern seen previously in auto-ubiquitination assays with only labelled pUb\textsuperscript{S12*} substrate (Figure 3.5A, lane 3). Similarly, unmodified Ub was not sufficient to observe parkin-Ub chain building when only 10% of the mixture was Ub* (Figure 3.6A, lane 3 and 4). The data show ideal reaction conditions require Ub or pUb variant that is completely labelled and at a minimal concentration of 20 μM to observe phosphorylation-dependent modulation of parkin activity in auto-ubiquitination assays (Figures 3.4, 3.5A and 3.6A).

The corresponding anti-parkin immunoblot does not discriminate between the presence or absence of fluorescent labels (Figure 3.6B) since the blot shows similar parkin activity levels to those observed previously in the case of 100% pUb\textsuperscript{S12}, 100% Ub and 10% pUb\textsuperscript{S65-k} (Figure 3.6B, lanes 1-4). It is important to note that significant activity was seen with 90% Ub* and 10% pUb\textsuperscript{S65-k} in the Ub\textsuperscript{IR} scan (Figure 3.6A, lane 6). In the corresponding anti-parkin immunoblot, the activity of parkin seems to appear near the level of unstimulated parkin in the presence of 90% Ub* and 10% pUb\textsuperscript{S65-k} (Figure 3.6B, lane 6). Inconsistencies seen with the anti-parkin immunoblots, which are not uncommon, in auto-ubiquitination assays highlight the importance of the use of Ub* and pUb* variants.
3.3.4 Enhancing Cys-pUb$^{S12}$ production and purification

All auto-ubiquitination assays were done with pUb$^{S12*}$ purified from the pUC19 backbone plasmid. However, the Cys-Ub was purified from the pMCSG7 backbone plasmid and was produced in significantly higher yield (360 $\mu$g per litre E. coli culture for Cys-Ub) (Figure 3.2). Therefore, we switched the production of Cys-pUb$^{S12}$ to the pMCSG7 backbone plasmid. Since the pUb$^{S65}$ in auto-ubiquitination assays was previously produced using the kinase PINK1 and not fluorescently labelled, we used the pMCSG7 backbone plasmid to produce Cys-pUb$^{S65}$ as well.

Figure 3.6: Optimizing the concentration of fluorescently labelled Ub or pUb variants in auto-ubiquitination assays. (A) Parkin auto-ubiquitination assays with unmodified Ub (Ub and Ub*), PINK1 phosphorylated Ub (pUb$^{S65*}$), pUb$^{S12}$ and pUb$^{S12*}$ were visualized by IR scan at $\lambda=800$ nm (M, molecular weight marker). Reactions all contain E1, E2, full-length parkin (E3), ATP and different Ub and pUb variants in stoichiometry as listed below the gel. (B) Replicated parkin auto-ubiquitination assays (identical reactions as in A) visualized by anti-parkin immunoblot.

Although, incomplete purification was observed following size-exclusion chromatography of Cys-pUb$^{S12}$ and Cys-pUb$^{S65}$ using the pMCSG7 backbone plasmid (Figure 3.1C,D), these Cys-pUb variants were subjected to anion-exchange chromatography (Figure 3.7). We found that pUb was the dominant band in the Phos-tag gels, but anion-exchange chromatography did not completely separate Cys-pUb$^{S12}$ or Cys-pUb$^{S65}$ from non-phosphorylated Cys-Ub. We suspected the formation of disulfide...
bonds between Cys-pUb and mistranslated Cys-Ub led to incomplete pUb purification. We added reducing agent (250 μM TCEP) during this purification step, and this led to essentially pure pUb (Figure 3.7).

![Figure 3.7: Nearly complete separation of Cys-pUbS65 and Cys-pUbS12 by anion-exchange chromatography. Lanes show unmodified Ub as the negative control followed by elution fractions from anion-exchange chromatography resolved by Coomassie-stained Phos-tag gels of (A) pUbS65 and (B) pUbS12 produced using the pMCSG7 backbone plasmid. Migration of Ub and pUb are shown beside the gels.](image)

3.4 Discussion

3.4.1 Fluorescently labelled phospho-ubiquitin is a chemical probe for elucidating signalling pathways

Ub* has been previously employed to study the autoinhibitory Ubl domain of parkin\(^{15}\). This method proved to be more efficient as observed by the distinct chain building on parkin as opposed to the use of anti-parkin immunoblots to study the effect of the Ubl domain\(^{15,20}\). Ub* also has a wider range of applications since it can be used to determine enzyme kinetics of parkin through time-course measurements and varying concentrations of substrate.

Previously, we were able to successfully produce several pUb variants in pure form as demonstrated by MS/MS and Phos-tag gels\(^{11}\). These variants were used in auto-ubiquitination assays that were visualized on anti-parkin immunoblots to test their effect
on parkin activation\textsuperscript{11}. Here we optimized a quantitative method to monitor parkin activity with fluorescently labelled Ub and pUb variants.

Since Ub does not contain any internal Cys residues, we introduced a single Cys residue in the -2 position to allow specific labelling of the single free sulfhydryl group by maleimide chemistry. Introduction of a single Cys residue reduced the purity of pUb variants significantly by hindering separation of pUb variants from higher molecular weight proteins. A possible explanation is conformational change in the pUb variant preventing dissociation from bacterial proteins or disulfide bond formation between the Cys-pUb variant and other proteins. Addition of increasing amounts of reducing agent (i.e., TCEP) or switching to an alternate reducing agent (i.e., DTT) could overcome this issue in the future. Future experiments will also enable elucidation of the role the Cys residue plays in obstructing pUb separation.

3.4.2 pUb\textsuperscript{S65} and pUb\textsuperscript{S12} represent different routes to stimulate parkin

Mutations in \textit{park2} (the gene encoding parkin) were identified in 50% of AR-JP patients, and defective parkin is thought to be the primary cause of AR-JP\textsuperscript{1,10}. It was only recently discovered that PINK1 is recruited to the mitochondria during oxidative stress to activate parkin E3 ligase activity by phosphorylating parkin at S65 in the Ubl domain\textsuperscript{2-4}. PINK1 was also found to phosphorylate the homologous site, S65, in Ub to stimulate parkin activity\textsuperscript{5-8}. The exact mechanism of parkin activity is still undefined. Initially, pUb\textsuperscript{S65}-induced parkin activation was found to be dependent on the stoichiometry of phosphorylation, with parkin showing no activity when pUb\textsuperscript{S65} was the sole Ub variant\textsuperscript{8}. Our previous studies demonstrate unambiguously that pUb\textsuperscript{S65} stimulates parkin activity at any stoichiometry\textsuperscript{11}.

The use of fluorescently labelled Ub and pUb is an effective method to identify the substrate responsible for chain building during parkin activation. This provides further insight into the mechanism of parkin activation as currently proposed models are contradictory\textsuperscript{5-7,9}. Some studies suggest that PINK1 phosphorylates parkin and Ub, which then proceeds to relieve autoinhibition and stimulate parkin leading to a feed-forward model of parkin activation\textsuperscript{5,7}. Other studies suggested phosphorylation of parkin primes
the enzyme for activation by pUb$^{S65}$. Although there has been considerable amount of research on pUb$^{S65}$, there is not much evidence for the effect of other experimentally observed phosphorylation sites of Ub on parkin$^{12,13}$. Studies with pUb$^{S20}$ and pUb$^{S57}$ found that these pUb variants fail to simulate parkin$^9$. We found that stimulated parkin activity is reduced in a dose-dependent manner with increasing phosphorylation levels of pUb$^{S20}$ in the Ub substrate pool and that pUb$^{S12}$ can ubiquitinate parkin$^{11}$.

In this study, we found that unmodified Ub can stimulate parkin and pUb$^{S65}$ significantly activates parkin in fluorescent auto-ubiquitination assays. We also observed a low level of clearly defined parkin activity with pUb$^{S12}$ as the only Ub variant in the reaction. Since a system for site-specific incorporation of phosphothreonine has yet to be developed, pUb$^{S12}$ was used to mimic phosphothreonine at position 12 in Ub. In a mixture of unmodified Ub and pUb$^{S12}$, parkin was activated as well, but to a greater extent than when pUb$^{S12}$ was the only Ub variant. In addition, fluorescently labelled unmodified Ub showed more Ub chain building than pUb$^{S12}$. We also observed that fluorescently labelled Ub or pUb variants is a more consistent method of determining parkin activity in auto-ubiquitination assays when compared with anti-parkin immunoblots. However, ideal auto-ubiquitination assay conditions require completely labelled Ub or pUb variant that is at a minimal concentration of 20 μM to observe phosphorylation-dependent modulation of parkin activity.

In all cases, pUb$^{S65}$ showed the highest activation of parkin as seen by increased chain building from fluorescently labelled unmodified Ub. We propose that increased amounts of pUb$^{S12}$ in the cell could perturb pUb$^{S65}$-induced activation of parkin, which is required for targeting mitochondrial outer membrane proteins for degradation. Experiments with precise ratios of fluorescently labelled Ub and pUb variants are currently underway to determine the exact stoichiometry of chain building on parkin when Ub and pUb variants are present in different ratios in the same reaction mixture. The varying effects of different phosphorylation sites on Ub towards parkin activation suggest that phosphorylation of Ub may be a signal for regulation of parkin and possibly other E3 Ub ligases in the human proteome.
3.5 Conclusion

Of the 100,000 experimentally observed phosphorylation sites in the human proteome, the upstream kinase for more than 90% of these sites remains unknown\textsuperscript{13,21}. Parkin activity depends on the location and stoichiometry of phosphorylation on its substrate Ub. Site-specific phosphorylation of proteins, such as Ub, enables identification of new routes to parkin activation. Identification of alternate routes of phosphorylation-dependent activation of proteins involved in disease, such as parkin in PD, can provide insight into the molecular mechanism of PD. We demonstrated that differentially phosphorylated Ub molecules are characteristically distinct or, in comes cases, incompetent substrates for parkin.

3.6 References


Chapter 4

4  Summary and future perspectives

4.1  Experimental summary

4.1.1  Goal

Upon oxidative stress in the mitochondria, the phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1) phosphorylates parkin at a specific serine residue (S65) in its ubiquitin (Ub)-like (Ubl) domain and the homologous site, S65, in the parkin substrate Ub\(^1\)\(^\text{a}^{-5}\). These phosphorylation events disrupt the autoinhibition on the E3 Ub ligase parkin and primes the enzyme for activation\(^1\)\(^,2\)\(^,4\)\(^,6\). There are 6 other experimentally determined phosphorylation sites on Ub (T7, T12, T14, S20, S57 and Y59) for which the upstream kinases remain unknown\(^7\)\(^,8\). These sites are not proteomic artifacts, but genuine phosphorylation sites conserved from yeast to human\(^7\). Ub phosphorylation sites seen by high-throughput proteomic data were verified using several different phosphoproteomic methods\(^7\). The overall goal of this thesis was to identify novel routes of parkin activation by producing site-specifically phosphorylated Ub (e.g., pUb\(^S12\)) and pUb\(^S20\)).

Genetic code expansion is an effective method to produce site-specifically phosphorylated proteins in *Escherichia coli (E. coli)*\(^9\). Site-specific phosphoprotein production using genetic code expansion is essential when the upstream kinases are unknown or when it is difficult to produce phosphoproteins in active form. Production of pUb at two previously uncharacterized sites (T12 and S20) was achieved in high yield and high purity using genetic code expansion. Several other experimentally determined phosphorylation sites on Ub (T7 and S57) were also produced in pure form.

Due to low protein yields from competition between peptide chain elongation with the aminoacylated suppressor tRNA and termination by release factor 1 (RF1), a RF1 deficient *E. coli* strain was engineered\(^9\)\(^-14\). The RF1 deficient *E. coli* strain had an increased O-phospho-L-serine (pSer) incorporation efficiency per cell, but was found to be toxic to cell fitness and viability\(^15\). Genetic code expansion strategies, such as the pSer
system, do not have the same fidelity as the canonical protein synthesis since mistranslation is a common by-product especially in RF1 deficient *E. coli*\textsuperscript{16}. Near-cognate mis-incorporation of glutamine was most commonly observed, and mis-incorporation of lysine, tyrosine and glutamic acid were present in lower abundance than pSer\textsuperscript{16}.

Codon skipping was a novel product of mis-translation observed during production of pUb variants using the pSer system in RF1 deficient *E. coli* cells. During purification of pUb\textsuperscript{S7}, we observed a +3 frameshifted protein (Δ7) that completely skipped the in-frame UAG codon. Codon skipping was also observed during purification of the Ubl domain of parkin phosphorylated at Ser65. However, simple purification procedures led to separation of pure phosphorylated Ub and Ubl from mis-translated proteins. We identified that for single pSer incorporation, the 2\textsuperscript{nd} generation pSer system and BL21(DE3) cells are ideal for expression.

Precise stoichiometric measurements of pUbs needed to stimulate parkin E3 Ub ligase activity were also determined using high purity pUbs. Stimulated parkin was inhibited in a dose-dependent manner by pUb\textsuperscript{S20} (Chapter 2) and unstimulated parkin was partially activated by pUb\textsuperscript{S12} (Chapter 2 and 3). In addition, pUb\textsuperscript{S65} was found to be a suitable substrate for parkin at all stoichiometries, and our fluorescence assay uncovered that unstimulated parkin showed significant activity when unmodified Ub is the only source of Ub in the reaction.

### 4.1.2 Limitations

The limitations to site-specific production of pUbs that are previously uncharacterized is potential interactions with other proteins and changes in folding compared to wild-type Ub. Introduction of the cysteine residue at the -2 position of Ub could also create previously absent interactions. During purification of several pUb variants, unsatisfactory separation was observed: Cys-pUb\textsuperscript{S12} and Cys-pUb\textsuperscript{S65} did not separate from higher molecular weight *E. coli* proteins. Increasing the amount of reducing agent allowed separation and produced essentially pure Cys-pUbs. Likewise, the production of pUb\textsuperscript{S12}
and pUb\textsuperscript{S20} in high yield and high purity provided key insights and uncovered novel alternate routes to parkin activation.

4.2 Future Directions

4.2.1 Potential therapeutic agents for parkin

The activation of parkin by pUb\textsuperscript{S65} is widely studied due to the discovery of its upstream kinase, PINK1. Here, we demonstrate the first potential inhibitor to parkin. It is well-known that the Ubl domain autoinhibits parkin \textit{in cis}. However, there has been no observation of parkin inhibition by a molecule \textit{in trans}. We found that increasing phosphorylation of pUb\textsuperscript{S20} inhibits parkin activity, which implicates this phosphorylated form of Ub to be the first inhibitor to parkin. Although we need to do additional experiments to validate our findings, preliminary data suggests pUb\textsuperscript{S20} as the only potential parkin inhibitor to date. Through auto-ubiquitination assays, pUb\textsuperscript{S20} prevents stimulation of parkin in a dose-dependent manner (Figure 2.7C). We recognize that \textit{in vivo} studies must be performed to validate these \textit{in vitro} results, but this thesis has made key advances in providing the first assignment of function to a Ub phosphorylation site other than S65. These data suggest that Ub phosphorylation may represent a new tool to control parkin activity. Our fluorescently labelled pUb variants are, therefore, chemical probes of the parkin pathway and represent a starting point for future drug therapy to modulate stimulation of parkin during mitochondrial membrane potential depolarization. In addition, activation of parkin when pUb\textsuperscript{S65} or pUb\textsuperscript{S12} are the sole Ub variants in the reaction suggests potential alternate routes to mitophagy (Figure 2.7A,B). Small molecules that mimic the structure of pUb\textsuperscript{S65} or pUb\textsuperscript{S12} could both potentially be used to control the activity of parkin in parkin-linked Parkinson’s disease (PD) patients.

4.2.2 Crystallization of phospho-ubiquitin variants

Further mechanistic insight into the activation – or lack thereof – of parkin can be obtained through crystal structure determination. Since the pUb variants have different effects on parkin activation, deciphering the crystal structure of pUbs and pUb-parkin complexes will provide insight into the location of parkin binding or the structural hindrance preventing parkin from binding. The work presented in this thesis suggests
direct interactions between parkin and pUbS12, and potential interactions between parkin and pUbS20. Structural data is currently lacking in this area, so this represents an exciting future direction.

4.2.3 Phospho-ubiquitin interactome

Production of pure pUb can also help identify potential phosphorylation-dependent Ub interactors through pull-down assays and pUb-interactor identification by mass spectrometry. The pull-down assays can be done with human embryonic kidney (HEK)-293 cells or SH-SY5Y neuronal cells. One recent example shows that pull-down assays done with the phosphomimetic S65E improves the interaction between Ub and Rad23, and thus results in a possible inactivation of Rad23’s ability to shuttle proteins for proteasomal degradation17. It is thought that Rad23 would eventually be depleted from cells leading to increased Ub conjugates resulting from reduced substrate processing17. Protein interactions of the other experimentally determined pUb variants are yet to be explored and represent an exciting area for future research.

4.3 Conclusion

This thesis provides insight into the roles of additional phosphorylation sites on Ub as potential substrates for parkin. Although the upstream kinases for many of these sites were unknown, genetic code expansion strategies were employed to site-specifically phosphorylate desired sites on Ub so that the stoichiometry and location of phosphorylation can be controlled in precise experiments monitoring parkin activity. Elucidation of the roles of additional phosphorylation sites in Ub provides insight into signalling in the ubiquitin proteasome system, suggests potential drug targets and ultimately future therapies for the treatment of PD. The downstream signalling effects of phosphorylated Ub can uncover new nodes for potential therapeutic intervention in PD-related pathways.

4.4 References


Curriculum Vitae

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**Presentations:**

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