# The mechanism of Parkin activation by PINK1 phosphorylation

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This dissertation is submitted for the degree of Doctor of Philosophy

> Supervised by: Dr David Komander

# Declaration

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

This dissertation does not exceed the prescribed word limit for the relevant Degree Committee.

Christina Gladkova

# Summary

## The mechanism of Parkin activation by PINK1 phosphorylation Christina Gladkova

Mutations in the E3 Ub ligase Parkin (PARK2) and the protein kinase PINK1 (PARK6) are genetically linked to Young-Onset Parkinson's Disease. The two enzymes orchestrate clearance of damaged, cytotoxic mitochondria through a specialized form of autophagy termed mitophagy. By phosphorylating ubiquitin (Ub) on the surface of damaged mitochondria, PINK1 generates a phospho-Ub receptor for autoinhibited cytosolic Parkin. Once localized to the mitochondrial surface, Parkin is activated by direct PINK1-mediated phosphorylation on its Ub-like domain (Ubl). To efficiently recruit the autophagy machinery, activated Parkin transfers Ub from upstream E2 Ub-conjugating enzymes to substrates on the mitochondrial surface via an E3  $\sim$  Ub intermediate.

Resolving the precise mechanism of Parkin activation by PINK1-mediated phosphorylation is the primary aim of this thesis. Additionally, building on previous work in which a novel phospho-Ub conformer was characterized crystallographically, the same conformer was found in unmodified Ub in this work. This striking finding is supported by further biochemical and biophysical investigation of the newly discovered equilibrium in unmodified Ub.

To address the mechanism of Parkin activation and open new avenues for translational research, both dynamic and crystallographic approaches were employed. Hydrogen-deuterium exchange mass spectrometry reveals that Parkin phosphorylation enables a conformational equilibrium between an autoinhibited and an active Parkin state. In the active state the phospho-Ubl domain binds to the Unique Parkin domain (UPD) of the Parkin core and thereby displaces the catalytic RING2 domain from its autoinhibitory position. Catalytic intermediates, such as the E2-bound state or the Ub-charged Parkin species shift the equilibrium in favour of the active conformer. Parkin-mediated substrate ubiquitination occurs by a flexibly tethered catalytic RING2 domain independently of the Parkin core, in line with its minimal substrate selectivity observed *in vitro*. The new activating interface between the Parkin phospho-Ubl and the Parkin core is revealed in a 1.8Å crystal structure of phosphorylated human Parkin lacking the flexible catalytic domain. Additionally to the phospho-Ubl, a conserved linker region, the activating element (ACT) aids displacement of the catalytic RING2 domain by mimicking RING2 autoinhibitory interactions with the Parkin core. This crystal structure explains patient mutations in the UPD phosphate-binding pocket as well as in the newly identified ACT.

Together, the clinically relevant molecular insights described in this thesis may facilitate the development of therapeutic or diagnostic tools for Parkinson's disease.

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# List of Publications

The publications listed below arise as a result of the work described here. Images and Methods have been adapted from Gladkova et al. (2018) attached in Appendix E, and Gladkova et al. (2017) attached in Appendix F.

#### Chapters 4, 5, 6:

<u>Gladkova, C.</u>, Maslen, S. L., Skehel, J. M., & Komander, D. (2018). Mechanism of parkin activation by PINK1. *Nature*, 559(7714), 410–414. http://doi.org/10.1038/s41586-018-0224-x

#### Chapter 3:

<u>Gladkova, C.\*</u>, Schubert, A. F.\*, Wagstaff, J. L., Pruneda, J. N., Freund, S. M., & Komander, D. (2017). An invisible ubiquitin conformation is required for efficient phosphorylation by PINK1. *The EMBO Journal*, 36(24), 3555–3572. http://doi.org/10.15252/embj.201797876

#### Chapter 3:

Schubert, A. F., <u>Gladkova, C.</u>, Pardon, E., Wagstaff, J. L., Freund, S. M. V., Steyaert, J., Maslen, S. L., and Komander, D. (2017). Structure of PINK1 in complex with its substrate ubiquitin. *Nature*, 552(7683), 51–56. http://doi.org/10.1038/nature24645

#### Chapter 4:

Gersch, M., <u>Gladkova, C.\*</u>, Schubert, A. F.\*, Michel, M. A., Maslen, S., & Komander, D. (2017). Mechanism and regulation of the Lys6-selective deubiquitinase USP30. *Nature Structural & Molecular Biology*, 24(11), 920–930. http://doi.org/10.1038/nsmb.3475

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# Chapter 1.

# Introduction

# 1.1. The interplay between ubiquitination and phosphorylation

Proteins carry out essential structural, enzymatic and signalling functions in cells. Protein half-life is governed by their rate of synthesis and degradation. Because some proteins are actively degraded via the Ubiquitin-Proteasome System (UPS), (Hershko and Ciechanover, 2003), and others are longer lived, protein half-lives range between 45 minutes and 22.5 hours (Eden et al., 2011).

Turnover at the time scale of hours is too slow to mediate responses which occur within minutes, such as triggering immune or metabolic pathways. To mediate responses on this fast timescale, proteins already present in the cell are regulated by post-translational modifications (PTMs). In addition to signal transduction, PTMs also regulate cell division, organization and fate. To this end, both reversible and irreversible PTMs exist: while the covalent coupling of a functional group or a protein to a substrate can be reversed (phosphorylation, ubiquitination, glycosylation, acetylation, etc.), a regulated post-translational protein cleavage can not.

The most common PTM is phosphorylation, canonically the attachment of a phosphate group to substrate Ser, Thr or Tyr residues by kinase enzymes. While phosphorylation can only serve as an on/off signal, ubiquitination can be fine-tuned. Based on Phospho-SitePlus and recent proteomic studies, ubiquitination is the second most frequent PTM (Hornbeck et al., 2015; Akimov et al., 2018; Kim et al., 2011). A small signalling protein

ubiquitin (Ub) is canonically attached to substrate Lys residues through an isopeptide bond either as a monomer or a series of functionally distinct polymers by Ub E3 ligases (Hunter, 2007). While initially associated with protein degradation via the UPS, many additional functions of Ub signalling have since been reported (Komander and Rape, 2012; Swatek and Komander, 2016).

Crosstalk, in which phosphorylation and ubiquitination of substrates affect downstream signalling mediated by either PTM, to achieve specific cellular outcomes is very common (Hunter, 2007). For example, the cell cycle is regulated by phosphorylation of the anaphase-promoting complex, an E3 ligase which degrades specific sets of substrates at cell cycle checkpoints (Pines, 2011). On the other hand, ubiquitination modulates kinase activity in Nuclear Factor  $\kappa$  enhancer Binding protein (NF- $\kappa$ B)-mediated immune signalling (Chen, 2005).

Strikingly, convergence of the two modifications has only recently been reported in triggering mitochondrial clearance by autophagy, termed mitophagy (Harper et al., 2018). Ubiquitin itself becomes a substrate for phosphorylation by the PTEN-induced putative kinase 1 (PINK1) on Ser65, generating a new signalling molecule - phospho-Ub (pUb). Although other Ub modifications have been reported through large-scale proteomic studies, only the functional context of Ser65 Ub phosphorylation is presently understood (Swatek and Komander, 2016). The role of Ub phosphorylation and Ub signalling mediated by the E3 ligase Parkin in triggering mitophagy is the subject of this thesis.

The motivation to study PINK1/Parkin-mediated mitophagy arises from links between faulty mitochondrial clearance and Parkinson's disease (PD). Genetic defects in both the kinase PINK1 and the E3 ligase Parkin, are linked to Young-Onset Parkinson's Disease (YOPD), (Pickles et al., 2018). While kinases are currently the most common drug targets, it is, among others, this link to disease which motivates detailed understanding of the Ub system, a prime target for therapy development (Wertz and Wang, 2018).

## 1.2. The ubiquitin system

Ubiquitination was first described in the context of protein degradation. In the search for an ATP-dependent protease, an ATP-dependent system for Ub conjugation to specific substrates was discovered instead (Hershko et al., 1983). This energetic requirement enabled tight control of signalling outcomes by selection of specific conjugation targets (Hershko and Ciechanover, 2003).

The E1 Ub-activating enzyme is at the apex of the Ub conjugation cascade (Fig 1.1 A). During the only ATP-dependent step, the E1 enzymes activate Ub molecules by forming



Figure 1.1.: The ubiquitin system. (A) ATP-dependent Ub activation by the E1 activating enzyme. At the end of the first step of the Ub cascade, activated Ub is attached to the E1 active site Cys residue via its C-terminus. (B) Ub transfer from the E1 activating enzyme to the catalytic Cys residue of the E2 conjugating enzyme occurs in a transthiolation step. (C) Two possible mechanisms of substrate ubiquitination by E3 Ub ligase enzymes. Either activated Ub is transferred to the E3 active site Cys residue in a transthiolation step before conjugation onto substrate Lys residues (HECT, RBR), or substrate ubiquitination by the E2 ~ Ub conjugate is directed by a trimeric E3:E2 ~ Ub:substrate complex (RING). (D) Ub conjugation is opposed by DUB enzymes acting through metallo- or a cysteine-based mechanisms. (E) Different Ub topologies which can be assembled onto substrates.

a covalent  $E1 \sim Ub$  thioester intermediate linked through the E1 active site Cys residue and the Ub Carboxyl terminus (C-terminus) (Schulman and Harper, 2009). The activated Ub is then transferred via the E2 Ub-conjugating enzymes for discharge onto substrates by E3 Ub ligase enzymes (Fig 1.1 B,C), (Stewart et al., 2016). The E3 enzymes form the numerous base of the Ub conjugation cascade and the members of this enzyme class divide into three families with distinct ubiquitination mechanisms (Zheng and Shabek, 2017): While Really Interesting New Gene (RING) E3 ligases facilitate Ub transfer between the E2 ~ Ub conjugate and the substrate Lys residue by formation of a trimeric complex (Fig 1.1 C, bottom), Homologous to the E6-AP Carboxy-Terminus (HECT) and RING-in-Between-RING (RBR) E3 ligases receive the activated Ub onto their active site Cys residue prior to substrate ubiquitination (Fig 1.1 C, top). Crucial regulators of Ub signalling are the deubiquitinating enzymes (DUBs) which remove substrate-linked Ub (Fig 1.1 D). There are several classes of DUB enzymes which differ in scaffold and cleavage mechanism (Mevissen and Komander, 2017).

Although the discovery of ubiquitination was linked to protein degradation, this and many other functions of the Ub system take advantage of the tunability of the Ub signal. Because Ub can be attached to any Lys substrate, any one of its own seven Lys residues or the Ub Amino-terminus (N-terminus) can serve as substrates for conjugation (Fig 1.2 A). This gives rise to at least eight distinct Ub polymers, a number which rises when considering branched Ub chains or ones of mixed topology (Fig 1.1 E), (Komander and Rape, 2012). While the absolute quantities of Ub conjugates depend on the cell type, roughly one sixth of conjugated Ub forms Ub chains. Mono-ubiquitination is the most common Ub signal with up to 60% of cellular Ub observed to form monoUb conjugates formation in common cell culture lines (Kaiser et al., 2011). The remaining cellular Ub is free and unconjugated.

It is the availability of a dazzling number of ubiquitination sites within the proteome combined with the possibility of conjugating Ub polymers of distinct topologies (Ye et al., 2012), which gives the Ub system its unique tunability to control almost all aspects of cellular function (Komander and Rape, 2012). The repertoire of signalling outcomes can be increased further still as Ub itself can be subject to other types of PTM, as is the case for Ub Ser65 phosphorylation in mitophagy (Swatek and Komander, 2016). First, the structure of Ub enabling the unique tunability will be explored, followed by examples of how different Ub signals are utilized in cells and a mechanistic discussion of the Ub-conjugating machinery.

# 1.3. The structure of ubiquitin

In cells, the 76 residue Ub protein is generated by cleavage of a head-to-tail linked polyUb gene product (Ozkaynak et al., 1984). Yeast Ub only differs from human Ub by three substitutions (P19S, E24D, A28S) demonstrating a high degree of Ub conservation. Ub adopts a  $\beta$ -grasp fold consisting of an  $\alpha$ -helix packed against a mixed parallel-antiparallel  $\beta$ -sheet (Hochstrasser, 2009). Functionally, the key features of Ub are the flexible C-terminal -LRGG tail through which Ub is conjugated, and the seven Lys residues (Lys6, 11, 27, 29, 33, 48 and 63) which together with the N-terminus serve as linkage sites for further ubiquitination (Fig 1.2 A). While each of the eight possible linkage types has been observed in a cellular context, the most abundant are Lys48-linked chains (Peng et al., 2003; Kim et al., 2011).



Figure 1.2.: Structural features of ubiquitin. (A) Amine functional groups on the Ub surface (PDB ID: 1UBQ), which serve as linkage sites for further ubiquitination. (B) Surface patches necessary for proper Ub recognition. Left: Position of the Ile44 patch (blue - Ile44, Leu8, His68, Val70) relative to the amine functional groups. Right: Position of the Ile36 (green - Ile36, Leu8, Leu71, Leu73) and Phe4 (orange - Phe4, Gln2, Thr12) patches relative to the amine functional groups.

Members of the Ub conjugation machinery or Ubiquitin-Binding Domains (UBDs) most commonly utilize a hydrophobic patch on the surface of Ub consisting of residues Ile44, Leu8, His68 and Val70 (Fig 1.2 B, left). All but the most conservative mutations of residues making up the Ile44 patch are detrimental to yeast growth (Roscoe et al., 2013). In fact, two further patches are utilized for specific Ub recognition in cells - the Ile36 patch (Ile36, Leu8, Leu71 and Leu73) as well as the Phe4 patch (Phe4, Gln2 and Thr12), (Fig 1.2 B, right), both of which are also necessary for proper yeast growth (Roscoe et al., 2013).

While these surface features define the many biological functions of Ub, its stable hydrophobic core confers unique biophysical properties. Ub is extremely stable - its melting temperature ( $T_m$ ) is above 95°C and Ub remains folded across a wide pH range (Jenson et al., 1980). Pioneering studies using a range of techniques have probed the biophysical properties of Ub, and, especially for Nuclear Magnetic Resonance (NMR) technique development, Ub became a model system (Jackson, 2006).

Coupled with the >300 deposited structures of Ub (Harrison et al., 2016), NMR dynamic studies on the microsecond timescale have identified the  $\beta 1$ - $\beta 2$  loop as hotspot of conformational flexibility. This hairpin contains the Leu8 residue, which can contribute to either the Ile44 or the Ile36 patch depending on conformation of the loop (Hospenthal et al., 2013; Lange et al., 2008; Phillips and Corn, 2015). Rotational motion was also observed in the well-packed Ub core (Lindorff-Larsen et al., 2005). Ub also became a model system to study energetics of protein folding: Ub could be stabilized by altering its surface contacts (Makhatadze et al., 2003), although mutagenesis was not able to stabilize the Ub core (Lazar et al., 1997). Despite significant efforts, no consensus has been reached over the Ub folding pathway, i.e. whether Ub populates a folding intermediate (Sosnick et al., 2004; Fernandez and Li, 2004). What the link is between the unique biophysical properties of Ub and its cellular function has also remained unclear.

With our ability to purify and characterize pUb modified on Ser65, an entirely new Ub conformer has been reported (see Section 1.11.3), (Wauer et al., 2015b; Dong et al., 2017). This conformation is markedly different from the common Ub fold described above and the availability of the C-terminal tail for conjugation as well as integrity of the interaction hydrophobic surfaces is disrupted. Either owing to the equilibrium, or the presence of a bulky, negatively charged phosphate group, pUb was incompatible with several conjugating and DUB enzymes (Wauer et al., 2015b; Huguenin-Dezot et al.,

2016). This discovery suggests further facets of Ub structure remain to be uncovered.

# 1.4. Ubiquitin-like modifiers

The Ub structural features and principles governing the Ub system extend to other related Ubiquitin-like (Ubl) modifiers. These are similar in the sense that all Ubl modifiers adopt the Ub  $\beta$ -grasp fold and share the ability to be conjugated to substrates through orthogonal enzymatic cascades (Cappadocia and Lima, 2017; Hochstrasser, 2009). The Ubl fold is also common as a domain present in larger proteins, often involved in the ubiquitination cascade, such as in the RBR E3 ligase Parkin. As it is inserted within the sequence, it does not possess a free C-terminus and cannot be conjugated to substrate Lys residues.

Ubl modifiers which can be conjugated to substrates divide into several orthogonal systems, with independent E1 activating and E2 conjugating enzymes. Interestingly, unlike Ub, the Ubl conjugation cascades do not culminate in a numerous base of E3 enzymes (Cappadocia and Lima, 2017; Hochstrasser, 2009). Their distinct surface properties and C-terminal sequences allow the Ubls <u>SUMO</u> and <u>NEDD8</u> to be distinguished from Ub at the level of Ubl activation by E1 enzymes (Whitby et al., 1998; Walden et al., 2003; Bohnsack and Haas, 2003). Although orthogonal, modes of E2-mediated Ubl conjugation (Reverter and Lima, 2005; Scott et al., 2014), non-covalent Ubl-binding by E2s (Knipscheer et al., 2007; Duda et al., 2007; Capili and Lima, 2007) and E3-mediated Ubl conjugation (Streich and Lima, 2016; Scott et al., 2014) share commonalities with the Ub-system, described in detail below.

While for some Ubl families, such as <u>FAT10</u>, URM1 and UFM1, the substrates are currently unknown, those for NEDD8 are largely limited to a family of multi-subunit Cullin RING E3 Ligases (CRLs), (Zheng and Shabek, 2017), and those for ATG12 and ATG8-like proteins are limited to components of the autophagy machinery (see Section 1.6.1) (Cappadocia and Lima, 2017). The <u>ISG15</u> cascade is upregulated in antiviral responses (Lenschow et al., 2007; Okumura et al., 2008), but its substrates remain elusive. After ubiquitination, the landscape of SUMOylation is the most extensive and has been characterized by several proteomic studies (Becker et al., 2013; Hendriks et al., 2014; Tammsalu et al., 2015). Like Ub, SUMO is also conjugated into polymers, however only

the topology and functions of Ub polymers will be discussed here.

# 1.5. Ubiquitin polymers

Although most ubiquitination events seem to result in the attachment of monoUb, in a physiological context the Ub interactome and function is affected by the formation of Ub polymers linked via the Ub Lys6, 11, 27, 29, 33, 48 and 63 or the N-terminus.

Out of the eight possible Ub polymers, the degradative role of the most common Lys48linked chains has been best characterized. The second most abundant Lys63-linked chain type has been implicated in multiple non-degradative functions. Most prominently, Lys63 chains are involved in the DNA damage response. Lys63-linked chains also mediate inflammatory signalling together with one of the better characterized so called 'atypical' Ub chains, Met1-linked linear chains (linked head-to-tail via the termini). This category encompasses all Ub chain types except for Lys48- and Lys63- linked chains. Although more is known about Lys11- and Lys6- linked chains, the lack of chain-specific tools has hindered characterization of linkage-specific functions of the remaining atypical Ub chains (Lys27-, Lys29- and Lys33- linked) (Michel et al., 2017; Zhang et al., 2017).

With the recent determination of synthetic Lys27 di- and trimeric structures (Pan et al., 2016, 2018), crystal structures of all chain types have now been determined (Michel et al., 2015; Kristariyanto et al., 2015; Komander and Rape, 2012). Since linkages are made up of the flexible Lys side chain and the flexible Ub C-terminal tail, a variety of conformations can be achieved by each chain type. A notable exception are Lys27-linked chains, as the side chain of Lys27 packs against the hydrophobic core and thus more precisely dictates the position of the Lys27  $\epsilon$  amine group. An alignment of all the unique crystallized conformations of each dimeric Ub on the proximal Ub (free C-terminus) gives an idea of the range of conformations accessible to the distal Ub (conjugated C-terminus) in each chain type (Fig 1.3). Nonetheless, NMR is the best way of assessing whether various polyUb chains favour particular conformations in solution (Varadan et al., 2004).

Broadly, Ub chains fall into two categories. Either the relative position of the proximal and distal Ub enables favourable interaction via the Ile44 or Ile36 hydrophobic patches



Figure 1.3.: Ubiquitin polymer structures. Exemplary structures for each resolved conformation. The proximal/distal Ub; Ile44, Ile36 and Phe4 patches are coloured according to the legend. Symbols mark diUb conformations adopted in complexes, as described in the legend.

('closed' Ub chains) to offset the high conformational entropy available when the two Ub molecules behave as beads on a string ('open' Ub chains), (Fig 1.3), (Komander and Rape, 2012; Castaneda et al., 2016a). In the first case, a diUb NMR spectrum would reveal two sets of resonances as the fixed positioning of the two Ub molecules affects chemical shifts in each moiety. Only minor deviations are observed for open chains, resulting in a single set of peaks.

Lys48-linked polyUb is the classic example of a closed Ub chain conformation. The two Ub moieties are able to interact either via their Ile44 patches or the Ile36 patch of the distal Ub interacts with the Ile44 patch of the proximal Ub (Varadan et al., 2002). Lys11-linked chains also adopt a range of conformations, however an interaction between the Ile36 patches is possible and renders Lys11 chains predominantly 'closed' in solution (Bremm et al., 2010; Matsumoto et al., 2010). Lys6-linked polyUb also forms a compact interface utilizing the Ile44 patch of the proximal Ub and the Ile36 patch of the distal Ub (Hospenthal et al., 2013). On the other hand, Lys63- and Met1- linked chains form 'open' polyUb structures (Komander et al., 2009b; Datta et al., 2014). More recently, characterization of Lys29- and Lys33-linked polyUb, linkage sites which are separated only by a turn of the Ub  $\alpha$ -helix, show that both adopt 'open' conformations (Kristariyanto et al., 2015; Michel et al., 2015). Only synthetically generated Lys27-linked chains were analysed by NMR, but consistently with the crystal structures an open conformation is reported. Interestingly, large perturbations in the proximal Ub arise due to the structural role of Lys27 (Castaneda et al., 2016b), as described above.

The plethora of available structurally distinct polyUb linkages allows players in the Ub system, 'writers' - E2/E3 ligase pairs, 'erasers' - DUBs and 'readers' - UBDs, to contribute to a large variety of biological processes, using a common Ub building block.

## 1.6. Roles of the ubiquitin system

Examples of the distinct roles for different Ub chain types and chain-specific enzymes in a number of cellular pathways will be discussed (Fig 1.4), before considering the mechanistic implications of chain specificity on individual elements of the Ub cascade. This functional variety can be achieved because specific substrate modification at a given site by mono- or a particular type of polyUb can lead to a number of outcomes -



Figure 1.4.: Roles of the ubiquitin system. Adapted from (Swatek and Komander, 2016) (A) Example linkage specific enzymes responsible for the conjugation of each of the eight Ub polymers. (B) Example biological processes utilizing each Ub chain type.

e.g. substrate degradation or changes in its interaction partners, activity, and localization.

Due to its particular importance to this work, the role of ubiquitination by the RBR E3 ligase Parkin and phosphorylation by the PINK1 kinase in mitophagy will be discussed in detail below (see Section 1.11).

#### 1.6.1. Degradative processes

The best described role of ubiquitination is in protein degradation. Protein quality control and turnover are necessary to prevent protein misfolding and aggregation, a hallmark of neurodegenerative disease (Berke and Paulson, 2003). Many processes, such as cell cycle regulation and NF- $\kappa$ B signalling, converge on irreversible degradation of effectors or effector inhibitors to execute their programme. In addition to proteasomal degradation necessary to regulate cytoplasmic protein levels, ubiquitination also controls membrane protein degradation via endocytosis and lysosomal degradation. Another major degradative pathway orchestrated by Ub/Ubl proteins is selective autophagy, a process which enables degradation of targets larger than single proteins, such as defunct organelles or protein aggregates.

#### The ubiquitin-proteasome system

Upon proteasomal inhibition with bortezomib or MG132, most polyUb chains accumulate, suggesting a role in proteasomal degradative signalling (Kim et al., 2011). While Lys63-linked chains are likely not involved, no consensus has been reached over the role for atypical Ub chains in proteasomal degradation (Swatek and Komander, 2016). The canonical degradative Ub signal are Lys48-linked Ub chains (Chau et al., 1989) and Lys48 is the only Ub Lys residue essential in yeast (Finley et al., 1994). Lys48-linked chains constitute the most abundant Ub chain type and consistently accumulate upon proteasomal inhibition (Kim et al., 2011; Kaiser et al., 2011). Substrate N-terminal sequences can govern which substrates are preferentially proteasomally degraded. The N-end rule suggests that positively charged or bulky hydrophobic N-terminal residues are preferentially recognized, ubiquitinated and degraded (Tasaki et al., 2012).

On the proteasome, Ub is recognized by one of several proteasomal subunits - Rpn10 (Deveraux et al., 1994), Rpn13 (Husnjak et al., 2008), and Rpn1 (Shi et al., 2016). While other subunits have also been suggested (Lam et al., 2002), the requirement for Lys48-linked chains has not been structurally explained (Finley, 2009). A seminal study identified substrates modified with Lys48 tetraUb chains as optimal proteasomal clients, although each Ub moiety in the chain was recognized independently (Thrower et al., 2000). With more recent determination of new chain types and architectures which mediate proteasomal degradation in cell cycle control (see Section 1.6.2), the proteasomal signal is being redefined to a threshold of Ub molecules present on the substrate necessary for efficient degradation (Swatek and Komander, 2016). Once recognized, Ub chains are remodelled and removed by DUB enzymes resident on the proteasome - Rpn11, Uch37 and USP14 (Finley, 2009).

#### Lysosomal degradation

Different types of Ub signals are required for endocytosis and lysosomal degradation of misfolded or activated receptors (Mukhopadhyay and Riezman, 2007). Monoubiquitination or Lys63-linked ubiquitination of receptors such as the EGFR (Haglund et al., 2003) or G-protein coupled receptors (Terrell et al., 1998) lead to receptor isolation in endosomes, which are subsequently routed to the lysosome via Endosomal Sorting Complex for Transport (ESCRT) by UBD-containing proteins, such as Eps15 (Raiborg and Stenmark, 2009). Interestingly, Eps15 was found to also interact with previously little-studied Lys33-linked chains generated on elements of *post*-Golgi membrane trafficking (Yuan et al., 2014).

#### Autophagy

Autophagy is another form of lysosome-mediated degradation - cargo is engulfed by the phagophore membrane, which is then closed to form an autophagosome. The mature autophagosome is trafficked to, and fused with, the lysosome (Cohen-Kaplan et al., 2016). Initially a Ub-independent non-selective form of autophagy induced upon nutrient starvation was discovered in yeast (Ohsumi, 2014). Subsequently autophagy specificity was found: instances were reported, in which a variety of large substrate cargo are selectively engulfed. Among others, these include invading bacteria, defunct organelles or aggregates - all larger than proteins which are degraded by the UPS (Khaminets et al., 2016). To enable this specificity, cargo is decorated with 'eat me' signals (e.g. Ub or Galectin-8 for bacterial autophagy), which are then linked to the phagophore membrane through a number of selective autophagy receptors (Randow and Youle, 2014). The process of selective autophagy therefore bears striking resemblance with the UPS. In contrast to the UPS, all Ub chain types accumulate in the brains of autophagy-deficient mice (Riley et al., 2010), leaving the question of which particular Ub chain types and architectures are required for selective autophagy unanswered.

Both non-selective and selective autophagy rely on the unique phagophore membrane. Phagophore identity is specified by lipid phosphorylation (e.g. the generation of phosphatidylinositol 3-phosphate) and incorporation of lipidated Ubl proteins into the phagophore membrane. While in yeast the autophagy-related gene 8 (ATG8) is the only lipidated Ubl, six orthologs are found in humans - the LC3s and GABARAPs. In the case of Ub-directed selective autophagy, the ATG8-containing phagophore forms around ubiquitinated structures marked for degradation. To this end, several selective autophagy receptors are utilized, such as p62 (Komatsu et al., 2007; Pankiv et al., 2007), OPTN, NDP52 (von Muhlinen et al., 2012), NBR1 (Kirkin et al., 2009), TAXBP1 and TOLLIP. These contain a LC3-Interacting Region (LIR) to interact specifically with the Ubl-containing phagophore membrane, and UBDs to bind the ubiquitinated cargo. Owing to their bidentate nature, selective autophagy receptors are thus able to anchor the LC3-decorated phagophore to ubiquitinated substrates and are therefore key for selective autophagy programmes.

Both selective and non-selective autophagy share a common mechanism of phagophore membrane generation (Hurley and Young, 2017):

The assembled and activated Unc-51-Like autophagy activating Kinase 1 (ULK1) complex phosphorylates and activates the VPS34 kinase-containing class III PI3K complex, which subsequently generates phosphatidylinositol 3-phosphate within the phagophore membrane. The phospholipid is specifically recognized by WD Repeat Domain, Phosphoinositide Interacting protein 2 (WIPI2) and defines the identity of the phagophore membrane (Polson et al., 2010; Dooley et al., 2014).

Incorporation of lipidated ATG8 proteins into the membrane is necessary to elongate and close the phagophore. Two Ubl-conjugation cascades are necessary to transfer activated ATG8 from the E2 ~ ATG8 complex onto substrate phosphatidylethanolamine lipids (Ichimura et al., 2000). ATG8 lipidation, carried out by the second cascade, is mediated by a ligase complex containing the ATG5-ATG12 conjugate produced by the first cascade. Firstly the attachment of Ub-like ATG12 to its substrate, ATG5 is catalysed by E1-like (ATG7) and E2-like (ATG10) enzymes. This constitutively generated conjugate associates with ATG16L1 to form the functional ligase complex for ATG8 lipidation. Additionally, ATG8 lipidation requires the same E1-like enzyme (ATG7), but a distinct E2 enzyme (ATG3). Lipidated ATG8-like Ubl proteins later become incorporated into the autophagosome and mediate both membrane elongation and specific cargo engulfment (Hanada et al., 2007; Kabeya et al., 2003).

Mitophagy, the process at the heart of this thesis is initiated by PINK1 and Parkin and converges into canonical selective autophagy mediated by OPTN and NDP52 (see Section 1.11.1).

## 1.6.2. Cell cycle regulation

An example non-degradative cellular process executed by Ub-mediated proteasomal degradation of an essential effector protein is the cell cycle. Progression through the cell cycle at several checkpoints is regulated by a multisubunit E3 ligase - the Anaphase Promoting Complex/Cyclosome (APC/C) (Pines, 2011). The ubiquitination activity of the APC/C is carried out by its catalytic RING domain together with the Lys11- linkage specific E2 enzyme UBE2S (Jin et al., 2008; Matsumoto et al., 2010; Williamson et al., 2009) or other, less chain-specific E2s (Meyer and Rape, 2014). The APC/C in fact synthesizes Ub chains branched at Lys11 and Lys48 residues which, unlike homotypic Lys11-linked chains, are efficiently recognized and degraded by the proteasome (Meyer and Rape, 2014; Grice et al., 2015). Irreversible checkpoint progression is governed by proteasomal degradation of substrates, specified by one of two APC/C substrate adaptors - Cell-Division Cycle protein 20 (CDC20) or CDC20 Homolog 1 (CDH1). During early mitosis the CDC20 adaptor is sequestered by the Spindle Assembly Complex (SAC). Upon its release CDC20 mediates the degradation of Securin and Cyclin B substrates, enabling chromatid separation. During the G1 phase, the CDH1 adaptor directs APC/C ubiquitination which is subsequently attenuated by phosphorylation during S-phase (Pines, 2011).

## 1.6.3. Inflammatory signalling

In signalling transduction, Ub chains can perform a scaffolding function, necessary to assemble functional signalling complexes. An interesting example are Lys63-linked chains which trigger assembly of the RIG I complex necessary for downstream antiviral signalling (Zeng et al., 2010; Xia et al., 2009).

However, the most well studied case is in canonical NF- $\kappa$ B signalling, which is triggered in response to external stimuli, such as Tumor Necrosis Factor (TNF) or related cytokines (Elliott, 2016). NF- $\kappa$ B signalling is also executed by proteasomal degradation - in this case of Inhibitor of  $\kappa$ B (I $\kappa$ B). Upon activation of the receptor, a series of factors are recruited: adaptors such as the TNF Receptor 1-Associated Death Domain (TRADD), kinases such as Receptor-Associated Protein kinase 1 (RIP1) and E3 Ub ligases. The RING ligases TNF Receptor Associated Factor 6 (TRAF6) and cellular Inhibitor of APoptosis (cIAP) synthesize Lys63- and Lys63-/Lys11- linked Ub chains, respectively (Deng et al., 2000; Dynek et al., 2010), while the Linear Ubiquitin Chain Assembly Complex (LUBAC) containing the active RBR E3 ligase HOIL-1-Interacting Protein (HOIP) synthesizes Met1- linked linear Ub chains (Tokunaga et al., 2009; Kirisako et al., 2006). Indeed, in a cellular context heterotypic or branched Met1-/Lys63- linked chains are found within the TNF receptor complex (Emmerich et al., 2013).

While it is accepted that Lys63-linked chains are responsible for recruitment of the Transforming growth factor- $\beta$ -Activated Kinase 1 (TAK1) to the receptor complex (Emmerich et al., 2013), Met1-linked Ub chains are essential in recruiting and partially activating the I $\kappa$ B Kinase complex (IKK) through one of its subunits - Nuclear factor- $\kappa$ B Essential Modifier (NEMO), (Rahighi et al., 2009). Full activation of the IKK complex at the receptor occurs by TAK1-mediated phosphorylation. The need for the two different Ub chain types to recruit the two independent interacting components provides yet another layer of TNF signalling regulation. Although more work is necessary to define the precise sequence and assembly of the receptor complex, the downstream signalling effect is well known: Phosphorylation of I $\kappa$ B by the activated IKK complex results in its degradation by the multi-subunit E3 CRL complex: the Skp, Cullin, F-box containing complex (SCF), unleashing the transcriptional activity of NF- $\kappa$ B. Downregulating the NF- $\kappa$ B pathway are several DUBs restricting both Lys63- and Met1- linked Ub chains: A20, CYLD and OTULIN (Elliott, 2016; Harhaj and Dixit, 2012).

### 1.6.4. DNA damage response

In addition to immune signalling, Lys63-linked chains also play a key role in the DNA damage response (Jackson and Durocher, 2013). Additionally, Lys6- and Lys33-linkages were upregulated upon UV genotoxic stress (Kim et al., 2011), while the formation of Lys27-linkages was promoted upon double-strand break induction (Gatti et al., 2015). Ubiquitin foci at sites of double-strand breaks are assembled by the E3 ligases RNF168 (Doil et al., 2009; Stewart et al., 2009) and RNF8 (Jackson and Durocher, 2013). Ub accumulation results in localization of repair promoting effectors such as 53BP1 or BRCA1, a RING E3 ligase complex which promotes further synthesis of Lys6- linked

chains (Wu-Baer et al., 2003; Christensen et al., 2007).

Two additional DNA repair pathways also necessitate Ub signalling: Replicative damage is signalled by site-specific Lys164 ubiquitination of the Proliferating Cell Nuclear Antigen (PCNA) by the E2 Rad6 and the E3 Rad18 (Hoege et al., 2002; Kim et al., 2009; Hibbert et al., 2011). Ubiquitinated PCNA replaces replicative polymerases with translesion polymerases able to overcome the damage (Moldovan et al., 2007; Freudenthal et al., 2010; Bienko et al., 2005). DNA cross-link repair is triggered by a complex associated with the Fanconi Anemia genetic disorder. The E3 ligase FANCL ubiquitinates the DNA repair proteins FANCD2/FANCI (Alpi et al., 2008), and recruits the FAN1 nuclease to mediate repair. Both pathways can be opposed by the USP1 DUB (Jackson and Durocher, 2013), whose stability is interestingly sensitive to UV radiation (Huang et al., 2006).

Moreover, the canonical tumour suppressor p53 gene responds to DNA damage in a Ub-dependent manner (Jackson and Durocher, 2013). Under steady-state conditions p53 is ubiquitinated (among others by the MDM2 ligase), and proteasomally degraded. In response to DNA damage, ubiquitination is suppressed and the p53 transcription factor is stabilized.

# 1.7. Mechanisms of ubiquitin conjugation

Within physiological context, chain specificity is determined by which chain types are both preferentially synthesized and cleaved on a given substrate. The difficulty of studying Ub signalling in cells has been due to the lack of chain-specific tools coupled with the large variation in the abundance of individual Ub chain types. Despite advances in Ub Mass Spectrometry (MS) (Ordureau et al., 2015b), the discovery and deployment of chain-specific DUB enzymes to determine which Ub chains are present on a substrate (Mevissen et al., 2013; Hospenthal et al., 2015), and continuing efforts to devise high-affinity chain-specific reagents (Michel et al., 2017), much of the present work and understanding is derived from *in vitro* biochemical and structural approaches. These have revealed that chain specificity is conferred by the ability of the enzymes carrying out either Ub assembly and disassembly to interact with two Ub molecules simultaneously. Linkage-specific ligation requires the ability to orient the acceptor Ub molecule towards the activated donor Ub C-terminus such that only one Lys is efficiently modified. Interestingly, the formed chain type is always determined by the conjugating enzyme which was last covalently bound to Ub via a thioester linkage. Therefore, the chain type of RING E3 ligase-mediated assembly is determined by the E2 conjugating enzymes, while HECT- and RBR-mediated specificity is E2 independent (Stewart et al., 2016). Similarly, chain-specific DUBs have defined distal/proximal Ub binding sites positioned relative to each other such that only conformations attainable by a particular chain type can bind across the active site. Once particular chain types are synthesized, a specific response is conferred by UBDs which are able to 'read' the conformational variation across the chain types. (Komander and Rape, 2012).

## 1.7.1. E1 ubiquitin-activating enzymes

The only component of the Ub conjugation cascade which must not confer any Ub substrate or chain specificity is the Ub-activating E1 enzyme. To this end, two E1 genes are encoded in the human genome (UBA1 and UBA6), (Schulman and Harper, 2009). Although the majority of active E2/Ub pairs can be processed by UBA1, selectivity of the UBA6 enzyme was reported upon its discovery (Jin et al., 2007). In addition to charging a subset of UBA1-compatible E2s, UBA6 is uniquely specific for the E2 UBE2Z (Jin et al., 2007; Gu et al., 2007). Both E1 enzymes are widely expressed and their activities comparable, suggesting their differential regulation might favour particular aspects of Ub signalling and confer a specific cellular response (Jin et al., 2007). Aspects of the UBA1 mechanism are highlighted below.

Although some Ub-like proteins utilize non-canonical E1 enzymes, such as the autophagyassociated E1 ATG7, a canonical model of Ub activation by E1 enzymes has emerged by studies across several Ub/Ubl cascades (Schulman and Harper, 2009). The catalytic cycle of the E1 enzyme has been well described - first the transferred Ub molecule is activated by adenylation (AMP is attached to the C-terminus). Subsequently the Ubl is transferred to the E1 active site Cys residue (Haas and Rose, 1982). Interestingly, before transthiolation transfer of the Cys-linked Ub to the E2 enzyme, another Ub adenylate conjugate is formed such that the transfer-competent E1 complex harbours two Ub molecules - Ub(a); adenylated Ub and Ub(t); transferred Ub (Fig 1.5), (Schulman and Harper, 2009; Schaefer et al., 2014).

Canonical E1 enzymes consist of a pseudo-dimer of two adenylation domains, only one of which is active, the IAD - inactive adenylation domain and AAD - active adenylation domain. Two insertions (First Catalytic Cys Half-domain (FCCH) and Second Catalytic Cys Half-domain (SCCH)), one in each adenylation domain, form the whole catalytic Cys domain. While the SCCH domain inserted into the AAD harbours the catalytic Cys residue, a C-terminal Ubiqutin-Fold Domain (UFD) domain is able to contact the incoming E2 enzyme (Fig 1.5 A). Crystal structures show that the adenylated Ub is contacted through an extensive interface, predominantly via its Ile44 patch and the Ub C-terminus (Arg72 forms an extensive charge interaction network), (Fig 1.6 A, bottom), (Lee and Schindelin, 2008; Misra et al., 2017; Lv et al., 2018). Subsequently, the adenylated Ub is transferred to the catalytic cysteine domain and contacted by a much weaker interface, which is still dependent on the Ile44 patch (Fig 1.6 A, top), (Huang et al., 2007; Schaefer et al., 2014). Binding of the incoming E2 enzyme is predominantly mediated by



Figure 1.5.: The E1 enzyme mechanism. (A) Schematic features of the monomeric Uba1 enzyme. AAD - Active Adenylation Domain, IAD - Inactive Adenylation Domain, FCCH - First Catalytic Cys Half-domain, SCCH - Second Catalytic Cys Half-domain, UFD - Ubiqutin-Fold Domain, 4HB - Four Helix Bundle. (B) A schematic depicting mechanism of Ub activation by the E1 enzyme based on resolved crystal structures of yeast orthologs. E1:Ub(a) complex - (Lee and Schindelin, 2008); E1-Ub(t):Ub (a) complex - (Schaefer et al., 2014); E1-Ub(t):Ub(a):E2 complex - (Olsen and Lima, 2013).

the UFD, whose flexibility is required to align the E2 and E1 active site Cys residues (Huang et al., 2007; Olsen and Lima, 2013; Lv et al., 2017). Generally, although crystal structures provide glimpses of the Ub activation mechanism, a large degree of flexibility is required in the system to bridge the significant distances between active sites within each crystallized complex (Fig 1.5 B), (Schulman and Harper, 2009).

## 1.7.2. E2 ubiquitin-conjugating enzymes

Linking the few E1 enzymes at the top of the ubiquitination cascade with the most numerous group of E3 ligases at the bottom, the ~40 E2 conjugating enzymes must exhibit a certain degree of modularity. While all must be able to interact with and discharge the E1 ~ Ub thioester, their specific activity in a physiological context will be determined by their diverse chain/substrate specificity as well as their interacting E3s (Stewart et al., 2016). While RING E3 enzymes simply catalyse aminolysis of the E2 ~ Ub thioester by substrate Lys residues, HECT and RBR enzymes discharge the E2 ~ Ub thioester onto their active site Cys residue in a transthiolation reaction.

In solution, E2~Ub conjugates are highly dynamic, adopting both extended, 'open' conformations as well as 'closed' conformation where the Ub Ile44 patch interacts with the E2 enzyme (Fig 1.6 B, left). Interestingly, E2 reactivity in solution towards free Lys (aminolysis) has been linked to their propensity to populate the closed conformation (Pruneda et al., 2011; Wenzel et al., 2011). Most E2s alone are not very reactive towards aminolysis in solution to prevent non-specific discharge, but some such as UBE2S, UBE2R1, UBE2N/UEV1A are sufficiently reactive towards the Lys  $\epsilon$ -amino group to mediate free Ub chain formation in solution. Commonly, Lys reactivity is promoted by interaction with E3s, which stabilize the closed conformation (Fig 1.6 B, left), (Wenzel et al., 2011; Plechanovová et al., 2012). Non-covalent binding of a second Ub molecule to E2's backside can also enhance reactivity towards Lys, for instance in the UBE2D family (Brzovic et al., 2006; Buetow et al., 2015).

A notable exception is the case of UBE2L3, which is not reactive towards aminolysis and can only undergo transthiolation reactions.  $E2 \sim Ub$  reactivity towards Cys residues is promoted in the open conformation during thioesterification by HECT or RBR E3 ligases which harbour a catalytic Cys residue of their own (Wenzel et al., 2011; Stewart


Figure 1.6.: Interactions of the conjugation machinery with Ub. Ile44 patch is shown as blue sticks, while Ile36 residues are coloured green. (A) E1-Ub(t):Ub(a) (Schaefer et al., 2014). (B) RNF4:UBE2D1:Ub (Plechanovová et al., 2012). (C) NEDD4L:UBE2D2-Ub (Kamadurai et al., 2009). (D) HOIP:UBE2D2-Ub (Lechtenberg et al., 2016).

#### et al., 2016; Kamadurai et al., 2009; Lechtenberg et al., 2016).

Unlike in HECT- and RBR-mediated assembly, E2 enzymes determine chain specificity in RING-mediated assembly. In fact, E2 enzymes are able to specialize for ubiquitination of particular substrates or Ub sites (Windheim et al., 2008). While the aforementioned APC/C-interacting UBE2S promotes Lys11-linked ubiquitination by providing an acidic binding site for the acceptor Ub molecule (Wickliffe et al., 2011), other strategies are employed to ensure Lys48- or Lys63-linked ubiquitination. The active E2 UBE2N functions in complex with an inactive partner, UEV1A which provides a binding site for the acceptor Ub, such that Lys63 is presented for ubiquitination by UBE2N, thus conferring specificity (Eddins et al., 2006). Another strategy, whereby an acidic loop is inserted in the proximity of the E2 catalytic site to orient the donor Ub with respect to the acceptor Ub Lys48 residue is used by the UBE2R1 (cdc34) E2 necessary to degrade substrates of the CRL SCF complex involved in NF- $\kappa$ B signalling (Kleiger et al., 2009; Chong et al., 2014). A noteworthy E2 member, selective of its substrate rather than a chain type site on Ub is UBE2W. UBE2W is able to specifically attach monoUb to disordered N-termini (Vittal et al., 2015; Tatham et al., 2013; Scaglione et al., 2013). Differences between the pKa of the N-terminus compared to Lys residues enables UBE2W to prefer peptide over isopeptide bond synthesis (Oregioni et al., 2017), and lack of a disordered Ub N-terminus imposes substrate monoubiquitination.

#### 1.7.3. E3 ubiquitin ligases

E3 Ub ligases form the numerous base of the Ub conjugation cascade and grossly divide into three families - RINGs, HECTs and RBRs (Zheng and Shabek, 2017). Although some E2 enzymes are able to assemble Ub chains independently, E3 enzymes promote assembly and direct ubiquitination to particular substrates. In the case of HECT and RBR ligases which form a Ub-thioester intermediate, they also direct linkages of the assembled chains.

Several models of polyUb assembly by E3 ligases have been proposed, extending the model for transferring monoUb (Fig 1.1), (Hochstrasser, 2006). The fundamental issue to be addressed is how longer polyUb chains arise in cells by processive E3 Ub ligase activity. Simply adding Ub molecules to the growing chain in sequential rounds of monoubiquiti-

nation is unlikely to result in long Ub chains (sequential model). Other, indexation and seesaw models have been proposed, where the Ub chain is built on either the E3 or a combination of the E2 and E3 active sites before being transferred to the substrate. Although these models could explain the remarkable processivity observed for some E3 ligases, little experimental evidence is available to distinguish between them (Hochstrasser, 2006).

Known E3 activity is based on a limited number of catalytic folds: While HECT ligases utilize a unique HECT domain, one canonical RING domain and one RING-like domain is utilized by RBR ligases to interact with incoming E2 conjugates and form the RBR  $\sim$  Ub thioester, respectively. Interestingly, a novel E3 ligase MYCBP2 capable of forming an E3  $\sim$  Ub intermediate before attaching Ub to substrate Thr residues through an ester bond also relies on a RING domain to recruit the E2  $\sim$  Ub thioester (Pao et al., 2018). To confer specific context-dependent ubiquitination in the cell, E3 ligase activity is regulated through formation larger complexes, autoinhibition or post-translational modification (including autoubiquitination).

#### 1.7.4. RING ubiquitin ligases

RING or U-box ligases form the largest ligase family with over 600 predicted members (Zheng and Shabek, 2017). While the structural necessity for Zn binding in RINGs is substituted by core polar interactions in U-box ligases, their folds are fundamentally the same (Hatakeyama et al., 2001). RING ligases catalyse Ub transfer from E2 enzymes by promoting Ub discharge onto substrates, and therefore do not control the type of Ub chains being assembled (Wenzel et al., 2011).

Early crystallographic studies have defined the conserved interface between E2 enzymes and a number of RINGs: cIAP/UBE2D2 - (Mace et al., 2008); cCbl/UBE2D3 - (Zheng et al., 2000); TRAF6/UBC13 (UBE2N) - (Yin et al., 2009), Ring1b/UBE2D3 - (Bentley et al., 2011). While E2 hydrophobic residues are inserted into a shallow grove on the surface (notably Phe63 in UBE2L3), additional polar contacts contribute to the interface (Fig 1.6 B, right). As this interface does not necessitate conformational changes in the RING or E2 and is distant to the E2 catalytic centre, it cannot account for the increase in Ub transfer efficiency by RING binding (Wenzel et al., 2011). The mechanism by which RINGs promote Ub transfer was revealed by further dynamic (Soss et al., 2013; Pruneda et al., 2012) and structural studies of RING complexes with E2  $\sim$  Ub thioester mimetics (Plechanovová et al., 2012; Dou et al., 2012; Pruneda et al., 2012). Replacing the E2 catalytic Cys residue with either Ser or Lys allowed stabilization of these intermediates (denoted as E2-Ub) and revealed that RINGs are able to interact with both the E2 and the donor Ub, notably by insertion of an Arg linchpin residue, to promote a closed, reactive conformation of the E2  $\sim$  Ub conjugate.

Although RING E3 ligases sometimes function as monomers, they are commonly part of larger oligomeric assemblies. Several RINGs, such as cIAP or TRAF6 homodimerize (Yin et al., 2009), thus bringing two functional E2 units together, while others form heterodimers where only one of the RING domains interacts with an E2 enzyme. The most common multimeric assemblies are based on the cullin scaffold and encompass the largest subfamily of RING ligases: the Cullin-RING ligases (CRLs), which includes SCF - the ligase complex which releases the NF- $\kappa$ B effector of TNF inflammatory signalling. Seven distinct cullin scaffolds bring in one of ~ 300 substrate adaptors, thereby modularly generating hundreds of potentially functionally diverse sets of ligase complexes in human cells. Additionally, CRL activity is regulated, for instance by reversible NEDDylation of the cullin scaffold (Buetow and Huang, 2016; Petroski and Deshaies, 2005), which is reversed by NEDD8 removal by the COP9 signalosome (Cope et al., 2002; Mevissen and Komander, 2017).

#### 1.7.5. HECT ubiquitin ligases

HECT Ub ligases are comprised of  $\sim 30$  members, such as NEDD4L and HUWE1, in humans (Zheng and Shabek, 2017). As this ligase family forms a covalent thioester intermediate with the transferred Ub, HECT Ub ligases are able to assemble specific Ub chains. While well studied examples such as E6-Associated Protein Ub-protein ligase (E6AP) and Neural precursor cell Expressed Developmentally Down-regulated protein 4-like (NEDD4L) assemble Lys48- and Lys63- linked chains respectively (Kim and Huibregtse, 2009), more recently HECT-mediated assembly of atypical chains has been leveraged to elucidate properties and functions of Lys29- (UBE3C), Lys33- (AREL1) and Lys6- (HUWE1) linked chains (You and Pickart, 2001; Michel et al., 2015; Kristariyanto et al., 2015; Michel et al., 2017). Intense study, especially of the Lys63-specific NEDD4 family of HECT ligases identified that a flexible linker between the two lobes of the HECT fold is key to mediate ubiquitination (Huang et al., 1999). Unlike RINGs, HECTs interact with their E2-Ub in an open conformation (Fig 1.6 C). While the E2 interaction is mediated through the N-lobe of the enzymes (Kamadurai et al., 2009), the flexibly tethered catalytic Cys-containing C-lobe is able to discharge the E2 ~ Ub thioester conjugate and in a second step ubiquitinate N-lobe bound substrates or the acceptor Ub (Kamadurai et al., 2013), oriented to favour Lys63- chain synthesis (Maspero et al., 2013).

#### 1.7.6. RBR ubiquitin ligases

RBR ligases constitute the most recently discovered and smallest subfamily of E3 Ub ligases, with 14 members such as Parkin and HOIP (Zheng and Shabek, 2017; Walden and Rittinger, 2018). RBRs contain flexibly tethered RING1 and "RING2" (structurally distinct from the canonical RING fold) domains intercalated by an In-Between RING (IBR) domain (Spratt et al., 2014). The seminal discovery of this ligase family stemmed from their ability to transfer Ub onto substrate Lys residues using the UBE2L3 E2 enzyme, which specifically discharges onto Cys residues (Wenzel et al., 2011). The RBRs were thus dubbed as RING1/HECT hybrids: although they contain a canonical RING unit, they also formed and  $RBR \sim Ub$  thioester intermediate via a catalytic residue on their RING2 domain, similarly to HECT ligases. The most striking feature shared by the best studied examples of this family - Parkin, HOIP and Human Homolog of Ariadne (HHARI) - is autoinhibition of the E3 ligase activity. Auxiliary domains, specific to each family member utilize the differences in relative positions of the RBR domains to form unique autoinhibitory interactions (Walden and Rittinger, 2018). Although Parkin does not display chain specificity (Ordureau et al., 2014), HOIP remarkably synthesizes Met1-linked, linear Ub chains only (Kirisako et al., 2006).

Owing to its link to YOPD, Parkin is one of the best characterized RBR ligases to date. Due to its significance for this work, the autoinhibitory features described by crystal structures of Parkin solved to date will be discussed in greater detail later (see Section 1.12), (Trempe et al., 2013; Riley et al., 2013; Wauer and Komander, 2013). Briefly, the RING2 catalytic domain is sequestered through a hydrophobic interaction with the Unique Parkin Domain (UPD), while the E2-binding site on the RING1 domains is occluded by the Repressor Element of Parkin (REP) and the Parkin Ubiquitin-like (Ubl) domain. Interestingly, crystallographic and biochemical analysis has shown that Parkin has the ability to non-covalently associate with pUb (phosphorylated on Ser65 by the PINK1 kinase), (Wauer et al., 2015a; Kumar et al., 2015; Sauvé et al., 2015; Kumar et al., 2017a). Parkin activity is unleashed by phosphorylation of its Ubl domain by PINK1 (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012), although the mechanism of this activation has remained elusive.

Physiologically active HOIP is associated with Heme-Oxidized IRP2 Ub Ligase 1 (HOIL-1L) and Shank-Associated RH Domain-Interacting protein (SHARPIN) in the LUBAC complex (Ikeda et al., 2011; Gerlach et al., 2011; Tokunaga et al., 2011; Fujita et al., 2018). Isolated HOIP is autoinhibited, although N-terminal truncation activates the construct *in vitro* (Smit et al., 2012; Stieglitz et al., 2012). Structural characterization of HOIP activity has explained its absolute specificity for synthesizing Met1- linked linear Ub chains required for NF- $\kappa$ B signalling (Walden and Rittinger, 2018). During assembly, HOIP binds both the donor and acceptor Ub molecules and orients them head-to-tail (Stieglitz et al., 2013). The latter interaction occurs via a HOIP-specific Linear Ub chain Determining Domain (LDD) - a C-terminal extension of the RING2 domain. A structure describing the interaction of the active domain configuration in HOIP with an E2 ~ Ub conjugate also resolves an allosteric binding site for an additional Ub molecule, bound analogously to pUb in Parkin (Lechtenberg et al., 2016), suggesting that this allosteric binding site might be utilized across the RBR family (Fig 1.6 D).

Lastly, crystal structures of HHARI, an RBR E3 ligase gene product essential for development (Aguilera et al., 2000; Lin et al., 2013), reveal yet another mechanism of autoinhibition. As in Parkin, the RING2 domain is sequestered by a domain unique to HHARI ligases, the Ariadne domain (Duda et al., 2013). In contrast to Parkin however, the E2 binding site is unobstructed in the autoinhibited structure and allows a specific, high-affinity interaction of HHARI with the UBE3L3 ~ Ub conjugate (Dove et al., 2017; Yuan et al., 2017; Martino et al., 2018). The analogous allosteric Ub/pUb interaction site available in Parkin and HOIP is occupied by a Ub-Associated Domain (UBA) domain, suggesting Ub-dependent mechanisms of activation. Interestingly, upon release, the HHARI RING2 domain has been shown to weakly interact with the donor Ub molecule (Dove et al., 2016) and both HHARI and another RBR ligase family member, TRIAD1 can be activated by CRLs (Kelsall et al., 2013). Insights from the E2-Ub-bound structures of HOIP and HHARI suggest that RBR E3 ligases interact with  $E2 \sim Ub$  conjugates in an open conformation (Fig 1.6 D). Since the conformations differ across the two available HHARI structures (Dove et al., 2017; Yuan et al., 2017), as well as in comparison to HOIP (Lechtenberg et al., 2016), it is unclear which permutation of the 'open'  $E2 \sim Ub$  conformation is required for interaction with Parkin.

# 1.8. Deubiquitinating enzymes

DUBs cleave polyUb precursors and disassemble Ub chains generated by the Ub conjugation cascade in order to edit Ub chains present on substrates, oppose Ub signalling or recycle Ub at the proteasome (Komander et al., 2009a; Mevissen and Komander, 2017; Clague et al., 2019). In humans, currently about 100 DUB enzymes have been described, falling into one metalloprotease or several Cys protease families, each of which harbours a distinct fold. Recent discoveries of the <u>MINDY</u> (Rehman et al., 2016), and <u>ZUP1</u> (Haahr et al., 2018; Kwasna et al., 2018; Hermanns et al., 2018) families continuously expand our understanding of the strategies employed by DUBs.

Generally, DUBs recognize a distal Ub in the chain through extensive interactions, with additional sites for the proximal Ub determining chain specificity. DUB  $Zn^{2+}$  metalloproteases belong to the <u>JAMM/MPN+</u> family and often function as subunits of larger molecular complexes, such as the proteasome or the COP9 signalosome which removes NEDD8 from cullin scaffolds (Cope et al., 2002; Mevissen and Komander, 2017). Cysteinebased proteases work via a papain-like enzyme mechanism, which generates a thioester intermediate to be hydrolysed in the second reaction step. Although their folds are distinct, the active site geometries have converged around the Ub C-terminus (Komander and Barford, 2008). Unlike the <u>Josephin</u> family, the <u>OTU</u> DUBs display remarkable chain specificities and are utilized as tools in linkage-specific analysis (Mevissen et al., 2013; Hospenthal et al., 2015). Uniquely, their fold prevents the <u>UCH</u> family recognition of a folded substrate protein (inc. Ub in the proximal site) (Popp et al., 2009), suggesting these only cleave Ub conjugated to peptides or unfolded proteins.

The <u>USP</u> family is the most numerous, with over 50 members. Either terminal or inserted auxiliary domains, including UBDs, mediate protein-protein interactions (Komander

et al., 2009a). Although the fold exhibits the same architecture in all solved USP structures (Mevissen and Komander, 2017), some variation is observed across the members: For instance USP7, implicated in affecting p53 stability, (Cummins and Vogelstein, 2004), undergoes an activating rearrangement upon distal Ub binding (Hu et al., 2002; Kim et al., 2019), while others such as CYLD have a pre-arranged active site (Komander et al., 2008). In terms of chain specificity, most members are promiscuous, although USP30 resident on the outer mitochondrial membrane shows a preference for Lys6-linked chains and CYLD for Met1- and Lys63- linked chains, restricting NF- $\kappa$ B signalling.

# 1.9. Ubiquitin-binding domains

UBDs are crucial to 'read' the Ub chains assembled and effect Ub signalling, exemplified by selective autophagy receptors, which bring the phagophore in close proximity of its cargo (Dikic et al., 2009; Komander and Rape, 2012). Interestingly, UBDs are also present as auxiliary domains in proteins with Ub-specific enzymatic activity, such as E3 ligases and DUBs (Zheng and Shabek, 2017; Mevissen and Komander, 2017). To achieve chain or substrate specificity, UBDs must have separate interaction surfaces to bind both the distal and the proximal Ub/substrate. Interestingly, although up to 60% of conjugated Ub is made up of monoubiquitination (Kaiser et al., 2011), the majority of structural efforts has been focussed on understanding of chain-specific UBDs. Many UBD folds have been identified - mostly they are based on an  $\alpha$ -helix with a Ub-interacting hydrophobic face or a Zn-finger with hydrophobic interaction surfaces, each of which can contact either the Ile44 or Ile36 patches on Ub (Dikic et al., 2009). An exception to this is the ZnF UBP domain, which confers the USP5 DUB unique specificity for unanchored Ub-chains (e.g. products of Ub gene synthesis) by recognizing the free C-terminal GlyGly motif of the proximal Ub, thus directing USP5 substrate specificity (Reyes-Turcu et al., 2006).

While most are non-linkage specific (Toma et al., 2015), some UBDs can recognize the distance/configuration which arises between two linked Ub molecules and therefore possess a linkage preference. This is the case for RAP80 whose two UIM domains are positioned to interact with Lys63- but not Lys 48-linked chains (Sims and Cohen, 2009; Sato et al., 2009a). A different strategy to recognize Lys63-linked chains is employed by the NZF zinc finger of TAB2/3, which serve as adaptors for recruitment of the TAK1 kinase to the NF- $\kappa$ B receptor complex. The relative positioning of the Ile44 patches of both the proximal and distal Ubs enabled by the Lys63 linkage is recognized (Kulathu et al., 2009; Sato et al., 2009b). While no structures of native Lys6-bound UBDs have been published to date, the ability of TAB2 to interact with Lys6-linked diUb suggests that Lys6-linked diUb can be bound in a similar manner to Lys63-linked Ub (Zhang et al., 2017). NZF domains present in larger proteins are versatile and also able to recognize Met1- (in HOIL-1L, an RBR E3 ligase), Lys29- and Lys33- (in TRABID, an OTU DUB) linked diUb by binding the Ile44 patch on the distal Ub molecule (Sato et al., 2011; Kristariyanto et al., 2015; Michel et al., 2015). An alternative strategy to recognize Met1-linked chains is employed by the UBAN domain of NEMO, an IKK complex subunit. The dimeric coiled-coil NEMO recognizes the Ile44 patch on the distal moiety, as well as the Ile36 patch on the proximal moiety allowing the elongated interaction interface to include the linkage site (Rahighi et al., 2009). For Lys48-linked chains the Ile44 patches, otherwise involved in generating a closed chain architecture, are contacted (Varadan et al., 2005; Rahighi et al., 2016).

# 1.10. Therapeutic manipulation of the ubiquitin system

Because Ub governs nearly all processes in the cell, small molecules able to modulate the Ub system are at the heart of numerous pharmaceutical campaigns (Wertz and Wang, 2018). Safe therapeutic intervention requires target specificity, therefore given the number of enzymes in each class of the conjugation machinery, targeting E3 ligases (>600) or DUBs ( $\sim 100$ ) is preferable. Additionally, DUB enzymes possess active site clefts specialized to thread the Ub C-terminus. Unlike active sites of DUBs or kinases, no obvious clefts in E3 ligases can be exploited by modular design of compounds. E3 ligase targeting therefore requires discovery of novel compound development strategies and validation methodologies (Wertz and Wang, 2018).

However, non-specific targets within the UPS family can also be explored: General proteasome inhibitors, such as Bortezomib have been approved for anti-cancer therapy (Wertz and Wang, 2018). Non-specific E1 inhibition could at least distinguish between orthogonal Ubl-pathways and potent E1 inhibition has been achieved by compounds which modify Ubl C-termini to block E1 active sites (Milhollen et al., 2014; Misra et al.,

2017). In addition to manifesting off-target effects, previous classes of E1 inhibitor compounds simultaneously modulate multiple biological aspects, such as NF- $\kappa$ B signalling and p53 transcriptional activity. This behaviour is expected and arises from targeting the Ub conjugation machinery apex.

Modulation of the MDM2/p53 interaction to affect p53 transcriptional activity is the culmination of many parallel discovery efforts. While direct modulation of MDM2 activity leads to side-effects (Scott et al., 2016), several groups have recently designed high affinity covalent inhibitors of USP7, a DUB regulating MDM2 stability (Turnbull et al., 2017; Kategaya et al., 2017; Lamberto et al., 2017; Gavory et al., 2018; O'Dowd et al., 2018). The relative success of targeting DUBs coupled with platforms to screen DUB activity in a high-throughput way (Ritorto et al., 2014) underlines the difficulties faced in targeting E3 ligases. Indeed, inhibition of USP30, the DUB opposing the PINK1/Parkin mitophagy pathway could have neuroprotective effects by promoting mitochondrial clearance. In line with its identification as a potential target, specific USP30 inhibitors have recently been reported (Kluge et al., 2018).

An alternative therapeutic use of E3 ligase activity lies in directing the degradation of non-native cellular substrates. After the discontinuation of thalidomide due to its teratogenicity, it was discovered that thalidomide and its analogous IMmunomodulatory Drugs (IMiDs) interact with the Cereblon protein (CRBN) substrate adaptor of CRL4 (Ito et al., 2010), and recruit transcriptional factors for ubiquitination by the complex and subsequent degradation. This facet of their function was later exploited for cancer therapy. Understanding of the coupling between each drug and particular transcription factor may extend IMiD use for therapy (Fischer et al., 2014; Donovan et al., 2018; Sievers et al., 2018; Matyskiela et al., 2018). While no other compounds to target E3 ligase activity in a biological context have been approved, a more targetted use of the idea on non-native substrate degradation is achieved through PROteolysis TArgeting Chimeras (PROTACs). Here, two independent E3- and substrate-interacting ligands are linked to generate a compound which promotes specific substrate degradation in a cellular context through physically coupling the E3 ligase and a non-native substrate (Bondeson and Crews, 2017).

## 1.11. Mitochondrial clearance

The Ub-dependent pathway of mitochondrial clearance by autophagy governed by the PINK1 kinase and the Parkin E3 ligase is linked to YOPD and constitutes the best studied mitochondrial clearance pathway (Pickrell and Youle, 2015; Harper et al., 2018). Furthering our molecular understanding of this system, detailed below, could aid translational work in the future given the crucial roles mitochondria play in cells. Efforts in this direction have already started to bear fruit with the recent report of USP30 inhibitors (Kluge et al., 2018).

Aside from energy production via Oxidative Phosphorylation (OXPHOS), which relies on an electrochemical potential across the inner mitochondrial membrane, mitochondria lie at the heart of many cellular processes such as innate immunity (via the RIG I pathway), and cell death (via Bax/Bak) (Pickles et al., 2018). While mitochondria carry their own  $\sim 16$  kB genome encoding mostly OXPHOS subunits, the majority of mitochondrial proteins are imported from the cytosolic translation machinery by the Translocase of the Outer Membrane (TOM) and Translocase of the Inner Membrane (TIM) complexes in an electrochemical potential-dependent manner (Neupert, 2015). A lack of coordination between transcription and protein import is tackled by the Mitochondrial Unfolded Protein Response (UPR<sup>mt</sup>), (Nargund et al., 2012). Without additional high fidelity quality control mechanisms, damage of mitochondrial proteins and DNA by Reactive Oxygen Species (ROS) leaked from the electron transport chain would result in defunct mitochondria and give rise to heterogeneity in the mitochondrial population (Pickles et al., 2018; Harper et al., 2018).

While Parkin ubiquitination is sufficient for proteasomal degradation of a subset of outer membrane proteins in a manner dependent on the p97 ATPase (Tanaka et al., 2010; Xu et al., 2011; Kim et al., 2013), other processes require an intimate interplay of PINK1 phosphorylation and Parkin ubiquitination to induce lysosomal degradation of depolarized mitochondria (Pickles et al., 2018). Either Mitochondrially Derived Vesicles (MDV) fuse directly with the lysosome (Soubannier et al., 2012; McLelland et al., 2014, 2016) or the mitochondria are selectively engulfed by the autophagosome in mitochondria-specific autophagy, mitophagy (Pickles et al., 2018; Harper et al., 2018). Mitophagy is dependent on components of the canonical autophagy pathway, for instance ULK1 (Kundu et al., 2008; Egan et al., 2011), and can engulf the whole organelle or couple to fission events such that only parts of the organelle are degraded (Yang and Yang, 2013; Yamashita et al., 2016).

Although PINK1/Parkin mitophagy may contribute to some cellular responses, such as the transition from brown to white adipocytes (Lu et al., 2018), clearance of paternal mitochondria following fertilization seems to only depend on ubiquitination by Parkin and the E3 ligase MUL1 without a requirement for PINK1 (Rojansky et al., 2016). Other programmes bypass the need for a Ub link to the autophagy machinery entirely: In reticulocyte maturation the mitochondrially anchored, LIR-containing autophagy receptor Nix is utilized to directly recruit the autophagosome (Sandoval et al., 2008; Schwarten et al., 2009; Novak et al., 2010). Hypoxia-induced mitophagy is mediated via a Nix-related mitochondrial autophagy receptor BNIP3 (Hamacher-Brady et al., 2007; Hanna et al., 2012). Mitochondrial degradation induced by yeast growth in a non-fermentable medium is mediated by the mitochondrially anchored autophagy receptor ATG32, which also directly recruits the autophagy machinery (Kanki et al., 2009; Okamoto et al., 2009). A growing body of evidence suggests that these alternative, PINK1/Parkin independent mitophagy pathways mediated by Nix, BNIP3 or other autophagy receptors contribute to basal mitophagy in the cell (Villa et al., 2018; Lee et al., 2018).

#### 1.11.1. PINK1/Parkin-mediated mitophagy

#### PINK1

The PINK1 kinase consists of 581 residues split across the following elements: an N-terminal mitochondrial targeting sequence (residues 1–34), a transmembrane helix (residues 94–110), a catalytic domain (residues 150–513) and a C-terminal region (residues 514–581). PINK1 is unique among kinases owing to its mitochondrial targeting sequence as well as unique insertions within the kinase catalytic domain (Woodroof et al., 2011; Manning et al., 2002). In addition to expression in the brain (Blackinton et al., 2007), PINK1 is interestingly found in other tissues, most prominently the heart, skeletal muscle, testis and glandular tissues (Berthier et al., 2011; Uhlen et al., 2015), (Human Protein Atlas available from www.proteinatlas.org). These findings suggest that neuronal cell types seem to be more susceptible to defects in PINK1 activity than others. YOPD-associated mutations were identified in three consanguineous families on chromosome 1, the ~1.8 kB PINK1 gene was implicated as it is expressed in the CNS and harboured two independent substitutions in the three initially examined families (Valente et al., 2015).

#### 2004).

#### Parkin

The Parkin RBR E3 ligase consists of 465 residues split across the following elements: an N-terminal Ub-like domain (residues 1-76), a linker region (residues 77-145), the UPD (Unique Parkin Domain) (residues 146-216), and the RBR module consisting of a RING1, IBR and a RING2 domain (residues 222-465). Parkin harbours several unique elements within the RBR module, including a short REP helix (residues 393-405) and a C-terminal helical extension of the RING2 domain (residues 454-465), (Kitada et al., 1998; Morett and Brok, 1999; Shimura et al., 2000). Parkin expression seems more restricted as compared to PINK1 and unlike PINK1, several isoforms of Parkin have been observed (Huynh et al., 2001). Originally, Parkin has been found in several brain regions (Kitada et al., 1998; D'Agata et al., 2000; Gu et al., 2000; Horowitz et al., 1999; Stichel et al., 2000). Later studies of developing mouse embryos as well as human tissues reveal an expression profile apparently restricted to brain, heart, skeletal muscle, kidney and testis (Huynh et al., 2001; Uhlen et al., 2015), (Human Protein Atlas available from www.proteinatlas.org). Originally, Parkin was linked to YOPD by the identification of five patients from several unrelated families carrying exon deletions in the the  $\sim 1.4$  kB Parkin gene located on chromosome 6.

For further discussion, see Section 1.11.2.

#### PINK1/Parkin-mediated mitophagy

Following the discovery of their link to Parkinson's disease (Valente et al., 2004; Kitada et al., 1998), studies in drosophila identified that PINK1 and Parkin are linked in a common pathway and are required for proper mitochondrial function (Greene et al., 2003; Clark et al., 2006; Park et al., 2006). Further study of PINK1/Parkin-dependent mitophagy was fuelled by its link to disease, and a host of subsequent molecular work, to a large extent relying on Parkin overexpression and chemically-induced mitochondrial depolarization, defined the interplay between these two enzymes in wholesale mitochondrial clearance (Cummins and Götz, 2017).

Under resting conditions, PINK1 is imported into the mitochondrial matrix in a potentialdependent manner by the TIM/TOM complexes (Jin et al., 2010). Once spanning both membranes, PINK1 is clipped in the matrix by the Mitochondrial Processing Peptidase (MPP) and PINK1/PGAM5-Associated Rhomboid-Like protease (PARL) at the inner mitochondrial membrane (Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011). Upon cleavage, PINK1 (truncated in the transmembrane helix, such that the former Phe104 residue is exposed at the N-terminus) is retrotranslocated to the cytoplasm and degraded via the N-end rule pathway (Fig 1.7, I), (Yamano and Youle, 2013). While PINK1 continuously samples the mitochondrial network through cycles of import, cleavage, and degradation, the RBR ligase Parkin resides in the cytosol. Structural and biochemical insights of the cytosolic form of Parkin demonstrate several elements of intramolecular Parkin autoinhibition (Trempe et al., 2013; Riley et al., 2013; Wauer and Komander, 2013), detailed in Section 1.12.1.



polarized, functional mitochondria<sup>1</sup> depolarized mitochondria (loss of potential)

Figure 1.7.: PINK1/Parkin dependent Mitophagy. Left: In functional polarized mitochondria, MPP+ and PARL cleave imported PINK1 and trigger N-end rule PINK1 proteasomal degradation (I). Right: Upon loss of mitochondrial potential across the inner mitochondrial membrane, PINK1 is stabilized at the TOM complex (II), and phosphorylates Ub conjugated to OMM proteins (III, red circle represents phosphate group). Phospho-Ub triggers autoinhibited Parkin (light blue) rearrangement and localization from the cytosol (IV). pUb-bound Parkin (medium blue) is subject to further direct PINK1-mediated phosphorylation and activation (V). Active phospho-Parkin (dark blue) conjugates further Ub to OMM proteins (VI), which can be phosphorylated and trigger additional Parkin localization in a positive feedback loop (VII). Unphosphorylated Ub chains are recognized by the autophagy machinery such that selective mitochondrial autophagy is triggered (VIII). OMM = outer mitochondrial membrane, IMS = inter-membrane space.

Upon loss of mitochondrial membrane potential, PINK1 is stabilized on the OMM in a complex with several subunits of the TOM complex (Fig 1.7, II), (Lazarou et al., 2012; Okatsu et al., 2013; Hasson et al., 2013). Here, PINK1 encounters its first phosphorylation substrate, Ub, and phosphorylates its Ser65 (Fig 1.7, III), (Kane et al., 2014; Kazlauskaite et al., 2014b; Koyano et al., 2014; Ordureau et al., 2014; Wauer et al., 2015b). PINK1/Parkin-mediated mitophagy is the first physiological context, in which a role for a specific chemical Ub modification has been identified (Swatek and Komander, 2016). While previously the idea necessitating initial Parkin recruitment to generate Ub conjugates required for PINK1 activity (Ordureau et al., 2014), a consensus in the field has been reached that PINK1 phosphorylates pre-existing Ub on the mitochondrial surface (Shiba-Fukushima et al., 2014; Pickles et al., 2018; Harper et al., 2018). This notion is supported as low levels of mitophagy can proceed in Parkin Knockout (KO) cells (Lazarou et al., 2015), suggesting other sources of Ub conjugates on the outer mitochondrial membrane exist. These are generated by other E3 Ub ligases such as MUL1 or others (Yun et al., 2014; Yonashiro et al., 2006). In Parkin overexpression systems, where mitochondrial potential is chemically uncoupled by Carbonyl Cyanide m-Chlorophenyl hydrazone (CCCP) or Oligomycin/Antimycin A (OA) treatment, up to 20% of mitochondrial Ub is phosphorylated (Ordureau et al., 2015a, 2014). Surprisingly, our group has shown that Ub phosphorylation on Ser65 enables Ub to access a previously uncharacterised conformer and affects its role to generate a unique signalling molecule (Wauer et al., 2015b), detailed in Section 1.11.3.

Ub phosphorylated on Ser65 serves as a high-affinity mitochondrial surface receptor for cytosolic Parkin (Fig 1.7, IV), (Wauer et al., 2015a; Kumar et al., 2017a; Sauvé et al., 2015; Kumar et al., 2015). Although pUb localizes Parkin to sites of mitochondrial damage, Parkin remains autoinhibited upon pUb binding (Wauer et al., 2015a; Kumar et al., 2017a). As a consequence of conformational changes upon pUb binding, the Parkin Ubl is released. The Parkin Ubl domain is also phosphorylated by PINK1 on Ser65 (Fig 1.7, V), suggesting a common substrate recognition mechanism (Shiba-Fukushima et al., 2012; Kondapalli et al., 2012). Ubl phosphorylation finally activates Parkin through a thus far unknown structural rearrangement.

Upon its activation at the surface of damaged mitochondria, phospho-Parkin ubiquitinates a plethora of mitochondrial substrates with no apparent substrate specificity (Fig 1.7, VI), (Sarraf et al., 2013; Ordureau et al., 2018). The Ub conjugated by Parkin to the mitochondrial surface which enables further PINK1 phosphorylation and results in a positive-feedback loop as additional Parkin molecules are recruited as a result of further pUb synthesis (Ordureau et al., 2014). A novel approach quantitating the kinetics of Parkin ubiquitination sites could not identify preferred substrates or degrons, although some sites on individual substrates were protected from modification, likely due to higher order assembly formation (Ordureau et al., 2018). Consistent with their high abundance on the mitochondrial surface, the most common Parkin substrates were Voltage-Dependent Anion Channel (VDAC) family members. In overexpressing systems, Parkin was found to assemble Lys6-, Lys11-, Lys48- and Lys63- linked chains (Ordureau et al., 2014), whereas in depolarized dopaminergic neurons, only an increase in Lys63linkages was observed (Ordureau et al., 2018).

Coincidentally with the abundance of VDAC ubiquitination, both VDAC and the specific autophagy receptor p62 were shown to be required for PINK1/Parkin-dependent mitophagy (Geisler et al., 2010). Subsequent studies identified p62 as crucial for the perinuclear clustering of depolarized mitochondria rather than mitophagy itself (Narendra et al., 2010; Okatsu et al., 2010), and screens assessing all five autophagy receptors identified OPTN and NDP52 as required, but redundant, for mitophagy (Lazarou et al., 2015; Heo et al., 2015) which was supported by previous cellular studies (Wong and Holzbaur, 2014). The involvement of OPTN is interesting as both OPTN and its activating kinase TBK1 are associated with another neurodegenerative disorder - Amyotrophic Lateral Sclerosis (ALS) (Maruyama et al., 2010; Cirulli et al., 2015; Freischmidt et al., 2015), further highlighting the necessary role of autophagy-mediated quality control for proper neuronal function. Novel quantitative approaches were able to resolve whether autophagy receptors interact with phosphorylated or unphosphorylated Ub on the mitochondrial surface, since both are present (Fig 1.7, VIII), (Ordureau et al., 2018). Interestingly and contrary to studies which suggest PINK1 is sufficient for triggering low levels of mitophagy (Lazarou et al., 2015), only unphosphorylated Ub chains seem to be bound by specific autophagy receptors (Ordureau et al., 2018).

Although the requirement of specific autophagy receptors has been shown, their canonical role for recruiting ATG8 proteins already inserted into a pre-formed phagophore has been contested in the context of mitophagy. KO of all eight ATG8 proteins permits mitophagy, but blocks downstream fusion with the lysosome (Nguyen et al., 2016; Pontano Vaites et al., 2017). Moreover, OPTN and NDP52 have recently been shown to

promote de-novo autophagosome biogenesis at mitochondrial sites via their LIR motifs, rather than recruitment of pre-formed phagophores (Padman et al., 2019). Additionally, a novel autophagy receptor, Prohibitin 2, has been identified on the inner mitochondrial membrane, only able to promote mitophagy once the outer mitochondrial membrane has been ruptured (Wei et al., 2017).

Among other DUBs, such as USP15 (Cornelissen et al., 2014) and USP8 (Durcan et al., 2014), the mitochondrially-anchored USP30 DUB has been found to oppose PINK1/Parkin-mediated mitophagy (Bingol et al., 2014; Cunningham et al., 2015; Marcassa et al., 2018). Whether Ub conjugate levels by these DUBs are regulated prior to, or following, the engagement of the Ub positive feedback loop initiated by PINK1/Parkin is currently unclear. The phosphatase opposing PINK1 activity is unclear, although several candidates such as PGAM5 and PTEN-L are being explored (Wang et al., 2018a). Interestingly, recently PINK1/Parkin mitophagy has been linked to a strong inflammatory phenotype, likely triggered by mtDNA haemorrhaged into the cytosol by defective mitochondria and sensed by the cGAS-STING cytosolic DNA sensor (Sliter et al., 2018). How inflammation is linked to the PD patient symptoms currently ascribed to mitophagy defects in cells is unclear.

#### 1.11.2. Disease links of PINK1/Parkin-mediated mitophagy

In most cases, Parkinson's disease arises sporadically and is caused by the death of dopaminergic neurons in Substantia nigra. Commonly, loss of 50-60% of dopaminergic neurons results in a 70-80% loss in dopamine levels (Pickrell and Youle, 2015). Additionally, a clear  $\alpha$ -synuclein aggregate (Lewy body) pathology is often observed in the brain. Currently, no curative treatment is available and symptoms are managed by dopamine replacement therapy. Moreover, no objective diagnostic tests are possible and instead tremor, slowness of movement, rigidity and loss of balance are assessed by physicians subjectively. Around 10% of PD cases have a genetic link to a small number of PD-associated genes (Corti et al., 2011) whose investigation could inform potential therapeutic or diagnostic approaches.

The first discovered PD-associated genetic alteration was the single A53T substitution in the SNCA gene (Polymeropoulos et al., 1997), encoding the  $\alpha$ -synuclein protein. Another dominant associated gene is the kinase LRRK2, the mutations in which are most prevalent among familiar PD (Corti et al., 2011). Parkin (Kitada et al., 1998), and PINK1 (Valente et al., 2004) are examples of recessively inherited PD-associated genes. Contrary to SNCA and LRRK2, whose alterations most commonly take the form of deletions and point mutations are concentrated in a few hotspots, Parkin carries the largest number of different pathogenic mutations across its whole sequence (Corti et al., 2011; Pickrell and Youle, 2015), (Fig 1.8 A). While present structural knowledge can explain a number of point mutations mostly predicted to disrupt the autoinhibited cytosolic state and likely lead to protein destabilization, a number of mutations across the Parkin gene remain unexplained (Fig 1.8 B). Meanwhile, Parkin mutations continue to be reported, as exemplified by the identification of two independent patients carrying a pathogenic S65N mutation (McWilliams et al., 2018). This mutation is of particular interest as it has no effect on the Parkin autoinhibited state, but instead prevents Parkin activation by phosphorylation, underlining the crucial importance of this event for physiological



Figure 1.8.: YOPD-associated Parkin mutations. Redrawn from (Pickrell and Youle, 2015). Red circle represents PINK1 phosphorylation site in the Ubl domain, while the yellow star represents the RING2 active site Cys residue. (A) Mutations in the indicated Parkin domains are mapped based on the Parkinson's disease mutation database, (Nuytemans et al., 2010a; Cruts et al., 2012) and (Pickrell and Youle, 2015). (B) Identified Parkin mutations, the mode of pathogenicity of which cannot be explained by the currently available Parkin structures.

PINK1/Parkin-mediated mitophagy.

In addition to PINK1 and Parkin mutations, further aspects link mitochondrial quality control and PD pathogenesis. Patients who consumed illegally synthesized opioid analgesic desmethylprodine (MPPP) containing a synthesis impurity (MPTP), experienced permanent parkinsonism symptoms (Davis et al., 1979; Laston et al., 1983). Later studies uncovered the basis for this extreme toxic specificity: The MPP+ metabolite originating from MPTP is selectively taken up by dopaminergic neurons where it inhibits mitochondrial complex I of the OXPHOS electron transport chain. This leads to the generation of free radicals which damage mitochondria specifically in dopaminergic neurons (Schapira et al., 1989; Cleeter et al., 1992). Indeed, MPP+ treatment is used as a PD model. In addition to this environmental evidence, analysis of tissue samples from PD sufferers suggests accumulation of defective complex I (Schapira et al., 1989). Similarly, PD patient samples accumulated mutations in the mitochondrial genome (Bender et al., 2006; Kraytsberg et al., 2006), and conversely, patients with error-prone mitochondrial polymerases are at a higher risk of developing PD (Luoma et al., 2004; Reeve et al., 2013).

In order to recapitulate PD-like phenotypes, Parkin or PINK1 KO mice must be challenged with UPR<sup>mt</sup> or a higher mitochondrial mutational load (Pickles et al., 2018). Parkin KO mice however develop hepatocellular carcinoma (Fujiwara et al., 2008). The role of Parkin as a tumour suppressor for a number of cancers has been well documented by an increased mutation of the Parkin genetic locus in tumours, although interestingly for some cancers Parkin function seems to be necessary (Veeriah et al., 2010; Bernardini et al., 2017).

#### 1.11.3. The structure of phospho-ubiquitin

Ubiquitin phosphorylated on Ser65 constitutes a unique molecular signal in mitophagy and could be used as a specific mitophagy marker in patient brain tissue staining (Hou et al., 2018). Strikingly, Ub phosphorylation induces an interconversion between two conformations of (pUb) (Wauer et al., 2015b). The exchange rate between the two conformers is very slow  $(2 \text{ s}^{-1})$ , (Wauer et al., 2015b), while the proportion of the two species is dependent on pH and temperature (Dong et al., 2017; Kazansky et al., 2018). At 25  $^{\circ}\mathrm{C}$  and physiological pH, approximately 30% of pUb assumes a novel conformer.

Upon phosphorylation both secondary and tertiary structures are preserved in the 'common' pUb conformer, while perturbations are observed in the vicinity of the phosphorylation site due to addition of the bulky, charged phosphate group (Wauer et al., 2015b). The novel 'C-terminally retracted' pUb conformer (pUb-CR) has the same tertiary structure, however the relative position of the last  $\beta$ 5-strand relative to the rest of the Ub core is shifted by two residue positions. Hydrophobic pockets in the Ub core occupied by Leu67, Leu69 and Leu71 in the common conformation are occupied by Leu69, Leu71 and Leu73 respectively in the CR conformer. This results in a retraction of the flexible C-terminal Ub tail and the extension of the loop preceding the last  $\beta$ 5-strand containing the phosphorylation site, Ser65 (Fig 1.9 A). Interestingly, in structures of the pUb-CR conformer (Dong et al., 2017; Gladkova, 2015) the Leu8 loop assumes the 'loop-in' conformation, such that Leu8 contributes to the Ile36 hydrophobic patch rather than the disrupted Ile44 patch (Hospenthal et al., 2013). The average loop conformation could therefore report on the extent of pUb-CR population.

The shifted hydrogen bonding pattern in the  $\beta$ -sheet in the pUb-CR conformation was initially characterized using NMR techniques to detect spatial (NOE) and hydrogenbonding (long-range HNCO) contacts between residues in the  $\beta$ -sheet (Wauer et al., 2015b). Later, a solution-based structure of both conformers confirmed the predicted hydrogen-bonding pattern (Dong et al., 2017), (Fig 1.9 B, C).

Although crystallographic analysis of the common pUb species was possible, and the structure exhibits no significant differences from unphosphorylated Ub (Wauer et al., 2015b), the pUb-CR conformer initially eluded crystallization. Leu67 shifts from a hydrophobic environment in common pUb to being solvent exposed in pUb-CR, therefore an introduction of a polar residue at position 67 was necessary to increase the proportion of pUb-CR. Indeed, the L67N pUb mutant exhibits only one set of peaks in the BEST-TROSY spectrum corresponding to pUb-CR and could be studied using X-ray crystallography (PDB ID: 50XH), (Gladkova, 2015; Gladkova et al., 2017). Monophosphorylation at Ser65 had to be ensured by mutation of Thr66 to Val due to PINK1 off-target phosphorylation of Thr66 in the L67N variant (Fig 1.9 D).



Figure 1.9.: The common and C-terminally retracted conformations of pUb. (A) Schematic representing both conformations of pUb. In the common conformation (left), the  $\beta$ 5-strand Leu residues (67, 69, 71) face the core in their corresponding hydrophobic pockets. In the CR conformation (right), the Leu residues have retracted by two positions, such that each Leu (69, 71, 73) resides in the (n-2) core hydrophobic pocket. This leads to an extended Ser65-containing loop and a retracted C-terminal tail. (B) The structure of the common pUb conformer closest to the ensemble average from PDB-ID: 5XK5. (C) The structure of the CR pUb conformer closest to the ensemble average from to trap the CR conformer. Asn67 position in the common conformer is disfavoured. (E) Rationale for design of the L71Y mutation to trap the CR conformer. Tyr71 position in the CR conformer is disfavoured.

An analogously stabilizing mutation of the common pUb conformation was designed by the substitution of Leu71 to Tyr (Fig 1.9 E). In the common conformation, Leu71 occupies a more spacious cavity than in pUb-CR, where a Tyr could not be accommodated. The BEST-TROSY spectrum of L71Y pUb only exhibited a single set of peaks, corresponding to the common pUb conformer (Schubert, 2018; Gladkova et al., 2017).

This new conformation of pUb alters biophysical but also biochemical properties of Ub to generate a novel signalling molecule. To-date the only specific receptor for pUb is Parkin, which interacts with the common pUb conformer. However, further roles for the pUb-CR in cells might be uncovered in the future.

#### 1.11.4. PINK1

Due to the role of PINK1 as a sensor of damaged mitochondria in PINK1/Parkindependent mitophagy and its targeting of an unusual Ub substrate, there is considerable interest in its mechanism. Activating PINK1 could promote basal mitophagy and is of therapeutic benefit. Thus far, pharmacological PINK1 activation *in vitro* or *in vivo* has only been achieved by utilizing a nucleotide analogue kinetin triphosphate instead of ATP (Hertz et al., 2013). Following the initial discovery of the PINK1 gene, the kinase was found to associate with the TOM complex in a dimeric state (Okatsu et al., 2013), which was later corroborated for isolated PINK1 in solution (Rasool et al., 2018).

Molecular work on PINK1 was accelerated by the discovery that unlike the human PINK1 ortholog, insect versions of the protein can be produced in *E. coli* (Woodroof et al., 2011). These insect orthologs possess a 40 - 50% sequence identity with human PINK1 and have been instrumental in understanding the molecular basis of YOPD mutations in PINK1 (Rasool et al., 2018). PINK1 is a highly divergent kinase. The domain structure of PINK1 is as follows: an N-terminal mitochondrial targeting sequence is followed by a putative transmembrane helix, an unusual kinase domain (containing the canonical N-and C-lobes) and a highly conserved, functionally required C-terminal region (Rasool and Trempe, 2018). The N-lobe of the PINK1 kinase domain is unusual because it contains three insertions conserved from insects to humans (Woodroof et al., 2011).

Recently a number of structures of the PINK1 catalytic domain and the C-terminal region were reported. The apo and nucleotide-bound structures of *Tribolium castaneum*, Red flour beetle, *Tc*PINK1 resolved the active state of PINK1 (Kumar et al., 2017b; Okatsu et al., 2018). Our group also reported a structure of active *Pediculus humanus*, Human louse, *Ph*PINK1 in complex with a substrate Ub T66V, L67N (TVLN) variant in the Ub-CR conformation (Schubert et al., 2017). Our biochemical studies showing that PINK1 preferentially bound to and phosphorylated this Ub variant (Schubert, 2018; Gladkova, 2015; Gladkova et al., 2017), were corroborated by the Ub binding mode represented in the structure (Schubert et al., 2017). The extended Ser65 loop of the CR conformer is necessary to reach the active site of the kinase.

All three structures demonstrated the importance of PINK1's C-terminal region for stabilizing the kinase domain. Additionally, a role for the C-terminal region in dimerisation was postulated (Schubert et al., 2017; Kumar et al., 2017b; Okatsu et al., 2018). The importance of PINK1 autophosphorylation on residues Ser228 and Ser230 (human PINK1 numbering) was underlined as phosphorylation of these residues was resistant to  $\lambda$  phosphatase treatment (Schubert et al., 2017) or substituted by phosphomimetic residues for other crystallographic studies (Kumar et al., 2017b; Okatsu et al., 2018). Changes upon Ub substrate binding include ordering of one of the insertions unique to PINK1 (to form a Ub-interacting subdomain), as well as conformational changes in the  $\alpha$ C helix (required for kinase activation), (Schubert et al., 2017; Rasool and Trempe, 2018).

Although only the Ub-CR conformation is able to make canonical contacts with the kinase activating loop, the kinetics of Ub TVLN phosphorylation were not formally characterized. However, investigation of wt Ub or the Parkin Ubl as substrates poses an interesting conundrum, as the Ser65 is buried in both (Rasool and Trempe, 2018): For typical phosphorylation reactions, a rapid equilibrium is set up between substrate bound and unbound states of the enzyme. Under these conditions the substrate dissociation constant ( $K_D$ ) can be approximated to the observed  $K_m$  for the enzyme-catalysed reaction. This is the case for the Parkin Ubl, which is phosphorylated ~100 times faster than Ub (Gladkova et al., 2017; Rasool et al., 2018), with a reported  $K_m$  of ~35 µM matching the reported  $K_D$  of ~43 µM (Rasool et al., 2018). In stark contrast, no binding between *Ph*PINK1 and wt Ub could be detected by ITC, suggesting a very high  $K_D$ . Interestingly, the measured  $K_m$  for wt Ub phosphorylation by *Tc*PINK1 was ~400 µM. The  $K_D$  measured for the interaction between *Ph*PINK1 and Ub TVLN was

in the same range of ~ 300  $\mu$ M. Based on NMR and ITC measurements, Ub TVLN indeed binds to PINK1 more strongly than wt Ub and is phosphorylated faster (Gladkova et al., 2017). The discrepancy between the K<sub>m</sub> and the predicted K<sub>D</sub> for wt Ub would suggests an unknown multistep phosphorylation mechanism for wt Ub, while Ubl phosphorylation is not governed by the same constraints. Although the analysis discussed here originated from two different PINK1 orthologs, the values should be comparable within an order of magnitude owing to good sequence conservation (Woodroof et al., 2011).

Additional substrates for PINK1 have been reported, such as Mitofusin 1 (Chen and Dorn, 2013), or Miro (Shlevkov et al., 2016), which do not share the Ub/Ubl fold. The mechanism of phosphorylation of these substrates is presently also unclear.

### 1.12. Parkin

Parkin is also under intense scrutiny - in fact, it is the best characterized member of its E3 RBR Ub ligase class. In humans, Parkin has 465 residues divided across several domains (Fig 1.10 A). The Parkin domain sequence is as follows: The N-terminal Ubl is followed by a  $\sim 60$  AA linker, the function of which has thus far not been explored. Found after the linker are the proteolysis resistant UPD domain and the RINGin-Between-RING (RBR) module. The RBR module consists of a canonical RING1 domain and two non-canonical IBR and RING2 domains tethered by a flexible linker. Interestingly, rather than being confined to a few hotspots, homo- and hetero-zygous PD-associated mutations are dispersed through each sequence element of Parkin (Fig 1.8).

As with other members of the RBR class, Parkin needs to undergo a number of catalytic steps for efficient substrate ubiquitination: binding the E2  $\sim$  Ub thioester conjugate through its conserved E2-binding interface on the RING1 domain (Plechanovová et al., 2012; Lechtenberg et al., 2016), discharging the E2  $\sim$  Ub thioester with the active site Cys present on the Parkin catalytic RING2 domain (Wenzel et al., 2011), forming a Parkin  $\sim$  Ub thioester (Lazarou et al., 2013), and finally positioning the thioester linkage to discharge Ub onto substrate Lys residues (Sarraf et al., 2013; Ordureau et al., 2018). Early cellular studies suggested the Parkin active site is Cys431 on the RING2 domain (Lazarou et al., 2013). This has been corroborated by subsequent biochemical analysis

(Wauer and Komander, 2013; Trempe et al., 2013; Riley et al., 2013).



Figure 1.10.: Parkin autoinhibition. (A) The domain structure of Parkin, with the catalytic and phosphorylated residues highlighted as shown in the legend. (B) Top: the serpentine arrangement of Parkin domains coloured as in A. Autoinhibitory elements are marked with red ovals. Bottom: The autoinhibited structure of Parkin (PDB ID: 5C1Z). Domains are coloured as in A, insets show catalytic Cys and E2 binding site occlusion. (C) Top: Parkin domain arrangement as in B, Ubl is released due to pUb (orange) binding. Remaining autoinhibitory elements are marked with red ovals. Bottom: The autoinhibited pUb-bound structure of Parkin (PDB ID: 5N2W). The linker helix between the RING1 and IBR domains is straightened to generate the pUb binding site. Domains are coloured as in A, insets show catalytic Cys and E2 binding site occlusion as in B. Note that in the crystal structure, the Ubl domain has remained in its autoinhibitory position despite its high mobility in solution.

In vitro, Parkin activity is inhibited (Wauer and Komander, 2013; Trempe et al., 2013; Riley et al., 2013), whereas in vivo significant activity could be observed upon mitochondrial depolarization (Lazarou et al., 2013). Parkin with an engineered Ser residue in place of the catalytic Cys431 readily forms a stable oxyester conjugate with Ub, demonstrating its ability to efficiently undergo the catalytic cycle and therefore a lack of autoinhibition under depolarized conditions. In line with these findings, Parkin is activated in the context of PINK1/Parkin mitophagy. Although Ub phosphorylation is required to generate the primary mitophagy signal and localize Parkin to sites of mitochondrial damage (Kazlauskaite et al., 2014b; Koyano et al., 2014; Kane et al., 2014; Ordureau et al., 2014; Wauer et al., 2015a), further Parkin phosphorylation by PINK1 is required to unleash its ubiquitination activity (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012). The activity of Parkin increases according to the following defined activation sequence: Parkin < Parkin : pUb < phospho-Parkin < phospho-Parkin : pUb. The same sequence was obtained by using measurements based on either mass spectrometry (Ordureau et al., 2014, 2015a) or Activity-Based Probes (ABPs) adapted from their standard use to measure DUB activity, (Ovaa and Vertegaal, 2018; Park et al., 2017). Although both approaches yielded the same activation sequence, the basal level of unphosphorylated Parkin activity is much lower when measured by a mass spectrometry, compared to the use of ABPs.

Although understanding of fully active phospho-Parkin in complex with pUb is missing, structural insights gained into the cytosolic and pUb-bound forms of Parkin reveal the modes of Parkin autoinhibition.

#### 1.12.1. The cytosolic form of Parkin

In 2013 our group and others solved high-resolution structures of the Parkin C-terminal fragment, encompassing the UPD and RBR domains (Trempe et al., 2013; Wauer and Komander, 2013; Riley et al., 2013). The most striking feature of these structures was the serpentine arrangement adopted by the Parkin domains (Dove and Klevit, 2013). This arrangement was corroborated by a low resolution structure of full-length Parkin, where the linker between the Ubl and UPD domains remained disordered (Trempe et al., 2013). In subsequent work, the linker was removed to yield high resolution structures of the domain arrangement in inhibited Parkin (Kumar et al., 2015; Sauvé et al., 2015),

(Fig 1.10 B).

The most C-terminal helix, which is unique to Parkin and extends from the conserved RING2 fold forms a highly hydrophobic interface with the UPD (Fig 1.10 B). Most strikingly, as a result of this interaction, the catalytic Cys431 of Parkin is buried and cannot carry out Ub-transfer. The interaction is enabled by a linker between the IBR domain and the RING2 domain, which wraps around the Parkin RING1 domain. The function of this linker is revealed by the structures coupled with conservation analysis: the Repressor Element of Parkin (REP) two-turn helix interacts with the canonical E2-binding site on the RING1 domain. Occlusion of the catalytic Cys by the UPD and of the E2-binding site by the REP element constitute two autoinhibitory elements which must be relieved for Parkin activity.

Improving the resolution of the Ubl binding site by linker deletion is consistent with data that the Parkin Ubl and C-terminal UPD-RBR fragment of Parkin interact with low µM affinity (Chaugule et al., 2011). High resolution structures (Kumar et al., 2015; Sauvé et al., 2015) show Ubl interacting through its Ile44 patch with the RING1 helix, which also contributes to E2 interaction (Fig 1.6 B, right; Fig 1.10 B, bottom right). Therefore, the determined Ubl binding site constitutes the third autoinhibitory element of Parkin.

These structures, published alongside the first HHARI structure (Duda et al., 2013), also characterized the Zn-binding modes of all four Zn-binding domains in Parkin (UPD, RING1, IBR and RING2). While the RING1 adopts a canonical cross-braced Zn fold, shared with RING E3 ligases, the IBR and RING2 domains both bind their two Zn ions sequentially. In contrast, the UPD domain forms a  $\beta$ -hairpin around its two Zn residues. The UPD domain, also dubbed the RING0 (Hristova et al., 2009), adopts a fold unique to Parkin - a property which has given rise to the name (Hampe et al., 2006; Wauer and Komander, 2013). Further analysis of the autoinhibited structures identified a putative phosphate binding pocket in the UPD, lined with residues Lys211, Lys161 and Arg163 (Wauer and Komander, 2013; Sauvé et al., 2015).

Resolving the structure of the individual Parkin domains explained the basis of most YOPD-associated Parkin mutations. These could be classified into several categories as outlined below: The first category comprises mutants affecting the structure and stability of the protein, and is exemplified by disruption of the Zn-interacting residues, such as Cys212, Cys289 and Cys441 (Hampe et al., 2006; Wauer and Komander, 2013; Sriram et al., 2005). The second category of mutants interfere with catalytic activity: C431F mutant blocks all activity completely, but this is also reduced when Glu444 and His433 constituting a putative catalytic triad are mutated (Trempe et al., 2013; Wauer and Komander, 2013; Riley et al., 2013). Lastly, mutants disrupt protein-protein interactions are found, such as the T240M/R mutants. These disrupt E2 binding, and result in lower Parkin activity (Shimura et al., 2000; Trempe et al., 2013).

Additionally, based on the resolved autoinhibitory elements, mutations in the UPD-RING2 (F146A, F463A), REP-RING1 (W403A) or Ubl-RING1 (L266K) interfaces were designed to bypass the need for Parkin activation by phosphorylation (Trempe et al., 2013; Wauer and Komander, 2013; Ordureau et al., 2014; Tang et al., 2017). Although the *in vitro* activity of the W403A variant was increased, its localization to mitochondria still required PINK1 activity (Trempe et al., 2013). Therefore, *in vivo*, these activating mutations are unlikely to function properly.

#### 1.12.2. Phospho-ubiquitin-bound Parkin

Coincidentally with the reports of Ub phosphorylation, a high-affinity interaction between pUb and Parkin was reported (Ordureau et al., 2014). This finding spurred further structural effort to elucidate the effect of this interaction. While others have identified the interface using mutagenesis and NMR spectroscopy (Sauvé et al., 2015; Kumar et al., 2015), our laboratory succeeded in solving a crystal structure of Parkin from *Pediculus humanus*, Human louse (*Ph*Parkin) in a covalent complex with pUb achieved through the use of a Ub Activity-Based Probe (ABP) (Wauer et al., 2015a), (see Section 6.1.1). Later, the same approach was used to capture human Parkin with a Ubl-UPD linker truncation bound to pUb (Fig 1.10 C), (Kumar et al., 2017a).

The pUb-bound Parkin structures retain all three autoinhibitory elements: the RING2 catalytic Cys is occluded (1); RING1 E2 binding site remains blocked by the REP (2); and the Ubl (3). pUb binds Parkin through a cleft on the surface generated by movement of the IBR domain (Fig 1.10 C). This movement is achieved through straightening of the linker helix between the RING1 and IBR domains (Wauer et al., 2015a). Movement of the IBR domain is in line with changes to the IBR position based on its crystallographic

environment (Trempe et al., 2013; Wauer et al., 2015a; Riley et al., 2013) and dynamic NMR measurements (Beasley et al., 2007). While pUb interacts with the RING1 and IBR domains via its Ile44 patch, a phosphate pocket is formed by residues His302, Arg305, Lys151, Tyr312. Gly284 lies in close proximity to the pUb binding site and its YOPD-linked substitution to Arg results in loss of pUb binding. G284R therefore constitutes the first discovered mutant which disrupts Parkin localization and activation (Wauer et al., 2015a). Later, the same binding mode was found in a structure of active HOIP in complex with a UBE2D2 ~ Ub thioester mimetic (Lechtenberg et al., 2016), suggesting this may be a common feature among RBR ligases.

As pUb is generated selectively on damaged mitochondria by PINK1, pUb triggers Parkin localization to mitochondria. In addition, the conformational change required to interact with pUb releases the Parkin Ubl domain from its autoinhibitory position. This pUb/Ubl switch has been supported by binding measurements, which report a higher pUb affinity for the C-terminal fragment of Parkin without the Ubl (Sauvé et al., 2015). Ubl binding to pUb-bound Parkin is also reduced (Wauer et al., 2015a). However, the Ubl release markedly increases the phosphorylation rate of the Parkin Ubl by PINK1, suggesting a need for the Ile44 patch in the phosphorylation reaction (Wauer et al., 2015).

The increased affinity of the phospho-Parkin : pUb complex for the E2  $\sim$  Ub conjugate mimetic over E2 alone suggested an additional Ub binding site on Parkin (Kumar et al., 2015). The presence of this site was corroborated by mutational analysis stemming from the crystal arrangement of the human Parkin : pUb complex, in which the Ubl-UPD linker was truncated (Kumar et al., 2017a). The same site was found to bind the donor Ub in the active HOIP structure (Lechtenberg et al., 2016), suggesting its conservation. The presence of this site could explain reports of *in vitro* unphysiological E2-independent Parkin activity (Zhang et al., 2000; Tsai et al., 2003; Chew et al., 2011).

#### 1.12.3. The active form of phospho-Parkin

Parkin is only fully activated upon Ubl phosphorylation by PINK1 (Ordureau et al., 2014, 2015a; Park et al., 2017), suggesting that phosphorylation induces release of the catalytic Cys431. Consistently, the affinity of phosphorylated Parkin for E2 is increased,

showing that in addition to displacement of the Ubl, the REP element is also disrupted (Sauvé et al., 2015). How these rearrangements are achieved by remote phosphorylation of Ser65 on the Parkin Ubl domain is currently unclear. However, previous work provides several clues about the identity of active Parkin. The phosphate binding pocket identified from autoinhibited Parkin structures encompasses two residues mutated in YOPD: -Lys211 and Lys161 (Fig 1.8 B). While these residues are not involved in pUb binding and subsequent structural changes, Parkin activation by PINK1 phosphorylation and its localization to mitochondria in the mutants are impaired *in cellulo*, suggesting the patient variants K211N and K161N likely disrupt full Parkin activation (Wauer et al., 2015a; Ordureau et al., 2014).

Both SAXS and Analytical Ultracentrifuagtion (AUC) analysis suggest a more extended form of active Parkin that remains monomeric (Sauvé et al., 2015; Aguirre et al., 2017). These reports are therefore inconsistent with Parkin dimerisation, a model that was proposed to resolve the distances between the  $E2 \sim Ub$  thioester linkage and the active site Cys predicted from the autoinhibited structures (Kumar et al., 2017a; Arkinson and Walden, 2018). Instead, both dynamic measurements suggest a more 'open' conformation of phosphorylated Parkin. While the later study (Aguirre et al., 2017), rationalizes this structure opening only in terms of Ubl domain release, RING2 release must also be accounted when analysing these measurements.

#### 1.12.4. Alternative Parkin activation?

While the most well studied function of Parkin is in mitophagy, a growing body of literature implicates Parkin-mediated ubiquitination in other cellular pathways, exemplified below. Intriguingly, these do not also involve PINK1, suggesting a PINK1-independent activation pathway would be necessary to carry out each cellular function.

In mitophagy, Parkin has been shown to associate with, but not ubiquitinate the Activating Molecule in BECN1-Regulated Autophagy protein 1 (AMBRA1) autophagy machinery component (Van Humbeeck et al., 2011). The involvement of Parkin in xenophagy, form of autophagy specialized to degrade intracellular pathogens, has also been demonstrated (Manzanillo et al., 2013). This link is particularly intriguing due to the evolutionary origin of mitochondria as bacterial endosymbionts, and patient data suggesting higher susceptibility to infection associated with Parkin mutations (Mira et al., 2004; Ali et al., 2006). Furthermore, involvement of Parkin has also been reported in NF- $\kappa$ B signalling, where its ubiquitination activity was observed on NEMO and RIP1 substrates (Müller-Rischart et al., 2013; Wang et al., 2018b). In this context, Parkin was also found to promote Met1- linked linear chain synthesis by LUBAC (Müller-Rischart et al., 2013), although Parkin is unable to synthesize this chain type *in vitro* (Ordureau et al., 2014). In post-mitotic neurons, Parkin has been shown to downregulate Wnt signalling (Rawal et al., 2009) and interact with components of the endocytic pathway (Fallon et al., 2006; Trempe et al., 2009). On the other hand, reports studying Parkin in dividing cells suggest Parkin is able to function together with the SCF complex and degrade Cyclin proteins essential for cell cycle progression (Staropoli et al., 2003). This activity could also substantiate the role of Parkin as a tumour suppressor (Veeriah et al., 2010; Gong et al., 2014a).

Some of these studies are carried out in an overexpression setting and their implications require closer scrutiny (Müller-Rischart et al., 2013). In other cases, the involvement of Parkin may be indirect. Recently MDVs containing ROS, whose generation relies on PINK1 and Parkin, have been shown to play an antimicrobial role (Abuaita et al., 2018). This pathway links reports of Parkin antimicrobial function to its canonical function in mitochondrial quality control. On the other hand, physical interaction of Parkin with components of the endocytic pathway has been demonstrated, supporting genuine PINK1-independent Parkin involvement. In either case, the question of Parkin activation must be resolved to understand how ubiquitination in a cellular context is enabled by Parkin.

## 1.13. Aims

Fast-paced research to date has uncovered many aspects of PINK1/Parkin-mediated mitophagy. While the basis of Parkin autoinhibition and mitochondrial localization through pUb binding has been well characterized, understanding of the active state of Parkin achieved through its phosphorylation is missing. Reports of Patients with S65N pathogenic Parkin mutations (McWilliams et al., 2018), as well as the requirement for Parkin phosphorylation in neuronal mitochondrial clearance (Ordureau et al., 2018),

suggests a crucial physiological role for phosphorylation-induced Parkin activation.

The primary aim of this thesis is to complete the molecular puzzle of Parkin activation. With a molecular understanding of all states of the Parkin activation sequence, the differences between them could be exploited by further translational work. Small molecules could be designed for specific pharmacological enhancement of mitophagy to benefit PD sufferers. Ideal drugs need to be able to target a variety of dysfunctional Parkin variants present in patient cells, and reinstate their ability to be activated by PINK1 or directly activate Parkin in a mitochondria-specific context. Conversely, Parkin inactivation in dividing cells could yield future cancer therapies.

Additionally, structurally resolving the last state of the Parkin activation sequence, activated Parkin, could explain the basis of further YOPD-linked mutations. In this respect, the two pathogenic mutations (K161N and K211N) lining a phosphate binding pocket in the UPD are most intriguing.

A three-pronged approach using biochemical (Chapter 4), dynamic (Chapter 5) and structural (Chapter 6) techniques was used to define the active state of phospho-Parkin. An emphasis was placed on the requirements necessary to achieve the fully active state: Which sequence elements mediate Parkin activation? Is phosphorylation sufficient, or are mechanistic intermediates required to achieve the fully active state of Parkin? What is the nature of the dynamic rearrangements responsible for the elongated nature of phospho-Parkin in solution? Ultimately this understanding was leveraged to obtain a high-resolution structure of the previously elusive active Parkin state.

During my thesis work, I also continued building on my previous work which culminated in structural characterization of the novel C-terminally retracted pUb conformer (Gladkova, 2015). Since this conformer is a superior PINK1 substrate, my particular aim was to investigate whether this conformer can also be accessed by unmodified Ub. C-terminal retraction prior to phosphorylation would explain how the buried Ser65 is exposed and justify the curious kinetic properties of PINK1-mediated phosphorylation. Could facets of this equilibrium impact the biochemical or biophysical properties of Ub? Together, this work has the potential to provide clinically relevant molecular insights to facilitate the development of therapeutic or diagnostic tools for Parkinson's disease and further our understanding of the Ub system.

# Chapter 2.

# Materials and Methods

Unless otherwise stated, methods are modified, extended and consolidated from Gladkova et al. (2017) and Gladkova et al. (2018).

# 2.1. Cloning

The vectors used in this work are detailed in Table 2.1, the most frequently used pOPIN vectors are described in (Berrow et al., 2007). PCR was performed using Phusion<sup>®</sup> polymerase (NEB). cDNA of *Thamnophis sirtalis* (*Ts*) Parkin was obtained from GeneArt<sup>TM</sup> (Invitrogen) with codon-optimization for bacterial expression. Amplified inserts were inserted into linearised vectors either using In-Fusion<sup>®</sup> (Clonetech) according to manufacturer's instructions, or by homologous recombination in *E. coli* as in (Jacobus and Gross, 2015). Briefly, PCR products were gel-purified and co-transformed into chemically competent *E. coli* with a linearised, phosphatase-treated vector of choice.

Site-directed mutagenesis was performed using primers designed with the QuikChange primer design tool (Agilent). Either a QuikChange protocol was followed, or overlap extension PCR was performed using QuikChange and terminal primers, enabling downstream In-Fusion or homologous recombination. For deletions and insertions, the Q5<sup>®</sup> site-directed mutagenesis kit (NEB) was used according to manufacturer's instructions. The constructs shown in the top portion of Table 2.2 were produced in this work, while the rest was obtained from the lab stock. *Hs*Ube1/PET21d was a gift from Cynthia Wolberger [Addgene plasmid # 34965, (Berndsen and Wolberger, 2011)].

| Vector  | Resistance | Tag                             | Protease<br>specific for<br>cleavage site | Overhang |
|---------|------------|---------------------------------|---|----------|
| pET17b  | Ampicillin | -                               | -   | -        |
| pGex6P1 | Ampicillin | GST (N)                         | 3C  | GPLGS-   |
| pET21d  | Ampicillin | His <sub>6</sub>                | -   | -        |
| pOPIN-K | Kanamycin  | $His_6$ -GST (N)                | 3C  | GP-      |
| pOPIN-S | Kanamycin  | $His_6$ -SUMO (N)               | SENP1                                     | -        |
| pTXB1   | Ampicillin | intein/CBD, in-frame fusion (C) | -   | -        |

**Table 2.1.: Vectors.** Vectors used throughout this thesis. CBD = Chitin Binding Domain. Cleavage sites specific for either 3C or SENP1 proteases are inserted between the tag and construct, these proteases are subsequently used during protein purification.

# 2.2. Protein expression

All proteins generated in this work were produced from Rosetta2 (DE3) pLacI *E. coli* cells in media supplemented with 35 µg/mL Chloramphenicol and 50 µg/mL Kanamycin or 100 µg/mL Ampicilin. Parkin, E2 and *Hs*UBE1 cultures were grown at 37°C in 2xTY medium, cooled to 18°C at  $OD_{600}$  of 0.6–0.8, and protein expression induced at  $OD_{600}$  of 0.8– 1.0 by the addition of 200 µM IPTG for E1 and E2 or 30 µM IPTG for Parkin. Additionally, Parkin cultures were supplemented with 200 µM ZnCl<sub>2</sub> upon induction. Cells were harvested by centrifugation at 4000 g after overnight growth at 18°C, resuspended in lysis buffer as specified below and frozen at -20 or -80°C.

Ub and Ub-intein variants were either grown as above, with 200  $\mu$ M IPTG at 37°C overnight, or using auto-induction medium. To generate auto-induction medium, ZY media (10g bacto-tryptone; 5g yeast extract per L) was supplemented with NPS (20x = 0.5M (NH<sub>4</sub>)SO<sub>4</sub>, 1.0M KH<sub>2</sub>PO<sub>4</sub>, 1.0M Na<sub>2</sub>HPO<sub>4</sub>), 5052 (50x = w/v 25% glycerol, 0.025% glucose, 1%  $\alpha$ -lactose), 1 mM MgSO<sub>4</sub>, and trace metals (1000x = 50 mM Fe<sup>3+</sup>, 20 mM Ca<sup>2+</sup>, 10 mM Mn<sup>2+</sup> and Zn<sup>2+</sup>, 2 mM Co<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, MoO<sub>4</sub><sup>2-</sup>, SeO<sub>3</sub><sup>2-</sup>, H<sub>3</sub>BO<sub>3</sub>).

Singly <sup>15</sup>N-labelled or doubly <sup>15</sup>N- and <sup>13</sup>C- labelled proteins were expressed in minimal medium (41 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 13.5 mM NaCl) supplemented with 1mM MgSO<sub>4</sub>, trace metals as above, vitamins (BME vitamin solution, sterile-filtered, Sigma)
| Construct                         | Species             | Boundaries | Mutations                              | Vector  |
|-----------------------------------|---------------------|------------|--|---------|
| Ub F4A                            | Homo sapiens        | 1 - 76     | F4A                                    | pET17b  |
| UBE2D3                            | Homo sapiens        | 2 - 147    | S22R, C85K                             | pGex6P1 |
| UBE2L3                            | Homo sapiens        | 1 - 154    | C86K                                   | pGex6P1 |
| Parkin                            | Homo sapiens        | 1 - 100,   |  | pOPIN-K |
| $\Delta$ 101 - 109                |                     | 110 - 465  |  |         |
| Parkin                            | Homo sapiens        | 1 - 115,   |  | pOPIN-K |
| $\Delta$ 116 - 123                |                     | 124 - 465  |  |         |
| Parkin R104A                      | Homo sapiens        | 1 - 465    | R104A                                  | pOPIN-K |
| <i>Ts</i> Parkin                  | Thamnophis sirtalis | 1 - 451    |  | pOPIN-K |
| * <i>Hs</i> Parkin (TEV)          | Homo sapiens        | 1 - 465    | ASGTTTQ (383-<br>388) NLYFOS           | pOPIN-K |
| * <i>Hs</i> Parkin (TEV)<br>Q347C | Homo sapiens        | 1 - 465    | ASGTTTQ (383-<br>388) NLYFQS           | pOPIN-K |
| * <i>Ts</i> Parkin (TEV)<br>M333C | Thamnophis sirtalis | 1 - 451    | KSPGATA (368-<br>374) ENLYFQS<br>M333C | pOPIN-K |
| Ub                                | Homo sapiens        | 1 - 76     |  | pET17b  |
| Ub                                | Homo sapiens        | 1 - 76     |  | pGex6P1 |
| Ub-intein                         | Homo sapiens        | 1 - 75     |  | pTXB1   |
| Ub TVLN                           | Homo sapiens        | 1 - 76     | T66V, L67N                             | pET17b  |
| Ub L71Y                           | Homo sapiens        | 1 - 76     | L71Y                                   | pET17b  |
| UBE1                              | Homo sapiens        | 1 - 1058   |  | pET21d  |
| UBE2D3                            | Homo sapiens        | 2 - 147    |  | pGex6P1 |
| UBE2L3                            | Homo sapiens        | 1 - 154    |  | pGex6P1 |
| Parkin                            | Homo sapiens        | 1 - 465    |  | pOPIN-K |
| Parkin K211N                      | Homo sapiens        | 1 - 465    | K211N                                  | pOPIN-K |
| Parkin Ubl                        | Homo sapiens        | 1 - 76     |  | pOPIN-S |
| PINK1                             | Pediculus humanus   | 115 - 575  |  | pOPIN-K |

Table 2.2.: Expressed constructs. Constructs expressed throughout this thesis. The top half lists construct produced in this thesis, while the bottom half lists constructs obtained from the lab stock. Constructs marked with (\*) are described in detail in Fig 6.5; in short, residues in the IBR-RING2 Parkin linker are substituted for residues forming the consensus TEV protease cleavage site.

and 1g / L <sup>15</sup>NH<sub>4</sub>Cl and 4g / L glucose or <sup>13</sup>C<sub>6</sub> glucose where indicated. Cultures were inoculated in the afternoon, grown overnight at 20°C, induced with 200  $\mu$ M IPTG at OD<sub>600</sub> of 0.5–0.6 on the next day, and following overnight growth at 18°C, cells were harvested by centrifugation at 4000 g, resuspended in lysis buffer and frozen at -20 or -80°C. 2xTY, ZY and minimal media were obtained from the LMB media and glass wash facility.

# 2.3. Protein purification

While constructs listed in Table 2.2 were produced in this work, proteins listed in Table 2.3 were obtained from the lab stock. Buffers used during protein production or chemical labelling were filtered either using a reusable Nalgene<sup>TM</sup> filter holder with 0.22  $\mu$ M cut-off membrane or SteriCup<sup>®</sup> with a 0.22  $\mu$ M cut-off (Milipore). All chromatographic steps were carried out on an Äkta Pure system (GE Healthcare) unless otherwise specified. Initial affinity capture was carried out by gravity flow using glass Econo-Columns<sup>®</sup> with a 2.5 or 5 cm diameter (BioRad). Before reliable protocols were established, and for purity assessment, fractions were analysed by SDS PAGE. Proteins were flash-frozen and stored at -80°C.

All buffers used during protein production or chemical labelling are detailed in Table 2.4.

#### 2.3.1. Ubiquitin

The Ub purification protocol used in our laboratory (Michel et al., 2018), is adapted from Pickart and Raasi (2005). Ub-expressing cells from a 2 L culture were resuspended to a final volume of 35 mL in Ub lysis buffer supplemented with 2 mg/mL Lysozyme (Sigma), 0.2 mg/mL DNaseI (Sigma) and incubated on ice for 20 - 30 min. Cells were lysed by sonication (3 min total, cycle = 10 s on/10 s off, 70% of 750W) and the lysate clarified by centrifugation at 46,000g for 35 min at 4°C. The supernatant was stirred on ice while 245 µL of 70% perchloric acid was added dropwise to reduce the pH. The resulting mixture was stirred on ice for a further 30 min to precipitate contaminating *E. coli* proteins and further clarified by centrifugation as above. The resulting supernatant

| Construct     | Boundaries       | Mutations           | Vector  |
|---------------|------------------|---------------------|---------|
| UBE2R1        | 2 - 235          |                     | pGex6P1 |
| UBE2S         | 2 - 222          |                     | pGex6P1 |
| UBE2N         | 2 - 152          |                     | pGex6P1 |
| UEV1A         | 2 - 147          |                     | pGex6P1 |
| cIAP          | 363 - 612        |                     | pOPINJ  |
| TRAF6         | 50 - 211         |                     | pOPINK  |
| HUWE1         | 3,993 - 4,374    |                     | pOPINS  |
| HOIP RBR-LDD  | 699 - 1,072      |                     | pOPINK  |
| USP30 (long)  | 64-357, 432-502  | F348D, M350D, I353E | pOPINK  |
| USP30 (short) | 64-178, 178-217, | F348D, M350D, I353E | pOPINK  |
|               | 357-432, 432-502 |                     |         |

**Table 2.3.:** Additional proteins used. Additional proteins used throughout this work obtained from the lab stock. GST-cIAP1 and GST-TRAF6 were a kind gift from Dr K. N. Swatek, while HUWE1 and HOIP RBR-LDD were a kind gift from Dr P. Elliott. All constructs stem from *Homo sapiens* sequences except for cIAP, which originates from *Mus musculus*.

was dialysed into Ub LO buffer.

The dialysate was applied to cation-exchange chromatography (HiLoad 26/10 SP Sepharose, GE Healthcare). Ub variants were eluted in Ub HI buffer as follows: 1 CV = 0-15%, 1 CV = 15%, 2 CV = 15-50% where CV stands for column volume and % are of Ub HI buffer. Peak fractions were pooled, concentrated using a spin concentrator (3 kDa MWCO, Amicon Ultra) and applied to Size Exlusion Chromatography (SEC), (HiLoad 16/60 Superdex 75 pg, GE Healthcare Life Sciences) in the buffer of choice: For use in biochemical assays, variants were exchanged into the Ub (biochem) buffer. For NMR variants were exchanged into a reducing agent-free NMR buffer. For generation of a non-native Lys-linked E2 conjugate, wt Ub was exchanged into CAPSO (conj) buffer.

#### 2.3.2. Ubiquitin-like domain of Parkin

Parkin Ubl- expressing cells from a 6 L culture were resuspended to a final volume of  $\sim 100$  mL in TALON binding buffer supplemented with 5% w/v glycerol, 2 mg/mL

Lysozyme (Sigma), 0.2 mg/mL DNaseI (Sigma), and one EDTA-free cOmplete<sup>TM</sup> Protease Inhibitor Cocktail tablet (Roche). Cells lysed by sonication and clarified as above were incubated with HisPur<sup>TM</sup> cobalt resin (2 mL of slurry per 6 L culture, Thermo Fisher Scientific) for 5-10 min prior to wash with 2 L of TALON binding buffer. For elution, TALON binding buffer was supplemented with 250 mM imidazole (from a 1 M stock, pH adjusted to 7.6). SENP1 for overhang-free removal of the His<sub>6</sub>-SUMO tag was added to pooled eluted fractions prior to overnight dialysis in TALON cleavage buffer at  $4^{\circ}$ C.

Following dialysis, the  $\text{His}_6$ -SUMO tag was captured by reusing the  $\text{HisPur}^{\text{TM}}$  cobalt resin. The flowthrough was concentrated as above and applied to SEC in NMR buffer containing 10 mM DTT.

#### 2.3.3. Parkin

HsParkin or TsParkin expressing cells from a 30 L culture were resuspended to a final volume of ~400 mL in reducing agent-containing GST lysis buffer supplemented with 2 mg/mL Lysozyme (Sigma), 0.2 mg/mL DNaseI (Sigma) and 80 µg/mL PMSF (added gradually during lysis). The suspension was homogenized using an EmulsiFlex-C3 (Avestin) for two passes at ~15,000 p.s.i. and cleared by centrifugation at 46,000g for 35 min at 4°C. The clarified lysate was incubated with Amintra glutathione resin (Expedeon) for 1 - 1.5 h (10 mL slurry per 30 L of culture), which was subsequently washed with 5 L of DTT-containing GST HI buffer. Resin, equilibrated with GST LO buffer, was then transferred into a 50 mL conical tube and GST-3C prescission protease added to remove the His<sub>6</sub>-GST tag during overnight cleavage at 4°C.

The flowthrough was collected and resin washed with GST NO buffer to achieve a final NaCl concentration of 50 mM. Pooled fractions were subjected to anion-exchange chromatography on a 6 mL Resource Q column (GE Healthcare) with a 0–25% linear gradient from ResQ LO buffer to ResQ HI buffer over 15 CV. Parkin eluted at a conductivity value of ~17.0 mS/cm. Due to a large proportion of co-purifying *E. coli* GroEL eluting at higher conductivity value of ~32.5 mS/cm, the Resource Q column was washed using 2 M NaCl and 0.5 M NaOH according to manufacturer's instructions after each use. The

| Namo                     | Composition                                    |      |                         |   |
|--------------------------|--|------|-------------------------|---|
| Name                     | Buffer   | pН   | Salt                    | Additive                                |
| Ub lysis                 | 25 mM Tris                                     | 7.4  | -                       | -                                       |
| Ub LO                    | $50 \mathrm{~mM}$ NaOAc                        | 4.5  | -                       | -                                       |
| Ub HI                    | $50 \mathrm{~mM}$ NaOAc                        | 4.5  | 1 M NaCl                | -                                       |
| Ub (biochem)             | 25  mM Tris                                    | 7.4  | $150 \mathrm{~mM}$ NaCl | -                                       |
| NMR                      | $18 \text{ mM Na}_2\text{HPO}_4,$              | 7.2  | $150 \mathrm{~mM}$ NaCl | $\pm10$ mM DTT                          |
|                          | $7 \mathrm{~mM} \mathrm{~NaH}_2 \mathrm{PO}_4$ |      |                         |   |
| CAPSO (conj)             | $25 \mathrm{~mM}$ CAPSO                        | 9.5  | $150 \mathrm{~mM}$ NaCl | $20~{\rm mM~MgCl}_2$                    |
| TALON binding            | 25  mM Tris                                    | 8.5* | 200  mM NaCl            | 2mM $\beta$ ME                          |
| TALON cleavage           | 25  mM Tris                                    | 8.5  | $300 \mathrm{~mM}$ NaCl | 2mM $\beta$ ME                          |
| GST lysis                | 25  mM Tris                                    | 8.5  | $300 \mathrm{~mM}$ NaCl | $\pm14.3\mathrm{mM}$ $\beta\mathrm{ME}$ |
|                          |  |      |                         | 10% w/v glycerol                        |
| GST HI                   | 25  mM Tris                                    | 8.5* | 500  mM NaCl            | $\pm10$ mM DTT                          |
| GST LO                   | 25  mM Tris                                    | 8.5* | 200  mM NaCl            | 10  mM DTT                              |
| GST NO                   | 25  mM Tris                                    | 8.5  | -                       | 10  mM DTT                              |
| ResQ LO                  | 25  mM Tris                                    | 8.5  | $50 \mathrm{~mM}$ NaCl  | $10~\mathrm{mM}~\mathrm{DTT}^{**}$      |
| $\operatorname{ResQ}$ HI | 25  mM Tris                                    | 8.5  | $50 \mathrm{~mM}$ NaCl  | $10~\mathrm{mM}~\mathrm{DTT}^{**}$      |
| Parkin storage           | 25  mM Tris                                    | 8.5* | 200  mM NaCl            | $10~\mathrm{mM}~\mathrm{DTT}^{**}$      |
| E1 storage               | 25  mM Tris                                    | 8.5  | $300 \mathrm{~mM}$ NaCl | 5  mM DTT                               |
|                          |  |      |                         | $5\%~{\rm w/v}$ glycerol                |
| Ub-intein                | 20  mM HEPES,                                  | 6.5  | $75 \mathrm{~mM}$ NaCl  | -                                       |
|                          | $50 \mathrm{~mM}$ NaOAc                        |      |                         |   |
| Ub-intein HI             | 20  mM HEPES,                                  | 6.5  | 500  mM NaCl            | -                                       |
|                          | $50 \mathrm{~mM}$ NaOAc                        |      |                         |   |

**Table 2.4.: Buffers used for protein purification.** Use of reducing agent is indicated in the text for each protein. The pH of \* marked buffers was adjusted to 7.4 for E2 purification. In \*\* marked buffers DTT was substituted with TCEP for coupling of Ub-VS or E2-Ub probes with Parkin and their storage.

resulting pooled fractions were concentrated using a spin concentrator (30 kDa MWCO, Amicon Ultra) and either phosphorylated as described below or applied to SEC as above in Parkin storage buffer. To obtain the best possible results, samples were immediately used for HDX MS or supplemented with a final 10% v/v glycerol and stored at a 30  $\mu$ M

concentration in PCR tubes for best performance in biochemical assays (Deng et al., 2004).

#### 2.3.4. GST-*Ph*PINK1

*Ph*PINK1 was expressed from 12 L of culture, lysed, clarified, bound to Amintra glutathione resin (Expedeon) and washed as described for Parkin above. Instead of overnight cleavage, the GST-fusion protein was eluted using pH-adjusted GST LO buffer supplemented with 15 mM reduced glutathione. The resulting protein-containing fractions were pooled, concentrated and applied to SEC on an Äkta Explorer system (GE Healthcare) before storage.

#### 2.3.5. *Hs*UBE1

This E1 purification protocol was developed based on (Tongaonkar and Madura, 1998). Firstly, N-terminal GST-Ub fusion protein was expressed in a 2 L culture using an auto-induction medium, lysed by sonication and clarified as described for Ub above. The resulting clarified lysate was incubated with Amintra glutathione resin (Expedeon) for 1 h and washed with 2 L of reducing-agent-free GST HI buffer. Subsequently, the resin was equilibrated with 2 mM ATP from a pH-adjusted stock (using NaHCO<sub>3</sub>) in 50 mM Tris (pH 8.5). This first step yields resin coupled to Ub molecules with C-termini free for activation and thioesterification by the E1 enzyme.

Secondly, HsUBE1- expressing cells from a 12 L culture were resuspended to a final volume of ~200 mL in reducing-agent-free GST lysis buffer supplemented with 2 mg/mL Lysozyme (Sigma), 0.2 mg/mL DNaseI (Sigma) and three EDTA-free cOmplete<sup>TM</sup> Protease Inhibitor Cocktail tablets (Roche). The cells were lysed and clarified using the EmulsiFlex-C3 (Avestin) as described above for Parkin. The resulting  $\beta$ ME-free lysate was supplemented with 10 mM ATP and 10 MgCl<sub>2</sub> and incubated with the GST-Ub fusion-bound glutathione resin at room temperature for 30 min. During this step, the E1 enzyme contained within the lysate adenylates and covalently binds Ub associated with the GST resin. Thus only active E1 enzyme is captured. The E1-bound resin was then washed with DTT-free GST HI buffer supplemented with 5 mM MgCl<sub>2</sub>. HsUBE1 was eluted in DTT-containing GST LO buffer, able to discharge the E1 ~ Ub thioester

conjugates due to its free thiol (DTT) component. E1-containing fractions were applied to an ion-exchange with a linear gradient and size exclusion chromatography in E1 storage buffer as described for Parkin above.

#### 2.3.6. UBE2L3 and UBE2D3

The E2 enzymes were affinity purified from a 6 L culture as described for Parkin above using buffers at pH = 7.4: TALON binding buffer for lysis, GST HI and GST LO buffers for affinity purification. The flowthrough and wash fractions were pooled and applied to SEC and the enzymes exchanged into CAPSO (conj) buffer for generation of a non-native Lys-linked E2 conjugate or Parkin storage buffer adjusted to pH = 7.4 for biochemical assays. Despite a change of pH across the PI of both proteins during SEC into CAPSO (conj) buffer, no aggregation or precipitation was observed.

#### 2.3.7. Ubiquitin activity-based probes

The Ub-intein/CBD fusion was purified and cleaved as described in (Wilkinson et al., 2005). Ub-intein/CBD expressing cells from a 6 L culture were resuspended to a final volume of ~100 mL in Ub-intein buffer supplemented with 0.2 mg/mL DNaseI (Sigma). No Lysozyme was used as this would digest the Chitin resin matrix required for Ub-intein purification. Cells lysed by sonication and clarified as above were incubated with Chitin resin (35 mL of slurry per 6 L of culture, NEB) in a shaking incubator at  $37^{\circ}$ C for 2 h prior to wash with 3 L of Ub-intein HI buffer. Resin was equilibrated with and suspended in 40 mL of Ub-intein buffer supplemented with 100 mM of sodium 2-mercaptoethanesulfonate (MesNa) and incubated overnight at room temperature. During this step, the rearranged intein product is discharged by MesNa in solution to generate a Ub MesNa thioester conjugate (Fig 2.1 A). To prevent Ub-MesNa hydrolysis, the pH is kept at 6.5 and no reducing agent is used. Following overnight cleavage, the flowthrough is collected and resin washed with Ub-intein buffer. Concentrated flowthrough was applied to SEC in Ub-intein buffer. The chitin resin was recycled up to 10 times according to manufacturer's protocol.

Ub-PA, Ub-2Br and Ub-2Cl probes used in Fig 6.2 were obtained from the lab stock (Fig 2.1 C).

#### Ubiquitin vinyl sulphone

The H-Gly-VS hydrochloride was a kind gift from H. Ovaa and B.-T. Xin (Leiden University).



Figure 2.1.: Generation of Ub activity based probes. (A) The Ub (1-75)intein-CBD fusion is expressed and captured using chitin resin. During an overnight MesNa cleavage step, intein rearrangement takes place. Analogously to protein splicing, the nucleophilic thiol group of the Cys residue directly C-terminal of the Ub Gly75 attacks the amide bond linking the two residues, resulting in rearrangement of the Ub (1-75)-intein-CBD fusion. The resulting thioester bond is then substituted MesNa in a transthiolation cleavage reaction to generate Ub-MesNa. (B) Ub-MesNa is used as a building block for the generation of a number of Ub ABPs by substitution of the MesNa group with amine-linked Gly76-mimicking compounds containing an electrophilic warhead group at the Ub C-terminus. In this work both Ub-VS (described in Section 2.3.7) and Ub-C3Br (described in Section 2.3.7) were generated. (C) The structures of Ub-PA, Ub-C2Cl and Ub-C2Br, analogously derived from Ub-MesNa are shown.

Ub-MesNa, stored in Ub-intein buffer at  $\sim 20 \text{ mg/mL}$ , was used to dissolve  $\sim 50 \text{ mg}$ H-Gly-VS hydrochloride.  $\sim 30 \text{ mg}$  of N-hydroxysuccinamide (Fluka) was added to the reaction to act as a catalyst promoting productive coupling of Ub-MesNa with H-Gly-VS over H-Gly-VS multimerization reactions (Borodovsky et al., 2002), (Fig 2.1 A, Fig 5.10). The pH was raised to ~8.5 by stepwise addition of ~60  $\mu$ L (final volume) of 4 M NaOH, mixing well following each addition. The resulting mixture was incubated at 37°C and the reaction progress was monitored by LC–MS analysis (see Section 2.7), since an increased pH sensitized Ub-MesNa to hydrolysis and promoted Ub-VS multimerization. The reaction was quenched by addition of 20 µL of 12 M HCl when the ratio of Ub-VS to hydrolysed Ub-MesNa product was close to 1:1, and formation of the doubly coupled, Ub-VS-VS species, was minimal. The resulting sample was diluted in 30 mL of Ub LO buffer and applied to cation-exchange chromatography (MonoS, 1 mL, GE Healthcare) with a 10–35% linear gradient between Ub LO and Ub HI buffers. Resulting fractions were analysed by LC-MS and Ub-VS containing fractions were pooled and applied to size exclusion chromatography as above in reducing-agent free Parkin storage buffer or Ub-intein buffer.

#### Ubiquitin bromopropylamine

Ub-MesNa, stored in Ub-intein buffer, was diluted to 5 mg/mL with Ub-intein buffer and mixed with 0.2 g/mL 3-bromopropylamine hydrobromide (Fluka) dissolved in PBS (pH 4.8) in a 1:1 (v/v) ratio, such that the final Ub-MesNa concentration was 445  $\mu$ M. The coupling of Ub-MesNa and 3-bromopropylamine hydrobromide was initiated by the addition of 50  $\mu$ L of 4 M NaOH to raise the pH to 10.5 and carried out on ice for 30 min. These relatively harsh conditions were able to minimize Ub-MesNa hydrolysis and yield almost only Ub-C3Br. The reaction was quenched by addition of 12  $\mu$ L of 12 M HCl and buffer exchanged using a disposable PD-10 desalting column (GE Healthcare) into Parkin storage buffer. For coupling of Q347C Parkin engineered to react with the pUb-C3Br probe (Wauer et al., 2015a), the Ub-C3Br was freshly produced each time.

# 2.4. Protein modification

All chromatographic steps were carried out on an Åkta Pure system (GE Healthcare). Buffers used for biochemical manipulation and assays are described in Table 2.5.

#### 2.4.1. Ubiquitin phosphorylation

Ub variants purified as described above were incubated with GST-PhPINK1 in phosphorylation buffer at pH = 7.4. At most a 100 : 1 ratio of Ub to kinase was used for Ub L71Y, with a higher ratio utilized for other, more easily phosphorylatable variants (see Section 3.5). Reaction progress at 25°C was monitored using LC-MS. While wt, F4A and TVLN Ub phosphorylation reached completion, despite a shift to  $37^{\circ}$ C, increased incubation time, and addition of PhPINK1 and ATP, Ub L71Y could not be phosphorylated to completion. Once there were no changes in the recorded spectra, the reaction mixture was dialysed against water, using a 3.5 kDa cut-off dialysis cassette (Thermo Scientific). As GST-PhPINK1 likely precipitates under these conditions, the reactions were not quenched. The dialysate was applied to an anion-exchange (1 mL MonoQ 5/50 GL, GE Life Sciences) column using a 5 mL loop. pUb was eluted by a 0 - 100 % linear gradient of 50 mM Tris (pH = 7.4) over a 40 CV. As unphosphorylated Ub did not bind the column and pUb variants eluted at very low conductivity between 1.30 - 2.00 mS/cm, it was paramount to use fresh deionized water each time. Protein-containing fractions did not require concentration and were directly applied to SEC using reducing agent-free NMR buffer for subsequent analysis.

#### 2.4.2. Parkin phosphorylation

Parkin obtained from anion-exchange as described above was mixed at a 100 : 1 molar ratio with GST-*Ph*PINK1 in phosphorylation buffer. The reaction was monitored using LC-MS and once a mass corresponding only to phospho-Parkin was observed, GST-*Ph*PINK1 was removed by incubation with Amintra glutathione resin (Expedeon). The flowthrough and three 1 mL wash fractions (ResQ LO) were diluted in ResQ LO buffer and separated using anion-exchange chromatography as above. The shallow 0–25% linear gradient allowed phospho-Parkin separation from ADP (which elutes at a conductivity value of 12.10 mS/cm) and ATP (which elutes at a conductivity value of 16.30 mS/cm). Subsequently, pooled and concentrated fractions were applied to SEC in Parkin storage buffer.

| Name            | Composition  |             |                         |                        |
|-----------------|--------------|-------------|-------------------------|------------------------|
| Iname           | Buffer       | $_{\rm pH}$ | Salt                    | Additive               |
| CAPSO (conj)    | 25  mM CAPSO | 9.5         | 150 mM NaCl             | $20 \text{ mM MgCl}_2$ |
| phosphorylation | 20  mM Tris  | $8.5^{*}$   | $150 \mathrm{~mM}$ NaCl | 10  mM ATP             |
|                 |              |             |                         | $10 \text{ mM MgCl}_2$ |
|                 |              |             |                         | 1  mM DTT              |
| ubiquitination  | 30  mM HEPES | 7.5         | 100  mM NaCl            | $10 \text{ mM MgCl}_2$ |
|                 |              |             |                         | 10  mM ATP             |

Table 2.5.: Buffers used for assays. \* For Ub phosphorylation pH = 7.4 was used

#### 2.4.3. Parkin modification with activity-based probes

#### Ubiquitin vinyl sulphone coupling to phospho-Parkin

For quantitative coupling of Parkin to in-house generated Ub-VS, phospho-Parkin was obtained from anion-exchange chromatography carried out with ResQ LO and HI buffers containing TCEP reducing agent rather than DTT. Initially when coupling reactions performed with phospho-Parkin stored in DTT-containing buffer were analysed on LC-MS it became apparent that Ub-VS is modified with a +154 Da adduct corresponding to DTT. The competition between coupling to DTT (high, mM concentration) or phospho-Parkin (low,  $\mu$ M concentration) caused incomplete phospho-Parkin coupling to commercial Ub-VS observed in our laboratory and others (Wauer et al., 2015a; Ordureau et al., 2014). In contrast, rapid, complete coupling of phospho-Parkin stored in TCEP-containing buffer was observed with in-house generated Ub-VS. At room temperature in TCEP-containing buffer, phospho-Parkin mixed with Ub-VS in a 1 : 4 molar ratio reacted to completion within 10 min, while a 1 : 2 mixture resulted in complete coupling within 30 min. Interestingly, Ub-VS could not be stored in TCEP-containing Parkin storage buffer as an inert adduct was formed following overnight chromatographic steps at 4°C.

For quantitative coupling, phospho-Parkin and in-house generated Ub-VS were mixed at a 1 : 3 molar ratio and incubated at room temperature. Reaction progress was monitored

by LC-MS analysis and upon completion the reaction was quenched by addition of DTT ( $\sim 30$  min). The resulting sample was purified using size exclusion chromatography in DTT-containing Parkin storage buffer.

#### UBE2L3-Ub amide-linked activity-based probe coupling to phospho-Parkin

The His<sub>6</sub>-UBE2L3-Ub amide-linked Activity-Based Probe (ABP) was a kind gift from Dr Satpal Virdee and Kuan-Chuan (Eric) Pao at the MRC Protein Phosphorylation and Ubiquitylation Unit.

Phospho-Parkin stored in TCEP-containing Parkin storage buffer was used for labelling. As TCEP is able to unproductively couple with the UBE2L3-Ub amide-linked ABP, TCEP concentration was reduced by two rounds of buffer exchange using a disposable PD-10 desalting column to a final concentration  $< 300 \,\mu\text{M}$  (GE Healthcare) in reducing-agent-free Parkin storage buffer. Reported coupling was carried out at relatively low pH of 7.5 with a low final concentration of phospho-Parkin (3  $\mu$ M) and the UBE2L3-Ub amide-linked ABP (15  $\mu$ M), (Pao et al., 2016). I was able to achieve  $\sim 40\%$  coupling by increasing the pH to 8.5 by buffer-exchange of the UBE2L3-Ub amide-linked ABP into a Tris-based buffer and increasing phospho-Parkin and UBE2L3-Ub amide-linked ABP concentrations to 7.5 and 30  $\mu$ M, respectively. Additional improvements could be observed by increasing the ABP concentration up to 90  $\mu$ M at a lower pH.

Batch coupling and subsequent purification for HDX MS experiments was carried out as follows: the TCEP concentration was reduced to 100  $\mu$ M, the UBE2L3-Ub amide-linked ABP was buffer-exchanged into Parkin storage buffer supplemented with 100  $\mu$ M and reaction carried out for 4 h at 30°C and not quenched to allow recovery of remaining active UBE2L3-Ub amide-linked ABP (Mr = 28,613). The resulting reaction mixture was applied to Ni-NTA Agarose (QIAGEN). Although the reacted His<sub>6</sub>-UBE2L3-Ubphospho-Parkin complex only associated with the beads weakly, it fortunately eluted in a later wash fraction than uncoupled phospho-Parkin. The covalent complex was applied to SEC in TCEP-containing buffer to preserve activity of the recovered probe.

#### Ubiquitin bromopropylamine coupling to Parkin in the presence of PINK1

Anion-exchange purified unphosphorylated Parkin variants containing the Q347C (*Hs*Parkin) and M333C (*Ts*Parkin) engineered sites were used, which enable coupling with the phosphorylated Ub-C3Br probe (Wauer et al., 2015a). Parkin was incubated with a 1 : 4 molar excess of freshly prepared Ub-C3Br in phosphorylation buffer. GST-*Ph*PINK1 was added in a 1 : 9 molar ratio to Parkin and the resulting mixture was incubated at a final Parkin concentration of ~ 70 µM for 1 h and monitored using LC-MS. During this incubation period, Ub-C3Br attached to the engineered reactive Cys on the IBR domain and both the conjugated Ub as well as the Parkin Ubl domain were phosphorylated by GST-*Ph*PINK1. As both phosphorylation events are cooperative (see Section 1.12.2), (Wauer et al., 2015a; Sauvé et al., 2015), the final phospho-Parkin-pUb complex is generated rapidly. GST-*Ph*PINK1 was subsequently removed using Amintra glutathione resin (Expedeon) and the complex purified as for phospho-Parkin above.

To generate the phospho-*Ts*Parkin-pUb-Ub complex used in Fig 6.3 and Fig 6.4, the anion-exchange step was performed with TCEP-containing ResQ LO and HI buffers prior to coupling with Ub-VS as described above.

#### 2.4.4. TEV-mediated Parkin cleavage

TEV-cleavable phospho-HsParkin or phospho-TsParkin linked to pUb through the engineered Cys residues were generated by incubation with GST-PhPINK1 as described above. As previously, GST-PhPINK1 was removed using Amintra glutathione resin (Expedeon), subsequently the flowthrough and three 100 µL wash fractions were pooled and subjected to His<sub>6</sub>-TEV cleavage overnight at 4°C. His<sub>6</sub>-TEV was subsequently removed using Ni-NTA Agarose (QIAGEN), sample diluted in ResQ LO buffer and applied to anion-exchange followed by size exclusion chromatography as described for Parkin above.

#### 2.4.5. Generation of non-native Lys-linked E2-Ub conjugates

E2 variants where the active site Cys has been replaced with a Lys residue - UBE2L3 (C86K), UBE2D3 (C85K) were purified and stored in CAPSO (conj) buffer. UBE2D3 (C85K) additionally carried the S22R 'backside' Ub binding mutation, such as UBE2D3 could not bind free Ub during the charging reaction or in subsequent use of the Lys-linked conjugate (Buetow et al., 2015). To improve reaction efficiency, all components were mixed at the highest possible concentrations and incubated with 10 mM ATP at  $37^{\circ}$ C overnight. The E2 was mixed with Ub in a 1 : 2 or 1 : 3 molar ratio to promote conjugation. *Hs*UBE1 ranging from 1 to 2.5 µM final concentration was added. As both reactions were incomplete, the resulting mixture was applied to SEC in Parkin storage buffer to separate the reactants and products. Owing to its larger size, UBE2L3 could not be separated from the Lys-linked UBE2L3-Ub and fractions containing both were pooled, concentrated and applied to a subsequent round of SEC. The two species could not be separated by the cation-exchange performed analogously to Ub-VS generation as described above.

### 2.5. Assays assessing the ubiquitination cascade

#### 2.5.1. E1 charging assay

For E1 and E2 charging assays with either wt Ub or Ub TVLN the following components were mixed in 100 µL final volume: 0.2 µM of HsUBE1, 4 µM E2 (UBE2L3 or UBE2D3) and 20 µM of either Ub variant. The zero time point was removed into LDS sample buffer (NuPAGE, Thermo Fisher Scientific), prior to ATP addition to the reaction mixture, instead ATP was added directly to the denatured mixture for the zero time point. Reactions were started by the addition of the remaining ATP and samples quenched at indicated time points with LDS sample buffer. The mixtures were resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon). A final sample was removed and quenched with DTT- containing sample buffer to determine the nature of the conjugates formed. Consistent with a thioester linkage being formed during both HsUBE1 and E2 charging, conjugates disappeared upon reducing agent treatment.

#### 2.5.2. E2-/E3-mediated assembly assay

While Parkin-mediated assembly was further optimized (see below), assemblies in Fig 3.10 and Fig 3.11 were performed as follows: For assembly assays performed in a 100  $\mu$ L final volume, *Hs*UBE1 and Ub variant concentrations were kept at 0.2 and 20  $\mu$ M respectively. E2-mediated assembly was performed with 4  $\mu$ M of active E2, while E3-mediated assembly was performed with 2  $\mu$ M of E2 and 5  $\mu$ M of GST-cIAP1, GST-TRAF6, HUWE1 and phospho-Parkin or 1  $\mu$ M of HOIP RBR-LDD. GST-cIAP1 and GST-TRAF6 were a kind gift from Dr K. N. Swatek, HUWE1 and HOIP RBR-LDD were a kind gift from Dr P. Elliott, and phospho-Parkin was purified as described above. Samples were removed at indicated time points as described above. In UBE2R1- and UBE2N/UBE2V1-mediated assemblies a visible number of conjugates were thioester-linked after 60 min of assembly as revealed by reducing agent treatment of the final sample.

#### 2.5.3. Parkin-mediated assembly assay

For Parkin assembly reactions, phosphorylated or unphosphorylated Parkin variants wt/R104A/ $\Delta$ 101-109/ $\Delta$ 116-123 *Hs*Parkin or wt *Ts*Parkin - were used at 4 µM unless otherwise indicated. Parkin variants were incubated with *Hs*UBE1 (0.2 µM), UBE2L3 (2 µM) and Ub (15 - 30 µM) unless otherwise indicated, in ubiquitination buffer at 37°C. Where indicated, a quantity of pUb corresponding to 10% of the Ub added for conjugation was supplemented. Either time points were removed as indicated or 2 h endpoints were examined. Reactions were quenched using LDS buffer containing DTT or  $\beta$ -mercaptoethanol and iodoacetamide to prevent Parkin oxidation and resulting smearing. For ubiquitination site determination on USP30, assembly reactions were resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon). Tryptic digest of extracted gel slices and site determination was performed by Sarah Maslen from the LMB Mass Spectrometry facility and analysed by Dr Malte Gersh as described in (Gersch et al., 2017). Otherwise ubiquitination activity was assessed by western blotting as described below.

Following initial examination of phospho-Parkin assembly reaction, an active PhPINK1 contaminating activity was discovered. This likely results from cleaved PhPINK1, as it was not retained by affinity capture and co-eluted with Parkin on both anion-exchange and SEC chromatography. In particular, this contamination prevented accurate deter-

mination of the Parkin assembly profile using LbPro as described in Section 4.4 and provided pUb-mediated Parkin activation in samples where pUb was not supplemented. As the extent of the contaminating PhPINK1 activity decreased with Ub concentration in the assays, this was reduced to 15 µM for LbPro analysis. Good inhibition was observed upon addition of 10 µM Staurosporine (Santa Cruz Biotechnology), but near-complete eradication of contaminating activity could only be achieved by the addition of 0.8 µM of Nb696 designed to stabilize the PhPINK1 : Ub TVLN complex (Schubert et al., 2017). Prior to downstream analysis, the ubiquitination (and contaminating kinase) activity was quenched by incubating the reaction mixture with 5 mM CaCl<sub>2</sub> and Apyrase (2 mU, Sigma-Aldrich) at 30°C for 1 h. For analysis of UBE2L3 ubiquitination architecture, the UBE2L3 concentration was increased to 4 µM.

For USP30 ubiquitination, 4  $\mu$ M of USP30 substrate was added to the assembly reaction. Since Staurosporine- and Nb696-mediated inhibition had not yet been optimized, USP30 ubiquitination assays were performed with a high concentration (120  $\mu$ M) of Ub S56A, as it had been noted that the Ub S65A variant significantly impairs phospho-Parkinmediated ubiquitination. *Hs*UBE1 was also substituted for mouse UBE1 for USP30 ubiquitination assays.

For generation of USP30<sup>c13</sup>-Ub(F4R), phospho-Parkin was incubated with the assembly machinery as above with 30 µM Ub in reaction buffer supplemented with 10 µM Staurosporine (20 mM Tris, pH 8.5, 200 mM NaCl, 10 mM MgCl2, 1 mM ATP, 0.7 mM DTT). Monoubiquitinated and free USP30 were partially separated through anion-exchange chromatography using ResQ LO and HI buffers (Resource Q, 1 mL, GE Healthcare). KG-TAMRA cleavage assay was performed by Dr Malte Gersch as described in Gersch et al. (2017).

#### Western Blotting analysis

Samples resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) were transferred to either a PVDF membrane (for Ub blot) or a nitrocellulose membrane (for USP30 blot) using a Trans-Blot Turbo system (Bio-Rad). Membranes were blocked in a 5% (w/v) milk solution in PBS-T (PBS + 0.1% (v/v) Tween-20) for 30 min and incubated overnight at 4°C with a primary antibody recognizing either Ub or USP30 in 5% (w/v) BSA in PBS-T

and 0.1% (w/v) sodium azide. The membrane was then washed with PBS-T, incubated for 1 h at room temperature with  $\alpha$ -mouse or rabbit IgG-HRP, detailed in Table 2.6, in 5% (w/v) milk in PBS-T, washed in PBS-T and visualized using the Amersham Western Blotting Detection Reagent (GE Healthcare) and a ChemiDoc Touch Imaging System (BioRad). The antibodies used are detailed in Table 2.6.

| Antibodies                            | Source            | Identifier       |
|---------------------------------------|-------------------|------------------|
| $\alpha$ -Ub (mouse)                  | Novus Biologicals | Ubi-1, NB300-130 |
| $\alpha$ -USP30 (rabbit)              | Atlas Antibodies  | HPA016952        |
| HRP-conjugated sheep $\alpha$ -mouse  | GE Healthcare     | NXA931           |
| HRP-conjugated sheep $\alpha$ -rabbit | GE Healthcare     | NA934V           |

Table 2.6.: Antibodies used. Antibodies used in this thesis.

#### Parkin reactivity by ubiquitin vinyl sulphone

Parkin variants (5  $\mu$ M) stored in either DTT- or TCEP-containing buffer were incubated with in-house generated Ub-VS (15  $\mu$ M) in a 1 : 3 molar ratio at room temperature. The reactions were quenched at indicated time points by addition of DTT- and iodoacetamidecontaining LDS buffer, resolved on a 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon).

#### 2.5.4. $E2 \sim Ub$ discharge assay

First, the UBE2D3 ~ Ub thioester-linked conjugate is generated in ubiquitination buffer supplemented with CaCl<sub>2</sub>. UBE2D3 was used as UBE2L3 was readily modified by Parkin, interfering with reliable readout by SDS-PAGE and the single-turnover nature of the assay. A 300 µL mixture of UBE2D3 (20 µM), HsUBE1 (20 nM) and Ub (80 µM) was incubated at 37°C for 10 min. A low concentration of HsUBE1 (20 nM) was used to enable efficient inhibition of the charging reaction by addition of 0.5 U of Apyrase (NEB) and subsequent incubation at 30°C for 30 min.

Following the addition of 1  $\mu$ M of wt or R104A phospho-Parkin to a diluted, Apyrasetreated charging reaction mixture (final UBE2D3 concentration was 9  $\mu$ M), samples were removed at indicated time points. The reaction progress was tracked by quenching samples by the addition of DTT-free LDS buffer. The final sample was collected at 11 min in DTT-containing LDS buffer to assess the extent of isopeptide-linked UBE2D3–Ub species formation. Samples were resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon). The gel band intensity was quantified on raw images using ImageJ by isolating the specific intensity of the UBE2D3 Ub thioester band as indicated, subtracting the background of the final reduced sample and normalising within each reaction.

# 2.6. Biophysical techniques

Differential Scanning Calorimetry (DSC) and Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS) experiments were performed with assistance from Chris Johnson at the LMB Biophysics facility.

#### 2.6.1. Ubiquitin differential scanning calorimetry

DSC experiments were chosen to assess the stability of Ub variants, as this technique is able to reach temperatures higher than 100°C, necessary to unfold the stable Ub core. Ub variants were dialysed into NMR buffer at high concentration (0.5 - 1.0 mM) using 3.5 kDa MW cut-off dialysis cassettes (Thermo Scientific) to enable accurate concentration determination using the low Ub extinction coefficient and UV<sub>280</sub> absorption. Subsequently samples were diluted to 50 µM. DSC was performed using a VP-capillary DSC instrument (Malvern Instruments). Samples were scanned from 20 to 115°C at a heating rate of 90°C/h in mid-feedback mode. Data were corrected for instrumental baseline using average buffer scans recorded immediately before and after Ub runs. After concentration normalisation, the intrinsic protein baseline between pre- and post-transitional levels was corrected using the progress function in the Origin software supplied with the instrument. Corrected endotherms were fitted to a non-two-state model allowing  $T_m$ ,  $\Delta H$ calorimetric (derived from peak integration) and  $\Delta H$  Van't Hoff (derived from line shape) to vary independently. Given that only a single measurement was carried out, the  $T_m$ error corresponds to calibration accuracy of the instrument, e.g.  $\pm 0.2^{\circ}$ C, while  $\Delta H$  calorimetric depends on the accuracy of the protein concentration measurements.

#### 2.6.2. Parkin thermal shift assays

Due to its lower  $T_m$ , Parkin melting curves were recorded on a Corbett RG-6000 real time PCR cycler (30 - 85°C with 7 s per 0.5°C). Samples contained 4 µM of indicated Parkin variant and 6x SYPRO<sup>TM</sup> Orange (Invitrogen) in ubiquitination buffer supplemented with 5 mM TCEP. Although an an excess of dye may lead to protein aggregation, 6x dye concentration yielded better results than 2x or 4x dye concentration in an initial screen. Melting curves were obtained as the maxima of the derivative of the fluorescence curve (dF/dT) plotted against temperature. All data were recorded in technical triplicate.

# 2.6.3. Analytical size exclusion chromatography binding studies

Binding studies of UBE2D3-Ub or UBE2L3-Ub with the phospho-Parkin-pUb covalent complex shown in Fig 6.1 were performed on an Äkta Micro system (GE Healthcare) using a Superdex 75 PC 3.2/30 column equilibrated in DTT-containing Parkin storage buffer. 25 µM of each covalent complex was mixed in a 1:1 molar ratio and incubated on ice for 30 min to allow complex formation. Protein-containing fractions were resolved on 4-12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon).

# 2.6.4. Size exclusion chromatography-multi angle light scattering

For Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS) experiments immediately following TEV cleavage on a site engineered before the RING2 domain, phospho-*Hs*Parkin was anion-exchanged prior to overnight TEV cleavage. Before the sample was applied to the system, TEV activity was attenuated by PEFA addition and after use the system was treated with an acid wash according to manufacturer's instructions. The masses of TEV-cleaved phosphorylated or unphosphorylated Parkin fragments in Fig 6.14 were determined by SEC-MALS. A Superdex 75 10/300 (GE Healthcare) column was connected to a Wyatt Dawn Heleos-II angle light scattering instrument coupled to a Wyatt Optilab rEX online refractive index detector. Protein samples (100 µL of 2.5 mg/mL) were loaded at 0.5 mL/min in Parkin storage buffer containing 5 mM TCEP. A BSA (2 mg/mL) scan carried out immediately prior to analysis was used to calibrate the instrument. The protein concentration was determined from the excess differential refractive index based on 0.19 RI per 1 g/mL. Determined protein concentration combined with the scattering intensity for each chromatogram enabled calculation of the absolute molecular mass using the ASTRA6 software (Wyatt technology). A higher protein concentration would have been necessary to more accurately determine the molecular mass of the cleaved, dissociated RING2 domain. In support of dissociation only in the phospho-Parkin sample, molecular masses calculated based on the UV signal and known extinction coefficients for both fragments match the experimentally determined molecular mass within error.

Fractions were collected using a manually synchronized fraction collector, resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon), the resulting gel image was manually aligned to the chromatograms based on the protein peak positions.

# 2.7. Liquid chromatography-mass spectrometry analysis

LC-MS analysis was carried out on an Agilent 1200 Series chromatography system coupled to an Agilent 6130 Quadrupole mass spectrometer. Samples were eluted from a Phenomenex Jupiter column (5 mL, 300 Å, C4 column, 150 x 2.0 mm) using an acetonitrile gradient + 0.2% (v/v) formic acid. Protein was ionized using an ESI source (3 kV ionization voltage), and spectra were analysed in positive ion mode with a mass range between 400 and 2,000 m/z. At most 1 µg of protein was injected for each analysis. Averaged spectra were deconvoluted using Promass (Novatia, LLC).

### 2.8. Limited proteolysis

The proteases used were purchased from Hampton Research as Proti-Ace (HR2-429) and Proti-Ace II (HR2-432) kits. 1 mg/mL protease stocks were created by dissolving freeze-dried proteases in 100 µL in water. The stock proteases were diluted 1 : 200 in Parkin storage buffer to yield a 5 µg/mL solution and mixed 1 : 1 (v/v) with a 1 mg/mL solution of Parkin variants as indicated. The cleavage reaction was incubated for 1 h at room temperature. In the initial screen several proteases were inactive. This could be due to deviation from manufacturer's instructions in the protease dilution step as the pH was increased from 7.5 to 8.5 (potentially affecting Pepsin,  $\alpha$ -Chymotrypsin, Trypsin) and NaCl concentration decreased from 500 to 200 µM (potentially affecting Endoproteinase Glu-C, Endoproteinase Arg-C, Thermolysin, Bromelain). Alternatively no efficient cleavage sites for these proteases are present on the Parkin surface for proteases other than Pepsin and Trypsin, both of which are known to efficiently cleave Parkin in optimized conditions. Reactions were quenched with DTT- and iodoacetamide-containing LDS buffer and resolved on 4–12% SDS gradient gels (NuPAGE, Invitrogen) and stained with Instant Blue SafeStain (Expedeon).

# 2.9. Crystallization and X-ray crystallography

Crystallization screens were performed by the sitting-drop vapour diffusion method in a 96-well plate MRC format (Molecular Dimensions) generated from commercial crystallization screens in-house. For non-covalent complex formation, proteins were mixed in a 1 : 1.3 molar ratio of Parkin to co-crystallized protein. Finally, samples were centrifuged at maximum table-top centrifuge speed at 4°C for 10 min before mixing with reservoir solution using a mosquito<sup>®</sup> LCP (TTP Labtech). Optimization fine screens were generated using the dragonfly<sup>®</sup> crystal set-up (TTP Labtech).

For the TEV-cleaved, pUb-linked phospho-HsParkin(1-382)-pUb complex, produced as described in section 2.4.4, initial crystals were found from crystallization experiments incubated at 18°C set up by mixing 100 nl of 4 mg/mL protein solution with 100 nl reservoir solution at room temperature (22°C). The crystallization condition of 12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, 12.5% (v/v) MPD, 0.03 M of each sodium nitrate, disodium hydrogen phosphate, ammonium sulfate, 0.1 M MOPS/HEPES-Na

(pH 7.5) was found from the MORPHEUS screen (Molecular Dimensions). Seeds were obtained from a fine screen and streak seeding was carried out in a hanging drop format from an 8 mg/mL protein solution. Larger crystals were obtained after 6 days in the original crystallization condition. Crystals were soaked in mother-liquor supplemented with 10% (v/v) glycerol before vitrification in liquid nitrogen.

Diffraction data were collected at the Diamond Light Source, beamline I-24 (0.9686 Å, 100 K), and processed using DIALS (Waterman et al., 2016). The crystal structure was determined by molecular replacement in Phaser (McCoy et al., 2007) by placing the structure of the human Parkin core truncated after the IBR obtained from PDB ID: 5N2W (Kumar et al., 2017a) and the structure of the human Parkin Ubl domain obtained from PDB ID: 5C1Z (Kumar et al., 2015). An initial model was built from a lower resolution 3.0Å dataset collected at ESRF, beamline ID23-2. Upon obtaining the high resolution data set, the structure was built at 1.80Å in multiple rounds of model building in Coot (Emsley et al., 2010) and PHENIX (Adams et al., 2011). Phenix ReadySet-derived geometry restraints for the 3CN warhead were used, with external restraints defining the linkage points: C-S bond length = 1.66Å ( $\sigma$ =0.02), C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle = 109.5° ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $123.5^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $122^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $116^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =  $120^{\circ}$  ( $\sigma$ =3), C $\alpha^{75}$ -C<sup>75</sup>-N<sup>76</sup> bond angle =  $120^{\circ}$  ( $\sigma$ =3). Restraining the C $\beta^{347}$ -S $\gamma^{347}$ -CB<sup>76</sup> bond angle =

Final Ramachandran statistics: 98.9% favoured, 1.1% allowed, and 0% outliers. Data collection and refinement statistics can be found in Table 6.1.

# 2.10. NMR techniques

Nuclear Magnetic Resonance (NMR) measurements were carried out by J. L. Wagstaff and S. M. V. Freund from the LMB NMR facility. I prepared all samples, assigned unphosphorylated and phosphorylated F4A Ub variants, assisted data analysis and plotted the derived data. For completeness, methods are reproduced below from Gladkova et al. (2017). We thank Tom Frenkel and facility staff at MRC Biomedical NMR Centre for 950 MHz NMR data collection. Nuclear magnetic resonance acquisition was carried out at 25°C on either Bruker Avance III 600 MHz, Bruker Avance II+ 700 MHz or Bruker Avance III HD 800 MHz spectrometers equipped with a cryogenic triple-resonance TCI probes unless otherwise stated. Topspin (Bruker) and NMRpipe (Delagio et al., 1995) were used for data processing and Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, UCSF, https://www.cgl.ucsf.edu /home/sparky/) was used for data analysis.

#### 2.10.1. **BEST-TROSY**

 $^{1}$ H,  $^{15}$ N 2D Band-Selective Excitation Short Transients-Transverse Relaxation-Optimised Spectroscopy (BEST-TROSY) experiments were acquired with in-house optimised Bruker pulse sequences incorporating a recycling delay of 400 ms and 1,024\*64 complex points in the  $^{1}$ H,  $^{15}$ N dimension, respectively. High-quality data sets were collected in approximately 9 min.

#### 2.10.2. Ub F4A backbone assignments

A triple <sup>1</sup>H-, <sup>15</sup>N-, <sup>13</sup>C-labelled Ub F4A sample was prepared and phosphorylated as described above.

Acquisition was carried out at 25°C on a Bruker Avance III 600 MHz spectrometer equipped with a cryogenic triple-resonance TCI probe. Backbone chemical shift assignments were completed using Bruker triple-resonance pulse sequences. HNCACB spectra were collected with 512\*32\*55 complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions, respectively. CBCA(CO)NH, HNCO and HN(CA)CO spectra were collected with 512\*32\*48 complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions respectively. All experiments were collected using non-uniform sampling (NUS) at a rate of 50% of complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions, respectively, and reconstructed using compressed sensing (Kazimierczuk and Orekhov, 2011).

Assignment of the common conformation peaks visible in pF4A <sup>1</sup>H - <sup>15</sup>N spectra at higher fields was aided by analysis of ZZ-exchange experiments (Latham et al., 2009), collected with 50-, 75-, 150-, 200-, 400- and 800- ms delays using the Bruker 950 MHz

Avance III HD spectrometer at the MRC Biomedical NMR centre for optimised sensitivity.

#### 2.10.3. CEST experiments

For Chemical Exchange Saturation Transfer (CEST) measurements, a Ub concentration of 1.5 mM was used.

Initial <sup>15</sup>N-pseudo-3D CEST experiments were collected at 700 MHz at 25, 37 and 45°C using established pulse sequences (Vallurupalli et al., 2012). At each temperature experiments were acquired with an exchange period of 400 ms and a weak B1 saturation field of either 12.5 or 25 Hz, which was calibrated according to (Vallurupalli et al., 2012) and applied in a range between 102 and 134 ppm at 184 or 92 frequency points, respectively. <sup>15</sup>N CEST profiles were plotted as  $I/I_0$  against applied B1 field, with the  $I_0$  value taken as first slice where the exchange period was omitted.

Higher resolution <sup>15</sup>N-pseudo-3D CEST experiments were then collected using Bruker 950 MHz Avance III HD spectrometer at the MRC Biomedical NMR centre. Here, experiments were collected at 45°C with an exchange period of 400 ms and weak frequency-swept B1 fields of 12.5, 25 and 50 Hz all at 12.5-Hz intervals for a total of 248 points. In order to optimise the experimental conditions and obtain exchange rates and invisible state populations, we modified the <sup>15</sup>N-pseudo-3D CEST experiments with amide proton to directly attached nitrogen-selective Hartmann–Hahn cross-polarisation periods to obtain highly selective pseudo-2D experiments (Pelupessy et al., 1999). Typically for each weak B1 saturation field, pseudo-2D CEST experiments were acquired with a relaxation delay of 5 s, 400-ms exchange time, 184 frequency-swept points and eight scans in ~2 h. To quantify the exchange rates and populations, we obtained <sup>15</sup>N-CEST profiles at five weak B1 saturation fields of 12.5, 20, 25, 37.5 and 50 Hz for a subset of exchanging peaks. Experiments were processed in Topspin 3.2 and the peak intensities simultaneously fitted using ChemEx (https://github.com/gbouvignies/chemex) as previously described (Vallurupalli et al., 2012).

#### 2.10.4. Calculating CSP

Weighted chemical shift perturbation calculations were performed using the following relationship:

weighted CSP = 
$$\sqrt{\left((\Delta^1 H)^2 + (\frac{\Delta^{15}N}{5})^2\right)}$$

where the  $\Delta$  denotes the difference in ppm of the chemical shift between the peaks of phosphorylated and unphosphorylated peaks of the same Ub or between different Ub variants.

#### 2.10.5. CLEANEX measurements

All CLEANEX experiments were collected at 800 MHz with a 3-s acquisition delay and mixing times of 5.2, 10.4, 20.8, 41.6, 83.2 and 166.4 ms using standard Bruker pulse programs. Backbone amide protons that exchanged with the bulk solvent were fitted using established methods (Hwang et al., 1998).

# 2.10.6. ${}^{15}N{}^{1}H$ - heteronuclear NOE measurements

 $^{15}\mathrm{N}\{^{1}\mathrm{H}\}$  - heteronuclear NOE (het NOE) measurements were carried out using standard Bruker pulse programs, applying a  $120^{\circ}$   $^{1}\mathrm{H}$  pulse train with a 5-ms inter-pulse delay for a total of 5-s interleaved on- or off-resonance saturation. The het NOE values were calculated from peak intensities according to the equation  $I_{\rm on}/I_{\rm off}$ .

### 2.10.7. $T_1/T_2$ relaxation experiments

For  $T_1$  and  $T_2$  experiments, data was collected as above using 600, 700 and 800 MHz spectrometers with varying relaxation delays as in (Phan et al., 1996).  $T_1$  and  $T_2$  were derived for each residue at each field by two-parameter exponential fits of resonance intensities plotted against the varied relaxation delays.

In subsequent analysis, the  $A_{ex}$  parameter is derived from a linear relationship between  $\left(R_2 - \frac{R_1}{2}\right)$  and  $B_0^2$ , where  $R_{1,2}$  are the reciprocal of  $T_{1,2}$ , and  $B_0^2$  is derived from the field strength.  $B_0$  values at each field are as follows: 600 MHz,  $B_0 = 14.1$ ; 700 MHz,  $B_0 = 16.4$ ; 800 MHz,  $B_0 = 18.8$ .  $A_{ex}$  can be converted to a meaningful exchange rate at each field by taking into account the  $B_0^2$ .

# 2.11. Hydrogen-deuterium exchange mass spectrometry measurements

Hydrogen-Deuterium Exchange Mass Spectrometry (HDX MS) measurements were carried out by S. L. Maslen and J. M. Skehel from the LMB MS facility. I prepared all samples, plotted and interpreted all data. For completeness, the general methodology is reproduced below from Gladkova et al. (2018).

### 2.11.1. Sample preparation

Initial experiments performed by Dr Tobias Wauer and not discussed here suggested the HDX MS profiles were sensitive to Parkin freeze-thaw. Although precipitation upon repeated free-thaw cycles could be reduced by supplementing Parkin storage buffer with 10% v/v glycerol, glycerol is not compatible with downstream reverse phase separation required for HDX MS.

Fresh Parkin samples were therefore prepared for each HDX MS experiment and only frozen once deuterium labelling was quenched as detailed below. In each case the compared states originated from one protein preparation with subsequent modifications carried out in parallel as described above. A technical triplicate (unless otherwise indicated) was performed for each condition and time point with very small deviations, as seen in panel D of Figs 5.3, 5.5, 5.7, 5.11. To compare the reproducibility of independent protein preparations and measurements, relative % Deuterium (D) uptake values from identical peptides measured in three biologically independent data sets were plotted (Fig 2.2). The analysis demonstrates a highly reliable protein preparation and measurement

protocol.

Complexes were formed on ice and incubated for 30 min to give a final Parkin concentration of 10  $\mu$ M. For all studies performed in Chapter 5, modified Parkin was mixed with pUb and Lys-linked UBE2L3-Ub conjugate non-covalently in a 1:1 ratio as indicated. Parkin modifications used in Chapter 5 include: Parkin phosphorylation (described in Section 2.4.2), phospho-Parkin covalent coupling to Ub-VS (described in Section 2.4.3) and phospho-Parkin covalent coupling to the His<sub>6</sub>-UBE2L3-Ub amide-linked ABP (described in Section 2.4.3). In Chapter 6, *Ts*Parkin constructs are covalently modified with Ub-C3Br as indicated, while *Hs*Parkin constructs were mixed with pUb non-covalently in a 1:1 ratio as indicated.

#### 2.11.2. General methodology

Deuterium-exchange reactions of Parkin and the different complexes were initiated by diluting the protein in  $D_2O$  (99.8% (v/v)  $D_2O$  ACROS, Sigma) in Parkin storage buffer supplemented with 1 mM TCEP to give a final  $D_2O$  percentage of 95%. For all experiments, deuterium labelling was carried out at 23°C unless otherwise stated at five time points: 0.3 s (3 s on ice), 3 s, 30 s, 300 s and 3,000 s in technical triplicate. For the covalently linked phospho-Parkin-UBE2L3-Ub complex, only three time points (3 s, 30 s, 300 s) were analysed as the amount of available complex was limited. The labelling reaction was quenched by the addition of chilled 2.4% (v/v) formic acid in 2 M guanidinium hydrochloride and immediately frozen in liquid nitrogen. Samples were stored at -80°C before analysis.

The quenched protein samples were rapidly thawed and subjected to proteolytic cleavage with pepsin followed by reversed phase HPLC separation. In brief, the protein was passed through an Enzymate BEH immobilized pepsin column, 2.1 x 30 mm, 5 µm (Waters, UK) at 200 µl/min for 2 min, the peptic peptides were trapped and desalted on a 2.1 x 5 mm C18 trap column (Acquity BEH C18 Van-guard pre-column, 1.7 µm, Waters). Trapped peptides were subsequently eluted over 11 min using a 3–43% gradient of acetonitrile in 0.1% (v/v) formic acid at 40 µl/min. Peptides were separated on a reverse phase column (Acquity UPLC BEH C18 column 1.7 µm, 100 mm x 1 mm; Waters) and detected on a SYNAPT G2-Si HDMS mass spectrometer (Waters) over an m/z of 300 to 2,000,



| Biological replicate:  |   |  |  |  |
|--|---|--|--|--|
| rep 1 (Fig 3.4)   rep 2 (Fig 3.5)   rep 3 (Fig 3.7,3.11)   |   |  |  |  |
| —— Peptides compar   | ed:   |  |  |  |
| 24-41 (32.5);<br>25-41 (33);<br>29-41 (35);<br>46-57 (51.5);<br>55-61 (58);<br>81-95 (88);   | FQLKEVVAKRQGVPADQL<br>QLKEVVAKRQGVPADQL<br>VVAKRQGVPADQL<br>AGKELRNDWTVQ<br>TVQNCDL<br>NATGGDDPRNAAGGC                                  |  |  |  |
| 81-102 (91.5);   | NATGGDDPRNAAGGCE-<br>-REPQSL  |  |  |  |
| 103-109 (106);<br>103-120 (111.5);<br>175-182 (178.5);<br>183-205 (194);   | TRVDLSS<br>TRVDLSSSVLPGDSVGLA<br>TLTQGPSC<br>WDDVLIPNRMSGECQSP-<br>HCPGTS   |  |  |  |
| 184-205 (194.5);   | DDVLIPNRMSGECQSPHC-<br>-PGTS  |  |  |  |
| 183-207 (195);   | WDDVLIPNRMSGECQSPH-<br>-CPGTSAE   |  |  |  |
| 184-208 (196);   | DDVLIPNRMSGECQSPHC-<br>-PGTSAFF   |  |  |  |
| 185-208 (196.5);   | DVLIPNRMSGECQSPHCP-<br>-GTSAEF  |  |  |  |
| 188-207 (197.5);   | IPNRMSGECQSPHCPGTSAE  |  |  |  |
| 188-208 (198);   | IPNRMSGECQSPHCPGTS-<br>-AEF   |  |  |  |
| 229-240 (234.5);<br>243-249 (246);<br>267-276 (271.5);<br>269-284 (276.5);<br>273-284 (278.5);<br>275-284 (279.5);<br>312-322 (317);<br>365-378 (371.5);<br>269-279 (272); | IATNSRNITCIT<br>DVRSPVL<br>YCVTRLNDRQ<br>VTRLNDRQFVHDPQLG<br>NDRQFVHDPQLG<br>RQFVHDPQLG<br>YNRYQQYGAEE<br>CRECKEAYHEGECS<br>CKEAYHEGECS |  |  |  |
| 308-378 (373);<br>368-379 (373.5);<br>391-400 (395.5);<br>444-450 (447);<br>454-465 (459.5);   | CKEATHEGECS<br>CKEAYHEGECSA<br>YRVDERAAEQ<br>EWCWNCG<br>NRVCMGDHWFDV  |  |  |  |

Figure 2.2.: Reproducibility of Parkin D uptake. D uptake for the phospho-Parkin : pUb complex from three biologically independent experiments.

with the standard electrospray ionization (ESI) source with lock mass calibration using [Glu1]-fibrino peptide B (50 fmol/ $\mu$ l). The mass spectrometer was operated at a source temperature of 80°C and a spray voltage of 2.6 kV. Spectra were collected in positive ion mode.

Peptide identification was performed by  $MS^{e35}$  using an identical gradient of increasing acetonitrile in 0.1% (v/v) formic acid over 11 min. The resulting  $MS^e$  data were analysed using Protein Lynx Global Server software (Waters, UK) with an MS tolerance of 5 ppm.

Mass analysis of the peptide centroids was performed using DynamX software (Waters). Only peptides with a score >6.4 were considered. The first round of analysis and identification was performed automatically by the DynamX software, however, all peptides (deuterated and non-deuterated) were manually verified at every time point for the correct charge state, presence of overlapping peptides, and correct retention time. Deuterium incorporation was not corrected for back-exchange and represents relative, rather than absolute changes in deuterium levels. Changes in H/D amide exchange in any peptide may be due to a single amide or a number of amides within that peptide.

# 2.12. Figure generation

All figures and schemes were assembled in Adobe Illustrator (CC 2015). SPARKY 3, UCSF, (https://www.cgl.ucsf.edu/home/sparky/) was used to generate all <sup>15</sup>N-<sup>1</sup>H correlation plots. All XY graphs, bar charts and heat maps were generated using Prism (version 7). Non-linear regression CLEANEX fits were also performed using Prism (version 7). Structural figures were generated using PyMol (http://www.pymol.org). The contrast on scanned gels was automatically adjusted across the whole image using Adobe Photoshop (2015). Unadjusted gels were used for band quantification by ImageJ (https://imagej.nih.gov/ij/). Sequences were aligned using Clustal Omega and visualized with ESPript3 (http://espript.ibcp.fr), (Robert and Gouet, 2014).

# Chapter 3.

# Retraction of the ubiquitin C-terminus

#### Key findings:

- A small population of wild-type (wt) Ub assumes a C-terminally retracted (Ub-CR) conformation.
- Shifting the resulting equilibrium by mutagenesis impacts Ub stability and dynamics.
- Ub-CR is incompatible with Ub signalling as chain assembly is impaired by the Ub-CR conformer.
- Although Ub-CR is required for efficient Ub phosphorylation by PINK1, an equivalent conformation could not be identified in a second substrate of PINK1, the Ubl domain of Parkin.

Recently, our laboratory reported that Ub phosphorylation at Ser65 establishes an equilibrium between two conformers. The more prominent conformer exhibits the canonical, 'common' Ub structure, while in the novel pUb-CR conformer, the C-terminus necessary for Ub conjugation is retracted due to strand slippage (see Introduction 1.11.3), (Wauer et al., 2015b). Subsequently, in my Part III thesis submitted as a requirement for my MSci degree (Part III Chemistry), I designed Ub variants to trap and study the new conformation (Gladkova, 2015).

Interestingly, the CR conformation-enhancing variants populated Ub-CR already in their unphosphorylated form and their rates of phosphorylation by PINK1 were significantly increased (Gladkova, 2015). To understand whether this effect is physiological, I extended

the search for the CR conformation equilibrium to unmodified Ub and characterized the equilibrium biophysically and biochemically during my PhD research.

# 3.1. Revisiting the ubiquitin structure

The ability of Ub-CR-enhancing variants to access the Ub-CR conformation without the need for phosphorylation (Gladkova, 2015) suggests an intriguing possibility that a small extent of C-terminal retraction is also possible in unmodified wt Ub. Modification of Ub by designer mutations or phosphorylation simply shift the already available equilibrium.

This conformation has not been observed to date, despite extensive studies of Ub dynamics in the past (see Introduction 1.3). Detection of Ub-CR may have been hindered either by the timescale of the exchange or a very low population of Ub-CR. For a slowly exchanging species (with respect to the NMR BEST-TROSY/HSQC pulse sequence), two resonances for each residue would be visible, such as in pUb. Should however the population of this species be below the detectable limit, no resonances could be confidently assigned above the noise level of the spectrum. A faster exchange would simply lead to peak shifting, with the peak position proportional to the population-weighted average of the two extreme resonance positions. Intermediately exchanging peaks would become exchange broadened and lost in the noise of a 2D correlation spectrum such as BEST-TROSY or HSQC.

In collaboration with Jane L. Wagstaff and Stefan M. Freund from the NMR facility at the MRC-LMB, we have turned to the use of Chemical Exchange Saturation Transfer (CEST) and other dynamic experiments to study the possibility of Ub-CR occupancy in unphosphorylated, wt Ub.

#### 3.1.1. Chemical Exchange Saturation Transfer (CEST) theory

CEST experiments were developed as an alternative to Carr-Purcell-Meiboom-Gill (CPMG). While the CPMG method is able to assess equilibria exchanging at a rate of 200 - 2000 s<sup>-1</sup> with the second conformation being populated to >0.5%, CEST is able to

assess equilibria exchanging more slowly, with an even smaller population of the second conformer. In a regular BEST-TROSY/HSQC experiment slow equilibria and highly uneven populations would produce a second very weak, 'invisible' peak as described above (Vallurupalli et al., 2012; Kay, 2016).

The principle of the CEST method lies in applying varying  $B_1$  frequencies to an exchanging sample and identifying those  $B_1$  frequencies which lead to attenuation of the height



Figure 3.1.: The theory of a Chemical Exchange Saturation Transfer (CEST) experiment. (A) A schematic representation of the saturation transfer observed during a CEST experiment. (B) A schematic representation of how the saturation transfer is measured in a <sup>15</sup>N-pseudo-3D CEST experiment leading to the observed CEST profile.

of a peak in an HSQC NMR spectrum. In an exchanging system, peak attenuation can either be due to direct resonance saturation of the main peak corresponding to the major conformer (Fig 3.1 A (left)) or due to resonance saturation of the 'invisible' weak peak corresponding to the second lowly populated conformer (Fig 3.1 A (right)). The latter saturation is reflected in attenuation of the main peak as the two species are in chemical exchange.

In a <sup>15</sup>N-pseudo-3D CEST experiment, a series of HSQC spectra are collected with a varying applied  $B_1$  field (Fig 3.1 B (top)). The main peak attenuation can be observed by taking a slice through each HSQC spectrum at a fixed <sup>1</sup>H frequency of the main peak to obtain an intensity plot of the <sup>15</sup>N chemical shift against signal intensity.

A series of intensity plots with varying applied  $B_1$  field exhibits attenuation corresponding to two distinct frequency regions. When the applied  $B_1$  field matches the <sup>15</sup>N chemical shift of the invisible peak, saturation is transferred to and accumulates at the main species leading to signal attenuation of the main peak (Fig 3.1 B (middle, red)). When the  $B_1$  field matches the <sup>15</sup>N chemical shift of the main peak, near-complete loss of intensity is observed (Fig 3.1 B (middle, purple)).

Taking a further slice at a fixed <sup>15</sup>N chemical shift of the main peak, across the series of intensity plots, a CEST plot is obtained (Fig 3.1 B (bottom)). This shows variation of the intensity of the main peak with the applied  $B_1$  field. In an exchanging equilibrium as described, a CEST profile contains information on the <sup>15</sup>N chemical shift of both the main and the invisible peaks. Further variation of the frequency width ('resolution') of the applied  $B_1$  can yield equilibrium parameters and the ratio of the two conformer populations. The small peak corresponding to the invisible conformer is hereafter referred to as the CEST peak.

The benefit of the CEST experiment is that in addition to characterizing the equilibrium parameters of the exchange, the derived <sup>15</sup>N chemical shift of the weak invisible peak can be used to compare its properties to other samples where the NMR behaviour and structure have well characterized.

#### 3.1.2. A C-terminally retracted species in unmodified ubiquitin

We initially performed <sup>15</sup>N-pseudo-3D CEST experiments as described above on a Ub sample at 25 °C, however no additional attenuation corresponding to a CR conformer was observed in the CEST profile (data not shown). We reasoned that since the population of the pUb-CR conformer is dependent on pH and buffer conditions, we might be able to alter the landscape by changing the experimental conditions, such as altering the temperature.

Strikingly, when the same CEST experiment was collected at 45 °C, we were able to observe a second set of peaks in the CEST profiles for 28 of the ~72 resonances typically observed in a Ub spectrum (Fig 3.2 (right)). These were present throughout the Ub  $\beta$ -sheet and the Ser65 and Gly47 loops of Ub. To assess whether the additional resonances corresponding to a lowly populated conformer were due to C-terminal retraction, we qualitatively compared the <sup>15</sup>N chemical shifts for the two peaks observed in the wt Ub CEST profiles with peakes observed for pUb and pUb-CR in an HSQC experiment performed at 45 °C to account for peak shifting due to temperature (Fig 3.2 (right), for full spectra see Appendix A.1).

There is good agreement between the <sup>15</sup>N chemical shift positions of all CEST peaks with the previously assigned pUb-CR resonances, including the  $\beta$ 5- and  $\beta$ 1-strands as well as the Ser65 loop (Wauer et al., 2015b). The consistent trend in the <sup>15</sup>N chemical shift positions suggests the new CEST peaks correspond to a conformer, in which individual residues occupy similar environments as in pUb-CR. An exact match cannot be expected as for both the common and CR conformations, local perturbations due to the presence of a phosphate group will cause additional perturbation (Dong et al., 2017; Kazansky et al., 2018).

A comparison of the <sup>15</sup>N offset of the two peaks in each CEST profile and the <sup>15</sup>N offset between the common and CR conformer peaks in the pUb HSQC spectrum provided an unbiased approach to determining the extent of the agreement (Fig 3.3 A). The absolute differences are well matched in the  $\beta$ 1- and  $\beta$ 5-strands as well as the Gly47 loop. Deviation is observed in the phosphorylated Ser65 and neighbouring Thr66 residues as expected due to local perturbations from the phosphate group. The Leu67 resonance is



Figure 3.2.: Selected Ub CEST profiles. CEST profiles observed at  $45^{\circ}$ C for indicated residues (right). Left, correlation to pUb in the <sup>15</sup>N dimension.
exchange broadened in the pUb-CR spectrum and therefore cannot be analysed.

Although the experiments were performed in a physiological buffer (25 mM NaPi (pH 7.2), 150 mM NaCl), NMR experiments are typically performed at 25°C. Ub CEST



Figure 3.3.: Ub CEST characterisation. (A) A comparison of the absolute difference in the <sup>15</sup>N dimension between the two peaks observed in wt Ub CEST profiles (purple) and the common and CR conformations in wt pUb (grey). (B) Temperature dependance of an example wt Ub CEST profile for Gln62. (C)  $B_1$  Fitted field dependance of an example wt Ub CEST profile Glu64. (D) Schematic representing the equilibrium of wt Ub.

at 25°C yields no additional peaks corresponding to the Ub-CR conformer, explaining why this species has been systematically missed in the past. Ub CEST at 37°C reveals smaller CEST peaks arising from Ub-CR, suggesting that this conformer is present at physiological temperature and may play a physiological role (Fig 3.3 B).

In order to obtain equilibrium parameters at  $45^{\circ}$ C for the observed exchanging species, we optimized a <sup>15</sup>N-pseudo-2D CEST experiment, in which selective excitation is applied to the specific <sup>15</sup>N frequency of any desired peak. Together with variation of the B<sub>1</sub> field, an equivalent CEST-profile could be obtained directly in less time with a higher resolution in the indirect <sup>15</sup>N dimension to allow accurate fitting (Fig 3.3 C). Several ranges of B<sub>1</sub> fields were collected across a number of peaks to yield a global exchange rate of 63.6 s<sup>-1</sup> and a population of 0.68 % of Ub-CR in unmodified wt Ub (Fig 3.3 D). At 37°C the population or the exchange rate are likely to be reduced, but could not be quantified for wt Ub (see Section 3.3.1).

The presence of Ub-CR in unmodified Ub was fascinating, especially given that Ub is regarded as a well-characterized biophysical standard. We set out to further define how the equilibrium affects Ub biophysical and functional behaviour.

## 3.2. Equilibrium can be modulated by distant mutations

With the CEST characterization of the Ub and Ub-CR equilibrium in wt Ub, we wanted to make use of the previously characterized extreme conformer trapping mutations to probe the equilibrium further. The TVLN Ub mutant favours the CR conformation in both phosphorylated and unphosphorylated Ub, while the L71Y mutant favours the common conformation even in phosphorylated Ub (see Introduction 1.11.3). In addition to these extreme conformation locks, we also wanted to identify mutations which would modulate the equilibrium more subtly.

Phe4 is part of the  $\beta$ 1-strand and its side chain packs against the  $\beta$ 5-strand in the common Ub conformation. Removal of this interaction may perturb the equilibrium and disfavour the common Ub conformation to a lesser extent than the TVLN Ub mutant

(Fig 3.4 A). This was found to be the case by comparing the BEST-TROSY spectra of F4A Ub or pUb to wt pUb. In the unphosphorylated case F4A Ub behaves as wt Ub, as seen both by the reporter Lys11 resonance as well as an unbiased heat-map of weighted chemical shift perturbations derived from reassignment of the F4A spectrum (Fig 3.4 B (left), C). For F4A Ub peak assignment, see Appendix A.2.

However, in the phosphorylated case, both conformers were observed only at high fields (800 Hz or 950 Hz spectrometers), with a 600 Hz spectrum only showing one set of peaks corresponding to the CR conformer. In the 800 Hz spectrum, the main set of peaks (87.9%) corresponded to F4A pUb-CR, while a smaller set of F4A pUb peaks overlaid well with the common conformer of wt pUb (Fig 3.4 B (right), D). The smaller set of peaks was assigned as the common conformation of F4A pUb by performing ZZ exchange



Figure 3.4.: F4A Ub favours the CR conformation. (A) Rationale for design of the F4A mutation to favour the CR conformer. Lack of interactions between Ala4 and the backbone favour the CR conformer (B) An inset from BEST-TROSY spectra showing the Lys11 peak in F4A Ub (left) or pUb (right) compared to a BEST-TROSY of wt pUb (C) A heat map showing a weighted chemical shift perturbation between F4A Ub, large perturbations are observed when compared to pUb-CR, suggesting the common conformer is populated. (D) A heat map showing a weighted chemical shift perturbation between the main set of peaks of F4A pUb, large perturbations are observed when compared to Ub and the common conformer of pUb, suggesting the Ub-CR conformer is populated preferentially.

experiments at 950 Hz from the assigned spectrum of F4A pUb at 600 Hz. This approach was previously used to show exchange between the peaks of the two pUb conformations (Wauer et al., 2015b). For F4A phospho-Ub peak assignment, see Appendix A.2.

#### 3.3. Measuring the equilibrium

Together with wt Ub and the previously characterized TVLN and L71Y conformer locks (see Introduction 1.11.3) the identification and characterization of the F4A mutation completed a set of Ub variants with apparently different equilibrium parameters. To study the observed equilibrium more closely in the set of Ub variants, CEST, more conventional NMR dynamic techniques as well as biophysical analysis was used.

#### 3.3.1. CEST experiments

First, we analysed the behaviour of all Ub variants in addition to wt Ub using CEST. CEST experiments analysing the unphosphorylated proteins reveal that the CR conformer is populated to varying extents. Qualitative pseudo-3D CEST experiments at 45°C can be used to directly compare CEST peaks for the Ub variants. When the <sup>15</sup>N chemical shifts of the residues are not greatly affected by the introduced mutations, a set of CEST profiles such as in Fig 3.5 can be obtained. A single peak position is found for all variants in the common conformation, while another peak position indicated the CR conformer.

Qualitatively, the F4A mutant populated Ub-CR to a greater extent than wt Ub, similarly to the case of phosphorylated Ub (as judged by the intensity of the pUb-CR peaks observed in a BEST-TROSY spectrum). In addition, common conformer CEST peaks can be observed in a subset of TVLN Ub resonances, suggesting that this 'locking' mutant continues to undergo exchange on a detectable timescale. The only variant which exhibits no measurable exchange is L71Y, this may not be detectable either due to a decrease of the population as compared to wt Ub, or due to a slower exchange that can no longer be characterized by CEST, similarity to the wt pUb case (see below). In fact, identification of F4A Ub as a more readily exchanging variant compared to wt Ub or either of the locking mutants allowed us to optimize the CEST methodology used in this study. Pulse sequences and in particular, the delay between scans, had to be optimized to account for the relatively slow exchange rates (relative to the scope of the CEST experiment) observed for the Ub equilibrium. Wt pUb could not be used to optimize the experimental protocol. Although pUb-CR is populated to a large extent, the exchange rate of 2 s<sup>-1</sup> is too slow and falls outside the scope of equilibria accessible by the CEST experiment.

With optimization of the <sup>15</sup>N-pseudo-2D CEST pulse sequence and collection of CEST profiles across several ranges of  $B_1$  fields, the equilibrium parameters were fitted for all of the exchanging Ub variants at 45°C (Table 3.1). Additionally, in the F4A mutant Ub-CR occupied a high enough fraction at 25°C that equilibrium parameters could be derived at this lower temperature. Upon cooling of the system, the rate constant decreased more than 2-fold while the population decreased more than 3-fold. Extending a similar trend to wt Ub at 25°C suggests why the Ub-CR conformer was not observed until now, as the populations and rates would be inaccessible to any other method. For fit quality, see Appendix A.3.



Figure 3.5.: CEST characterisation of Ub variants at  $45^{\circ}$ C. CEST profiles for the Leu71 and Ile13 residues for analysed Ub variants. The Tyr71 peak in the L71Y mutant has a different <sup>1</sup>H chemical shift and is therefore not shown. Note that the lower baseline for Leu71 in F4A Ub corresponds to exchange broadening of the peak due to local motion in the common conformer compared to the other variants.

| Ub   | °C | $k_{ex}~(s^{-1})$ | % Ub-CR          |
|------|----|-------------------|------------------|
| wt   | 45 | $62.6 \pm 4.0$    | $0.68 \pm 0.03$  |
| F4A  | 45 | $111.4 \pm 2.2$   | $4.63 \pm 0.06$  |
| F4A  | 25 | $46.0 \pm 8.5$    | $1.28 \pm 0.21$  |
| TVLN | 45 | $203.5 \pm 17.4$  | $99.35 \pm 0.03$ |
| L71Y | -  | N.D.              | N.D.             |

Table 3.1.: Exchange rate and population of Ub-CR by ubiquitin variants. No CEST peaks were observed for L71Y Ub.

### 3.3.2. Clean Chemical Exchange Transfer (CLEANEX) experiments

With knowledge of the equilibrium parameters in the set of Ub variants, we wondered whether some hallmarks of the equilibrium could be observed in other, more conventional, NMR experiments at 25  $^{\circ}$ C.

CLEANEX experiments report on protein dynamics on the millisecond timescale through measurement of chemical exchange of unexcited backbone amide protons with excited solvent protons. The relative peak intensity increases with increased exchange between the solvent and amide protons from a flat baseline. Residues forming flexible loops or residues found at the termini of secondary structure elements (with backbone amide protons not involved in secondary structure hydrogen bonding) are more likely to exchange in a CLEANEX experiment. Therefore, exchange can only be measured in solvent accessible residues which are not buried in the core or involved in a robust hydrogen bonding network.

In addition, spectra collected at different solvent/amide proton mixing times can yield a fitted solvent exchange rate constant. Interestingly, the measured solvent exchange rate constants for Ub at 25 °C (Table 3.2) are of the same order of magnitude as equilibrium rate constants extended to 25 °C from CEST measurements at 45 °C based on the example of F4A Ub. The CLEANEX measurement of overall residue dynamics may therefore contain a small contribution from the sparsely populated Ub-CR conformation.

Rate and extent of solvent exposure across identical structural elements in different Ub variants can be correlated with intramolecular flexibility of the given region, such as strand slippage. We performed CLEANEX analysis and fitting on the characterized Ub variants and wt Ub. In particular, we were interested whether differences can be observed between wt Ub and L71Y Ub, as this would indicate a contribution of Ub-CR to global wt Ub dynamics. Figure 3.6 shows the typical saturation profiles observed from a CLEANEX experiment, while Table 3.2 lists the derived exchange rates for residues exchanging in all Ub variants. Additional residues exchanging only in individual Ub variants are shown in Appendix A.4.

Given that the Leu8 loop position is distinct between the common and CR conformation of Ub and pUb (see Introduction 1.11.3), we selected this as the first region of interest (Figure 3.6 A). Both Leu8 and Gly10 in TVLN Ub display most solvent exchange, while

| residue | wt $k_{ex}~(s^{-1})$                                      | TVLN $k_{ex}$ (s <sup>-1</sup> )                                | F4A $k_{\rm ex}~(s^{-1})$                                    | L71Y $k_{ex}$ $(s^{-1})$                        |
|---------|---|---|--|---|
| Gln2    | $0.43 \pm 0.06$   | $7.81 \pm 0.47$   | $3.00 \pm 0.18$  | $0.60 \pm 0.13$                                 |
| Leu8    | $16.37 \pm 0.66$  | $19.52\ \pm 0.91$   | $22.10 \pm 1.52$   | $11.05 \ \pm 0.32$                              |
| Gly10   | $16.56 \pm 0.44$  | $28.24 \ \pm 0.42$  | $19.63 \ \pm 0.50$   | $12.10\ \pm 0.55$                               |
| Lys11   | $9.33 \pm 0.41$   | $11.10 \pm 0.22$  | $11.51 \pm 0.55$   | $8.41 \pm 0.29$                                 |
| Thr12   | $16.64 \pm 0.44$  | $29.83 \pm 0.89$  | $16.13 \pm 0.95$   | $13.90 \pm 0.10$                                |
| Thr14   | $1.06 \pm 0.13$   | $1.82 \pm 0.31$   | $2.03 \pm 0.46$  | $0.91 \ \pm 0.07$                               |
| Glu16   | $0.21\ \pm 0.08$  | $0.37 \pm 0.09$   | $0.53 \pm 0.18$  | $0.16 \ \pm 0.07$                               |
| Ser20   | $0.49 \pm 0.09$   | $1.23 \pm 0.09$   | $0.75 \pm 0.23$  | $0.59 \pm 0.06$                                 |
| Gly47   | $3.22 \hspace{.1in} \pm \hspace{.1in} 0.17 \hspace{.1in}$ | $7.06 \hspace{0.1 cm} \pm \hspace{0.1 cm} 0.13 \hspace{0.1 cm}$ | $3.63\ \pm 0.06$   | $3.00 \hspace{0.1 cm} \pm \hspace{0.1 cm} 0.17$ |
| Gln49   | $2.87 \ \pm 0.12$   | $6.14 \hspace{0.1in} \pm \hspace{0.1in} 0.28 \hspace{0.1in}$    | $3.35 \hspace{.1in} \pm \hspace{.1in} 0.17$                  | $2.43 \ \pm 0.13$                               |
| Asp52   | $0.39 \pm 0.13$   | $0.37 \pm 0.05$   | $0.44 \ \pm 0.07$  | $0.32 \pm 0.09$                                 |
| Asn60   | $0.62\ \pm 0.14$  | $4.90 \hspace{0.1 cm} \pm \hspace{0.1 cm} 0.21 \hspace{0.1 cm}$ | $0.94 \hspace{0.1in} \pm \hspace{0.1in} 0.09 \hspace{0.1in}$ | $0.55 \ \pm 0.11$                               |
| Lys63   | $1.99\ \pm 0.14$  | $13.22 \ \pm 0.51$  | $2.91 \ \pm 0.22$  | $1.78\ \pm 0.13$                                |
| Leu73   | $12.22 \ \pm 0.15$  | $7.11 \pm 7.39$   | $15.13 \pm 0.30$   | $15.75 \ \pm 0.43$                              |
| Arg74   | $41.59 \ \pm 2.25$  | $3.81 \hspace{.1in} \pm \hspace{.1in} 0.23 \hspace{.1in}$       | $45.99 \ \pm 1.61$   | $38.18 \pm 1.12$                                |
| Gly75   | $120.3 \pm 24.97$   | $129.7 \pm 31.47$   | $145.5 \pm 42.56$  | $97.78 \pm 19.54$                               |
| Gly76   | $4.57 \pm 0.09$   | $9.35 \pm 1.44$   | $5.43 \pm 0.07$  | $4.81 \pm 0.10$                                 |
|         |   |   |  |   |

Table 3.2.: CLEANEX-derived solvent exchange rates for residues exchanging in all analysed Ub variants. All residues exchanging in each variant are shown in Appendix A.4. CLEANEX profiles for residues in bold are shown in Fig 3.6.



Figure 3.6.: CLEANEX characterisation of Ub variants. CLEANEX saturation profiles for selected residues in studied Ub variants.

remaining most protected in L71Y. Seemingly the solvent exchange rate (flexibility) of the  $\beta$ 1- $\beta$ 2 loop reports on the extent of the Ub-CR population. In both cases, wt Ub displays more flexibility than L71Y Ub, suggesting a possible contribution from Ub-CR. As both F4A and TVLN Ub show more solvent exchange than wt Ub, all following the same trend, this effect is unlikely to be due to local perturbation caused by the introduced point mutations.

A further region of interest, which is predicted to become more flexible due to loss of hydrogen bonding in Ub-CR, is the Ser65-containing loop (Figure 3.6 B). Again, most solvent exchange is seen in TVLN Ub. While wt Ub and L71Y Ub cannot be distinguished in this region, F4A exhibits intermediate solvent exchange for both Asn60 and Lys63 residues shown.

In the C-terminus (Figure 3.6 C), we expect the opposite effect - stabilization in TVLN Ub, which is indeed observed for both Leu73 and Arg74. Solvent exchange in the C-terminus of F4A Ub seems to be consistently higher than for wt Ub, although the CEST-derived equilibrium exchange rates suggest more rigidity due to Ub-CR contribution should be observed. This may be due to a consequence of increased C-terminal local motion in the common conformation of F4A, as suggested also by exchange broadening of the Leu71 residue in CEST (Fig 3.5, left). The Arg74 residue is further away from the Leu71 mutation site than Leu73 and may therefore be a more accurate representation of the behaviour of the C-terminal tail. However, Arg74 exchange is faster in wt Ub than L71Y Ub, suggesting that wtUb may exhibit similar additional motion in the C-terminal tail as suggested for Ub F4A above.

Further structural differences are observed in the flexible Gly47 loop connecting the  $\beta$ 3- and  $\beta$ 4-strands (Figure 3.6 D). These are again recapitulated by increased flexibility of the loop in TVLN Ub. This region is remote from any of the introduced mutations and the derived rate constants can distinguish between wt Ub and L71Y Ub, suggesting a small Ub-CR contribution to wt Ub dynamics in this region may be observed.

In summary, the presented CLEANEX data for residues measured in all Ub variants suggest that TVLN Ub is most flexible compared to wt Ub (Fig 3.7). F4A Ub falls between wt and TVLN Ub, while L71Y Ub tends to be generally less flexible than wt Ub as observed by a comparison across a number of residues (Leu8, Gly10, Lys11, Thr12,



Figure 3.7.: CLEANEX exchange rates relative to wt Ub. The hydrogen bonding pattern for common Ub is shown for L71Y Ub and F4A Ub, while the Ub-CR hydrogen bonding pattern is shown for TVLN Ub. Residues are coloured by their relative solvent exchange rate as compared to wt Ub.

Glu16, Gln49, Asp52). Although care must be taken to distinguish the effects from perturbation due to the introduced point mutations, CLEANEX seems to be able to capture some aspects of the equilibrium between Ub and Ub-CR in a conventional bulk measurement, where contribution from each conformer is averaged.

No statistical significance analysis was performed on the differences in fitted exchange rates or maximum values of solvent exchange, as no repeat biological or technical measurements were performed due to the long duration of the reported CLEANEX experiments. While the observed CLEANEX trends are in agreement with our observations by CEST, identification of the magnitudes and sources of variation in the CLEANEX analysis is required for conclusive quantitative analysis of the data.

#### 3.3.3. <sup>15</sup>N <sup>1</sup>{H} heteronuclear NOE experiments

In contrast to CLEANEX, <sup>15</sup>N <sup>1</sup>{H} heteronuclear NOE (hetNOE) experiments report on the nano- or pico- second motion of the protein backbone. Peak intensity is measured following NOE saturation (flexibility leads to a peak intensity decrease) and its relative intensity to a control spectrum is reported. Especially well suited for study by this method is motion of protein termini. We looked at the flexibility of the C-terminus in the common and CR conformers of both the phosphorylated and unphosphorylated Ub variants to gain more confidence by analysing more Ub species (Fig 3.8). For hetNOE variation across the whole sequence of individual Ub variants, see Appendix A.5.

We observe that in all measurable Ub-CR conformers the C-terminus is rigidified and phosphorylation has no effect on its behaviour on this short timescale (Fig 3.8, right). The Ub-CR population occupied by all variants is similar, as no signifiant differences in C-terminal hetNOE values across the Ub-CR conformers was observed. Common conformers showed more flexibility in Leu73 and especially in Arg74 compared to Ub-CR. Interestingly phosphorylation of the common conformer lock leads to a further increase in flexibility of Arg74.

This analysis is in agreement with stabilization of the C-terminus observed at longer time scales by CLEANEX (Section 3.3.2), as well as previously carried out analysis of



Figure 3.8.: hetNOE of the Ub C-terminus. hetNOE values for the C-terminus of Ub variants in the common conformations are shown on the left, while values for Ub variants in the CR conformation are shown on the right.

the wt pUb equilibrium (Wauer et al., 2015b).

#### 3.3.4. Melting temperature analysis

It was previously reported that wt pUb is significantly destabilized compared to wt Ub (Wauer et al., 2015b; Gladkova, 2015). Since Ub is a very stable molecule, we wondered whether this destabilization is caused by the presence of a less stable conformer rather than merely addition of the phosphate group. We therefore analysed the melting temperature ( $T_m$ ), for all phosphorylated an unphosphorylated Ub variants using Differential Scanning Calorimetry (DSC). Since the temperature increase during the stability measurement is slow relative to the conformer exchange determined from CEST (1.5°C per minute versus an exchange rate of ~ 60 s<sup>-1</sup>),  $T_m$  may represent another bulk measurement with potential to capture aspects of the observed equilibrium.

In line with previous analysis that TVLN Ub in the CR conformation is more flexible than wt Ub in the common conformation, we observe least stability for the phosphorylated



Figure 3.9.: Melting temperature analysis of Ub variants. Baseline and buffer adjusted DSC profiles are shown.  $T_m$  is derived from the position of the peak.  $\Delta H_{cal}$  is derived from the area under each curve, while  $\Delta H_{VH}$  is calculated based on the derived  $T_m$ .  $\Delta H_{cal}$  assumes a two-state transition between the folded and unfolded states. For derived values, see Table 3.3.

TVLN variant (Table 3.3). The stability of L71Y Ub is similar to wt Ub, suggesting that this mutation has little additional effects on the Ub fold other than preventing the Ub-CR conformation upon phosphorylation. F4A exhibits intermediate stability. Interestingly, an increased propensity of the unphosphorylated Ub variant to occupy the CR conformation leads to a smaller relative destabilization of the phosphorylated form (Table 3.3). This might suggest that the destabilization observed upon phosphorylation of wt Ub is indeed due to significant population of the pUb-CR conformer.

| variant |           | $\begin{array}{c} T_{m} \ \pm \ 0.2 \\ (^{\circ}C) \end{array}$ | $\Delta T_m$ (°C) | Ub-CR<br>population (%) | $\begin{array}{c} \Delta H_{cal} \\ (kcal \ mol^{-1}) \end{array}$ | $\frac{\Delta H_{\rm VH}}{\rm (kcal\ mol^{-1})}$ |
|---------|-----------|---|-------------------|-------------------------|--|--|
| L71Y    | Ub<br>pUb | 95.5<br>81.8  | 13.7              | N.D.                    | 89<br>72   | 90<br>71   |
| wt      | Ub<br>pUb | 96.5 $89.4$   | 7.1               | 0.68                    | 94<br>85   | 91<br>75   |
| F4A     | Ub<br>pUb | 88.7<br>82.4  | 6.3               | 4.63                    | 84<br>83   | 77<br>67   |
| TVLN    | Ub<br>pUb | 83.1 $80.9$   | 3.1               | 99.35                   | 77<br>67   | 69<br>68   |

Table 3.3.: Melting temperature analysis of Ub variants.  $\Delta T_m$  shows the destabilization of each variant upon phosphorylation; Ub-CR population shows the percentage Ub-CR population in each unphosphorylated variant at 45°C. The ratio between  $\Delta H_{cal}$  and  $\Delta H_{VH}$  (Van't Hoff) suggest a simple two-state transition for all Ub variants (Johnson, 2005).

### 3.4. Impact of the equilibrium on the ubiquitination cascade

Previously, our group and others have shown that Ub phosphorylation at Ser65 impedes the Ub cascade and when conjugated, resists de-conjugation by DUB enzymes (Wauer et al., 2015b; Huguenin-Dezot et al., 2016). With the availability of the unphosphorylated Ub-CR locking TVLN Ub mutant, we set-out to characterize the extent to which C-terminal retraction, as opposed to phosphorylation in the common conformer impacts the ubiquitination cascade. Ubiquitin harbours two exposed hydrophobic surface patches - the Ile44 and the Ile36 patch. The Ile44 patch is commonly used by a number of Ub-interacting proteins (see Introduction 1.7). The Ile44 patch made up of residues Leu8, Ile44, His68 and Val70 is disrupted in Ub-CR (TVLN Ub) as the 'loop-in' conformation of the Leu8 is assumed and the  $\beta$ 5-strand is shifted. This results in Leu8 contributing to the Ile36 hydrophobic patch on the surface of Ub while His68 and Val70 are moved up by two positions with respect to Ile44. These changes result in disruption of a continuous surface hydrophobic patch (Fig 3.10 A).

Given the Ub Ile44 hydrophobic patch necessary both for Ub adenylation and thioesterification by the E1 enzyme is disrupted in Ub-CR, we first investigated the effect of TVLN Ub on E1 activity. For its two separate activities, the Ub is engaged by two E1 domains sequentially, the adenylation and the Cys domain. Although the E1 enzyme undergoes large-scale rearrangements during its catalytic cycle, structures of the Ub-engaged adenylation domain as well as biochemical studies suggest that the Ile44 patch plays a crucial role in Ub activation (Lee and Schindelin, 2008; Olsen and Lima, 2013; Lv et al., 2017; Misra et al., 2017; Singh et al., 2017), (see Introduction 1.7.1). Surprisingly however, TVLN Ub formed a thioester conjugate with E1 (Fig 3.10 B), with subsequent transthiolation onto all of the studied E2 enzymes (Fig 3.10 B, C).

While the Ile44 patch seems to be important for E1 interaction, and is disrupted in Ub-CR, Gly10 as well as Arg72 have emerged as crucial E1-interacting residues on Ub (Lee and Schindelin, 2008; Olsen and Lima, 2013; Lv et al., 2017; Misra et al., 2017; Singh et al., 2017). Although in Ub-CR the Leu8 loop (containing Gly10) assumes the 'loop-in' conformation while in E1-bound Ub the Leu8 loop exhibits the 'loop-out' (Hospenthal et al., 2013), increased flexibility in this loop presumably allows Gly10 to assume the same position as in the common Ub conformation (See section 3.3.2). Interestingly Arg74 replaces Arg72 in the Ub-CR conformation, maintaining this crucial contact. Although weakened binding of Ub-CR to the E1 Ub adenylation site might be possible, this binding mode places the Ub C-terminus out of reach of the nucleotide binding site. The ability of the E1 to permit Ub-CR activation may either rely on the <0.6% population of the common conformer and weaker affinity for the CR conformer in Ub TVLN, or significant



Figure 3.10.: The effect of TVLN Ub on E1 and E2 enzymes. (A) The Ile44 patch (dark blue surface) in the common, grey and CR, light blue conformers. (B) Charging of either UBE2D3 (left) or UBE2L3 (right) with hUbe1 in the presence of either wt or TVLN Ub. Bandshift suggest conjugate formation, while susceptibility to DTT indicates a thioester linkage. (C) E2-mediated assembly of Ub chains by UBE2R1 (left), UBE2S (middle) or UBE2N/UBE2V1 (right). Conjugates resistant to DTT are isopeptide linked.

flexibility of the E1 enzyme.

Next, we noted that TVLN Ub can be charged onto all of the tested E2 enzymes - UBE2D3, UBE2L3, cdc34/UBE2R1, UBE2S and UBE2N (Fig 3.10 B). Discharge of the E2 ~ Ub thioester onto a substrate primary amine group depends on the ability of the E2 ~ Ub conjugate to adopt the closed conformation which is mediated through the Ile44 patch (Metzger et al., 2014; Stewart et al., 2016). Therefore, chain synthesis



Figure 3.11.: The effect of TVLN Ub on E3 enzymes. (A) The detrimental effect of TVLN Ub on assembly by the GST-RING enzymes cIAP and TRAF6. (B) The detrimental effect of TVLN Ub on assembly by the HECT domain of HUWE1, the RBR domain of HOIP and phosphorylated Parkin.

by E2 enzymes ought to be impaired by Ub-CR. We have examined several linkage specific E2s where the position of the donor Ub depends on the Ile44 patch, although the strategy to position of the acceptor Ub varies according to synthesized chain type (Chong et al., 2014; Wickliffe et al., 2011; Eddins et al., 2006), (see Introduction 1.7.2). In all cases, synthesis of free Ub chains was impaired, while auto-mono-ubiquitination remained possible. This may be as autoubiquitination arises from ubiquitination of the closest available Lys residue without the need to position the acceptor Ub.

In line with the TVLN Ub effect on E2-mediated assembly, inhibition in assembly by the RING ligases cIAP and TRAF6 was observed (Fig 3.11 A). Discharge from the  $E2 \sim Ub$  thioester complex is directed by the RING domains, which promote the closed conformation of the  $E2 \sim Ub$  thioesters and are therefore also dependent on the Ile44 patch (Metzger et al., 2014; Pruneda et al., 2012; Yin et al., 2009).

For HECT and RBR E3 ligases, transthiolation form the E2  $\sim$  Ub thioester onto the active site Cys of the E3 enables subsequent discharge onto substrates from the E3 ligase itself. In contrast to the requirement of the closed conformation for RING-catalysed Ub transfer, transthiolation by both HECT and RBR ligases requires an open conformation of the E2  $\sim$  Ub conjugate (Kamadurai et al., 2009; Wenzel et al., 2011; Lechtenberg et al., 2016; Dove et al., 2016, 2017; Yuan et al., 2017). While this doesn't involve an Ile44 patch interaction with the E2, additional contacts of the Ub to the E3 or substrate may require this versatile hydrophobic patch on the Ub surface. This is consistent with our observation that TVLN Ub blocks assembly by the HECT domain of HUWE1, the RBR domain of HOIP as well as the full-length active, phosphorylated RBR ligase Parkin (Fig 3.11 B).

Taken together, while Ub transfer onto substrate primary amines is impaired by all enzymes tested, it is somewhat surprising that the Ub activation and transthiolation reactions remain unimpaired with TVLN Ub. As lysine ubiquitination is impaired in our *in vitro* set-up, we did not investigate the effect of Ub-CR on deubiquitination or UBDs.

# 3.5. Impact of the equilibrium on the rate of ubiquitin phosphorylation

While Ub-CR seems to have adverse effects on the ubiquitination cascade, we were keen to investigate whether this conformation plays a different constructive physiological role. TVLN Ub is phosphorylated by the PINK1 kinase much faster than wt Ub (Gladkova, 2015). This could either be due to fortuitous manipulation of the phosphorylation sequence and increase of interaction with the PINK1 kinase or due to an increased population of the Ub-CR conformation which exposes the Ser65 residue. Please note the experiment presented in this section is included for clarity and completeness. The NMR phosphorylation rate measurement stems from previously described work (Schubert, 2018; Gladkova, 2015), and was carried out by Dr Alexander F. Schubert.

Analysis of the phosphorylation rate by PINK1 revealed that for the Ub variants, the phosphorylation rate is proportional to the extent of population of Ub-CR as determined by CEST. Namely the order of phosphorylation rate is as follows: TVLN, F4A, wt and L71Y Ub (Fig 3.12). This correlation would suggest that for phosphorylation of Ub, the transition between the Ub-CR and common conformations is rate limiting (conformational selection). A structure of *Ph*PINK1 bound to TVLN Ub solved by Dr Alexander F. Schubert suggests that PINK1 is only able to contact the  $\beta$ 5-strand with its activation loop when the Ub-CR conformation is adopted (Schubert et al., 2017).

While no CEST peaks can be observed for L71Y, full phosphorylation of this variant is achieved. As structural characterisation of the PINK1:(TVLN)Ub complex suggest that the Ub-CR population is necessary, we postulate that either Ub-CR must be accessible to L71Y Ub or another slower mechanism of phosphorylation is employed. An example of such an alternative mechanism with a higher energy barrier could be loosening of the the whole  $\beta$ 5-strand instead of a strand 'slippage'. These cases could be distinguished by screening and design of improved common conformation locking constructs or observation of PINK1 phosphorylation *in situ* by CEST NMR.



Figure 3.12.: The effect of Ub-CR on phosphorylation by PINK1. The rate of phosphorylation of the analysed Ub variants. The conversion is observed by NMR and quantified for several resonances. Adapted from (Gladkova et al., 2017).

# 3.6. Dynamic analysis of the Parkin ubiquitin-like domain

The Parkin Ubl domain was reported as a target for PINK1 (Kazlauskaite et al., 2014b) prior to Ub being identified as a target. Analysis of both domains suggests that the secondary structure is conserved, while sequence variation is observed in the Ser65 loop. Therefore, unusually, phosphorylation in this case seems relatively independent of the local sequence (Fig 3.13 A). The phosphorylation rate of the Parkin Ubl domain was analysed by Dr Alexander F. Schubert alongside analysis of the Ub-variants. Strikingly we observed that the Parkin Ubl domain is phosphorylated at a rate identical to TVLN Ub (Fig 3.12, blue).

Comparison of the Parkin Ubl structure available from crystallographic studies of autoinhibited Parkin with wt Ub suggested that the Ser65 loop adopts the same conformation in Ub and in the Parkin Ubl despite sequence diversity. In both cases, the Ser65 residue is buried and makes hydrogen bonding contacts to the backbone of residue 62 (Fig 3.13 B). Although the CR conformation for the Parkin Ubl domain has not been reported, we postulate that the Parkin Ubl must access a conformation where Ser65 is exposed to enable phosphorylation at this residue. Such a phenomenon would explain near-identical phosphorylation rates between the Parkin Ubl and TVLN Ub, where phosphorylation rate seems to be limited by a common factor in both cases. From measurement of wt Ub and Parkin Ubl phosphorylation rates by TcPINK1 (Rasool et al., 2018) turnover numbers in the orders of magnitude of 10<sup>4</sup> and 10<sup>6</sup> are derived for wt Ub and the Ubl respectively. These suggest that while PINK1-mediated Ubl phosphorylation is faster than average enzyme-mediated catalysis (Bar-Even et al., 2011), the diffusion limit is not reached by two orders of magnitude.

To investigate the possibility that the Parkin Ubl adopts a defined alternative conformation akin to Ub-CR, we carried out <sup>15</sup>N-pseudo-3D CEST experiments at 10°C, 25°C and 37°C, but no peaks for the Ubl were observed. Due to a lower stability of the Parkin Ubl (Aguirre et al., 2017), we were not able to analyse the Ubl sample at concentrations matching our 1.5 mM Ub sample, nor were we able to collect the full <sup>15</sup>N-pseudo-3D CEST at 45°C. However given the phosphorylation rate and the predicted occupancy of the alternative conformation we argue that this would have been visible under the experimental conditions we employed. However it may be that the timescale of the exchange is inaccessible by our CEST experiments, or that the second conformation is not as defined as Ub-CR and the <sup>15</sup>N shift position for the alternative peaks may be averaged, leading to loss of signal.

Next, we wanted to investigate whether contributions from additional conformations to bulk Parkin Ubl dynamics could be observed by CLEANEX measurements, analogously to Ub (Fig 3.13 C). Overall, we observed that rates of exchange were generally higher



Figure 3.13.: Dynamic behaviour of the Parkin Ubl domain. (A) Sequence alignment of the *H. sapiens* Parkin Ubl and Ub. Secondary structure displayed on the top is from an autoinhibited structure of Parkin PDB-ID: 5C1Z. (B) The same view of the aligned (RMSD = 0.998) structures of Ub (PDB-ID: 1UBQ), top and Parkin Ubl (PDB-ID: 5C1Z), bottom. The Ser65 loop assumes an identical conformation with the Ser65 hydrogen bonded to the backbone of residue number 62 in both structures. (C) CLEANEX rates of solvent exchange across the Parkin Ubl sequence. When comparing to Appendix A.4, note the difference in scale. (D) Exchange contribution, the  $A_{ex}$ constant, derived for each residue across the Parkin Ubl sequence derived from T2 relaxation measurements.

than with wt Ub (compare Fig 3.13 C to Appendix A.4), however, the pattern of exchange across the secondary structure elements resembled wt Ub rather than TVLN Ub. Apart from the C-terminus, the most flexible region was the  $\beta$ 2-strand rather than the Ser65 loop, like in TVLN Ub. More residues were seen to exchange (27) compared to wt Ub (16), but exchange rates measured in the  $\beta$ 5-strand were not comparable to TVLN Ub.

As no additional conformation was found using CEST or implicated from CLEANEX bulk measurements, we proceeded with dynamic characterization of the Parkin Ubl using relaxation measurements. To probe a timescale faster than accessible by our CEST experiment, we looked at dynamics by CPMG at 10°C and were unable to observe contributions from an alternative conformation. Further, we analysed  $T_1$  and  $T_2$  relaxation times analogously to (Phan et al., 1996) at both 10°C and 25°C. Relaxation parameters are analysed for each residue at three different spectrometer fields and the exchange contribution, which would arise from intramolecular motion, can be extracted in the form of an exchange parameter,  $A_{ex}$ . More variation of  $A_{ex}$  across the Parkin Ubl residues was observed at 10°C, as at higher temperatures all residues exchanged relatively quickly (Fig 3.13 D).

Largest exchange contribution is observed in the  $\beta 1$ - $\beta 2$  hairpin, the Ser65 phosphorylation loop as well as the  $\beta 5$  strand. This secondary structure pattern of the motion is similar to the pattern seen in chemical shift perturbation due to switching between the common to the CR conformation of Ub (compare with Fig 3.4). While motion in these regions may lead to exposure of Ser65 for phosphorylation analogously to Ub, we are unable to deduce the identity of the potential alternative conformation sampled by the Parkin Ubl from this type of measurement.

Surprisingly, when the order parameter (correlation between  $T_1$  and  $T_2$  relaxation times) was analysed at 25°C for Parkin Ubl and Ub, both were found to be highly ordered (data not shown), suggesting that the relative destabilization of the Ubl fold may arise due to local motion but not inherent instability of the domain. Line shape analysis yielded broader peaks for the Parkin Ubl, suggesting more exchange due to local motion (data not shown). Perhaps introducing mutations to constrain motion of the Parkin Ubl, analogously to TVLN and L71Y mutations in Ub, and analysing their motion and phosphorylation behaviour could shed a light on how Ser65 is exposed in the Parkin Ubl. It may also be the case that, unlike Ub, the Parkin Ubl phosphorylation mechanism utilizes an induced fit

mechanism by PINK1 rather than conformational selection as seems to be the case for Ub.

#### 3.7. Conclusion and discussion

Our past efforts to mimic the Ub-CR conformation of pUb revealed that mutations can induce this conformer in unphosphorylated Ub. Although the mutations that were introduced to this end were non-conservative, their ability to assume the Ub-CR conformation suggested this conformer lies on the energy landscape of Ub. Together with our knowledge of the exchange rate between pUb and pUb-CR, we used this observation as a starting point to look for a slowly exchanging, lowly populated, second Ub-CR conformer in unmodified Ub. CEST experiments were able to reveal the Ub-CR population after temperature increase to 45 °C. At 45 °C Ub-CR is populated to 0.68 % with a rate of exchange  $62.6 \text{ s}^{-1}$ . Although the Ub structure has been very well studied in the past both in solution and by crystallography, Ub-CR was identified for the first time.

In addition to previously characterized Ub variants (TVLN Ub to mimic Ub-CR and L71Y to stabilize the common conformer), we designed and characterized the F4A Ub mutant. Together with wt Ub, this set of Ub variants revealed the contribution of the Ub-CR conformer to bulk measurements, such as CLEANEX or melting temperature analysis. This behaviour enabled validation of the results obtained by CEST and opened avenues for further exploration of the physiological relevance of Ub-CR.

To assess whether the ubiquitination cascade can also utilize the proportion of Ub in the CR conformation, we investigated the impact of TVLN Ub on activation by the E1 enzyme, Ub transfer to the E2 enzyme as well as E3-independent or E3-mediated assembly. We found perhaps surprisingly that Ub activation and transfer onto the E2 catalytic Cys remains robust for our Ub-CR mimicking TVLN variant. Discharge onto substrate primary amine groups was either impaired or redirected towards auto-ubiquitination.

Our well-characterized collection of Ub variants was able to reveal that the extent of population of Ub-CR is rate limiting for Ub phosphorylation by PINK1, suggesting a conformational selection mechanism. This could explain why the reported insect PINK1  $K_m$  for wt Ub phosphorylation is ~ 300 µM although the  $K_D$  could not be measured by

ITC. For simple cases of kinase-mediated phosphorylation these values match, suggesting a more complex reaction mechanism is required to phosphorylate Ub (see Introduction 1.11.4).

It might be interesting to systematically analyse the structural context of phosphorylation sites identified from large-scale proteomic studies, which seem inaccessible or occluded. A similar mode of strand slippage or conformational selection may be employed to phosphorylate these sites across the proteome. This strategy may allow modification of structurally important residues, which in turn may result in greater consequences for the behaviour of the phosphorylated protein.

Additional investigation of the Parkin Ubl phosphorylation rate suggested an overall more dynamic structure for the Ubl, a second validated physiological PINK1 substrate. We found that the Ubl is indeed more dynamic in the Ser65 loop, the  $\beta$ 5- and  $\beta$ 1-strands, but no Parkin Ubl-CR conformation could be detected. As Ser65 remains buried in the structure of the Parkin Ubl domain, we postulate that a conformational rearrangement remains necessary for phosphorylation of this residue. However this may not be motion between two defined states, as we observed for Ub and may occur on timescales inaccessible by our NMR experiments. Further investigation is required to determine whether an analogous conformation which exposes Ser65 of the Parkin Ubl domain might be populated. Suggestive evidence might be obtained by investigation of a common conformer lock akin to the L71Y Ub variant by mutating the Gln71 residue of the Ubl to a Tyr to Trp.

Optimization of the CEST method was crucial for our ability to investigate the conformational equilibrium in Ub. Unlike other dynamic NMR methods such as CPMG, CEST yields information on the <sup>15</sup>N chemical shift of alternative conformer residues. Information about the approximate expected coordinates of the Ub-CR peaks was available to us from our previous characterization of cases, where the equilibrium has been perturbed either by phosphorylation or introduced mutations. It was our previous characterization of these reference states that allowed us to identify the alternative conformation observed by CEST in wt Ub as Ub-CR. An approach where perturbations to the structure can be used to identify reference states, which can be used to explain conformations sampled by the native fold may be extended to CEST studies of other protein folds, such as SOD1 (Culik et al., 2018) or indeed, other members of the Ub-like protein family. Our characterization of the equilibrium together with structural characterization of the two endpoints of the motion lends itself well to *in silico* analysis of this behaviour. At the time of writing, a study was published using force field calculations to generate an energy landscape with the solved crystal structures as starting points (Röder and Wales, 2018). For wt Ub, it is predicted that above 36 °C a new structural ensemble encompassing the crystallized, canonical Ub-CR conformation becomes accessible, and therefore significantly lowering the energy of the Ub-CR conformation. This is consistent with our observation of Ub-CR only at higher temperatures. The computational method utilized in this study is able to derive parameters which match our experimentally determined exchange rates and derived free energy barrier (19.32 kcal/mol at 45°C). Notably, the mechanism of strand slippage is predicted to be concerted *in silico*, and the exchange rates observed for wt Ub, explaining why the observed phosphorylation rate was much lower for this mutant. Such a slow exchange rate would be inaccessible by the dynamic NMR methods employed in this work but may enable PINK1-mediated phosphorylation.

The most striking aspect of the discovery of Ub-CR in wt Ub is that despite a number of NMR studies at various conditions were not able to reveal this motion in the protein, e.g. (Lange et al., 2008). Interestingly, the destabilization and NMR spectra of the *S. cerevisiae* Ub variants L67S and L67N respectively have been reported previously (Loladze et al., 2001, 2002), but a lack of secondary structure assignment prevented identification of the Ub-CR conformation. Recently, a method has been developed to study Ub folding following a sudden change of pressure in a native, aqueous buffer using NMR spectroscopy. A folding intermediate has been found, with largest chemical shift deviations from the native state in the  $\beta$ 5-strand (Charlier et al., 2018). This intermediate also possessed an ordered Arg74 and therefore likely corresponds to Ub-CR.

The Ub-CR conformer is sparsely populated in near-physiological solution conditions in the NMR measurements at 45 °C. It might be interesting to measure in-cell proportion of Ub-CR as this may depend on a more complex environment (Smith et al., 2015), as for instance dependence on pH has already been observed for the wt pUb equilibrium (Dong et al., 2017; Kazansky et al., 2018). The proportion of Ub assuming the CR conformer may also change with conjugation to a substrate or variation of other mechanical parameters in a native environment. To date, only the behaviour of free phospho-Ub chains was investigated (Wauer et al., 2015b; Kazansky et al., 2018).

Although a function of the Ub-CR conformer has already been found in the catalytic Ser65 phosphorylation by PINK1, other, non-catalytic functions may exist. Namely since the canonical hydrophobic interaction surface is disrupted, either Ub-CR or pUb-CR could be specifically recognized in the cell. Such a scenario may be more likely in the case for pUb-CR, given its prevalence. To-date however, no specific pUb receptor aside from Parkin, which recognized the common pUb conformer, has been identified.

### Chapter 4.

### Parkin activity in vitro

#### Key findings:

- Two conserved elements were identified within the previously unstudied  $\sim 70$  AA Ubl-UPD Parkin linker.
- The activating element (ACT) is required for Parkin activation by phosphorylation.
- Parkin shows low processivity and ubiquitination site-specificity in vitro.

Because the autoinhibited and pUb-bound states of Parkin have been characterized at atomic resolution (Trempe et al., 2013; Wauer and Komander, 2013; Riley et al., 2013; Kumar et al., 2015; Sauvé et al., 2015; Wauer et al., 2015a; Kumar et al., 2017a), I focussed on resolving the missing active state of Parkin phosphorylated by PINK1 in the context of PINK1/Parkin-mediated mitophagy. In parallel to dynamic and structural approaches to capture the active form of Parkin (Chapters 5 and 6), I investigated the Parkin activation sequence biochemically. This approach helped define elements necessary for Parkin activity as well as characterize which Ub conjugates are assembled by Parkin. An insight into processivity and site specificity can be used to infer the mechanism of Parkin activity.

#### 4.1. Reconstituting the Parkin activation sequence

Building on previously published work from our laboratory and others, I was able to recapitulate the Parkin activation cascade using several biochemical assays. An endpoint of a multiple turnover Parkin ubiquitination assembly assay shows slight activation upon pUb addition and a much greater activation by phosphorylation (Fig 4.1 A). The

assembly assay measures the ability of Parkin to bind the incoming  $E2 \sim Ub$  conjugate, discharge the conjugate onto its catalytic Cys residue (in a transthiolation step), and finally mediate transfer of the activated Ub onto substrates. Although a method to reconstitute substrate ubiquitination has been reported (Kazlauskaite et al., 2014a), other biochemical assays are required to tease apart the intermediate steps necessary for Parkin activity.

The use of Parkin active site Cys-mediated reactivity using a Ub-VS Activity-Based Probe (ABP) as a surrogate for Parkin activity readout was developed in our lab (Wauer and Komander, 2013). ABPs originally developed to capture Cys-based DUBs were extended for use with Parkin, whose catalytic cycle is based on identical Cys chemistry. The extent of Parkin-Ub conjugate formation reports on the ability of Parkin to attack the C-terminal electrophilic group of the activated Ub by productively positioning the



Figure 4.1.: Full Parkin activation upon phosphorylation. (A) An assembly assay with Parkin or phospho-Parkin with or without pUb. The assembly efficiency of phospho-Parkin is greatly enhanced compared to unphosphorylated Parkin. The effect of pUb activation is relatively small. No difference between the two phospho-Parkin lanes likely stems from pUb generated by a PINK1 contamination in the phospho-Parkin preparation even when pUb is not supplemented (see Methods 2.5.3). (B) A Ub-VS probe-reactivity assay comparing Parkin activated with pUb and phospho-Parkin. Phosphorylation enables Ub-VS reactivity. (C) An UBE2D3 ~ Ub discharge assay comparing Parkin activated with pUb and phospho-Parkin is able to efficiently discharge the UBE2D3 ~ Ub conjugate. Band marked with (\*) corresponds to the time-dependant formation of ubiquitination product, such as a Lys-linked UBE2D3-Ub conjugate of diUb.

nucleophilic catalytic residue in proximity of the Ub C-terminus (see Introduction 1.7.6). In short, Ub-VS reactivity reports on accessibility of the RING2 catalytic residue and donor Ub binding. As previously observed (Ordureau et al., 2014), only phospho-Parkin and not Parkin activated with pUb is able to react with the Ub-VS and therefore possess an exposed catalytic Cys (Fig 4.1 B).

Lastly, I optimized an E2 ~ Ub discharge assay to measure Parkin transthiolation activity. In this single turnover assay, discharge of a pre-assembled E2 ~ Ub thioester conjugate upon addition of Parkin is measured by disappearance of the E2 ~ Ub species. While Parkin activated by pUb is not able to discharge the DTT-labile E2 ~ Ub conjugate, Ub transfer is observed for phospho-Parkin (Fig 4.1 C). As Ub is both received and discharged onto the available phospho-Parkin or E2 substrates within the short time-course, the lifetime of the Parkin ~ Ub intermediate seems to be very short. A similar phenomenon has been observed for HOIP, suggesting that a highly reactive E3 ~ Ub conjugate might be a common feature of the RBR ligase family (Lechtenberg et al., 2016).

In all of the assays, the increase in activity upon Parkin phosphorylation is markedly more pronounced than activation by pUb addition. This striking activation is the subject of further biochemical, dynamic and structural characterization in this work.

#### 4.2. New conserved sequence elements in Parkin

Together with sequence analysis, the established *in vitro* activity assays were used to interrogate the function of a previously unstudied linker region in Parkin activation by phosphorylation.

Upon identification of the disease-linked Parkin gene, the N-terminal Ubl domain was identified (Kitada et al., 1998), while later work described the C-terminal RBR Parkin module and identified its activity as an E3 Ub ligase (Shimura et al., 2000). Only subsequent proteolytic approach described the Zn-binding UPD (also known as the RING0 domain), preceding the RBR module (Hristova et al., 2009). This discovery paved the way to obtaining high-resolution crystal structures describing the C-terminal UPD-RBR portion of



Figure 4.2.: Parkin conservation in vertebrates. Sequence alignment of human (H. sapiens), pig (S. scrofa), mouse (M. musculus), chicken (G. gallus), snake (T. sirtalis) and zebrafish (D. rerio) Parkin. Secondary structure displayed on the top is from a structure of Parkin bound to pUb, PDB-ID: 5N2W. Domains are coloured as follows: Ubl - green, UPD - dark blue, RING1 - blue, IBR - sky blue, REP - red, RING2 domain - cyan. Highlighted are the phosphorylation site, phosphate-binding pocket residues and the active site residue.

autoinhibited Parkin (Wauer and Komander, 2013; Trempe et al., 2013; Riley et al., 2013).

Obtaining high resolution structures which also contained the N-terminal Ubl domain required replacement of the native region linking the Ubl domain to the UPD (68 residues) with a truncated 8 residue version of this linker (Kumar et al., 2015; Sauvé et al., 2015). Because this linker had previously been reported as unstructured and susceptible to proteolysis (Hristova et al., 2009), its function was not subsequently systematically investigated. Based on sequence alignments of Parkin across both vertebrate and invertebrate species, this linker region was classified as unconserved (Trempe et al., 2013; Kumar et al., 2015; Wauer et al., 2015a), (Appendix Fig B.1).

As a starting point to investigate the potential function of the linker region connecting the Ubl to the UPD-RBR module of Parkin, I performed a sequence-based Parkin alignment of example vertebrate organisms (Fig 4.2). In addition to the known Ubl, UPD and RBR domains, two short elements conserved across vertebrates were identified in the linker region following the Ubl. The first conserved region spans residues 101-110 (light green, Fig 4.2) and the second spans residues 116-123 (purple, Fig 4.2). While the first element is amphiphatic and consists of alternating polar and non-polar residues, the second stretch is solely hydrophobic.

Notably, a point mutation, R104W, in the first conserved region has been found in two PD sufferers from independent backgrounds (Chaudhary et al., 2006; Varrone et al., 2004). While the incidence of this point mutation is not sufficiently high to confirm clinical pathogenicity (Yi et al., 2018), its link to early-onset PD suggests this element might be important for the cellular function of Parkin. No disease-linked mutations are observed in the second conserved region.

## 4.3. The ACT element is required for proper Parkin activity

Initially, the deletion of both regions was assessed using a Ub-VS probe reactivity assay. While deletion of the first element blocked Cys-based probe reactivity, no effect of removing the second element was observed using this minimalistic assay (Fig 4.3 A). In a



Figure 4.3.: The ACT element is required for Parkin activity. (A) A Ub-VS probe-reactivity assay comparing phospho-Parkin lacking either the ACT (residues 101-109) or GLAVIL (residues 116-123). Removing the ACT prevents reactivity with Ub-VS. (B) An assembly assay comparing the same variants as in A. Note that the used wt phospho-Parkin was purified following the mutants and its basal level of activity is relatively higher. While  $\Delta 101-109$  (ACT) does not assemble any conjugates,  $\Delta$ 116-123 shows comparable activity to wt phospho-Parkin. Parkin was used at a 5  $\mu$ M concentration in this assay. (C) Thermal denaturation experiments reveal only minor destabilization of phosphorylated or unphosphorylated Parkin R104A compared to wild type variants. (D) An assembly assay with phospho-Parkin variants, wt or R104A. The assembly efficiency of phospho-Parkin R104A is greatly reduced compared to wt phospho-Parkin. Parkin was used at a 5  $\mu$ M concentration in this assay. (E) phospho-Parkin R104A exhibits decreased reactivity towards Ub-VS. (F) An UBE2D3  $\sim$  Ub discharge assay for wt or R104A phospho-Parkin shows a delayed Ub handover in phospho-Parkin R104A. (G) Quantification of two replicates of the UBE2D3  $\sim$  Ub discharge assay as in E.

more complex assembly assay using the phosphorylated linker deletion variants, very few conjugates are assembled when the first region is removed, while removing the second region permits Ub assembly by the phosphorylated Parkin variant (Fig 4.3 B; note that the observed variation is consistent with differences in Parkin activity across different independent preparations). Since the first region was necessary for phospho-Parkin activity, this was termed the activating element (ACT), as the second region has no effect in the Ub-VS reactivity or assembly assay it was named according to its residue composition, the GLAVIL element.

To explore whether the disease-linked R104W patient mutation disrupts the activating function of the ACT element, further biochemical analysis was necessary. Unfortunately the R104W Parkin variant was not soluble in *E. coli* and instead the R104A mutant was analysed. No difficulties were encountered when producing the R104A variant, consistent with an identical melting temperature for the R104A variant compared to wt Parkin (Fig 4.3 C). The behaviour of the R104A Parkin variant suggests that introducing the large hydrophobic Trp residue in the linker compromised solubility of the R104W variant, rather than folding defects induced due to ACT disruption.

Similarly to  $\Delta$ ACT, the phosphorylated R104A variant was impaired in both Ub assembly (Fig 4.3 D), as well as Ub-VS reactivity (Fig 4.3 E). Although these activity defects were smaller for R104A than for  $\Delta$ ACT, they could be the basis for the disease-contribution of the R104W variant. Additionally, the effect on E2 ~ Ub discharge was measured (Fig 4.3 F, G) and consistently, the phosphorylated R104A variant discharges the E2 ~ Ub less efficiently than wt phospho-Parkin. Together, defects in Ub-VS reactivity and E2 ~ Ub discharge suggest that the ACT element either contributes to binding the incoming donor Ub, or exposure of the catalytic Cys residue in activated Parkin. These two effects might be hard to disentangle biochemically as recent reports suggest the catalytic RING2 forms part of the donor Ub binding interface on RBR enzymes (Dove et al., 2016). While exposure of the catalytic Cys residue could be measured by oxidation assays, akin to Kulathu et al. (2013), any Ub-related readout will convolute the two possible ACT functions.

At the time of writing, the pathogenicity of reported disease-linked Parkin mutations was assessed based on clinical data and functional analysis *in cellulo* (Yi et al., 2018). The expression of Parkin variants relative to wt was measured to assess whether mutations affect stability of Parkin in a cellular environment, while the extent of mitochondrial clearance in response to depolarization reported on whether mutations affect Parkin mitophagy function. The R104W mutant was included in the analysis and while its expression levels matched that of wt Parkin, mitochondrial clearance was significantly reduced (50%). In a separate study, no effect on substrate ubiquitination by an overex-pressed R104W variant was observed (Bernardini et al., 2019). This is likely due to the high cellular levels of the protein which retains some residual activity.



Figure 4.4.: The Parkin chain assembly profile. (A) A method developed in our laboratory was used to analyse the composition of Parkin *in vitro* assembly. Asymmetric cleavage of conjugated Ub can either provide information on assembled Ub chain length or the number of substrate ubiquitination sites. (B) Analysis of assembled Ub reveals that Parkin preferentially mono-ubiquitinated its substrates or assembled short chains. Little difference in architecture is observed upon pUb addition. Note that the small amount of pUb in the (-pUb) condition is indicative of a PINK1 contamination of the phospho-Parkin preparation. (C) Analysis of UBE2L3, a mock substrate. Up to four ubiquitination sites can be observed. Only the most abundant charge state, z = +21 is shown.

#### 4.4. The Parkin chain assembly profile

In vitro reconstituted Parkin assemblies were used to characterize the Ub conjugates assembled by Parkin in collaboration with Dr Kirby N. Swatek and Joanne Usher, using novel MS approach developed in the laboratory by Dr Swatek.

While Absolute Quantification proteomics (AQUA) has revealed Parkin's ability to assemble both canonical (Lys48- and Lys63-) and non-canonical (Lys6- and Lys11-) linked Ub conjugates (Ordureau et al., 2014), the architecture of the assembled Ub chains could not be determined. The new approach pioneered in the laboratory to address this problem relies on isolating the assembled conjugates from unreacted Ub and subsequent asymmetric cleavage of the Ub molecules (Swatek et al., 2018). Ub is cleaved such that a GlyGly remnant is retained on the ubiquitination substrate, similarly to tryptic peptide digest. The proportion of monoubiquitination relative to assembled Ub chains can be inferred by observing the distribution of GlyGly-modified species using intact MS (Fig 4.4 A). Utilizing this method, optimized assemblies were analysed for Ub chain architecture using MS by Dr Kirby N. Swatek, and mock-substrate (UBE2L3) ubiquitination by Joanne Usher.

Analysis of assembled Ub by phospho-Parkin revealed a 1:2 ratio of GlyGly-modified Ub to unmodified Ub (Fig 4.4 B). Little difference in the architecture of conjugated Ub is observed upon addition of pUb to active phospho-Parkin. The most likely distribution of Ub species corresponding to this ratio consists of mono- and di- ubiquitination, suggesting that Parkin does not assemble long Ub chains, unlike the processive RBR HOIP (Stieglitz et al., 2013). Together with its ability to conjugate multiple chain types this might suggest the absence, or presence of a weak binding site for the acceptor Ub by Parkin.

Analysis of ubiquitination sites on a mock substrate within the assembly, the E2 enzyme UBE2L3, is consistent with low Parkin preference for ubiquitination sites and short chain synthesis (Fig 4.4 C). Up to four GlyGly remnants are observed on a single UBE2L3 molecule, consistent with Parkin-mediated multi-monoubiquitination and the distribution of ubiquitinated UBE2L3 species within the assembly (Fig 4.1 A).

#### 4.5. USP30 as a Parkin substrate

As a deubiquitinase anchored to the outer mitochondrial membrane, USP30 might be a particularly interesting Parkin substrate. USP30 preferentially cleaves Lys6-linked chains on the mitochondrial surface (Bingol et al., 2014; Cunningham et al., 2015; Gersch et al., 2017; Sato et al., 2017), a non-canonical linkage also assembled by Parkin *in vitro*. As such, USP30 can oppose Parkin activity on the mitochondrial surface and restrict PINK1/Parkin-mediated mitophagy (Cunningham et al., 2015; Gersch et al., 2017). Although USP30 has not been identified in proteomic screens searching for ubiquitination sites altered in a Parkin dependent manner upon mitochondrial depolarization (Sarraf et al., 2013), approaches utilizing immunoprecipitation of USP30 from depolarized Parkin overexpressing cells yield USP30 ubiquitination sites upon depolarization and Parkin overexpression (Bingol et al., 2014).

In collaboration with Dr Malte Gersch, *in vitro* Parkin-mediated ubiquitination of USP30 was recapitulated and the interplay between the assembly and disassembly activity of Parkin and USP30 explored.

Firstly, ubiquitination of USP30 was reconstituted in an *in vitro* Parkin assembly assay. While on the level of Ub, an equimolar amount of active USP30 is able to efficiently counteract Parkin activity, some ubiquitination of the active USP30 species is retained (Fig 4.5 A). When subjected to a tryptic digest and subsequent MS, three USP30 ubiquitination sites previously found upon Parkin overexpression and mitochondrial depolarization in cells - Lys235, Lys289, Lys310 - were recapitulated (Bingol et al., 2014).

With three modification sites observed and up to three ubiquitinated species resolved in the assembly (Fig 4.5 A), USP30 multi-monoubiquitination likely occurs, similarly to the UBE2L3 mock substrate (Fig 4.4 C). Although not site selective, monoubiquitination at any of the identified sites would likely disrupt USP30 deubiquitinase activity, as the distal Ub binding site would be disrupted (Gersch et al., 2017), (Fig 4.5 B).

Restricting USP30 activity by ubiquitination could promote accumulation of Ub conjugates on damaged mitochondria to trigger mitophagy, conferring functional importance of these ubiquitination events. We therefore set out to investigate whether non-residue specific modification of USP30 might result in a specific outcome - diminished deubiqui-


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Figure 4.5.: Parkin-mediated ubiquitination of USP30. (A) An assembly assay with phospho-Parkin with wt (active) or C77A (inactive) USP30 included as a substrate. Immunoblotted for USP30 (left) and Ub (right). Active USP30 is able to oppose Parkin-mediated assembly. \* marks a co-purifying USP30 cleavage product. (B) The binding interface between USP30 and the distal Ub of a Lys6-linked diUb, PDB ID: 50HK. Mutations chosen to disrupt this interface are highlighted. (C) Assembly assays with phospho-Parkin and active USP30 using indicated Ub variants. Assembled F4A, F4R and T12E Ub resist USP30 cleavage and can be used to generate active ubiquitinated USP30 species. (D) Anion-exchange based purification of an assembly reaction as in C with F4A Ub (left). Cleavage assay of a Ub-based substrate shows no effect of Parkin-mediated USP30 ubiquitination on activity (right, performed by Dr Gersch).

tinase activity. Akin to an approach where substrates are enzymatically modified with a L73P Ub variant resistant to deubiquitinase activity (Békés et al., 2013), we designed several Ub variants which might be resistant to USP30 cleavage based on a structure of a trapped USP30-Ub catalytic intermediate (Gersch et al., 2017). While the conserved hydrophobic Ile44 patch is used by USP30 to contact Ub, this would likely also be utilized by Parkin to interact with the donor Ub (Dove et al., 2016). We therefore focussed on disrupting the interaction of USP30 with the Ub C-terminus (Leu71, Arg72) as well as its contacts with residues of the Phe4 patch, Phe4 and Thr12 (Fig 4.5 B), (see Introduction 1.3).

While modifications of the C-terminus were not compatible with Parkin activity, F4A, F4R and T12E Ub variants enabled cleavage-resistant modification of USP30 (Fig 4.5 C). USP30 and USP30-Ub(F4R) were isolated from a preparative assembly and their relative cleavage activities were tested on a mock Ub substrate. No difference was observed in Ub substrate cleavage rates (Fig 4.5 D). Please note that the cleavage experiment presented in the right panel was performed by Dr Gersh and is included for completeness. Monoubiquitination of USP30 is unlikely to result in proteasomal degradation as longer Ub chains are required for protein degradation (Swatek and Komander, 2016). Consistently, monoubiquitinated forms of USP30 were relatively stable in cells (Liang et al., 2015).

Therefore, the role of Parkin USP30 ubiquitination, besides amplifying the mitophagy signal, remains unclear. This data however contributes to the notion that Parkin builds monoUb conjugates or short chains on its substrates and it is not site or substrate specific, supported also by extensive analysis of Parkin substrates on the mitochondrial surface (Ordureau et al., 2018).

## 4.6. Conclusion and discussion

In summary, several biochemical approaches were used to dissect Parkin activity *in vitro*. The Parkin activation cascade could be recapitulated biochemically on the level of the transthiolation reaction by the optimized  $E2 \sim Ub$  discharge assay.

Most strikingly, sequence conservation analysis focussing on vertebrate species uncovered two new conserved elements of Parkin - the ACT and the GLAVIL elements. Conservation analysis across both vertebrates and insects suggests a conserved N-terminal extension of the insect sequence, and weaker conservation in the Ubl-UPD linker (see Appendix Fig B.1). The hydrophobic nature of the N-terminal extension could account for both ACT and GLAVIL functions in *Hs*Parkin, although no exactly matching sequence is present.

The ACT element was shown to be necessary for full Parkin activation by phosphorylation. A PD-linked substitution of Arg104 in the ACT element is able to disrupt its activating function and diminishes Parkin activity by either disrupting donor Ub binding or preventing exposure of the catalytic RING2 Cys residue. Further analysis of the role of the ACT element is required to determine the mechanism of its activating function. Identification of this element is crucial to inform constructs used to capture the active state of Parkin crystallographically - truncation of the conserved regions of the linker may hinder this effort.

No patients harbouring a mutation in the GLAVIL motif were reported thus far. Databases such as ExAC and dbSNP report minor frequency alleles with mutations in the GLAVIL motif (G118A, L119M, I123N), no homozygous alleles were reported (Sherry et al., 2001; Lek et al., 2016). The function of the GLAVIL element remains unclear from the biochemical analysis presented here. No effect on activity of phosphorylated Parkin has been observed. Since the resolution of the only structure containing the full-length linker region is of a very low resolution (6.5 Å), the GLAVIL element may contribute to Parkin autoinhibition. This could be resolved by performing further biochemical assays using an unphosphorylated  $\Delta$ GLAVIL Parkin variant.

Analysis of Parkin chain assembly on two mock substrates and the Ub chain architecture produced by Parkin *in vitro* suggests low Parkin selectivity with regards to a ubiquitination site and predominant monoubiquitination or short chain synthesis. Synthesis of short chains only suggests that Parkin alone *in vitro* is not processive, supporting a sequential ubiquitination model (see Introduction 1.7.3). Other factors or constraining Parkin ligation activity to the two-dimensional mitochondrial surface may play a role in the physiological function of Parkin. It is therefore paramount that these observations are confirmed, ideally in a cellular setting.

# Chapter 5.

# **Dynamics of Parkin in solution**

#### Key findings:

- HDX MS is an optimal method to study Parkin dynamics in solution and recapitulates the known structural consequences of pUb binding.
- Parkin phosphorylation sets up a conformational equilibrium by favouring a new, rearranged active state.
- The activating interface formed between the phospho-Ubl and UPD domains releases the catalytic RING2 domain necessary for ubiquitination activity.
- Trapped catalytic Parkin intermediates shift the Parkin equilibrium towards the active conformer.
- The ACT element is rigidified in the Parkin catalytic intermediates which favour the active state.

Two states of the Parkin activation sequence have been characterized crystallographically thus far: The autoinhibited cytosolic state (Fig 5.1 A), (Trempe et al., 2013; Wauer and Komander, 2013; Riley et al., 2013; Sauvé et al., 2015; Kumar et al., 2015) and the mitochondrially localized pUb-bound state of Parkin, in which autoinhibitory elements persist (Fig 5.1 B), (Wauer et al., 2015a; Kumar et al., 2017a).

As understanding of the active state of Parkin is missing despite significant efforts (Fig 5.1 C), I set out to investigate this last step of the Parkin activation sequence. In the first instance, I wanted to characterize the changes to the Parkin structure induced by Ubl phosphorylation and elucidate the function of the ACT element by studying the dynamics of the whole activation sequence in solution. Due to its size, as well as the reported and



Figure 5.1.: The Parkin activation sequence. (A) Schematic representing the domain arrangement in autoinhibited Parkin, as described previously (Wauer and Komander, 2013; Riley et al., 2013; Trempe et al., 2013; Kumar et al., 2015; Sauvé et al., 2015). (B) Schematic representing the domain arrangement in pUb-bound Parkin, as described previously (Wauer et al., 2015a; Kumar et al., 2017a) (C) Open Parkin structure predicted for active Parkin (Harper et al., 2018). Autoinhibitory features are highlighted by red ovals.

predicted Parkin domain rearrangements (see Introduction 1.12), I chose to make use of Hydrogen-Deuterium Exchange Mass Spectrometry (HDX MS) in collaboration with Sarah L. Maslen and J. Mark Skehel at the LMB MS facility.

# 5.1. Hydrogen-Deuterium Exchange Mass Spectrometry (HDX MS) theory

HDX MS is a structural technique which allows dynamic investigation of proteins that may not be suitable for analysis by NMR due to their large size, crystallography or Cryo-Electron Microscopy (Cryo-EM) due to protein production hurdles, intrinsic protein dynamics or size. Readout by electrospray MS only requires small amounts of sample, and in an industrial setting HDX MS is routinely used for batch quality control in addition to more established biophysical methods (Houde et al., 2011). This technique also serves as a good complimentary approach to structural characterization by any other method, or may serve as the basis of construct design for further study (Marcsisin and Engen, 2010; Iacob and Engen, 2012).



Figure 5.2.: Hydrogen-deuterium exchange mass spectrometry (HDX MS) theory. (A) Schematic representation of the in-solution isotope exchange. Reaction is quenched at each indicated time point. (B) Schematic representation of the types of hydrogens present in proteins and their exchange behaviour. (C) A schematic representing how peptides are generated from the deuteriated sample under quench conditions. (D) Schematic representation of an example comparative HDX MS experiment investigating the effect of pUb binding on Parkin. Deuterium-exchanged amide hydrogens are indicated with black dots. A subset of peptides resulting from analysis of each condition differ in the amount of exchanged deuterium. (E) Left: Schematic representation of the comparative analysis of an individual peptide, as shown in D. The centroid (weighted average) of the isotopic distribution observed for each peptide is used as a measure of deuterium uptake. Right: Graphical representation used to plot peptides obtained across the whole AA sequence.

In a typical HDX experiment, a continuous labelling method is used. Proteins are preequilibrated on ice (to allow non-covalent complex formation), and labelling is initiated by dilution of the complexes in D<sub>2</sub>O-containing buffer, such that the final concentration of D<sub>2</sub>O exceeds 95 % v/v (Fig 5.2 A). During this step, both the backbone amide hydrogens, as well as side chain functional group hydrogens exchange with the deuterium from the solvent (Fig 5.2 B). Once samples are removed at indicated incubation times, exchange is quenched by the addition of formic acid (final pH=2.5) and 2M Guanidinium Chloride (GdmCl) before flash freezing.

As the side chain protons exchange rapidly, they are fully deuteriated at all time points. In contrast, the rate of exchange of backbone amide hydrogen protons is determined by their context within the protein structure assumed in solution. Solvent accessibility is the main determinant of the rate of exchange, i.e. the hydrophobic core amides are unlikely to exchange unless an unfolding event takes place. The rate of exchange is also adversely affected when amide hydrogens are involved in hydrogen bonding/secondary structure. Overall therefore, flexible loops on the surface of the protein will exhibit the highest degree of exchange  $(10 - 1000 \text{ s}^{-1})$ , whereas buried secondary structure elements will exhibit the least exchange  $( < 0.02 \text{ s}^{-1})$ , (Marcsisin and Engen, 2010). The time scale of the experiment is set within this dynamic range to distinguish between fast and slow exchanging backbone amide hydrogens. While all side chain groups are assumed to fully back-exchange in the subsequent peptide separation and analysis steps (Fig 5.2 C), no correction is made for the rate of backbone amide hydrogen back-exchange (exchange is therefore reported as 'relative D uptake').

During analysis, samples are kept at 'quench conditions', e.g.: low pH and 0°C. As low pH is required to minimize back-exchange during the cleavage step, pepsin protease is used and its cleavage aided by denaturation with 2M GdmCl. The peptic peptides are then desalted and separated at 0°C. Peptides are analysed by electrospray MS and annotated according to chromatographic retention times defined by peptide fragmentation of the non-deuteriated sample (Fig 5.2 C). Downstream processing and data analysis are tailored to the desired application of the method (Morgan and Engen, 2009; Houde et al., 2011).

Comparative HDX MS is most useful for analysis of the changes in protein dynamics upon given stimuli, such as Parkin binding to pUb or Parkin phosphorylation. Two samples are analysed in parallel and the difference in deuterium uptake between the two is analysed. Care must be taken that the correct reference state is isolated and used for comparison, otherwise result interpretation may be ambiguous. Solvent accessibility, and therefore deuterium uptake, can be prevented directly through complex formation or indirectly through induced conformational changes (Fig 5.2 D).

When performing comparative HDX MS analysis, only peptides which are observed in both conditions across all time points are considered. This leads to the loss of peptides where the pepsin cleavage pattern has changed or peptides contain different PTM sites (e.g. due to Parkin phosphorylation on Ser65). The isotopic distribution for each peptide is considered as an envelope curve whose centroid (weighted average) is determined. Increase in the centroid mass across time points may therefore not be an integer number of Da. Unbiased representation of the large dataset which includes many overlapping peptides is challenging (Fig 5.2 E):

A difference plot shows a point for each peptide (ordered by the starting AA position) and the difference (in Da) compared to peptides of the reference state. The x-axis in this case represents a pseudo-sequence and may be heavily biased towards regions covered by more peptides. Further bias is introduced as points for non-covered regions will be absent from the representation. Alternatively, a heatmap can be generated from a fractional uptake value assigned to each AA. This value is generated by taking the relative uptake value of the shortest peptide covering a given AA. Increased deuterium uptake when compared to the reference state is defined as a positive difference value (represented in red), while a decrease in deuterium uptake is defined as a negative difference value (represented in blue). A heatmap representation, although unbiased in sequence, can be falsely perceived to increase the resolution of the experiment and may be misleading. This phenomenon arises when neighbouring AAs are covered by different short overlapping peptides (Fig 5.2 E), (Houde et al., 2011). The fractional uptake values assigned to each AA can be plotted onto known protein structures to aid data interpretation.

## 5.2. Phospho-ubiquitin binding

To assess whether Parkin conformational changes in response to the activation sequence can be captured by HDX MS measurements, we initially focussed on the effect of pUb binding to Parkin, as this step has been characterized crystallographically and to some extent by NMR (Wauer et al., 2015a; Sauvé et al., 2015; Kumar et al., 2015, 2017a). Protection of the pUb binding site in the RING1 and IBR is expected from the known pUb binding interface together with exposure of the Ubl domain due to its release from the RING1 autoinhibitory binding site upon pUb binding.

A heatmap plot showing the difference in deuterium uptake in the Parkin:pUb and Parkin samples (Fig 5.3 A), shows exposure of both sides of the Ubl-RING1 autoinhibitory interaction - the Ubl domain (region (1) in Fig 5.3) and a portion of the RING1 domain (region (1r) in Fig 5.3). In addition, protection is observed in the UPD, RING1 and IBR domains (region (3) in Fig 5.3). Using a model based on the previously determined structure of the human Parkin-pUb complex (Kumar et al., 2017a; Sauvé et al., 2015), we observe that all protected regions correspond to the known pUb-binding site (Fig 5.3 B). A difference plot representation is able to reveal the same features together with small exposure of the REP and RING2 (Fig C.2; Appendix C.1). This small exposure accounts for the low level of Parkin activation which can be achieved by pUb binding (see Section 4.1), (Kazlauskaite et al., 2014b; Wauer et al., 2015a; Sauvé et al., 2015; Kumar et al., 2015).

Closer inspection of the peptide plots corresponding to regions of interest (Fig 5.3 D) reveals that with the exception of region (1r), the peptide corresponding to the Ubl binding site on the RING1 domain (resi 267-276), all analysed parts of the sequence are dynamic in the autoinhibited state (positive slope on deuterium uptake over time), (Morgan and Engen, 2009). pUb binding causes a switch in the behaviour of region (1r) from rigid to dynamic, as the corresponding helix becomes solvent exposed upon Ubl release.

In this proof-of-concept experiment, we have shown that HDX MS was able to reveal predicted consequences of non-covalent pUb binding, in solution in the context of full-length human Parkin. We were able to observe direct protection of the pUb-binding site from solvent exposure. Additionally, increased solvent exposure of both sides of the autoinhibitory Ubl interface could be observed. This confirms its release predicted from binding studies between the Ubl and the pUb-bound C-terminal UPD-RBR fragment of Parkin (Sauvé et al., 2015; Wauer et al., 2015a). This recapitulates the known transition between the autoinhibited and pUb-bound Parkin structures and shows that comparative



Figure 5.3.: Parkin binding to pUb. (A) Heatmap representing the difference in uptake resulting from pUb association. The sequence coverage was 92.3 % with 139 detected peptides. (B) Data for t = 30 s as in A, mapped onto a ParkinpUb complex structure-based model (PDB ID: 5N2W). The Ubl domain is shown as dissociated according to NMR measurements (Sauvé et al., 2015). For all time points and corresponding difference plots see Fig C.1 and Fig C.2, Appendix C.1. (C) Schematic representation of the cumulative measured effect of pUb-binding. (D) Individual peptide plots showing relative (uncorrected for back-exchange) deuterium uptake by peptides in regions of interest. Each point for the technical replicate experiments is shown. For clarity, data taken at identical time points are offset in the x-axis.

HDX MS studies are a powerful tool to characterize the Parkin activation sequence.

Our ability to trap and isolate distinct Parkin states becomes the challenge for Parkin activation analysis by comparative HDX MS.

# 5.3. Parkin phosphorylation

To understand Parkin activation induced by Ubl phosphorylation, we next looked at the difference between phosphorylated and unphosphorylated pUb-bound Parkin samples by HDX MS.

Overall, the phospho-Parkin:pUb complex appears to be more dynamic than the unphosphorylated Parkin:pUb complex. Most dramatically, the RING2 C-terminal helix, mediating the autoinhibitory contacts of the RING2 and UPD domains, becomes much more dynamic (region (6) in Fig 5.4 A-C, Fig 5.3 D). Displacement of this domain exposes the catalytic Cys and explains the great increase in Parkin activity upon phosphorylation (see Section 4.1), (Kondapalli et al., 2012; Kazlauskaite et al., 2015). Unusually, and unlike for Ubl displacement from its autoinhibitory position, we only see very little exposure of the reciprocal autoinhibitory interface on the UPD (region (6r) in Fig C.4, Appendix C.2).

The Ubl domain also becomes more dynamic upon phosphorylation (region (1) in Fig 5.4 A-C). In contrast little change is seen in the autoinhibitory Ubl binding site on the RING1 domain, suggesting that the additional observed Ubl exposure does not result from further displacement from the autoinhibitory site induced by Ubl phosphorylation (region (1r) in Fig C.4, Appendix C.2). Ubl phosphorylation is known to destabilise the isolated Ubl domain (Wauer et al., 2015a; Sauvé et al., 2015; Aguirre et al., 2017, 2018), which may be the cause of the increased Ubl solvent exposure seen in our HDX MS measurement.

Interestingly, the pUb binding site (region (3) in Fig 5.4 A-C) becomes more protected upon Ubl phosphorylation. This reflects a previously reported ~10-fold increase in affinity of pUb to phosphorylated Parkin compared to unphosphorylated Parkin (Kumar et al., 2015; Sauvé et al., 2015), which is a result of negative cooperativity between pUb and autoinhibitory Ubl binding in unphosphorylated Parkin (Fig 5.4 D, left). The linker



Figure 5.4.: Parkin phosphorylation. (A) Heatmap representing the difference in uptake resulting from Ubl phosphorylation. The sequence coverage was 92.3 % with 139 detected peptides. (B) Data for t = 30 s as in A, mapped onto an 'open' Parkin-pUb model based on PDB ID: 5N2W. In addition to the Ubl domain, the RING2 domain and the REP are shown as dissociated from the Parkin core. For all time points and corresponding difference plots see Fig C.3 and Fig C.4, Appendix C.2. (C) Schematic representation of the cumulative measured effect of Ubl phosphorylation. (D) Left: Model showing negative cooperativity between pUb and Ubl binding (red arrows), mediated by the RING1-IBR linker (red oval). Right: Phosphorylated Ubl does not compete with pUb binding, and triggers RING2 displacement by an unknown mechanism

helix between the RING1 and IBR domain assumes a unique conformation specific to either binding partner explaining this behaviour. Affinity of the isolated phospho-Ubl domain for the Parkin RING1 autoinhibitory binding site is significantly lower compared to the unphosphorylated case (Fig 5.4 D, right), (Sauvé et al., 2015; Wauer et al., 2015a). This decrease in affinity effectively removes the competition between the phospho-Ubl and pUb and leads to the increased protection of the pUb-binding site observed in our HDX MS experiment.

Neither further exposure of the phospho-Ubl domain or further protection of the pUbbinding site represent the phosphorylation-dependent conformational changes which lead to RING2 exposure and therefore Parkin activation. The only remaining significant feature is protection of a further portion of the UPD domain (region (4) in Fig 5.4 A-C). However, by comparative analysis of the Parkin:pUb and phospho-Parkin:pUb samples, we are unable to identify the binding partner or conformational change responsible for this protection. Further evidence is necessary to assess whether and how protection of region (4) can lead to exposure of the RING2 domain in activated, phosphorylated Parkin.

# 5.3.1. Role of the Unique Parkin Domain (UPD) in Parkin activation

Region (4) is located within the UPD domain, which harbours patient mutations recetly confirmed as pathogenic: K211N and K161N (Yi et al., 2018). The additional UPD protection observed upon Parkin phosphorylation encompasses both of these residue positions, as well as Arg163 (Fig 5.5 A). Together Lys211, Lys161 and Arg163 form a phosphate-binding pocket on the Parkin Ubl domain, which was first identified upon determination of the Parkin autoinhibited structures in 2013 by analysis of sulphate molecules bound to the crystallized protein surface (Wauer and Komander, 2013). The UPD phospho-pocket is in close proximity to the RING2 autoinhibitory interface with the UPD mediated by the RING2 C-terminal helix.

To study the importance of the UPD phospho-pocket for the dynamics of Parkin activation, we perturbed the phosphorylated, pUb-bound Parkin complex by introducing the pathogenic patient mutation K211N and performed comparative HDX MS analysis. *In vitro*, this Parkin mutant is incompatible with Parkin activation by phosphorylation and is inactive (Wauer et al., 2015a), (Fig 5.5 B) and has been reported to suppress



Figure 5.5.: Effect of the K211N pathogenic patient mutation on Parkin activation. (A) Inset showing the sulphate-bound UPD phospho-pocket in an autoinhibited Parkin structure (PDB ID: 4BM9) and coloured as in Fig 5.4 B. The UPD domain is shown as a transparent surface while the RING2 C-terminal helix is shown as cartoon-only. (B) Ub-VS probe-reactivity assay comparing Parkin variants. The K211N mutant is not activated by phosphorylation. (C) Heatmap representing the difference in uptake resulting from Ubl phosphorylation. The sequence coverage was 87.4 % with 79 detected peptides. (D) Peptide plot showing relative deuterium uptake for the C-terminal helix of the RING2 domain. Each point for the technical replicate experiments is shown. For clarity, data taken at identical time points are offset in the x-axis. (E) Model showing two interconverting states predicted to make-up the dynamic phospho-Parkin:pUb complex. One shows similar hallmarks of autoinhibition as previous structures, while the other is inferred from the dynamic behaviour observed for wt and K211N phospho-Parkin:pUb complexes.

mitophagy and Parkin localization in HeLa cells (Ordureau et al., 2014).

The most striking difference, when compared to the wt phosphorylated complex is in the RING2 C-terminal helix. Following introduction of the K211N mutation, the RING2 domain no longer becomes solvent exposed upon phosphorylation (region (6) in Fig 5.5 C, D). This behaviour suggests that protection of the UPD phospho-pocket (region (4)) upon Parkin phosphorylation is mechanistically involved in displacing the RING2 domain.

In contrast, the phosphorylated Ubl domain experiences higher deuterium incorporation than seen in the wt phospho-Parkin:pUb complex (region (1) in Fig 5.5 C, D). We expect the two effects identified previously to cause phospho-Ubl solvent exposure to be independent of the introduced K211N mutation - low affinity of the phospho-Ubl for the RING1 autoinhibitory interface and destabilization of the Ubl domain upon phosphorylation. Further exposure of the phospho-Ubl in the K211N mutant therefore indicates an unassigned additional mode of protection from solvent exposure in the wt phospho-Parkin:pUb complex. This mode of Ubl protection which is prevented in the K211N mutant is therefore dependent on the UPD phospho-pocket.

Taken together with the effect of Parkin phosphorylation on dynamics of the system, the K211N mutant analysis suggests the following model (Fig 5.5 E): The phospho-Parkin:pUb complex represents a dynamic ensemble of two interconverting states. Dynamics of the 'autoinhibited' phospho-Parkin state can be analysed directly (by analysing the K211N mutant), while dynamics of the active phospho-Parkin state have not yet been directly revealed. From our HDX analysis we postulate that in this 'active' state the phospho-Ubl may interact with the UPD phospho-pocket (both appear to be relatively protected in phosphorylated Parkin). As the UPD phospho-pocket is in close proximity of the UPD binding site for the RING2 C-terminal helix, it is likely that it is this interaction between the phospho-Ubl and the UPD that results in displacement of the RING2 catalytic domain from its autoinhibitory position. This model also explains why no/little exposure of the UPD interface reciprocal to the RING2 C-terminal helix autoinhibitory binding site is seen in the phosphorylated sample. Both the inactive and the proposed active Parkin state require a binding site in that region. Although such an active state has been previously proposed solely based on the *in vitro* inactivity of the phosphorylated Parkin K211N mutant (Wauer et al., 2015a), our HDX MS analysis provides the first structure-based evidence for its existence.

## 5.4. Parkin binding to the $E2 \sim Ub$ conjugate

Analysis of the phosphorylated Parkin complex provided indirect evidence for the presence of a distinct active Parkin conformation. During the RBR catalytic cycle the catalytic Cys must discharge the  $E2 \sim Ub$  thioester, form a Parkin  $\sim Ub$  conjugate, and orient its thioester linkage for discharge by substrate Lys residues. Any of these steps are inconsistent with the previously determined crystal structures of inactive Parkin states and would only be enabled by the active state.

The E2 ~ Ub-bound state may provide further insight into the active Parkin domain arrangement, as this is likely to be favoured by the phospho-Parkin:E2 ~ Ub complex for optimal catalytic activity. Additionally, a dynamic insight into Parkin : E2 ~ Ub binding will enable comparison of the Parkin : E2 ~ Ub transfer mechanism with that of previously characterized RBRs HOIP and HHARI (Lechtenberg et al., 2016; Dove et al., 2017; Yuan et al., 2017; Dove et al., 2016).

In cell-based assays, PINK1/Parkin-mediated mitophagy has been reported to depend on a number of different E2 enzymes (Geisler et al., 2014; Hasson et al., 2013; Lazarou et al., 2013). Several have been confirmed *in vitro*: UBE2 -D2, -E1, -J2, -L3 (Ordureau et al., 2014; Lazarou et al., 2013). Since UBE2L3 has been shown to specifically transfer Ub onto HECT or RBR active site Cys residues (Wenzel et al., 2011), and exhibits highest affinity for most RBRs (Martino et al., 2018), we have chosen to analyse a non-covalent complex of phospho-Parkin:pUb with UBE2L3-Ub conjugate mimetics by HDX MS. However, for example the UBE2D ~ Ub and the UBE2L3 ~ Ub conjugates behave differently in solution (Pruneda et al., 2011; Dove et al., 2016) and it may be insightful to also investigate how Parkin interacts with other E2s in the future.

Two strategies have been employed to mimic the native phospho-Parkin:UBE2L3 ~ Ub:pUb complex to obtain a good HDX MS sample. In the first instance, we stabilized the UBE2L3 ~ Ub conjugate by replacing the thioester bond with an amide linkage (Plechanovová et al., 2012). Secondly we analysed a trimeric covalent complex of

phospho-Parkin-E2-Ub trapped by using a recently developed UBE2L3-Ub Activity-Based Probe (ABP), (Pao et al., 2016).

#### 5.4.1. Non-covalent complex with UBE2L3-Ub

The amide-linked UBE2L3 conjugate was generated by replacing the UBE2L3 active site Cys residue with a Lys residue. In the presence of MgATP, the E1 enzyme is able to activate Ub, which can then be transferred onto the engineered E2 active site Lys (Plechanovová et al., 2012). The longer Lys side chain misaligns the two enzymatic active sites and results in slower Ub transfer (Fig 5.6). Although the Lys substitution extends the linkage between the E2 and Ub, affinity of ~1  $\mu$ M was reported for the interaction between Lys-linked UBE3L3-Ub and a non-covalent phospho-Parkin:pUb complex (Kumar et al., 2015). This method could be improved by synthesising a near-native conjugate by replacing the active site Cys with 2,3-diaminopropionic acid (DAP) by genetic code expansion (Ambrogelly et al., 2007; Huguenin-Dezot et al., 2018).

Both components of the UBE2L3  $\sim$  Ub complex have a previously identified binding site on Parkin: The canonical E2 binding site is present in the Parkin RING1 domain (see Introduction 1.7.4), which is identical to the interface observed to other E2-Ub conjugate mimetics binding to RING domains (Yin et al., 2009; Dou et al., 2012; Plechanovová et al., 2012; Pruneda et al., 2012). The donor Ub is able to weakly bind the Parkin



Figure 5.6.: Generating the Lys-linked E2-Ub conjugate. (A) Schematic representation of the Lys-linked E2-Ub conjugate. Upon Cys to Lys substitution, the active sited become misaligned and a longer linkage results. (B) Timecourse of Lys-linked E2-Ub conjugate generation for UBE3L3.

RING2 (Dove et al., 2016) and an additional IBR donor Ub binding site was postulated from crystals contacts of pUb-bound truncated Parkin (Kumar et al., 2017a). RING2 binding to the donor Ub would result in shifting the phospho-Parkin equilibrium towards the active state, in which the RING2 domain is dislodged and able to participate in this interaction. A shift towards the active Parkin state is indeed confirmed by further exposure of the RING2 C-terminal helix in the phospho-Parkin:UBE2L3-Ub:pUb sample (region (6) in Fig 5.7 A-C). Consistent with the active Parkin model (Fig 5.5 E), additional protection is also observed in the UPD phospho-pocket (region (4) in Fig 5.7 A-C).

Strikingly, the dynamic behaviour of the Ubl domain markedly changes upon UBE2L3-Ub association with the phospho-Parkin:pUb complex in comparison to any previously analysed sample. The phospho-Ubl is now protected from solvent exchange suggesting formation of a new interface (region (1) in Fig 5.7 A-D). We observed no binding between the isolated phospho-Ubl (1-76) domain and UBE2L3 by NMR (Fig C.7, Appendix C.3), suggesting that the newly formed phospho-Ubl interface is intramolecular. In this analysis, a decrease in dynamics of both reciprocal sides of the phospho-Ubl binding to the UPD phospho-pocket can be directly observed, strongly supporting the active state model.

Upon UBE2L3-Ub association, two further regions are protected - the ACT element (region (7) in Fig 5.7 A-C) and the linker helix between the RING1 and IBR domains (region (8) in Fig 5.7 A-D). A comparison with Lys-linked UBE2D2-Ub conjugate binding to HOIP (Fig 5.7 E) reveals that protection of region (8) could be caused by a similar position of donor Ub in both complexes. Direct superimposition is not possible due to relative displacement of the IBR domains. To accommodate for the donor Ub, either the Parkin IBR would have to swing further outwards, causing rearrangement of the pUb binding site (Fig 5.7 E, red arrows), or the donor Ub position would have to be adjusted accordingly (Fig 5.7 E, purple arrow). Donor Ub binding in this region of the Parkin structure has also been postulated from a crystal contact observed between the Parkin:pUb core and the Ubl position of a neighbouring molecule (Kumar et al., 2017a).

At the time of preparation of this thesis, an NMR-derived model of Lys-linked UBE2L3-Ub bound to Parkin UPD-RBR was published (Condos et al., 2018). This model places the E2 on the canonical RING-mediated E2 binding site. A more detailed examination of our difference plots for the non-covalently bound complex also revels this binding site (region (10) in Fig C.6, Appendix C.3). NMR analysis identifies that the donor Ub as



Figure 5.7.: phospho-Parkin:pUb binding to the Lys-linked E2-Ub conjugate. (A) Heatmap representing the difference in uptake resulting from association with Lys-linked E2-Ub conjugate. The sequence coverage was 83.9 % with 89 detected peptides. (B) Data for t = 3 s as in A, mapped onto an 'open' Parkin-pUb model as in Fig 5.4 B. For all time points and corresponding difference plots see Fig C.5 and Fig C.6, Appendix C.3. (C) Schematic representation of the cumulative measured effect of association with E2-Ub on the active Parkin model. (D) Peptide plots showing relative deuterium uptake for regions of interest. Each point for the technical replicate experiments is shown. For clarity, data taken at identical time points are offset in the x-axis. (E) Overlay of RING1 domains from HOIP bound to a Lys-linked UBE2D2-Ub conjugate (PDB ID: 5EDV), (Lechtenberg et al., 2016) with the 'open' Parkin-pUb model, RMSD = 3.65. Left: both structures shown with accessory domains from the same view. Parkin is coloured as in B, while the HOIP RBR domains are coloured according to the RBR portion of the domain diagram in A. Right: Overlay of the core RING1-IBR domains of HOIP: Ub and Parkin: pUb with red arrows showing necessary displacement.

the most flexible component of the complex. In agreement with structure-mapping of our HDX MS data, the NMR data suggest donor Ub binding to the IBR domain of Parkin is slightly shifted compared to the interface observed in HOIP, and corresponds to movement of the donor Ub (Fig 5.7 E, purple arrow).

Protection of the ACT element (region (7)) is very interesting, as this disordered linker between the Ubl and UPD domains is conserved and its disruption results in phospho-Parkin inactivity (see Section 4.3). This protection suggests that the ACT element is involved in formation of a new activating interface required for Parkin catalytic activity. The ACT can either be involved in an intramolecular interface or aid binding to either component of the UBE2L3-Ub complex.

Although the differences in relative deuterium uptake upon UBE2L3-Ub association are relatively small ( $\pm 5$ %), the analysed non-covalent phospho-Parkin:pUb:UBE2L3-Ub complex, provided a glimpse of the active Parkin state. Consistent with the model, the UBE2L3-Ub-bound complex favoured the active Parkin state. Both sides of the proposed activating interface are now protected - the UPD phospho-pocket and the phospho-Ubl. Open questions remain about the cause of protection, and function, of the ACT.

### 5.4.2. Covalent complex with UBE2L3-Ub

To improve the data quality from the non-covalent UBE2L3-Ub association experiment, we employed the recently developed approach of generating a covalent trimeric HECT/RBR-E2-Ub complex using an E2-Ub ABP (Pao et al., 2016; Byrne et al., 2017). Covalent association of the ABP with the RING2 catalytic Cys sterically prevents any autoinhibitory association between the RING2 and the UPD domains. This completely disfavours the autoinhibited state of Parkin, and the active state is expected to be solely populated in this sample.

The  $His_6$ -UBE2L3-Ub amide-linked ABP was a kind gift from Dr Satpal Virdee and Kuan-Chuan (Eric) Pao at the MRC Protein Phosphorylation and Ubiquitylation Unit.



Figure 5.8.: E2-Ub activity based probe labelling. (A) An amide-linked UBE2L3-Ub ABP. Note that the reaction centre is removed by two positions from the electrophilic site on a native  $E2 \sim Ub$  thioester conjugate. (B) Time course Parkin labelling with probe as in A under specified conditions.

The donated E2-Ub ABP was generated by functionalization of the Ub C-terminus using intein chemistry (Wilkinson et al., 2005) and attaching a soft electrophile to the C-terminus using click chemistry. After the active site Cys nucleophile of an E2 enzyme is trapped, the electrophile is regenerated to form an E2-Ub probe capable of irreversibly trapping ligases containing an active site Cys nucleophile (Fig 5.8 A), (Pao et al., 2016). Optimal Parkin E2-Ub probe-labelling conditions were found to be in excess of the ABP, pH = 8.5 and with minimum presence of TCEP (Fig 5.8 B). We postulate that the low labelling efficiency stems form displacement of the reactive centre position compared to the native thioester electrophile along the relatively rigid E2-Ub ABP linker. Using the hexahistidine tag, we were able to obtain sufficiently clean sample for comparative HDX MS study of the covalent trimeric complex.

On comparison of the pUb-associated trimeric complex with phospho-Parkin:pUb, differences observed are similar to those seen in analysis of non-covalent binding of the Lys-linked E2-Ub conjugate. As expected for a complete shift towards the active state, magnitudes of change and signal-to-noise ratio is significantly improved in this analysis. In line with the active Parkin features observed previously, the phospho-Ubl (region (1) in Fig 5.9 A,B) and the UPD phospho-pocket (region (4) in Fig 5.9 A,B) remain protected while the RING2 domain is displaced and exposed (region (6) in Fig 5.9 A,B).

The donor Ub site protection (region (8) in Fig 5.9 A,B) is recapitulated by the trimeric complex and additionally, the E2 binding site on the RING1 domain can also be seen at later time points (region (10) in Fig 5.9 A,B). Comparison of the difference plots for

the covalent and non-covalent phospho-Parkin:pUb complexes with E2-Ub reveals that a common region of the RING1 domain is exposed in both conditions upon association with E2-Ub (approx residues 284-311 marked (?) in Appendix Fig C.6, Fig C.9 and Fig C.11). This is likely due to an unknown rearrangement necessary to accommodate the



Figure 5.9.: HDX MS analysis of the covalent trimeric phospho-Parkin-UBE2L3-Ub complex. (A) Heatmap representing the difference in uptake resulting from formation of a covalent trimeric conjugate. The sequence coverage was 91.4 % with 156 detected peptides. (B) Data for t = 300 s as in A, mapped onto an 'open' Parkin-pUb model as in Fig 5.4 B. For all time points and corresponding difference plots see Fig C.8 and Fig C.9, Appendix C.4. (C) Heatmap representing the difference in uptake arising upon comparison of the phospho-Parkin-UBE2L3-Ub:pUb complex generated using the E2-Ub ABP with the phospho-Parkin-Ub:pUb complex generated using a Ub ABP (see Section 5.5). The sequence coverage was 91.4 % with 156 detected peptides. (D) Data for t = 300 s as in C, mapped onto an 'open' Parkin-pUb model as in Fig 5.4 B. For all time points and difference plots see Fig C.10 and Fig C.11, Appendix C.4.

E2-Ub complex.

The ACT element protection is recapitulated in the earliest analysed time point (3 s), but no further insight can be gleaned about its reciprocal binding site from this analysis.

# 5.5. Ubiquitin-charged Parkin

Prior to obtaining the E2-Ub ABP for HDX MS analysis, we set out to quantitatively generate and analyse a covalent complex trapping the subsequent catalytic state of Parkin - the Parkin  $\sim$  Ub thioester conjugate, which forms upon Ub discharge from the E2  $\sim$  Ub before Ub is finally transferred onto substrate Lys residues by Parkin. To generate a Parkin  $\sim$  Ub thioester mimic, we used a Ub-derived ABP. Although originally these Ub ABPs were designed to react with the catalytic Cys residues of deubiquitinases (Borodovsky et al., 2002), the phospho-Parkin active site Cys is also able to react with a particular ABP - the Ubiquitin Vinyl Sulphone (Ub-VS), (Ordureau et al., 2014; Wauer et al., 2015a).

Similar to trimeric phospho-Parkin-UBE2L3-Ub complex formation, we predicted that covalent attachment of Ub to the RING2 catalytic Cys would fully favour the active Parkin state due to steric clashes in the inactive state. As a result of low Parkin processivity, substrate ubiquitination likely occurs in the phospho-Parkin-Ub:pUb state, in accordance with the sequential model (Le Guerroue and Youle, 2018). Analysis of the dynamic behaviour of this state may therefore shed light on the apparent lack of Parkin substrate and linkage specificity (see Section 4.4), (Sarraf et al., 2013; Ordureau et al., 2018, 2014).

#### 5.5.1. Generation of Ub vinyl sulphone and Parkin coupling

Ub ABPs consist of Ub (1-75), which is linked to an exchangeable electrophile (a warhead) through a linker mimicking the terminal Gly76 of Ub. As the electrophilic warheads are used to irreversibly trap active site Cys nucleophiles, preserving the correct geometry of the active site is key. A semi-recombinant synthetic approach is used to generate the



Figure 5.10.: Generation of Ub-VS and Parkin labelling. (A) Reaction scheme showing coupling of H-Gly-VS with Ub-MesNa. The reaction is catalysed by NHS to improve yield over side reactions. (B) Side-reactions leading to the generation of the most-prominent side products. The reaction is quenched when hydrolysed Ub-MesNa accounts for approximately 50% of the product, with minimal formation of other side-products. (C) LC-MS spectrum of final Ub-VS sample. (D) LC-MS spectrum of final Parkin-Ub sample. (E) Time course of Parkin charging with Ub-VS in TCEP-containing buffer.

Ub ABPs: Ub(1-75)-intein polyprotein is recombinantly produced and following protein splicing, the polyprotein is cleaved by addition of sodium 2-mercaptoethanesulfonate (MesNa) to generate Ub-MesNa (Fig 5.10 A, middle). The Ub-MesNa thioester is used as a building block to generate a family of Ub ABPs. Various electrophilic warheads, (e.g. H-Gly-VS to generate Ub-VS (Borodovsky et al., 2001), or Propargylamine to generate Ub-PA (Ekkebus et al., 2013)) are coupled to Ub-MesNa by substituting the MesNa group (Fig 5.10 A), (Wilkinson et al., 2005; Borodovsky et al., 2002).

Currently, Ub-VS is commercially available only in quantities sufficient for biochemical experiments, while H-Gly-VS is commercially unavailable. In order to obtain a sufficient quantity of Ub-VS for quantitative Parkin labelling for HDX MS analysis, we established a protocol for Ub-VS generation. Larger amounts were needed as the previously reported Parkin Ub-VS labelling efficiency was only 50 % (Ordureau et al., 2014; Wauer et al.,

2015a).

The H-Gly-VS was a kind gift from Dr Huib Ovaa and Dr Bo-Tao Chin at the Leiden University Medical Centre.

The vinyl sulphone is a highly reactive electrophile, and, in addition to Ub-MesNa hydrolysis, side reactions of Ub-VS hydrolysis as well as polymerization of the Gly-VS groups occur at the coupling pH=8.5 (Fig 5.10 B). Subsequent purification of the coupled mixture yields mg quantities of pure Ub-VS probe per coupling reaction on the established reaction scale (Fig 5.10 C), (see Methods 2.3.7).

When the Parkin coupling product was analysed by Liquid Chromatography-Mass Spectrometry (LC-MS) in DTT-containing Parkin storage buffer, it was serendipitously found that Ub-VS is able to rapidly form an adduct with DTT. This competing reaction has likely caused the low Ub-VS Parkin labelling efficiency observed in the past (Ordureau et al., 2014; Wauer et al., 2015a). Subsequent replacement of DTT in the Parkin storage buffer with TCEP allowed complete and rapid Parkin labelling with the in-house generated Ub-VS ABP (Fig 5.10 D, E).

### 5.5.2. Dynamics of the phospho-Parkin $\sim$ Ub covalent mimetic

Covalent association with Ub through the active site Cys results in similar magnitudes of change as observed for the covalent trimeric phospho-Parkin-UBE2L3-Ub complex, as in both cases a complete shift towards the active Parkin state is expected. Consistent with the active Parkin state model, the phospho-Ubl (region (1) in Fig 5.11) and the UPD phospho-pocket (region (4) in Fig 5.11) remain protected while the RING2 domain is displaced and exposed (region (6) in Fig 5.11). Comparison with phospho-Parkin-UBE2L3-Ub:pUb (Fig 5.9 C,D) in fact shows no differences in the Ubl or UPD domains between the two covalent complexes, suggesting that the active Parkin intramolecular interface remains invariant upon UBE2L3 association. Interestingly the UBE2L3-containing covalent complex exhibits additional flexibility at the RING2 C-terminus, suggesting that a part of the C-terminal secondary structure may be incompatible with E2 binding (region (6) in Fig 5.9 C,D).



Figure 5.11.: HDX MS analysis of the covalent phospho-Parkin-Ub complex. (A) Heatmap representing the difference in uptake resulting from formation of a phospho-Parkin-Ub conjugate. The sequence coverage was 83.9 % with 89 detected peptides. (B) Data for t = 3 s as in A, mapped onto an 'open' Parkin-pUb model as in Fig 5.4 B. For all time points and corresponding difference plots see Fig C.12 and Fig C.13, Appendix C.5. (C) Schematic representation of the cumulative measured effect of conjugate formation on the active Parkin model. (D) Peptide plots showing relative deuterium uptake for regions of interest. Each point for the technical replicate experiments is shown. For clarity, data taken at identical time points are offset in the x-axis.

Protection of the RING1 or IBR domains is not observed in the phospho-Parkin-Ub complex (regions (8) and (10) in Fig 5.9 C,D), as this is a result of UBE2L3 binding and

positioning of the donor Ub by the E2. This suggests that in absence of UBE2L3, the donor Ub conjugated to the RING2 does not interact with the donor Ub binding site on the IBR domain. As a consequence, the RING2-Ub portion of the complex seems to be flexible and does not interact with the Parkin core.

This may explain the substrate and linkage promiscuity of Parkin ubiquitination (see Section 4.4), (Ordureau et al., 2018; Sarraf et al., 2013; Ordureau et al., 2014). In this model Ub would be discharged on the first encountered substrate Lys residue within the dynamic radius accessible by the RING2 ~ Ub tethered by a ~35 AA linker (from the end of the IBR domain to the identified RING2-Ub interaction site (Dove et al., 2016)). Predicted dissociation of the RING2 from the body of Parkin in the active state and in the absence of  $E2 \sim Ub$  could be confirmed by studying the effect of Parkin C-terminal truncation on its dynamics or directly showing RING2 dissociation in phosphorylated Parkin.

The two covalent complexes have been analysed in separate HDX MS experiments, however in both cases several peptides are lost from the RING2 domain. This is a consequence of pepsin miscleavage in the samples, which may result from RING2 binding to the donor Ub and therefore being protected from cleavage (Dove et al., 2016). Additionally, peptides containing the modified active site Cys compared to the control sample will be lost from analysis. This behaviour is in part also reflected in protection of a short RING2 peptide (400-409), (region (9) in Fig 5.11).

Similarly to the previous complex, the ACT is protected from solvent exchange (region (7) in Fig 5.11 A-D). Absence of the UBE2L3 from this sample rules out ACT association with the E2 enzyme. Ambiguity as to whether the ACT is involved in donor Ub binding or contributes to the intramolecular active Parkin interface remains. Distinguishing between these two cases may be difficult, as any activity read-outs simultaneously require donor Ub-binding and the ability of Parkin to assume the active conformation (see Section 4.4). Direct effects on measured binding to the E2 ~ Ub conjugate or Ub may also be obscured by affecting the equilibrium between Parkin active and inactive states. Identification of a minimal Parkin construct capable of ACT solvent protection in an HDX MS experiment or structural analysis of the active Parkin state would shed further light on the ACT function.

Overall, with fewer confounding features compared to  $E2 \sim Ub$  conjugate association, we can consider the phospho-Parkin-Ub complex a direct dynamic snapshot of the active Parkin state with the hallmarks of phospho-Ubl and UPD protection; and RING2 exposure due to its displacement.

## 5.6. Conclusion and discussion

HDX MS has emerged as an ideal method to study the domain rearrangements during Parkin activation and the Parkin catalytic mechanism. By studying the dynamics of trapped states along the Parkin activation cascade, we were able to build up a picture of how Parkin transfers Ub from the E2  $\sim$  Ub onto substrates (Fig 5.12). pUb binding displaces the Parkin Ubl domain, priming it for phosphorylation by PINK1 (Kazlauskaite et al., 2015). The phospho-Ubl domain enables Parkin to access a new conformational equilibrium, where only one of the states resembles the previously characterized inactive Parkin structures (Wauer and Komander, 2013; Trempe et al., 2013; Riley et al., 2013; Kumar et al., 2015; Sauvé et al., 2015; Wauer et al., 2015a; Kumar et al., 2017a). The active state is enabled by phospho-Ubl-mediated intramolecular domain rearrangement where phospho-Ubl binding to the UPD phospho-pocket displaces the RING2 catalytic domain. As this active state is competent to receive donor Ub from the E2  $\sim$  Ub thioester conjugate and transfer the Ub onto substrates, it is favoured by subsequent catalytic steps.

Initially, we were able to confirm that HDX MS can recapitulate the effect of pUb binding to Parkin. From crystallographic analysis of truncated Parkin, which has been covalently linked to pUb, the binding site for pUb created by rearrangement of the RING1-IBR linker was discovered (Wauer et al., 2015a; Kumar et al., 2017a). NMR and binding analyses on individual domains have revealed that pUb binding decreases the affinity towards the Parkin Ubl through the RING1 autoinhibitory binding site (Wauer et al., 2015a; Sauvé et al., 2015). HDX MS was able to recapitulate the pUb binding site, conformational changes and Ubl exposure in solution and in the context of the full-length native protein without the need for truncations, domain isolation or covalent pUb attachment (Fig 5.12 A,B). This proof-of-concept experiment verified HDX MS as a powerful method to study Parkin domain rearrangements.

The effects of Parkin phosphorylation, taken together with analysis of the K211N patient pathogenic mutation, pointed to the presence of an alternative Parkin conformer (i.e. the active state). A new intramolecular interface is formed between the phospho-Ubl and the UPD phospho-pocket (Fig 5.12 C,D). Because the phosphorylated state was the most dynamic condition analysed as the inactive and active states are in exchange, the new interface could not be directly observed upon phosphorylation only. These findings are consistent with AUC and SAXS analyses, which find that phospho-Parkin adopts, on average, a more open conformation than unphosphorylated Parkin (see Introduction 1.12.3), (Sauvé et al., 2015; Aguirre et al., 2017). Further stabilization of catalytic intermediates shifted the equilibrium in favour the active state and allowed direct observation of the active state.

Mimicking the Parkin ~ Ub thioester by trapping the phospho-Parkin-Ub:pUb complex using a Ub ABP has given us the opportunity to observe the new intramolecular interfaces of active Parkin directly. Protection of the phospho-Ubl and the UPD phospho-pocket together from solvent exchange with exposure of the RING2 domain correspond to phospho-Ubl association with the UPD phospho-pocket which leads to RING2 displacement in the active Parkin state (Fig 5.12 D).

Binding of the UBE2L3  $\sim$  Ub thioester was analysed using two UBE2L3  $\sim$  Ub thioester mimetics yielding similar results (Fig 5.12 E). In addition to the new active state intramolecular interfaces, UBE2L3 binding to the canonical RING1-E2 interface is observed. The donor Ub contacts the IBR domain in the trimeric complex and protects it from solvent exchange. Similar IBR interfaces interact with the donor Ub in the crystallized HOIP:UBE2D2-Ub complex (Lechtenberg et al., 2016), although recent NMR modelling suggests an adjustment of the donor Ub position as compared to HOIP complex (Condos et al., 2018).

The donor Ub site is not protected in the phospho-Parkin-Ub:pUb complex and no additional protection is seen on the Parkin core domains (UPD, RING1, IBR). This suggests that the RING2-Ub portion of the protein dissociates from the Parkin core and behaves independently, which may explain the relatively low substrate and chain specificity of Parkin-mediated ubiquitination (Fig 5.12 F), (Ordureau et al., 2018; Sarraf et al., 2013; Ordureau et al., 2014). Consistent with this idea, *in vitro* addition of a model Parkin substrate Miro or free Lys leads to an additional increase in gross Parkin



Figure 5.12.: HDX MS-derived model of Parkin activation. Available crystal structures describe the first, autoinhibited state (PDB ID: 4BM9, 4I1H, 4K95, 4K7D, 4ZYN, 5C1Z) as well as the pUb-bound state (PDB ID: 5CAW and 5N2W). Further states are based on previous NMR or the presented HDX MS analysis.

turnover but not in accelerating the transthiolation step of  $E2 \sim Ub$  discharge (Park et al., 2017). This discrimination suggests that substrate association does not alter the phosphorylation-dependent activation sequence of Parkin described here.

A good hallmark of the increased tendency to populate the active Parkin state, is exposure of the REP element (region (5) in Figs 5.3, 5.4, 5.7, 5.9, 5.11). The REP element peptide

is very dynamic and reaches saturation even in the autoinhibited state. Any perturbation to the autoinhibited structure that favours the active state leads to saturation of the peptide at an earlier time point. In the phospho-Parkin-Ub:pUb complex, the REP peptide (391-400) experiences such a high deuterium uptake, its secondary structure can be considered absent under physiological conditions (Fig 5.11 D).

At the time of preparation of this thesis chapter, an alternative model of Parkin activation was proposed (Condos et al., 2018). In this model, the Ubl domain is only dissociated from the Parkin core when phosphorylated (not explaining how PINK1 could access the bound Ubl for phosphorylation). Additionally, the catalytic Cys is only efficiently exposed in the UBE2L3-Ub-bound state in this model. In contrast, our data clearly show near-complete exposure of the Ubl domain already upon pUb binding. Although we also observe further exposure of the RING2 domain upon binding to the UBE2L3-Ub conjugate, we do not consider association with UBE2L3-Ub as necessary for Parkin to assume the active state. Phosphorylation is sufficient to enable access to the active Parkin state, as it alone exposes the catalytic Cys residue sufficiently for Ub-VS reactivity. In support of our model, unphosphorylated Parkin remains inactive towards the E2-Ub ABP (Pao et al., 2016), and is inactive in the E2 ~ Ub discharge assay (Fig 4.1 C), suggesting that UBE2L3-Ub binding is not sufficient to induce the active state. UBE2L3-Ub binding to phosphorylation further towards the active state, which is enabled by Parkin phosphorylation.

In both of the trapped analysed catalytic intermediates, the ACT element (see Section 4.3) is protected. The two common elements among these states is the presence of the donor Ub and preference for assuming the active Parkin state. The ACT element can therefore aid donor Ub binding or be involved in stabilizing the activating intramolecular interface between the phospho-Ubl and the UPD phospho-pocket. To gain atomic-level insight into the new Parkin intramolecular interface and pinpoint the mechanism of the activating function of the ACT element, crystallographic studies of the active Parkin conformer are necessary.

# Chapter 6.

# The structure of active Parkin

#### Key findings:

- Removal of the flexible RING2 catalytic domain stabilized the activating Parkin interface.
- A high resolution structure of pUb-bound *H. sapiens* phospho-Parkin(1-382) describes the active form of Parkin.
- The phospho-Ubl domain binds to an activating interface on the UPD, displacing the RING2 catalytic domain from its autoinhibitory position.
- The amphipathic ACT element mimics RING2 autoinhibitory interactions to stabilize activated Parkin.
- Disease-linked mutations in the UPD and ACT disrupt the active state of Parkin.

All published structures of Parkin represent an autoinhibited form of the enzyme (Trempe et al., 2013; Wauer and Komander, 2013; Riley et al., 2013; Sauvé et al., 2015; Kumar et al., 2015; Wauer et al., 2015a; Kumar et al., 2017a). The observed autoinhibitory elements/interfaces are incompatible with the RBR catalytic cycle (see Introduction 1.12). Parkin autoinhibition is alleviated by phosphorylation on Ser65 in the Parkin Ubl domain by PINK1, however the phosphorylation-induced mechanism of activation is unknown (Kondapalli et al., 2012). A S65N mutation does not affect the known Parkin autoinhibited state, but is likely disease causing in two recently identified individuals (McWilliams et al., 2018). Parkin activation by phosphorylation is therefore key in a physiological context.

Our HDX MS analysis suggests that an activated, rearranged, Parkin species is populated upon phosphorylation and remains in an equilibrium with an inactive state. In this species, the phospho-Ubl domain likely interacts with the UPD and the RING2 domain is displaced (see Chapter 5). Our biochemical and HDX MS data suggest a role for the ACT, a conserved unstructured linker region, which has either previously been omitted or not resolved in structural analyses. The ACT either forms an activating intramolecular interface or binds to the transferred (donor) Ub (see Chapters 4 and 5).

In order to elucidate the molecular details of the predicted Parkin rearrangement upon phosphorylation, I have integrated the biochemical and HDX MS assays described in Chapters 4 and 5 with approaches previously developed in our laboratory to capture the pUb-bound state of Parkin (Wauer et al., 2015a). This effort resulted in a viable strategy to capture the molecular detail of the active Parkin state.

# 6.1. Parkin crystallization challenges

In parallel to analysis of trapped Parkin states by HDX MS, the following have also been used for crystallization trials: phospho-Parkin, phospho-Parkin:E2 ~ Ub, phospho-Parkin-Ub. Due to a limiting amount of the His<sub>6</sub>-UBE2L3-Ub amide-linked Activity-Based Probe (ABP), we have not yet attempted to crystallize the phospho-Parkin-UBE2L3-Ub trimeric covalent complex. Despite current advances in Cryo-Electron Microscopy (Cryo-EM), determination of a Parkin structure by Cryo-EM is currently impractical due to its size of ~ 52 kDa (Renaud et al., 2018; Khoshouei et al., 2017).

#### 6.1.1. Covalent attachment of the phospho-ubiquitin

While non-covalent pUb addition to crystallized Parkin was tested, in a majority of cases pUb was covalently linked to an engineered Cys residue on the IBR domain of human Parkin (*Hs*Parkin) constructs (Wauer et al., 2015a), (Fig 6.1 A), (see Methods 2.3.7).

This approach was developed in our laboratory by Dr Tobias Wauer. He found that in the presence of PINK1 and MgATP, Parkin from the human louse, *Pediculus humanus* (*Ph*Parkin) very efficiently reacted with the Ub-C3Br ABP. Interestingly the Ub-C3Br probe displays restricted reactivity towards deubiquitinases due to its displaced reactive centre (Borodovsky et al., 2002). A crystal structure of PhParkin in a covalent complex with Ub-C3Br generated in the presence of PINK1 and MgATP revealed that the phosphorylated Ub ABP reacted with an unconserved Cys reside on the IBR domain of PhParkin, enabling pUb binding to its dedicated interface (Wauer et al., 2015a; Sauvé et al., 2015; Kumar et al., 2015) and explaining the lack of requirement for native linkage length. Engineering a Cys residue at an equivalent position in HsParkin enabled efficient reactivity and, when combined with a truncation in the UPD-Ubl linker, enabled further crystallographic analysis of the covalently linked HsParkin-pUb complex (Kumar et al., 2017a).



Figure 6.1.: Generation of the covalent Parkin-pUb complex. (A) Reaction scheme showing coupling of the Ub-C3Br ABP with an engineered Cys on utilized Parkin IBR domains in the presence of PINK1 and MgATP. (B) Analytical SEC profile of the phospho-Parkin-pUb complex (blue), Lys-linked UBE2D3-Ub (purple) and an equimolar ratio (black). (C) As in B with UBE2L3-Ub. Lack of co-elution suggests an intermediate-affinity interaction (>30  $\mu$ M).

Despite its utility for crystallization studies, the covalent link may strain the complex as the linkage site on the IBR domain has not been optimized. Two pieces of evidence suggest that the ABP-mediated covalent linkage of Parkin to pUb may induce non-native behaviour. Firstly, the phospho-Parkin-pUb covalent complex is inactive *in vitro* unlike phospho-Parkin when pUb is added in stoichiometric amounts (data not shown). Secondly, binding analysis of the phospho-Parkin-pUb covalent complex to the Lys-linked E2-Ub conjugate by analytical Size Exlusion Chromatography (SEC) shows weak or no association (Fig 6.1 B,C). The finding is in stark contrast to SEC and ITC binding analysis of the Lys-linked UBE3L3-Ub conjugate preformed with a non-covalent phospho-Parkin:pUb complex where a sub-micromolar  $K_D$  of 0.88  $\pm$  0.18 µM was reported (Kumar et al., 2015). The inactivity of the covalent phospho-Parkin-pUb likely results from its inability to efficiently interact with the E2 ~ Ub thioester conjugate, although the reason for the lack of interaction is unclear.

#### 6.1.2. Complex dynamics and scalability

The benefits of constraining the possible conformations of the phospho-Parkin-pUb complexes used for crystallography broadly outweigh the concerns of using a covalent pUb linkage due to its past utility. Constraining motion is essential, as our HDX MS analysis revealed that phosphorylated Parkin accesses the active state through an equilibrium between the active and inactive states. The dynamic nature of phosphorylated Parkin likely explains why no crystals of full-length phosphorylated Parkin were obtained, despite use of a covalent linkage with the pUb.

The HDX MS analysis informed on our ability to favour the active state by trapping catalytic Parkin intermediates. Trapping two non-covalent interactions presents a crystallographic challenge and no phospho-Parkin-containing crystals formed with either the UB2D3- or UBE2L3- Lys-linked conjugate when non-covalent pUb was added. Similarly, although the phospho-Parkin-Ub complex linked through the Parkin active site Cys can only populate the active Parkin conformer, HDX MS analysis suggests dissociation of the RING2-Ub unit from the Parkin core domains (UPD-RING1-IBR), (see Section 5.5). This additional conformational flexibility may explain the absence of crystal formation of this complex.
Systematically sampling the wide range of complexes and modifications available for full-length HsParkin becomes practically challenging for a number of reasons. To generate a sufficient amount of protein for crystallization trials (~2mg), 30 litres of *E. coli* culture are required with our expression system. Mass spectrometric analysis of assembly reactions with phospho-Parkin revealed that contaminating PINK1 activity remains after purification and further contamination by co-purifying *E. coli* GroEL chaperone was also observed. Crystal drop screening is made more challenging by the presence of inorganic crystals. These are relatively common as full-length Parkin denaturation releases eight molar equivalents of  $Zn^{2+}$  ions.

#### 6.2. Exploring Parkin orthologs

To reduce potential conformational flexibility in the system and improve yield while preserving the hallmarks of the activated state observed by HDX MS, I chose to explore possible Parkin orthologs. Although PhParkin has been analysed previously, it and other insect Parkin orthologs may not faithfully recapitulate the HsParkin activation mechanism despite possessing a pUb-binding site. The ACT, instrumental for HsParkin activation (Fig 4.3), is absent from insect variants. It is possible that an analogous function to the ACT is carried out by the N-terminal hydrophobic sequence extension in insect Parkin variants (Appendix Fig B.1), especially as N-terminal overhangs have been shown to activate Parkin (Burchell et al., 2012). Moreover, PhParkin possesses a phosphomimetic Asp residue in place of the phosphorylatable Ser at the analogous 65 residue position.

Parkin variants likely to preserve an identical activation mechanism to HsParkin while containing deletions to reduce flexibility were found in more closely related, vertebrate species. Parkin orthologs from the Common garter snake, *Thamnophis sirtalis* (*Ts*Parkin) and Zebrafish, *Danio rerio* (*Dr*Parkin) possess multiple AA deletions in the flexible Ubl-UPD linker while retaining the conserved ACT element and Ser65 (Fig 6.2 A, Fig 4.2). While both orthologs were tested in crystallization trials, *Ts*Parkin was favoured due to its shorter Ubl-UPD linker.

#### 6.2.1. Enzymatic and dynamic properties of TsParkin

To test whether TsParkin can be activated by binding to phospho-Ub or phosphorylation to the same extent as HsParkin, two biochemical assays were employed. Under comparable conditions, the ability of TsParkin to generate free Ub chains and autoubiquitination seems impaired when compared to HsParkin (Fig 6.2 B). Despite the lower turnover, significant activation upon phosphorylation on the level of Ub dimers is seen. The relatively small amount of activation upon pUb addition is recapitulated in TsParkin. To compare the reactivity profile to HsParkin, a Ub ABP assay was carried out (Fig



Figure 6.2.: Thamnophis sirtalis Parkin (A) Portion of Parkin alignment from Fig 4.2. (B) TsParkin assembly assay (left) compared to HsParkin (right) from the same experiment: diUb formation is promoted by phosphorylation although overall activity is lower. (C) TsParkin ABP reactivity assay: phospho-TsParkin only reacts with Ub-VS analogously to phospho-HsParkin. (D) Heatmap representing the difference in uptake resulting from formation of a phospho-TsParkin-Ub conjugate for t = 3 s on ice. 139 peptides were detected with sequence coverage of 90.7 %. Top numbering shows TsParkin domain boundaries. For the corresponding difference plot, see Fig D.1, Appendix D.

6.2 C). TsParkin only reacts with Ub-VS with a similar proportion to HsParkin in DTT-containing buffer (see Section 5.5).

To further assess the ability of TsParkin to populate the active state when covalently bound to pUb, the effect of Ub-VS addition on the dynamics of the phospho-TsParkinpUb covalent complex was investigated by HDX MS (Fig 6.2 D), analogously to HsParkin (Fig 5.11 A). Analysis of the shortest time point upon reaction reveals a very similar profile as was observed for HsParkin, suggesting that TsParkin is able to populate the same active state. The phospho-Ubl domain (region (1)), ACT (region (7)) and UPD phospho-pocket (region (4)) are protected from solvent exchange. In the TsParkin analysis a clear protection of the RING2 domain is seen as a result of interaction with donor Ub (region(9)), (postulated in Section 5.5.2). The C-terminal helix of the RING2 domain is exposed (region (6)), suggesting further displacement from the autoinhibitory position as phospho-TsParkin-Ub-pUb is locked in the active state.

Despite the truncations in the Ubl-UPD linker, recapitulation of any of the available phospho-*Ts*Parkin complexes did not yield *Ts*Parkin-containing crystals. In contrast, a higher level of co-purification of the *E. coli* GroEL chaperone lead to the appearance of poorly diffracting GroEL crystals. The same GroEL crystal morphology was observed in (Kiser et al., 2007).

#### 6.2.2. Limited proteolysis of *Ts*Parkin

I reasoned that further reduction in flexibility of the system could be achieved through limited proteolysis of phospho-Parkin. Since hydrophobic interfaces are exposed upon partial/full activation, optimizing the expressed construct for the activated state leads to protein insolubility. For instance removing the RING2 domain to lock phospho-Parkin in the active state renders the expressed, unphosphorylated, inactive state insoluble as the hydrophobic residues on the UPD would be exposed (Sriram et al., 2005). Limited proteolysis on the other hand, would allow construct adjustment, following expression of the inactive state and the activation by *in vitro* phosphorylation.

Crosslinking could offer another avenue to stabilize the system, however 'trapping' the phospho-Parkin equilibrium in such a way would only yield a proportion of molecules crosslinked in the active state, further reducing the yield. Additionally heterogeneities might arise even within the fraction of phospho-Parkin molecules trapped in the active state limiting downstream utility.

Limited proteolysis on either phospho-*Ts*Parkin-pUb (Fig 6.3 A) or phospho-*Ts*Parkin-Ub-pUb (Fig 6.3 B) was carried out using a variety of proteases. Some proteases known to cleave Parkin in other settings (e.g. Pepsin or Trypsin) were inactive, however Actinase, Proteinase K, Elastase, Subtilisin and Papain were able to cleave Parkin in this assay.

The most interesting cleavage profiles were obtained from Elastase and Papain (red rectangles, Fig 6.3). Both phospho-*Ts*Parkin-pUb and phospho-*Ts*Parkin-Ub-pUb were cleaved broadly into two fragments, corresponding to a single cleavage event. Phospho-*Ts*Parkin-pUb was cleaved into a large fragment just below the 49 kDa marker and a small fragment just above the 6 kDa marker (red arrows, Fig 6.3). The phospho-*Ts*Parkin-Ub-pUb was cleaved into a large fragment also just below the 49 kDa marker and a smaller fragment around 16 kDa (red arrows, Fig 6.3). Therefore covalent addition of Ub-VS to the RING2 active site increased the molecular weight (MW) of the smaller fragment approximately by the size a Ub, suggesting that the small fragment corresponds to the RING2 domain.



Figure 6.3.: Limited proteolysis of activated *Thamnophis sirtalis* Parkin (A) Limited proteolysis of the phospho-*Ts*Parkin-pUb covalent complex performed by various proteases as indicated. (B) As in A, for the phospho-*Ts*Parkin-Ub-pUb covalent complex. Red arrows and rectangles mark a change of cleavage pattern after Ub-VS conjugation of phospho-Parkin.

Consequently the large fragment is of great interest, as it encompasses the phospho-Ubl, ACT, UPD, RING1, IBR and the covalently linked pUb likely forming the intramolecular interface specific to active Parkin. If stable, crystallographic analysis of this fragment would eliminate the presence of the RING2(-Ub) unit flexibly tethered to the Parkin core.

# 6.3. A phosphorylation-dependent Parkin cleavage site

#### 6.3.1. Limited proteolysis using Elastase

To confirm the cleavage pattern suggested by the initial limited proteolysis experiment and determine whether this is specific to activated, phospho-TsParkin complexes, limited proteolysis was carried out using a range of TsParkin complexes as substrates (Fig 6.4 A). Elastase was used as the protease, as this yielded the cleanest cleavage pattern in the previous experiment (Fig 6.3). Separate reactions were used for analysis by intact-mass Liquid Chromatography-Mass Spectrometry (LC-MS) of the fragments.

Cleavage of unphosphorylated TsParkin only yields two fragments as was the case for phospho-TsParkin complexes (Fig 6.3). In contrast to phospho-TsParkin, the smallest fragment appeared larger and was shifted up above the 6 kDa marker, while the large fragment was found at the 38 kDa marker. The principal peak at a mass of 38,196.8 Da was flanked by peaks at 37.985.5 Da and 38,382.6 Da (Fig 6.4 B). In each case the spacing between the flanking peaks and the principal peak corresponded to two AA specific to the UPD-RBR linker, allowing unambiguous identification of the large fragment as C-terminal, spanning residues 116-452. LC-MS analysis of the smaller fragment yielded a mass of 11,049.8 Da (Fig 6.4 C). This mass could either correspond to residues 1-96 including the N-terminal GP cloning overhang (see Methods, Table 2.1) or to residues 359-452. Assignment of the large fragment as C-terminal points against the second possibility, and identifies the smaller fragment as the N-terminal 96 residues. Remarkably, this suggests that the predominant cleavage event in unphosphorylated TsParkin occurs within the conserved ACT element and is likely followed by C- or N- terminal elastase cleavages.



Figure 6.4.: Elastase-mediated limited proteolysis of TsParkin complexes

(A) TsParkin, phospho-TsParkin, phospho-TsParkin-pUb, phospho-TsParkin-Ub-pUb are subjected to limited proteolysis by Elastase from the Proti Ace kit (Hampton). A subset of bands were identified by intact-mass LC-MS (red rectangles). (B) Deconvolution of the larger peak on the LC-MS spectrum of cleaved TsParkin, obtained mass corresponds to residues 116-452. (C) Deconvolution of the smaller peak on the LC-MS spectrum of cleaved TsParkin, obtained mass corresponds to residues 1-96 including the N-terminal GP overhang. (D) Deconvolution of the smaller peak on the LC-MS spectrum of cleaved phospho-TsParkin-Ub-pUb, obtained mass corresponds to residues 389-452, where the catalytic Cys 418 has been modified with Ub-VS (8,625 Da). (E) Deconvolution of the smaller peak on the LC-MS spectrum of cleaved phospho-TsParkin-pUb, obtained mass corresponds to residues 389-452.

Multiple bands are observed upon cleavage of the activated phospho-*Ts*Parkin complexes, and only the most prevalent smallest fragments could be analysed by LC-MS (Fig 6.4 D, E). Cleavage of phospho-*Ts*Parkin and phospho-*Ts*Parkin-pUb yields the same size prevalent smallest band, which is shifted up in the phospho-*Ts*Parkin-Ub-pUb complex. The predominant small-fragment mass of 7,509.7 Da corresponded to the C-terminal RING2 389-452 residues (Fig 6.4 E), which was increased to 16,132.8 upon reaction with Ub-VS corresponding to addition of the Ub-VS probe to the catalytic Cys (8,625 Da), (Fig 6.4 D). The larger fragments of these cleavage reactions could not be identified by LC-MS, but the predominant large band likely corresponds to the phospho-Ubl-ACT-UPD-RING1-IBR(-pUb) fragment while other fragments likely arise from overcleavage and are annotated in the legend of Fig 6.4.

Strikingly, this means that there is a change in the dominant cleavage site upon activation by phosphorylation. This is likely due to the fact that the preferred ACT cleavage site is protected in activated Parkin complexes, while other sites simultaneously become more accessible. Not only can the limited proteolysis assay be used to read-out the state of the Parkin equilibrium, but it is a valid way to achieve construct adjustment upon activation for crystallization trials.

## 6.3.2. Preparation of $\Delta$ RING2 phospho-Parkin using TEV protease

In situ proteolysis by addition of elastase to crystal drops was unsatisfactory: at higher protein concentrations required for crystallography trials, overcleavage was more prominent leading to sample heterogeneity or loss of the desired phospho-Ubl-ACT-UPD-RING1-IBR(-pUb) fragment. To better control phospho-Parkin cleavage, a Tobacco Etch Virus (TEV) protease cleavage site was inserted following the IBR domain of both TsParkin and HsParkin (Fig 6.5 A).

To prepare the  $\Delta$ RING2 fragment of interest encompassing the intramolecular interface unique to active Parkin, a multistep purification protocol was developed (Fig 6.5 B,C). In the first step, full-length Parkin containing the TEV protease cleavage site and the engineered IBR Cys for pUb linkage is affinity and anion-exchange purified (see Methods 2.3.3). This is then incubated with GST-*Ph*PINK1, MgATP and the Ub-C3Br ABP. In this second step, both the Parkin Ubl domain and the Ub-C3Br ABP are phosphorylated while the pUb ABP probe is covalently attached to the engineered IBR Cys residue. Following this step, the reaction is affinity purified to remove GST-PhPINK1. In the third and final step, His<sub>6</sub>-TEV protease is added to the phospho-Parkin-pUb covalent complex to initiate cleavage of the RING2 domain at the engineered TEV site. Following cleavage, the mixture is affinity purified to remove the His<sub>6</sub>-TEV protease and applied to anion and size-exclusion chromatography to yield the final sample. The cleaved RING2 domain does not co-elute with the fragment of interest (phospho-Ubl-ACT-UPD-RING1-IBR(-pUb)) on size-exclusion chromatography, suggesting loss of the RING2 autoinhibitory binding site on the Parkin core (Fig 6.5 D), (see Methods 2.4.4).



Figure 6.5.: TEV protease-mediated cleavage yields a  $\Delta$ RING2 phospho-Parkin fragment. (A) Insertion of the TEV protease cleavage site following the IBR domain for both *Ts*Parkin and *Hs*Parkin. (B) Schematic representation of TEV protease-cleaved phospho-Parkin(-pUb) sample preparation. (C) Coomassie analysis of TEV protease-cleaved phospho-*Hs*Parkin-pUb preparation. (D) Preparative SEC profile showing dissociation of the cleaved RING2 domain from TEV-cleaved *Ts*Parkin.

#### 6.3.3. The impact of RING2 removal on Parkin dynamics

Our previous HDX MS analysis showed that covalently linking the Parkin RING2 active site Cys to the Ub-VS ABP reveals the hallmarks of active Parkin, as this covalent complex can not access the autoinhibited conformer (see Section 5.5.2). We postulate that upon removal of the RING2 domain, the remaining phospho-Ubl-ACT-UPD-RING1-IBR(-pUb) N-terminal fragment should recapitulate the intramolecular interface of the new active conformer, and display identical dynamics to the Ub-charged species. With the availability of a clean phospho-Parkin(1-382)-pUb sample, the impact of RING2 removal on Parkin dynamics can be assessed and compared to the known hallmarks of



Figure 6.6.: The  $\Delta$ RING2 phospho-Parkin fragment recapitulates active Parkin. (A) Top: heatmap representing the difference in uptake resulting from formation of a phospho-*Ts*Parkin-Ub-pUb as in Fig 6.2 D, repeated for clarity. Bottom: heatmap representing the difference in uptake resulting from cleavage and removal of the RING2 domain from phospho-*Ts*Parkin-pUb for t = 3 s on ice. 131 peptides were detected with sequence coverage of 95.4 %. For the corresponding difference plots see Figs D.1 and D.2, Appendix D. (B) Top: heatmap representing the difference in uptake resulting from phospho-*Hs*Parkin-Ub formation in the presence of non-covalent pUb as in Fig 5.11 A, remeasured at t = 3, 30, 300 s. 124 peptides were detected with sequence coverage of 93.6 %. Bottom: heatmap representing the difference in uptake resulting from cleavage and removal of the RING2 from phospho-*Hs*Parkin measured at t = 3, 30, 300 s. 117 peptides were detected with sequence coverage of 99.3 %. For the corresponding difference plots see Figs D.3 and D.4, Appendix D.

active Parkin revealed upon Ub-VS addition.

A comparative HDX MS experiment was carried out, where both the  $\Delta$ RING2 and the Ub-VS phospho-Parkin complexes were compared with the relevant phospho-Parkin state. A single shortest time point was analysed for *Ts*Parkin (Fig 6.6 A), while three longer time points were analysed for *Hs*Parkin (Fig 6.6 B). In both cases, the two relative uptake heatmap plots look nearly identical in the two conditions. All hallmarks of the active state are recapitulated: Ubl protection (region (1)), ACT protection (region (7)) and UPD phospho-pocket protection (region (4)).

Therefore, the  $\Delta$ RING2 fragment recapitulates the relevant active Parkin state and determining its structure would be of great interest. Interestingly, it also removes ambiguity with regards to the function of the ACT element - donor Ub is absent from the  $\Delta$ RING2 sample and ACT protection persists. This strongly suggests the ACT is part of the new active Parkin intramolecular interface. Additionally, together with the lack of association of the cleaved RING2 with the core of Parkin upon purification following TEV cleavage, this data further suggests independent behaviour of the RING2-Ub unit in active Parkin, as no site was additionally exposed upon its removal.

#### 6.4. Structure of phospho-*Hs*Parkin(1-382)-pUb

Following crystallization trials with  $\Delta$ RING2 complexes from both *Ts*Parkin and *Hs*Parkin, crystals appeared for the phospho-*Hs*Parkin(1-382)-pUb covalent complex.

Several conditions in a 100 nl sitting drop initial screen yielded crystals. Although some conditions resulted in needle-like crystals, which were not amenable to systematic optimization and yielded no diffraction (Fig 6.7 A), others found in the Morpheus screen were three dimensional (Gorrec, 2009). Fine screening identified the crystal form as readily reproducible (crystals grew in <48 h) and suggested the original condition was most preferred for yielding larger crystals with a well-defined morphology (Fig 6.7 C). Small crystals produced by fine-screening yielded initial diffraction to  $\sim 3\text{\AA}$  (Fig 6.7 B). To maximize potential utility of the structure, we sought to improve the diffraction resolution by increasing crystal size. Streak seeding using a cat's whisker (shed by the



Figure 6.7.: Crystals obtained of phospho-HsParkin(1-382)-pUb. P = precipitant, A = additive, B = buffer. (A) Initial hit (100 nL sitting drop format) in the indicated JBScreen Classic 6 screen (Jena Bioscience) condition at [protein]=4 mg/ml. (B) Initial hit (100 nL sitting drop format) in the indicated Morpheus screen condition at [protein]=4 mg/ml (Gorrec, 2009). (C) Fine screen at yields best crystals in the original condition (500 nL sitting drop) at higher [protein]. (D) Larger, weakly polarizing crystals obtained following streak seeding in a hanging drop format in the original condition.

domestic cats (*Felis catus*) Muffin or Mittens) in a hanging drop setting yielded crystals over 100 µm in length (Fig 6.7 D).

#### 6.4.1. Overall structure

Improved crystals diffracted to 1.8Å and resulted in a high resolution structure of the phospho-*Hs*Parkin(1-382)-pUb covalent complex with only one molecule per Asymmetric Unit (ASU) in the P3<sub>2</sub>21 space group (Fig 6.8 A, B), (Table 6.1), (PDB ID: 6GLC). Overall electron density was clear (Fig 6.8 C, left), especially in the core (Fig 6.8 C, right).

The Parkin core domains (UPD, RING1 and IBR shown with a surface representation in Fig 6.8 A) are arranged identically to the previously determined pUb-bound Parkin structures (Wauer et al., 2015a; Kumar et al., 2017a), pUb is contacted by the same interface and joined to the IBR domain by a covalent 3CN linkage, originating from the pUb-C3Br C-terminal warhead. This similarity allowed molecular replacement using



Figure 6.8.: The structure of phospho-*Hs*Parkin(1-382)-pUb. (A) Crystal structure at 1.8 Å resolution of phosphorylated covalent complex of *Hs*Parkin and pUb where the RING2 domain has been removed by TEV cleavage. Domains are coloured as previously. Core Parkin domains are shown in a cartoon and surface representation. Phospho-Ser residues are shown in a ball and stick representation, while the 3CN covalent linkage between the IBR domain Cys347 residue and the ABP warhead is shown as sticks. (B) Schematic representing the domain arrangement in A. (C) A composite omit density map generated with simulated annealing shown for the single complex in the asymmetric unit. 2|Fo|-|Fc| electron density is shown at  $1\sigma$ , detail for the the phospho-Ser residues is shown to the right (D) Density as in C, for the ACT interacting with the UPD hydrophobic interface, left. The isolated rainbow coloured peptide is shown in additional orientations, right.

search models consisting of the pUb-bound UPD-RING1-IBR (Parkin core) fragment from PDB ID: 5N2W (Kumar et al., 2017a), and the Ubl domain from PDB ID: 5C1Z (Kumar et al., 2015).

In stark contrast to any previous Parkin structures, the domain arrangement in phospho-HsParkin(1-382)-pUb is poised to carry out the E3 RBR catalytic cycle and our structure therefore represents active Parkin. The canonical E2-binding site on the RING1 domain is free to interact with an incoming  $E2 \sim Ub$  thioester conjugate, as the autoinhibitory interactions with the Ubl domain and the REP element are absent (Fig 6.8 A). Instead, the phospho-Ubl domain forms a new activating interface with the UPD on the opposite face of the core UPD-RING1-IBR module. The Ubl phospho-Ser65 residue on the Ubl domain interacts with the previously identified phospho-pocket on the UPD domain (Wauer and Komander, 2013), (see Section 5.3.1).

Additional peptide density interacting with the hydrophobic residues on the UPD is observed (Fig 6.8 D, left). Additional electron density could be assigned to the Ubl-UPD linker, as this linker is the only thus far unassigned portion of the crystallized construct. Although the solvent-channel exposed density is not as clear as in core parts of the structure, two residues are fully resolved in the unbiased omit map at  $1\sigma$  contour level (position 3 and 4 in Fig 6.8 D, right). The AA in position 3 branched at the  $\gamma$ position (Leu, Asp or Asn) and AA in position 4 AA is branched at the  $\beta$  position (Thr or Val) and together they mandate directionality to the bound portion of the linker. Additionally, it is likely that the hydrophobic UPD pocket is contacted by hydrophobic residues (corresponding to positions 3,6 and 8). Only the two conserved regions within the Ubl-UPD linker contain stretches of hydrophobic residues: ACT = SLTRVDLS and GLAVIL = SVGLAVIL (Fig 6.2 A). The Leu and Thr of the ACT element are therefore the only unique sequence fulfilling the requirements for positions 3 and 4. Furthermore the ACT element places Val in position 6 (rotamer is not fixed) and Leu in position 8, satisfying the requirement for hydrophobic interactions with the UPD. The context of placing ACT into the density is also sensible: density for the positively charged Arg104 guanidino-group is in proximity for the negatively charged Asp60 of the phospho-Ubl capable of electrostatic interactions, while Thr103 is within hydrogen bonding distance of Asp60.



Figure 6.9.: Mapping the HDX MS-derived active interface onto the phospho-HsParkin(1-382)-pUb structure. Data from the changes in hydrogendeuterium exchange upon conjugation of the Ub-VS ABP with the active site RING2 Cys as in Fig 5.4 for t = 3 s is mapped onto the phospho-HsParkin(1-382)-pUb structure.

In addition to revealing the active Parkin domain arrangement, our structure also unambiguously shows that the ACT contributes to the activating interface by contacting both the UPD and the phospho-Ubl. This placement of the ACT is in very good agreement with its protection from deuterium uptake upon stabilization of the the active interface when Parkin is conjugated with Ub via the active site (see Section 5.5.2). Mapping this data onto the newly obtained structure of phospho-HsParkin(1-382)-pUb reveals that protection of the Ubl (region (1)), the ACT (region (7)) and the UPD phospho-pocket (region (4)) are due to the new activating Parkin interface, while no significant changes occur in the rest of the structure (Fig 6.9).

As we do not see a full connectivity linking the phospho-Ubl domain to the ACT element (28 AA) and the ACT element to the UPD (34 AA), it cannot be excluded that either the Ubl or the ACT interact *in trans* with a complex from a neighbouring ASU. Since the interface is recapitulated by our HDX MS measurements carried out at a relatively low Parkin concentration ( $\sim 1 \mu$ M), it is likely that at least in solution the interaction is intramolecular.

|                               | phospho-Parkin (1-382) - phospho-Ub |
|-------------------------------|-------------------------------------|
| Data collection               |                                     |
| Space group                   | P 3 <sub>2</sub> 2 1                |
| Cell dimensions               |                                     |
| a, b, c (Å)                   | 83.93, 83.93, 105.12                |
| $\alpha, \beta, \gamma$ (°)   | 90, 90, 120                         |
| Resolution (Å)                | 72.69 –1.80 (1.84 – 1.80)           |
| R <sub>merge</sub>            | 0.065 (0.773)                       |
| 1/ σľ                         | 13.80 (2.40)                        |
| Completeness (%)              | 100.00 (99.30)                      |
| Redundancy                    | 6.7 (6.7)                           |
|                               |                                     |
| Refinement                    |                                     |
| Resolution (Å)                | 59.79 –1.80                         |
| No. reflections / test set    | 40229 / 2020                        |
| $R_{ m work}$ / $R_{ m free}$ | 0.180 / 0.205                       |
| No. atoms                     |                                     |
| Protein                       | 3039 (398 aa)                       |
| Ligand/ion                    | 41                                  |
| Water                         | 165                                 |
| <i>B</i> -factors             |                                     |
| Protein                       | 45.05                               |
| Ligand/ion                    | 66.19                               |
| Water                         | 46.94                               |
| R.m.s. deviations             |                                     |
| Bond lengths (Å)              | 0.008                               |
| Bond angles (°)               | 1.19                                |

\*Values in parentheses are for highest-resolution shell.

**Table 6.1.:** Data collection and refinement statistics for phospho-HsParkin(1-382)-pUb.

#### 6.4.2. The new active Parkin intramolecular interface

The activating Parkin interface requires the phospho-Ubl, UPD and the ACT element. Analysis of the active phospho-HsParkin(1-382)-pUb structure in light of the previously resolved pUb-bound truncated unphosphorylated HsParkin structure (referred to as 'inactive Parkin'), (PDB ID: 5N2W), (Kumar et al., 2017a) helped us gain understanding of the mechanism by which the new active domain arrangement dislodges the catalytic RING2 domain from its autoinhibitory interaction with the UPD and maintains accessibility of the catalytic Cys (Fig 6.10).



Figure 6.10.: Comparison with the  $HsParkin\Delta 61$ -pUb covalent complex. (A) Overlay of phospho-HsParkin(1-382)-pUb (top left) with a covalent HsParkin-pUb complex with 61 residues deleted in the Ubl-UPD linker (PDB ID: 5N2W), (Kumar et al., 2017a). RMSD (core) = 0.73 Å. The Ubl position changes by over 52.7 Å (Distance between the two Ser65 residues, dotted line). (B) An open book view of the RING2:UPD autoinhibitory interface (middle) in the  $HsParkin\Delta 61$ -pUb complex. On either side, interacting residues on each domain either side are shown in a ball and stick representation. (C) An open book view of the Ubl-ACT-UPD activating interface (middle) in the phospho-HsParkin(1-382)-pUb complex as in B.

The two structures align very well on the core domains (UPD, RING1 and IBR) with an RMSD of 0.73 Å (Fig 6.10 A). Most striking is the motion of the Ubl domain upon phosphorylation between the autoinhibitory interface in unphosphorylated Parkin and its activating binding-site upon phosphorylation. The distance separating the Ser65 residues in both structures is 52.7 Å (dotted line, Fig 6.10 A, right). The position of the bound, linked pUb molecules is nearly identical in both structures, while a small tilt of the tip of the UPD towards the pUb molecule in the phosphorylated Parkin structure can be observed. When all resolved Parkin structures are aligned, the relative position of the UPD with respect to the RING1 domain varies within the range seen in this alignment (not shown).

Binding of the phospho-Ubl to the UPD is mutually exclusive with the RING2:UPD autoinhibitory interaction seen in unphosphorylated Parkin due to steric clashes. However the two UPD binding sites do not overlap exactly as phospho-Ubl binding does not utilize the same hydrophobic interface as RING2 binding (see Introduction 1.12.1), (Fig 6.10 A, right). In contrast, the ACT element utilizes its amphiphatic nature to interact with the hydrophobic UPD pocket vacated by the RING2 and use solvent-exposed polar residues to contact the phospho-Ubl.

According to PISA analysis (Krissinel and Henrick, 2007), the autoinhibitory RING2:UPD interface buries an area on the UPD of ~600 Å<sup>2</sup> in autoinhibited Parkin (Fig 6.10 B). Hydrophobic residues of the C-terminal RING2 helix (Met458, Trp462, Phe463 and Val465) interact with the highly hydrophobic UPD interface. While the phospho-Ubl covers an area of more than 800 Å<sup>2</sup> on the UPD, only a few UPD residues contribute to both RING2 and phospho-Ubl binding in phosphorylated Parkin (Lys161, Arg163, Val164 and Leu176), (Fig 6.10 C). ACT binding to the UPD buries a further area of ~340 Å<sup>2</sup>, and in contrast to the phospho-Ubl binding interface, all 12 ACT-interacting residues on the of UPD in active Parkin also interact with the RING2 in inactive Parkin.

Analysis of the UPD-interacting surfaces in autoinhibited unphosphorylated Parkin and active phosphorylated Parkin shows that while the phospho-Ubl likely dislodges the RING2 from its autoinhibitory position, the ACT element is required to efficiently compete with the RING2 and sustain its exposure required for Ub transfer. This key role of the ACT element explains why  $\Delta$ ACT phospho-Parkin does not react with the activesite Ub-VS ABP as the active state is severely disfavoured in the mutant (see Section 4.3).

#### 6.4.3. Detail of the activating phospho-Ubl-UPD interface

Positioning of the phospho-Ubl on the UPD domain seems to be primarily governed by the optimized binding between phospho-Ser65 and the positively charged UPD phosphopocket consisting of residues Lys161, Arg163 and Lys211 (Fig 6.11 A). This electrostatic interaction is supported by an intricate hydrogen bonding network, where each oxygen of the phospho-Ser65 head group is engaged in at least one hydrogen bond, and additionally Lys161 also coordinates Asp62 of the Parkin phospho-Ubl domain (Fig 6.11 A).

The importance of this binding interface is emphasized by several mutations found in patients suffering from early-onset PD. K211N and K161N mutations have recently been reclassified as pathogenic (Yi et al., 2018) and two early-onset PD sufferers carrying a likely disease-causing S65N variant have recently been identified (McWilliams et al., 2018).

Removal of the key interaction of the phospho-Ser65 with the UPD phospho-pocket residues means that the phospho-Ubl is not able to bind to the UPD to dislodge the RING2 domain. This is reflected by both in the inability of K211N phospho-Parkin to react with the Ub-VS ABP (Fig 5.5 B) or assemble Ub conjugates *in vitro* (Wauer et al., 2015a). Disruption of this interaction also explains the increased deuterium uptake in the phospho-Ubl upon introduction of the K211N mutation in our HDX MS measurements (Fig 5.5 C) - the active state which protects the phospho-Ubl from solvent exchange cannot be accessed in this mutant. Neither K211N nor K161N Parkin localize to mitochondria under conditions inducing mitophagy (Ordureau et al., 2014; Yi et al., 2018), which can be explained by the observed *in vitro* activity defects. Parkin activity is vital to deposit Ub on mitochondrial substrates which can serve, upon phosphorylation by PINK1, as a localization signal for further Parkin recruitment. Absence of this positive feedback leads to insufficient mitochondrial localization of Parkin from the cytosol.

Further interaction between the phospho-Ubl and the UPD is mediated via the hydrophobic Ile44 patch of the Parkin Ubl (Fig 6.11 B). In the Parkin Ubl this patch consists of Ile44, Val70 and His68 and interacts with UPD residues 170-176, 197 and 198. His68 of the phosho-Ubl is engaged in a hydrogen bond with a conserved Asp219 residue in the linker between the UPD and RING1 domains of Parkin, while backbone-mediated hydrogen bonding also links Gly47 of the phospho-Ubl and Leu176 of the UPD.

As the major form of pUb contains both Ser65 and an identically positioned Ile44 hydrophobic patch, our analysis of the binding site does not exclude the possibility of pUb binding to the site identified here (Fig 6.11 B). One noticeable difference is in the position of the flexible Leu8 loop, the loop is not as extended as in the major form of pUb (e.g. the pUb moiety from the structure described here). Moreover the Leu8 loop of the phospho-Ubl is able to sandwich the positively charged Arg6 (substituted to Lys in Ub) between Asp219 and Gln171 in the Parkin core. Although only Parkin activity experiments with Ub-like residue substitutions in the Ubl would be able to reveal the extent to which these might weaken phospho-Ubl binding, the most likely factor favouring phospho-Ubl binding is the intramolecular nature of this interaction. Rearrangement nec-



Figure 6.11.: Detail of the activating phospho-Ubl interface. (A) Front view of the activating interface between the phospho-Ubl and the UPD is shown, interacting residues are shown as sticks and phospho-Ser65 residues are shown in a ball and stick representation. Label colours correspond to domain colouring. Hydrogen bonds are shown as dashed lines. Ile44 is coloured blue. (B) Back view of the activating interface. The major form of pUb (orange), co-crystallized with our structure is superimposed onto the phospho-Ubl. Common Ub/Ubl elements are labelled in black, while other labels are coloured as in A.

essary for this intramolecular interface to form also positions the ACT in proximity of its binding site to more effectively compete with the RING2 domain. If present, competition between phospho-Ubl and pUb could not impede the formation of our crystals, as pUb is covalently linked to the IBR and therefore constrained to the canonical pUb binding site.

#### 6.4.4. Detail of activating ACT binding site

In addition to covering the hydrophobic interface utilized by RING2 in the inactive unphosphorylated Parkin structures (Fig 6.10), the ACT also interacts with the phospho-Ubl domain, overall strengthening the activating interface (Fig 6.12 A).

The ACT forms a  $3_{10}$  helical turn where the carboxyl group of Ser101 makes a hydrogen bond with the Arg104 backbone amide hydrogen, three residues downstream. This allows the ACT to simultaneously place three hydrophobic residues, Leu102, Val105 and Leu107, into the UPD hydrophobic pocket and make polar contacts with the phospho-Ubl. Strikingly, both the direction and position of the ACT backbone as well as the UPD hydrophobic pockets occupied by Leu102, Val105 and Leu107 mimic the way in which residues Trp462 and Phe463 of the RING2 C-terminal helix bind to the UPD in autoinhibited Parkin (Fig 6.12 B,C).

Hydrogen bonds formed by the backbones of Ala46 and Leu102 and the side chains of Thr103 and Asp60 anchor the ACT to the phospho-Ubl (Fig 6.12 A). Further crucial electrostatic interactions hold Arg104 in proximity of Asp60. The side chain of the Ala46 residue packs tightly against the ACT, while Gly47 packs predominantly against the UPD, placing the Gly47 loop of the phospho-Ubl at the centre of the interface. Somewhat interestingly, the same loop mediates substrate-kinase contacts in the *Ph*PINK1:TVLN Ub complex (Schubert et al., 2017) and an A46T mutation in the Parkin Ubl reduces mitophagy by 60% (Yi et al., 2018). We have not ascertained whether this could be due to a difference in affinity for PINK1 or disruption of the activating interface, but importantly this variant seems to also be found in multiple individuals (including homozygotes) unaffected by YOPD, rendering its effect on the PINK1/Parkin-mediated mitophagy likely benign (Yi et al., 2018; Lek et al., 2016).



Figure 6.12.: Detail of activating ACT binding site (A) An inset showing ACT binding to the UPD in the activated Parkin structure, the amphiphatic nature of the ACT places hydrophobic residues at the hydrophobic interface, while polar residues contact the phospho-Ubl. (B) An inset showing RING2 binding to the UPD in the inhibited Parkin structure, same view as in A. Hydrophobic residues face the pocket, trapping the catalytic Cys431. (B) An overlay of both elements interacting with the UPD.

The importance of ACT contacts with both the Ubl and the UPD for stabilizing the active state of Parkin can help elucidate the basis of disease for carriers of the R104W Parkin variant (so far, no homozygotes for this variant were found in the population), (Chaudhary et al., 2006; Varrone et al., 2004; Nuytemans et al., 2010b; Cruts et al., 2012; Yi et al., 2018; Lek et al., 2016). Arg104 contacts Asp60 in the phospho-Ubl, its loss explains our findings that a single R104A point mutation decreases Parkin reactivity with the Ub-VS ABP and reduces the activity of phospho-Parkin *in vitro* (see Fig 4.3). The R104A mutation disrupts the interface between the ACT and phospho-Ubl sufficiently to disfavour the active state and lower the activity. Partial inactivation would impact on the rate of Parkin mitochondrial localization under mitophagy-inducing conditions, akin to the UPD phospho-pocket mutants. Recently, the R104W mutant has been shown to reduce mitophagy by 50% relative to wt Parkin, while maintaining the same level of expression, consistently with its role in the structure (Yi et al., 2018).

#### 6.4.5. $E2 \sim Ub$ binding to active Parkin

An independent, parallel approach to capture the active Parkin interface by determining a phosphorylated  $\Delta RING2$  Parkin structure was utilized by the Gehring laboratory, who determined a stucture of phospho-UBE2L3-Parkin $\Delta RING2$  : pUb using Parkin from Bactrocera dorsalis, the Oriental fruit fly (BdParkin), (Sauvé et al., 2018).

Similarly to other insect orthologs of Parkin, BdParkin contains an N-terminal extension of 29 AA containing multiple hydrophobic residues and the UBL-UPD linker is unconserved between BdParkin and HsParkin (the ACT is absent), (Appendix Fig B.1). In contrast to PhParkin, a Ser residue is present at position 65 in BdParkin. To generate a crystal-yielding construct, both the N-terminal extension and the Ubl-UPD linker were truncated, and the N-terminus fused to the H. sapiens UBE2L3 enzyme by a 10 AA linker. This construct was produced and phosphorylated *in vitro*. Upon structure determination of the non-covalent complex with pUb at 4.8 Å, the RING2 domain was missing. To obtain an improved resolution structure at 3.8 Å TcPINK1 was co-expressed with BdParkin truncated as above with additional deletion of the RING2. As co-translational phosphorylation is possible in this system, the protein is not rendered insoluble. The short fusion linker mandates E2 interaction *in trans* with the neighbouring ASU complex.

The resulting structure of phospho-UBE2L3- $BdParkin\Delta RING2$ : pUb shows the same domain arrangement as the structure of phospho- $HsParkin\Delta RING2$ -pUb described here the phospho-Ubl is bound to the UPD phospho-pocket in the activating interface, while pUb and the core Parkin domain are arranged as in the previous pUb-bound structures (Wauer et al., 2015a; Kumar et al., 2017a), (Fig 6.13 A). A major difference stems from the absence of the ACT element. Although this element is not present in the BdParkinsequence, sequences containing hydrophobic residues in either the N-terminus or the Ubl-UPD linker that could serve a similar purpose to the ACT were deleted from the crystallized construct. Interestingly, Gehring and colleagues show that the UBE2L3-fused, truncated BdParkin remains active in solution. This could either be due to the fact that the activation of BdParkin does not require the ACT element as suggested by sequence analysis or that the need for some activation elements are partially bypassed by the dimerisation of the fusion constructs necessitated by the short linker.

The E2-binding site revealed by the phospho-UBE2L3-BdParkin $\Delta$ RING2 : pUb structure matches the conserved RING1 binding site. In fact, modelling of the E2-Ub conjugate onto our structure based on the structure of HOIP in complex with Ub and the UBE2D2-Ub Lys-linked conjugate via alignment of the RING1 domains reveals a near-identical E2 orientation (Lechtenberg et al., 2016), (Fig 6.13 B). The UBE2L3 residues interacting with Parkin are identical in UBE2D2 apart from one substitution of Glu60 in UBE2L3 for Ala in UBE2D2 (Sauvé et al., 2018). The donor Ub likely adopts a slightly different position in  $E2 \sim$  Ub-bound Parkin as suggested by our HDX measurements and NMR modelling of inhibited Parkin in complex with Lys-linked UBE2L3-Ub (see Section 5.4.1),



Figure 6.13.: E2 ~ Ub binding to active Parkin. (A) A structure of the phospho-UBE2L3-*Bd*Parkin $\Delta$ RING2 in a non-covalent complex with pUb reveals the UBE2L3 binding site on the RING1 domain of activated Parkin (Sauvé et al., 2018). (B) Model of Lys-linked UBE2D2-Ub conjugate binding to phospho-*Hs*Parkin(1-382)-pUb described here based on HOIP (PDB ID: 5EDV), (Lechtenberg et al., 2016). RMSD of RING1 domains = 3.72. The donor Ub must be slightly rearranged for binding to Parkin (see Section 5.4.1). (C) An overlay of the two active Parkin structures based on the core domains revelas an identical domain arrangement. RMSD of core = 0.76. The ACT is absent from the *Bd*Parkin structure and a small displacement of the IBR domain is seen upon covalent linkage of the pUb.

(Condos et al., 2018).

Interestingly, the phospho-UBE2L3-*Bd*Parkin $\Delta$ RING2 : pUb structure is the first Parkin structure determined where pUb was not covalently linked using the 3CN linkage (see Section 6.1.1). An overlay of the the two active Parkin structures on their core domains shows an identical position of the pUb, including an intermolecular  $\beta$ -sheet formed between the pUb C-terminus and an IBR domain strand (Wauer et al., 2015a; Sauvé et al., 2018), (Fig 6.13 C). In contrast, however, the IBR domains are displaced in the overlay between the two structures by ~1.5Å based on distances between the structural Zn atoms. Although small, this displacement may explain the decreased affinity between the covalent complex and the Lys-linked E2-Ub conjugates (see Section 6.1.2).

#### 6.4.6. The role of the RING2 in active Parkin

Upon determination of the active structures and NMR modelling of the Lys-linked UBE2L3-Ub conjugate binding (Gladkova et al., 2018; Sauvé et al., 2018; Condos et al., 2018), questions in the field have arisen whether the Parkin ubiquitination mechanism is concerted and  $E2 \sim Ub$  conjugate association also brings the subsequently formed RING2 ~ Ub in close proximity of the substrate, or whether after transfer the RING2 ~ Ub is able to dissociate from the Parkin core and ubiquitinate even more distant substrates within the reach of its 35 AA tether, according to the sequential model (Le Guerroue and Youle, 2018).

Exposure in the C-terminal helix of the RING2 domain upon Parkin phosphorylation has been observed by HDX MS in our experiments as well as in recent studies of E2-/E2-Ub-bound Parkin (Sauvé et al., 2018; Condos et al., 2018). While this suggests RING2 dissociation from the autoinhibitory interface, rebinding to other parts of Parkin cannot be excluded. Our ability to control RING2 removal allowed a comparison between the effect the truncation with the effect of Ub conjugation to Parkin by HDX MS. This comparison shows near-identical changes in deuterium uptake in both samples across two Parkin orthologs and suggests a RING2 binding site on the core of Parkin has not been missed by out previous analyses (Fig 6.6). To show that the RING2 domain behaves independently only upon Parkin activation by phosphorylation, I made further use of our constructs with an engineered TEV protease cleavage site. I prepared TEV protease-cleavable phosphorylated or unphosphorylated Parkin and cleaved both with TEV protease overnight. Although the IBR-REP linker is likely more exposed in the phosphorylated sample, we predicted that this condition would also result in cleavage of unphosphorylated Parkin. The resulting samples were applied to Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS) to examine the elution behaviour and apparent fragment masses after cleavage (Fig 6.14).

Only one peak was observed in the unphosphorylated Parkin chromatogram corresponding to the expected full-length Parkin mass of 52 kDa. Cleavage in this sample was confirmed in the resulting fractions. These also clearly show co-elution of the RING2 with the N-terminal Parkin fragment, indicative of tight association with the RING2 in the unphosphorylated, inactive state. In contrast, and as observed previously by preparative SEC (Fig 6.5 D), the phosphorylated sample eluted in two peaks with masses of 45 and 12 kDa respectively matching the predicted N- and C- terminal fragment masses well.



Figure 6.14.: RING2 dissociates from Parkin upon phosphorylation. SEC-MALS profiles for TEV protease-cleaved phosphorylated or unphosphorylated Parkin samples. The RING2 co-elutes with unphosphorylated Parkin, but dissociates from phosphorylated Parkin.

Our HDX MS experiments suggest that the RING2 contacts the donor Ub, as also predicted from homology with HHARI (Dove et al., 2016), (Fig 6.6). However, based on comparison with the HOIP structure in complex with the Lys-linked UBE2D2-Ub conjugate (Lechtenberg et al., 2016), little interaction is expected between the RING2 and the E2 enzyme. Together, these results suggest that once the Ub is transferred to the Parkin catalytic RING2 domain, the RING2 ~ Ub unit dissociates from activated Parkin and transfers Ub onto substrates within the reach its ~ 35 AA tether. In line with an independently operating RING2 ~ Ub unit, Parkin chain and substrate specificity is low (Ordureau et al., 2018; Sarraf et al., 2013; Ordureau et al., 2014). This is in stark contrast with HOIP, where the donor Ub is held by non-covalent interactions with an extension unique to HOIP to orient the N-terminus towards the active site for linear Ub formation (Stieglitz et al., 2013).

#### 6.5. Conclusion and discussion

Our structure of phospho-*Hs*Parkin(1-382)-pUb finally sheds light on the activating intramolecular interfaces observed by HDX MS described in the previous Chapter. Together, our new structural and dynamic understanding resolve a long-standing question in the PINK1/Parkin-mediated mitophagy and RBR fields, i.e.: what is the mechanism of Parkin activation by PINK1-mediated phosphorylation on Ser65 (Dove and Klevit, 2013).

The phospho-Ubl domain moves by >50Å from its autoinhibitory to its activating position and by binding to the UPD displaces the catalytic RING2 domain and stabilizes exposure of the catalytic Cys. Furthermore we determine the function of the Parkin ACT element, which is necessary to efficiently prevent autoinhibitory RING2 association with the UPD. Mutations in Ser65, the phospho-Ubl binding pocket as well as the ACT are found in patients with early-onset PD and can be explained by our activated Parkin structure. (Pickrell and Youle, 2015; Yi et al., 2018; McWilliams et al., 2018; Chaudhary et al., 2006; Varrone et al., 2004).

Our approach seems to have several benefits compared to the approach used to obtain the structure of phospho-UBE2L3- $BdParkin\Delta RING2$ : pUb. No truncations of the protein were necessary beyond removal of the flexible RING2 domain and since the N-terminus

of the phospho-Ubl in our structure is not tethered, it seems to be involved in fewer crystal contacts. Most significantly, our structure represents the *Homo sapiens* protein and could therefore aid future translational research.

With this, the last structural insight necessary to understand the PINK1-dependent Parkin activation sequence in molecular detail, there is a possibility of rationally designing small molecules which could favour either the activated or the autoinhibited Parkin state by recognizing unique sites on each one. Favouring the active Parkin state could benefit a subset of early-onset PD sufferers by reversing the effects of particular mutations or potentially promote mitophagy to delay symptom progression in sporadic PD sufferers. On the other hand, loss-of-function Parkin mutations (deletions as well as point mutations) have been associated with several cancers, suggesting Parkin tumour-suppressor function (Veeriah et al., 2010; Gong et al., 2014b). In these cases, favouring the auto-inhibited state of Parkin could offer new therapeutic strategies.

Going forward, a reliable high-throughput activity and conformational read-out must be designed for Parkin. Recently, activity based probes able to detect changes in Parkin activity within the natural dynamic range have been designed (Park et al., 2017). However, as these may cross-react with screened compounds they may not provide an ideal screening tool for targeting Parkin activity. A FRET-based assay has been developed to read-out Parkin conformation (Tang et al., 2017). This assay may be misleading, as it is based on mimicking the conformation of the non-physiological F146A activating mutation, which would not favour the native active state, as F146A also weakens ACT binding which contributes to the phospho-Ubl interface (Trempe et al., 2013; Tang et al., 2017).

Instead, a structure-based FRET assay could be designed to determine the fractional occupancy of the Ubl in the active or inactive states. As introduction of bulky dyes on the often multifunctional surfaces or Parkin could lead to artefacts, a simpler assay could arise from utilizing the changes in proteolytic behaviours between active and inactive states of Parkin described here. Ideally, screened compounds should act indiscriminately of the particular mutations carried by patients with Parkin defects where Parkin protein is present. This added complexity calls for a screening method with minimal post-translational handling of the produced protein, such as the proposed proteolysis assay.

Although the PINK1-mediated Parkin activation mechanism has now been resolved, outstanding questions remain about the RBR Parkin mechanism. The conformation of the Parkin-bound  $E2 \sim Ub$  conjugate seems to be unique and the molecular details of the RING2 interaction with the donor Ub have also eluded characterization. Together insight into these states would help build up a picture of the Parkin transfer complex and potentially explain RING2-based pathogenic patient mutations, such as T415N, G430D and P437L (Yi et al., 2018). The use of a covalent pUb linkage, especially to help determine the  $E2 \sim Ub$ -bound complex requires careful consideration, as it seems that covalent pUb conjugation disrupts native  $E2 \sim Ub$  binding. Use of the UBE2L3-Ub amide-linked ABP could be coupled with carboxypeptidase treatment to remove the disordered, hydrophobic C-terminal segment formerly anchoring the RING2 to the UPD (Pao et al., 2016).

Previous models suggested that Parkin autoinhibition could be resolved by cooperation of two autoinhibited Parkin molecules (Arkinson and Walden, 2018; Kumar et al., 2017a). Several aspects remain unaddressed by this model. In the suggested dimer, the E2 binding site remains occluded by the autoinhibitory Ubl and REP binding in both molecules while the RING2 catalytic Cys is not sufficiently solvent exposed to mediate Ub transfer (Kumar et al., 2017a). We have not observed dimerization in any of our HDX MS or SEC-MALS analysis and no complementation was observed between inactive and E2-binding deficient Parkin (Sauvé et al., 2018), contradicting the cooperative model.

Functionally, our analysis presents several interesting questions. Conservation analysis identified two conserved regions within the Ubl-UPD linker in vertebrates - the ACT and the GLAVIL motifs. While the ACT has a defect in our minimal *in vitro* reconstituted assays, is protected in our dynamic analysis and resolved in the crystal structure, we have not been able to obtain any leads on the potential function of the GLAVIL motif. Given its purely hydrophobic nature, it could modulate a protein-protein interaction either with a specific substrate, anchoring protein or a PINK1-independent activator. As its function has not become apparent in our minimalistic systems, it may be necessary to look in a more complex setting. Assessing the the phenotype of GLAVIL disruption in a cellular system could determine whether its function lies within the PINK1/Parkin-mediated mitophagy pathway or contributes to a different Parkin function.

Several phosphorylation sites have been identified in the Ubl-UPD Parkin linker before the importance of Ser65 phosphorylation for PINK1/Parkin-dependent mitophagy had been established. Phosphorylation of Ser101 in the resolved ACT element has been ascribed to Casein Kinase-1 (CK-1) through an *in vitro* phosphorylation assay performed with wt or S101A mutant Parkin as a substrate (Yamamoto et al., 2005). Phosphorylation of Parkin at this site would likely disrupt ACT binding and prevent efficient Parkin activation by PINK1. Phosphorylation by c-Abl on the UPD residue Thr143 might similarly impact on ACT but not RING2 binding (Ko et al., 2010). Phosphorylation in later portions of the linker, such as Ser131 has been ascribed to CDK5 using a similar *in vitro* assay as was used for CK-1 (Avraham et al., 2007). Although of unknown function, this phosphorylation site has emerged as constitutive, and was found in other cellular studies as well (Yamamoto et al., 2005; Kondapalli et al., 2012). Other sites such as Ser108 or Ser116 have been identified by high-throughput screening and not validated (Hornbeck et al., 2015). While phosphorylation at Ser108 might also affect the ACT element function, phosphorylation at Ser116 might affect the unknown GLAVIL function.

Regulation of Parkin activity by phosphorylation in the linker, hierarchically above Ser65 phosphorylation by PINK1, could provide a switch to shut down PINK1/Parkindependent mitophagy. Interestingly, the beige-to-white adipocyte transition, mediated by PINK1/Parkin-dependant mitophagy, seems to be arrested by PKA phosphorylation in a similar manner (Lu et al., 2018). Beige adipocytes are generated from white adipocytes upon stress (e.g. cold) and contain a higher mitochondrial mass necessary to generate heat. To reverse the beige-to-white transition which occurs once stress conditions are removed, Parkin-dependent mitophagy is downregulated by PKA-mediated phosphorylation of either Parkin directly or a Parkin interactor (Lu et al., 2018). This system could potentially utilize a linker region phosphorylation site in Parkin (e.g. Ser101 or Ser108) and it will be interesting to determine the exact phosphorylation substrate and site to establish how activation by Ser65 phosphorylation can be reversed by additional phosphorylation events.

### Chapter 7.

## **Conclusion and outlook**

In conclusion, conformational equilibria assumed by both ubiquitin (Ub) and Parkin were identified and characterized in this work. Both dynamic processes are required for proper progression of PINK1/Parkin-mediated mitophagy.

The transition of Ub into the novel C-terminally retracted conformation exposes its Ser65-containing loop and is necessary for PINK1-mediated phosphorylation. This phosphorylation event results in a unique pUb signal accumulation on sites of PINK1 activity/mitochondrial damage. pUb is recognized by autoinhibited cytosolic Parkin and upon its translocation from the cytosol, Parkin undergoes PINK1-mediated phosphorylation of the Ser65 residue of its Ubl domain. The phosphorylation event enables Parkin to access a second, dramatically rearranged species, resolved here in a 1.80Å structure. The phospho-Ubl moves by more than 50Å compared to its autoinhibited binding site and contacts the UPD domain, such that the catalytic domain is displaced from its autoinhibitory position, thus activating Parkin.

The resolved structure of the activated human phospho-Parkin core finally completes the picture of the PINK1-mediated Parkin activation mechanism. Moreover, the structure explains the likely disease basis for several mutations found among YOPD patients. One of these lies in a newly identified sequence motif conserved in vertebrates, the ACT element. While the ACT element is resolved in the activated phospho-Parkin structure and is necessary for Parkin activation, no clues were obtained about the function of the second identified motif (GLAVIL), which is also conserved among vertebrates. No patients harbouring a mutation in the GLAVIL motif were reported thus far. Additionally, our HDX MS data suggest that GLAVIL is not involved in the catalytic mechanism, it

could therefore serve in Parkin functions outside of mitophagy or mediate protein-protein interactions with an unknown subset of partners.

Moreover, due to a clear link between mitophagy defects and Parkinsonism, this work has implications for Parkinson's disease diagnosis and therapy. Boosting PINK1/Parkinmediated mitophagy could delay dopaminergic neuron death to maintain patient dopamine levels or even provide preventative treatment. Additionally, current diagnosis is subjective - tremor, slowness of movement, rigidity and loss of balance is assessed by physicians individually. Surveying the levels of PINK1/Parkin mitophagy, presumed to increase prior to neuronal death, could be developed into a reliable diagnostic tool, in particular for this pathological process key to the development of YOPD.

Such a tool could either quantify the amount of pUb, a signal specific to PINK1/Parkinmediated mitophagy, or assess activity levels of PINK1 or Parkin. Molecular understanding of the active form of Parkin offers avenues to measure occupancy of the active Parkin conformer. While this work enables rational design of FRET-based conformational assays, it also offers an elegant proteolytic strategy to report on the Parkin conformational equilibrium. To pharmacologically boost Ub signalling in mitophagy, it is necessary to either boost Parkin activity or downregulate one of the several reported opposing DUB enzymes. Although DUB inhibition is becoming a more routine and mitochondrial USP30 is already being explored as a target, no DUB is linked to disease as firmly as PINK1 and Parkin. As this work completes the molecular picture of Parkin activation it enables unique opportunities for designing interactors specific to either the active or inactive Parkin conformers. The resolved structure defines several interfaces and structural elements unique to active Parkin, the stabilization of which could shift the Parkin equilibrium towards the active state in patients who carry defective Parkin variants. More generally, patients who require a mitophagy boost to prevent further dopaminergic neuron loss could also benefit from this effort.

Off-target effects could converge on the remaining members of the RBR E3 Ub ligase family. To avoid interference with the catalytic mechanisms of any of these ligases, targeting the Parkin activation sequence, as opposed to enzymatic activity, would be beneficial. In this case, targeting Parkin to stabilize its active conformation must also avoid disruption of the autoinhibited states of other RBR family members. Although it is unclear why all well studied RBR ligases require an elaborate and energy-demanding activation mechanism, it is conceivable, that their exposed catalytic Cys are highly reactive and their spatio-temporal activation must be precisely defined. While the activation sequence of Parkin by PINK1-mediated phosphorylation was finally resolved here, more work remains necessary to address the activation mechanism of HOIP and HHARI. Which binding interactions activate HOIP in the context of the LUBAC assembly machinery? How is the RING2 displaced from the Ariadne domain in HHARI? A common theme of carefully balanced affinities between activating and the autoinhibitory elements interacting with common surfaces unique to the each RBR might emerge. Here, the use of HDX MS was developed as a powerful tool to study these rearrangements.

We were able to use HDX MS to track the release of the RING2 catalytic domain upon Parkin phosphorylation, necessary to bridge the distance of over 50Å between the E2  $\sim$  Ub thioester linkage and the active site Cys in the autoinhibited structures. Interestingly, flexibility is necessary to hand over the activated Ub species by several Ub-handling enzymes. Resolved E1 structures explain how both adenylated and thioesterified Ub molecules interact with the enzyme, however the motion necessary to bridge the two E1 active sites remains obscure. Similarly, CRLs require a defined movement that allows the RING-bound activated E2  $\sim$  Ub conjugates to reach both auto-NEDDylated Lys residues, as well as substrate Lys residues bound at the opposite end of the cullin scaffold. Lastly, in the recently identified novel E3 ligase MYCBP2, a transthiolation relay mechanism is utilized, which depends on the highly mobile, structurally unresolved mediator loop to shuttle activated Ub between its two catalytic sites. All of these cases could benefit from analysis using HDX MS to resolve the dynamics required for Ub handover.

Lastly, the most surprising outcome of this work lies in the identification of a new conformer accessed by wt, unmodified Ub in solution. Despite intense Ub study by NMR and other biophysical techniques over the years, this conformation has been systematically missed due to its low population and relatively slow exchange rate. Our collaborative work shows that this conformation is necessary for PINK1-mediated Ub phosphorylation. The proposed phosphorylation mechanism could explain discrepancies in reported  $K_D$ and  $K_m$  constants for PINK1-mediated Ub phosphorylation and binding. Furthermore, the proposed phosphorylation mechanism posits an interesting question of how the Parkin Ubl or other substrates are phosphorylated by this unusual kinase. This aspect of the work could also be exploited therapeutically: The Ub conformational equilibrium is rate-limiting for PINK1-mediated phosphorylation of wt unmodified Ub. Therefore, enriching the Ub-CR conformation when the Ub substrate binds PINK1 could provide another viable avenue to boost mitophagy therapeutically.

Together, the clinically relevant molecular insights described in this thesis may facilitate the development of therapeutic or diagnostic tools for Parkinson's disease. This is therefore the first step in bridging years of basic research with further translational work on the PINK1/Parkin-mediated mitophagy pathway.

## List of Abbreviations

| $\beta ME$       | $\beta$ -mercaptoethanol.  |
|------------------|--|
| BdParkin         | Parkin from Bactrocera dorsalis, Oriental fruit fly.   |
| <i>Dr</i> Parkin | Parkin from <i>Danio rerio</i> , Zebrafish.  |
| <i>Hs</i> Parkin | Parkin from <i>Homo sapiens</i> , Human.   |
| <i>Ph</i> PINK1  | PINK1 from <i>Pediculus humanus</i> , Human louse.   |
| PhParkin         | Parkin from <i>Pediculus humanus</i> , Human louse.  |
| TcPINK1          | PINK1 from <i>Tribolium castaneum</i> , Red flour beetle.                                    |
| <i>Ts</i> Parkin | Parkin from <i>Thamnophis sirtalis</i> , Common garter snake.                                |
| AAD              | Active Adenylation Domain.   |
| ABP              | Activity-Based Probe.  |
| ALS              | Amyotrophic Lateral Sclerosis.   |
| AMBRA1           | Activating Molecule in BECN1-Regulated Autophagy protein 1.                                  |
| APC/C            | Anaphase Promoting Complex/Cyclosome.  |
| AQUA             | Absolute Quantification proteomics.  |
| ASU              | Asymmetric Unit.   |
| ATG              | Autophagy-Related Gene.  |
| AUC              | Analytical Ultracentrifuagtion.  |
| BEST-TROSY       | Band-Selective Excitation Short Transients-<br>Transverse Relaxation-Optimised Spectroscopy. |
| C-terminus       | Carboxyl terminus.   |
| CCCP             | Carbonyl Cyanide m-Chlorophenyl hydrazone.   |
| CDC20            | Cell-Division Cycle protein 20.  |
| CDH1             | CDC20 Homolog 1.   |
| CEST             | Chemical Exchange Saturation Transfer.   |
| cIAP             | cellular Inhibitor of APoptosis.   |

| CK-1    | Casein Kinase-1.                               |
|---------|--|
| CLEANEX | CLEAN chemical EXchange transfer.              |
| CNS     | Central Nervous System.                        |
| CPMG    | Carr-Purcell-Meiboom-Gill.                     |
| CRBN    | Cereblon protein.                              |
| CRL     | Cullin RING Ligase.                            |
| Cryo-EM | Cryo-Electron Microscopy.                      |
| CV      | Column Volume.                                 |
| D       | Deuterium.                                     |
| DSC     | Differential Scanning Calorimetry.             |
| E6AP    | E6-Associated Protein Ub-protein ligase.       |
| ESCRT   | Endosomal Sorting Complex for Transport.       |
| FCCH    | First Catalytic Cys Half-domain.               |
| FRET    | Förster Resonance Energy Transfer.             |
| GdmCl   | Guanidinium Chloride.                          |
| HDX MS  | Hydrogen-Deuterium Exchange Mass Spectrometry. |
| HECT    | Homologous to the E6-AP Carboxy-Terminus.      |
| hetNOE  | $^{15}$ N $^{1}$ {H} heteronuclear NOE.        |
| HHARI   | Human Homolog of Ariadne.                      |
| HOIL-1L | Heme-Oxidized IRP2 Ub Ligase 1.                |
| HOIP    | HOIL-1-Interacting Protein.                    |
| HSQC    | Heteronuclear Single Quantum Coherence.        |
| HUWE1   | HECT, UBA and WWE domain-containing protein 1. |
| IκB     | Inhibitor of $\kappa B$ .                      |
| IAD     | Inactive Adenvlation Domain.                   |
| IBR     | In-Between RING.                               |
| IKK     | $I\kappa B$ Kinase complex.                    |
| IMiD    | Immunomodulatory Drug.                         |
| ITC     | Isothermal Titration Calorimetry.              |
|         | v  |
| КО            | Knockout.   |
|---------------|---|
| LC-MS         | Liquid Chromatography-Mass Spectrometry.  |
| LDD           | Linear Ub chain Determining Domain.   |
| LIR           | LC3-Interacting Region.   |
| LUBAC         | Linear Ubiquitin Chain Assembly Complex.  |
| MDV           | Mitochondrially Derived Vesicle.  |
| MesNa         | sodium 2-mercaptoethanesulfonate.   |
| MINDY         | Motif Interacting with Ub-containing novel DUB fam-<br>ily.                       |
| MPP           | Mitochondrial Processing Peptidase.   |
| MS            | Mass Spectrometry.  |
| MW            | molecular weight.   |
| MWCO          | Molecular Weight Cut-Off.   |
| N-terminus    | Amino-terminus.   |
| NaPi          | Sodium phosphate buffer.  |
| NEDD4L        | Neural precursor cell Expressed Developmentally<br>Down-regulated protein 4-like. |
| NEMO          | Nuclear factor- $\kappa B$ Essential Modifier.                                    |
| $NF-\kappa B$ | Nuclear Factor $\kappa$ enhancer Binding protein.                                 |
| NMR           | Nuclear Magnetic Resonance.   |
| NOE           | Nuclear Overhouser Effect.  |
| NZF           | Npl4-like Zinc Finger.  |
| OA            | Oligomycin/Antimycin A.   |
| OTU           | Ovarian Tumor Protease.   |
| OXPHOS        | Oxidative Phosphorylation.  |
| PARL          | PINK1/PGAM5-Associated Rhomboid-Like protease.                                    |
| PCNA          | Proliferating Cell Nuclear Antigen.   |
| PD            | Parkinson's disease.  |
| PINK1         | PTEN-Induced Putative Kinase 1.   |

| PISA     | Protein Interfaces, Surfaces and Assemblies service at<br>the European Bioinformatics Institute. |
|----------|--|
| PROTAC   | PROteolysis TArgeting Chimera.   |
| PTM      | Post-Translational Modification.   |
| pUb      | phospho-Ub.  |
| RBR      | RING-in-Between-RING.  |
| REP      | Repressor Element of Parkin.   |
| RING     | Really Interesting New Gene.   |
| RIP1     | Receptor-Associated Protein kinase 1.  |
| RNF      | RING finger.   |
| ROS      | Reactive Oxygen Species.   |
| SAC      | Spindle Assembly Complex.  |
| SCCH     | Second Catalytic Cys Half-domain.  |
| SCF      | Skp, Cullin, F-box containing complex.   |
| SEC      | Size Exlusion Chromatography.  |
| SEC-MALS | Size Exclusion Chromatography-Multi Angle Light Scattering.                                      |
| SHARPIN  | Shank-Associated RH Domain-Interacting protein.  |
| SOD1     | Superoxide Dismutase 1.  |
| $T_{m}$  | melting temperature.   |
| TAK1     | Transforming growth factor- $\beta$ -Activated Kinase 1.   |
| TCEP     | tris (2-carboxyethyl) phosphine.   |
| TEV      | Tobacco Etch Virus.  |
| TIM      | Translocase of the Inner Membrane.   |
| TNF      | Tumor Necrosis Factor.   |
| TOM      | Translocase of the Outer Membrane.   |
| TRADD    | TNF Receptor 1-Associated Death Domain.  |
| TRAF6    | TNF Receptor Associated Factor 6.  |
| Ub       | ubiquitin.   |
| Ub-C3Br  | Ubiquitin Bromopropylamine.  |
| Ub-CR    | C-terminally retacted Ub.  |
| Ub-VS    | Ubiquitin Vinyl Sulphone.  |

| UBA                          | Ub-Associated Domain.                           |
|------------------------------|---|
| UBAN                         | Ubiquitin Binding in ABINs.                     |
| UBD                          | Ub-binding domain.                              |
| Ubl                          | Ubiquitin-like.                                 |
| UCH                          | Ub C-terminal Hydrolase.                        |
| UFD                          | Ubiqutin-Fold Domain.                           |
| UIM                          | Ub Interaction Motif.                           |
| ULK1                         | Unc-51-Like autophagy activating Kinase 1.      |
| UPD                          | Unique Parkin Domain.                           |
| $\mathrm{UPR}^{\mathrm{mt}}$ | Mitochondrial Unfolded Protein Response.        |
| UPS                          | Ubiquitin-Proteasome System.                    |
| USP                          | Ub-Specific Protease.                           |
| v/v                          | volume/volume.                                  |
| VDAC                         | Voltage-Dependent Anion Channel.                |
| w/v                          | weight/volume.                                  |
| WIPI                         | WD Repeat Domain, Phosphoinositide Interacting. |
| wt                           | wild-type.                                      |
| YOPD                         | Young-Onset Parkinson's Disease.                |

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## Colophon

This thesis was made in  $L^{A}T_{E}X$  using a modified version of the "hepthesis" class (Buckley, 2017). The  $T_{E}X$  was edited and compiled using TeXstudio 2.12.8.

## Appendix A.

# Full NMR-derived plots for ubiquitin variants



## A.1. Full CEST profiles

Figure A.1.: Full Ub CEST profiles. Full spectra akin to Fig 3.2.



Figure A.2.: Full Ub CEST profiles. Full spectra akin to Fig 3.2. An additional peak, Leu56 is shown.

### A.2. Full F4A Ub and pUb spectrum assignment



Figure A.3.: F4A Ub assignment. De-novo derived BEST-TROSY assignment.



Figure A.4.: F4A pUb assignment. pUb-CR peaks were assigned using ZZ-exchange experiments at 950 MHz from the F4A pUb-common assignment at 600 MHz.

### A.3. CEST fit quality for Ub variants



Figure A.5.: CEST fitting for Ub variants. Fitted field dependence of example Ub variant CEST profiles. Fit values are summarized in Table 3.1.

### A.4. CLEANEX solvent exchange for all residues



Figure A.6.: CLEANEX rates of solvent exchange across the whole sequence of Ub variants. A subset of values and fits is reported in Table 3.2 and Fig 3.3.2.

### A.5. HetNOE values for all residues



Figure A.7.: hetNOE values across the whole sequence of unphosphorylated Ub variants. A subset of values and fits is reported in Fig 3.8.



Figure A.8.: hetNOE values across the whole sequence of phosphorylated Ub variants. A subset of values and fits is reported in Fig 3.8.

# Appendix B.

## Parkin conservation



Figure B.1.: Sequence alignment of Parkin insect orthologues. Sequence alignment of HsParkin with Parkin from  $Drosophila\ melongaster\ (Dm$ Parkin), PhParkin used in Wauer et al. (2015a), and BdParkin used in Sauvé et al. (2018). Secondary structure elements shown for human Parkin: 1-382 from PDB ID: 6GLC (Gladkova et al., 2018), 383-465 from PDB ID: 5N2W, (Kumar et al., 2017a). Phosphate binding pocket, the active site and the Ser65 phosphorylation site are labelled. Insect Parkin variants all have a hydrophobic N-terminal extension of conserved length preceding the N-terminal Ubl domain. Although the ACT and GLAVIL elements are missing, some conservation among the insect species is present in the Ubl-UPD linker. PhParkin contains an Asp residue at a position analogous to Ser65. Domains are coloured as in 4.2.

# Appendix C.

# Additional Parkin dynamics plots

### C.1. Parkin binding to pUb



Figure C.1.: Strucural mapping of HDX MS pUb-binding data for all time points. Structural representation of all measured time points as in Fig 5.3 B.



**Figure C.2.:** A HDX MS difference plot of pUb binding to Parkin. Difference plot as described in Fig 5.2 E. Difference (in Da) between the Parkin and the Parkin:pUb complex is plotted as a connecting line for individual time points (0.3 s - orange; 3 s - red; 30 s - cyan, 300 s - blue, 3000 s - black). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Exposure of the Ubl and the Ubl autoinhibitory binding site on the RING1 is visible, as seen in the heat map in Fig 5.3 A, regions (1) and (1r). Similarly, protection of the pUb binding site is also observed, region (3). Additional regions of interest - REP exposure (5) and a small effect on RING2 exposue (6) is more prominent in the difference plot compared to the heat map. Peptides for which plots are shown in the main text are indicated.





Figure C.3.: HDX MS structural mapping of Parkin phosphorylation for all time points. Structural representation of all measured time points as in Fig 5.4 B.



Figure C.4.: A HDX MS difference plot of Parkin phosphorylation. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-Parkin:pUb and the Parkin:pUb complexes is plotted as a connecting line for individual time points (0.3 s - orange; 3 s - red; 30 s - cyan, 300 s - blue, 3000 s - black). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Exposure of the phospho-Ubl (1) and the C-terminus (6) as well as protection of the pUb-binding site (3) and the UPD phospho-pocket (4) is seen as in the heat map in Fig 5.4 A. In addition to exposure of the REP element (5) and the reciprocal REP binding site (5r) on the RING1 domain can be seen in the difference plot. Similarly a small portion of the reciprocal RING2 binding site on the UPD is also exposed (6r). As seen in Fig 5.3 D, the reciprocal Ubl binding site on RING1 is only slightly more exposed (1r), suggesting increased Ubl solvent exposure mostly results from Ubl destabilization upon phosphorylation. Peptides for which plots are shown in the main text are indicated.

#### C.3. UBE2L3-Ub non-covalent binding to Parkin



Figure C.5.: HDX MS strucural mapping of UBE2L3-Ub non-covalent binding to the phospho-Parkin:pUb complex for all time points. Structural representation of all measured time points as in Fig 5.7 B.



Figure C.6.: A HDX MS difference plot of UBE2L3-Ub binding to the phopsho-Parkin:pUb complex. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-Parkin:pUb:UBE2L3-Ub and the phospho-Parkin:pUb complexes is plotted as a connecting line for individual time points (0.3 s - orange; 3 s - red; 30 s - cyan, 300 s - blue, 3000 s - black). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Exposure of the C-terminus (6), the REP (5) and protection of the Ubl (1), the UPD phospho-pocket (4), the ACT (7) and the donor Ub site (8) is seen as in the heat map in Fig 5.7 A. Protection from solvent exposure in the RING1 E2 binding site is also seen more clearly in the difference plot (10). Similarly, and as for the effect of Parkin phosphorylation, in addition to exposure of the REP element (5), exposure of the reciprocal REP binding site on the RING1 domain is present (5r). Further exposure of a part of the RING2 reciprocal binding side on the UPD is also observed (6r). Interestingly a significant exposure in the RING1 domain (marked ?, resi 275-299) is seen, which may be a consequence of binding of either component of the UBE2L3-Ub complex. Peptides for which plots are shown in the main text are indicated.



Figure C.7.: A lack of binding between the Parkin phospho-Ubl and UBE2L3 observed by NMR. Top left: A BEST-TROSY spectrum of <sup>15</sup>N labelled Parkin phospho-Ubl at 40  $\mu$ M. Top right: A BEST-TROSY spectrum of a 1:1 mixture of <sup>15</sup>N labelled Parkin phospho-Ubl (40  $\mu$ M) and unlabelled UBE3L3. Bottom left: A BEST-TROSY spectrum of a 1:2 mixture of <sup>15</sup>N labelled Parkin phospho-Ubl (40  $\mu$ M) and unlabelled UBE3L3. Bottom left: A BEST-TROSY spectra.

Spectral overlay is indicative of the absence of any binding (sensitive to up to µM affinity). No systematic peak shifts or changes in relative peak intensity upon addition of unlabelled UBE3L3 are seen.



#### C.4. UBE2L3-Ub-Parkin trimeric covalent complex

Figure C.8.: HDX MS structural mapping of generation of a covalent trimeric phospho-Parkin-UBE3L3-Ub:pUb complex for all time points. Structural representation of all measured time points as in Fig 5.9 B.



Figure C.9.: A HDX MS difference plot resulting form generation of the trimeric phospho-Parkin-UBE2L3-Ub:pUb complex. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-Parkin-UBE2L3-Ub:pUb and the phospho-Parkin:pUb complexes is plotted as a connecting line for individual time points (3 s - red; 30 s - cyan, 300 s - blue). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Exposure of the C-terminus (6), the REP (5) and protection of the Ubl (1), the UPD phospho-pocket (4), the ACT (7), the donor Ub site (8) and the E2 binding site (10) is seen as in the heat map in Fig 5.9 A. As in previous difference plots, in addition to exposure of the REP element (5), exposure of the reciprocal REP binding site on the RING1 domain can be seen clearly in the difference plot (5r). As is the case with the non-covalent UBE2L3-Ub complex with phospho-Parkin:pUb, exposure in the RING1 domain is seen (marked ?).



Figure C.10.: HDX MS strucural mapping of covalent attachment of UBE2L3. Structural representation of all measured time points as in Fig 5.9 D.



Figure C.11.: A HDX MS difference plot reporting on covalent attachment of UBE2L3. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-Parkin-UBE2L3-Ub:pUb and the phospho-Parkin-Ub:pUb (as described in Section 5.5) complexes is plotted as a connecting line for individual time points (3 s - red; 30 s - cyan, 300 s - blue). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

This difference effectively describes covalent association with UBE2L3, which results in positioning of the donor Ub in the donor Ub binding site. As in both cases Parkin is fully in the active state, the N-terminal portion of the difference plot does not show many significant differences. Protection of the E2 and donor Ub sites is now clearly visible ((10) and (8) respectively). Interestingly, the RING1 exposure associated with UBE2L3-Ub binding remains seen in this analysis (marked ?). This may suggest a possible need for rearrangement/displacement of the RING1 loops upon UBE2L3-Ub binding resulting in increased solvent exposure. Major exposure in the RING2 domain is shifted away from the very C-terminus (Better seen in the heatmap in Fig 5.9 C which accounts for non-covered residues.), suggesting a possible rearrangement of the RING2 C-terminal secondary strucures upon UBE2L3 binding (6).


## C.5. The phospho-Parkin-Ub:pUb complex

Figure C.12.: HDX MS strucural mapping of Parkin Ub charging. Structural representation of all measured time points as in Fig 5.11 B.



Figure C.13.: A HDX MS difference plot reporting on Parkin Ub charging. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-Parkin-Ub:pUb and the phospho-Parkin:pUb complexes is plotted as a connecting line for individual time points (0.3 s - orange; 3 s - red; 30 s - cyan, 300 s - blue; 3000 s - black). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Exposure of the C-terminus (6), the REP (5) and protection of the Ubl (1), the UPD phospho-pocket (4) and the ACT (7) is seen as in the heat map in Fig 5.11 A. As previously, in addition to exposure of the REP element (5), exposure of the reciprocal REP binding site on the RING1 domain can be seen clearly in the difference plot (5r). Early time point protection in the RING2 (9) likely corresponds to the donor Ub binding site on RING2.

## Appendix D.

## Dynamics of activated crystallization constructs



Figure D.1.: A HDX MS difference plot reporting on TsParkin Ub charging. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-TsParkin-Ub-pUb and the phospho-TsParkin-pUb covalent complexes is plotted as a connecting line for a single time point corresponding to 0.3 s - orange. Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left.

Protection of the Ubl (1), the UPD phospho-pocket (4) and the ACT (7) is seen as in the heat maps in Fig 6.2 D and Fig 6.6 A, top. Exposure of the reciprocal REP binding site on the RING1 domain can be seen in the difference plot (5r), although at this time point does not exceed the significance limit. Highly significant protection is seen for 47 residues in the RING2 domain (9), corresponding to the RING2 protection upon conjugation with the donor Ub postulated from HsParkin data in Fig 5.11.



Figure D.2.: A HDX MS difference plot reporting on TsParkin RING2 removal. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-TsParkin $\Delta$ RING2-pUb and the phospho-TsParkin-pUb covalent complexes is plotted as a connecting line for a single time point corresponding to 0.3 s - orange. Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left. Note that the sequence only up to resi 367 is covered due to removal of the RING2.

Protection of the Ubl (1), the UPD phospho-pocket (4) and the ACT (7) is seen as in the heat map in Fig 6.6 A, bottom. Exposure of the reciprocal REP binding site on the RING1 domain can be seen in the difference plot (5r). Notably the residues contributing to changing regions and the magnitudes of change are almost indistinguishable from the effects of Ub charging (Fig D.1). The resulting  $\Delta$ RING2 protein therefore recapitulates active Parkin well.



Figure D.3.: A HDX MS difference plot reporting on *Hs*Parkin Ub charging. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-*Hs*Parkin-Ub:pUb and the phospho-*Hs*Parkin:pUb non-covalent complexes is plotted as a connecting line for individual time points (3 s - red; 30 s - cyan, 300 s - blue). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left. This experiment constitutes a biological repeat measurement from Fig 5.11, difference plot in Fig C.13.

Protection of the Ubl (1), the UPD phospho-pocket (4) and the ACT (7) is seen as in the heat map in Fig 6.6 B, top. Exposure of the REP (5) and the reciprocal REP binding site on the RING1 domain can be seen in the difference plot (5r). Protection is seen for 32 residues in the RING2 domain (9), corresponding to the RING2 protection upon conjugation with the donor Ub postulated from a previous biological replicate measurement in Fig 5.11. The C-terminus is also further exposed (6), as the equilibrium is now arrested in the active state.



Figure D.4.: A HDX MS difference plot reporting on *Hs*Parkin RING2 removal. Difference plot as described in Fig 5.2 E. Difference (in Da) between the phospho-*Hs*Parkin $\Delta$ RING2:pUb and the phospho-*Ts*Parkin:pUb non-covalent complexes is plotted as a connecting line for individual time points (3 s - red; 30 s - cyan, 300 s - blue). Cumulative difference for each peptide is indicated by grey bars, while the experimental error (standard deviation of centroid positions across conditions, replicates and time points) is shown in light grey. Dotted lines represent the threshold for 98% confidence limit in significance of the changes observed (Houde et al., 2011). The residue range and length of significantly changing regions is indicated on the bottom, each assigned region is interpreted on the top left. Note that the sequence only up to resi 382 is covered due to removal of the RING2.

Protection of the Ubl (1), the UPD phospho-pocket (4) and the ACT (7) is seen as in the heat map in Fig 6.6 B, bottom. Exposure of the reciprocal REP binding site on the RING1 domain can be seen in the difference plot (5r). Notably the residues contributing to changing regions and the magnitudes of change are almost indistinguishable from the effects of Ub charging (Fig D.3). The resulting  $\Delta$ RING2 protein therefore recapitulates active Parkin well.

## Appendix E.

## Mechanism of parkin activation by PINK1

## LETTER

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### Mechanism of parkin activation by PINK1

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Mutations in the E3 ubiquitin ligase parkin (PARK2, also known as PRKN) and the protein kinase PINK1 (also known as PARK6) are linked to autosomal-recessive juvenile parkinsonism (AR-JP)<sup>1,2</sup>; at the cellular level, these mutations cause defects in mitophagy, the process that organizes the destruction of damaged mitochondria<sup>3,4</sup>. Parkin is autoinhibited, and requires activation by PINK1, which phosphorylates Ser65 in ubiquitin and in the parkin ubiquitin-like (Ubl) domain. Parkin binds phospho-ubiquitin, which enables efficient parkin phosphorylation; however, the enzyme remains autoinhibited with an inaccessible active site<sup>5,6</sup>. It is unclear how phosphorylation of parkin activates the molecule. Here we follow the activation of full-length human parkin by hydrogendeuterium exchange mass spectrometry, and reveal large-scale domain rearrangement in the activation process, during which the phospho-Ubl rebinds to the parkin core and releases the catalytic RING2 domain. A 1.8 Å crystal structure of phosphorylated human parkin reveals the binding site of the phospho-Ubl on the unique parkin domain (UPD), involving a phosphate-binding pocket lined by AR-JP mutations. Notably, a conserved linker region between Ubl and the UPD acts as an activating element (ACT) that contributes to RING2 release by mimicking RING2 interactions on the UPD, explaining further AR-JP mutations. Our data show how autoinhibition in parkin is resolved, and suggest a mechanism for how parkin ubiquitinates its substrates via an untethered RING2 domain. These findings open new avenues for the design of parkin activators for clinical use.

Work in the past decade has shown how PINK1 and parkin initiate mitophagy, and many steps in this process are mechanistically well understood<sup>3,4</sup>. It has further been suggested that targeted activation of either PINK1 or parkin could increase mitochondrial turnover and impede the progression of Parkinson's disease. A detailed understanding of the underlying molecular mechanisms of these processes is therefore essential.

Parkin requires an elaborate activation mechanism. The first crystal structures of parkin<sup>7–9</sup> revealed several distinct mechanisms of autoinhibition (Fig. 1a, Extended Data Fig. 1a). Most strikingly, the active site Cys on the catalytic RING2 domain, which receives ubiquitin from the E2 enzyme, is obstructed by an interface with the UPD (also known as RING0) (Extended Data Fig. 1a). The RING2–UPD interface is highly hydrophobic<sup>7–9</sup> (Extended Data Fig. 1b), and it is not clear how this intramolecular interaction can be opened.

Activation of parkin is mediated by the mitochondrial outer membrane (MOM) Ser/Thr protein kinase PINK1, which phosphorylates Ser65 in ubiquitin (generating phospho-ubiquitin)<sup>10-14</sup> and in the parkin Ubl domain<sup>15-17</sup>. A current model for PINK1-mediated activation of parkin suggests that PINK1 phosphorylates ubiquitin attached to MOM proteins, and autoinhibited, cytosolic parkin is recruited with nanomolar affinity to sites of PINK1 activity<sup>3,5,13,18-21</sup>. Binding of phospho-ubiquitin induces conformational changes in parkin that lead to the release of the Ubl domain from the parkin core, and enable PINK1 to phosphorylate the parkin Ubl domain<sup>3,5-7,13,18-22</sup> (Fig. 1a, Extended Data Fig. 1c). Notably, in structures of parkin bound to phospho-ubiquitin<sup>5,6</sup>, parkin is still autoinhibited; the E2 binding site remains blocked by the repressor (REP) element, and RING2 and its catalytic Cys remain obstructed by the UPD (Fig. 1a, Extended Data Fig. 1c).

Indeed, full activation of parkin requires phosphorylation of its Ubl. A parkin S65A mutant is not retained at mitochondria, is unable to trigger mitochondrial ubiquitination and mitophagy, and thus is physiologically inactive<sup>13,15,17,21,23</sup>. Biochemically, parkin phosphorylation enhances activity to a greater extent than binding of phosphoubiquitin<sup>13,21,24</sup>, and parkin phosphorylation, but not phosphoubiquitin binding, enables ubiquitin activity-based probes (Ub-ABPs) to access the active site Cys<sup>5,13,25</sup>. How Ubl phosphorylation is able to activate parkin, and in particular, how it can disrupt the RING2–UPD interface, has remained unknown, and this has led to various models of parkin activation<sup>3,5,6,8,9,26</sup>.

We reconstituted activation of full-length human parkin by PINK1, and followed domain rearrangements by hydrogen-deuterium exchange mass spectrometry (HDX-MS)<sup>27</sup> (Fig. 1b-e, Extended Data Figs. 2, 3). HDX-MS reports on the relative rate of exchange of backbone amide hydrogens with deuterium, based on the strength of hydrogen bonding and solvent accessibility in the folded protein, and distinguishes peptides in a protein's core (which show no or little exchange with solvent over time) from those at an exposed surface (which show high or increasing exchange with solvent over time). The power of the method lies in its ability to compare identical peptides between different states along an activation cascade, revealing peptides that become exposed and thus interfaces that are opened (red in Fig. 1b-e), and regions in the protein that become protected and form new interfaces (blue in Fig. 1b-e). For parkin, this allowed us to confirm previously reported conformational changes upon phospho-ubiquitin binding<sup>5,6</sup>, whereby the parkin Ubl is released and becomes exposed to solvent (numbers 1 and 2 in Fig. 1b), the phospho-ubiquitin binding site becomes protected (3), and RING2, REP (4) and UPD are essentially unperturbed (Fig. 1b, Extended Data Fig. 3a).

Phosphorylation of parkin initiates release of REP and RING2 (4, 5), especially at later time points, but the phosphorylated Ubl also remains flexible and in exchange with solvent (1) (Fig. 1c, Extended Data Fig. 3b). The behaviour of phospho-Ubl changes markedly when a covalent, non-dischargeable E2-ubiquitin conjugate is added to the sample—now, the C-terminal RING2 peptide at the UPD interface is exposed to solvent (5), and the phosphorylated Ubl becomes protected (1), indicating the formation of a new interface (Fig. 1d, Extended Data Figs. 2, 3c). Finally, charging of the catalytic Cys of RING2 by ubiquitin was assessed using phosphorylated parkin covalently modified with the Ub-ABP ubiquitin-vinylsulfone (Ub-VS)<sup>5,13</sup> (see Methods, Extended Data Fig. 2a-c). 'Charged' phospho-parkin reiterates the conformational changes observed in the phospho-parkin E2-Ub-bound sample (Fig. 1e, Extended Data Fig. 3d), showing that the ubiquitin-modified RING2 had been fully released from the parkin core (5). Overall, the HDX-MS experiments indicated that there were considerable rearrangements of Ubl and RING2, with loss of old and formation of new intramolecular interfaces on the parkin core (Fig. 1, Extended Data Figs. 2, 3).

Unexpectedly, a section of the linker between Ubl and UPD was protected during rebinding of the phospho-Ubl (6) (Fig. 1d, e, Extended Data Figs. 2, 3). This region of parkin, spanning amino acids 75–145,

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Fig. 1 | Domain rearrangements in parkin, resolved by HDX-MS. a, Cartoon of parkin activation. Left, parkin is autoinhibited by several mechanisms (red circles)<sup>7-9</sup>. Middle, binding of phospho-ubiquitin (pUb) to parkin releases the Ubl domain, but most mechanisms of autoinhibition remain<sup>5,6</sup>. Right, after Ubl phosphorylation, parkin is fully active (green circles), but a structure of active parkin has not been reported. Also see Extended Data Fig. 1. b-e, HDX-MS difference maps with the shortest peptides covering any given region, coloured from blue (more protected from exchange compared to previous state) to red (more accessible to solvent exchange). Peptides for grey regions could not be analysed (see Extended Data Fig. 3). The five columns per sample indicate different time lengths for hydrogen-deuterium exchange (0.3 s, 3 s, 30 s, 300 s and 3,000 s). All experiments were performed with human full-length parkin, as technical triplicates. See Extended Data Figs. 2, 3 for raw data and structural mapping, respectively. b, Difference between parkin and parkin bound to phospho-ubiquitin. c, Difference between parkin–phospho ubiquitin and phospho-parkin-phospho-ubiquitin. d, Difference between phospho-parkin-phospho-ubiquitin, and phospho-parkin-phospho-ubiquitin bound to a non-dischargeable UBE2L3-ubiquitin (Ub) complex (see Methods). e, Difference between phospho-parkin-phospho ubiquitin and phospho-parkin-phospho-ubiquitin charged with Ub-VS (see Methods)

has remained unstudied as it is disordered in full-length parkin<sup>7</sup> and was removed in subsequent structures of human and rat parkin<sup>6,18,19</sup>

The Ubl–UPD linker contains two connected, short sections of highly conserved residues that are flanked by a variable number of unconserved residues (Extended Data Fig. 4). A minimal linker is present in *Thamophis sirtalis* (*Ts*) parkin (garter snake parkin, sequence identity to human parkin 73%, Extended Data Fig. 4), and *Ts*parkin



Fig. 2 | Structure of the phosphorylated parkin core. a, Schematic for obtaining a crystallizable phosphorylated parkin core. Scissors indicate the introduction of a TEV protease cleavage site after the IBR domain (amino acid 382). b, Crystal structure at 1.80 Å of the human phosphorylated parkin core lacking RING2, bound to phospho-ubiquitin. Phosphorylated residues are shown in ball-and-stick representation. A cartoon representation similar to a is shown to the right. Also see Extended Data Table 1.

was used for comparative studies. HDX-MS revealed highly similar changes upon ubiquitin charging in phosphorylated *Ts*parkin when compared to human parkin (Extended Data Fig. 5a, with Fig. le). Moreover, limited proteolysis of full-length *Ts*parkin revealed that auto-inhibited, unphosphorylated *Ts*parkin was cleaved first in the Ubl–UPD linker, whereas phosphorylated *Ts*parkin was cleaved first in the IBR–RING2 linker, and was not efficiently cleaved in the Ubl–UPD linker (Extended Data Fig. 5b). After cleavage of phospho-*Ts*parkin, RING2 was no longer stably associated with the parkin core (Extended Data Fig. 5c). Together, these data again strongly suggest that the unstudied Ubl–UPD linker ECOMEs ordered in activated parkin, whereas REP and RING2 are dislodged, and RING2 becomes mobile.

We realized that crystallographic analysis of active parkin was likely to be impeded by a mobile RING2 domain, and this inspired new construct design. Parkin is insoluble when expressed without the RING2 domain (data not shown), probably owing to the exposed, hydrophobic UPD (Extended Data Fig. 1b). Hence, we engineered a tobacco etch virus (TEV) cleavage site into the IBR-RING2 linker (Fig. 2a, see Methods). This enabled us to remove the RING2 domain upon phospho-ubiquitin binding and Ubl phosphorylation (Extended Data Fig. 5d). Notably, Ub-VS-charged Tsparkin and Tsparkin lacking RING2 (Tsparkin ARING2) displayed identical difference HDX-MS profiles, indicating that removal of the mobile RING2 had no effect on the remaining molecule (Extended Data Fig. 5e). For human parkin, the resulting covalent phospho-parkin  $\Delta RING2$  - phospho-ubiquitin (hereafter phospho-parkin-phospho-ubiquitin) complex was crystallized, and resulted in a 1.8 Å structure (Fig. 2b, Extended Data Table 1, Extended Data Fig. 6).

The structure of phospho-parkin–phospho-ubiquitin (Fig. 2b) revealed a near-identical organization of the parkin core (UPD– RING1–IBR) bound to phospho-ubiquitin, as compared to previous structures (r.m.s.d. 0.73 Å with human parkin–phospho-ubiquitin, PDB 5N2W<sup>6</sup>) (Extended Data Fig. 7a), and there were no large conformational changes in individual domains. Modelling of an open E2–Ub conjugate structure<sup>28</sup> reveals sensible interfaces (Extended Data Fig. 7b)

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Fig. 3 | The parkin UPD-phospho-Ubl interaction. a, Structural detail of the binding site between parkin phospho-Ubl (green) and UPD (blue). Key residues are shown, and phospho-Ser65 is highlighted. Grey spheres indicate Zn atoms, and hydrogen bonds are shown as dotted lines. b, Ub-VS probe reactivity of the RING2 catalytic Cys residue with parkin-phospho-ubiquitin, phospho-parkin (pparkin), or phosphoparkin(K211N). The experiment was done in duplicate with identical results; for gel source data, see Supplementary Fig. 1. c, HDX-MS analysis of phospho-parkin-phospho-ubiquitin in comparison to phosphoparkin(K211N)-phospho-ubiquitin. The C-terminal peptide profiles are compared (see Extended Data Fig. 7d for overall data). The RING2 C terminus remains solvent-protected in the phospho-parkin(K211N) background. Technical triplicates are shown for all time points. d, Superposition of parkin-phospho-ubiquitin (PDB 5N2W<sup>6</sup>), and phospho-parkin-phospho-ubiquitin showing the relative positions of the RING2 (cyan surface) and phospho-Ubl (green surface), respectively, on the UPD domain.

and corroborates the ubiquitin binding site observed in HDX-MS<sup>6</sup> (Fig. 1d, Extended Data Fig. 3c). Notably, the phosphorylated Ubl domain was bound to the UPD,

Notably, the phosphorylated Ubl domain was bound to the UPD, and had moved by more than 50 Å from its position in autoinhibited parkin (Figs. 2b, 3a, Extended Data Fig. 7a). The interface between phospho-Ubl and UPD is mediated by a common interaction site of ubiquitin-fold modifiers, the hydrophobic Ile44 patch of the Ubl, and engulfs the elongated UPD domain covering a surface of more than

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800 Å2; this interface can be recapitulated in HDX-MS data (Fig. 1d, e, Extended Data Fig. 7c). Furthermore, the interaction places phosphorylated Ser65 into a positively charged pocket on the UPD (Fig. 3a). The phospho-acceptor pocket is lined by Lys161, Arg163 and Lys211, which contact the phosphate group and form four hydrogen bonds. We had previously noted this putative phosphate-acceptor binding site<sup>8</sup>, the importance of which is highlighted by two mutations found in patients with AR-JP (K211N and K161N)<sup>1,2</sup> that also abrogate the function of parkin in mitophagy<sup>13,29</sup>. Mechanistically, phosphorylated parkin with a K211N mutation blocking the phospho-acceptor pocket vas no longer modified by Ub-VS5 (Fig. 3b). HDX-MS confirmed that phospho-parkin-phospho-ubiquitin(K211N) showed little sign of RING2 release and had the strongest relative solvent protection in the C terminus, where RING2 binds the UPD (Fig. 3c, Extended Data Fig. 7d). This indicated that the catalytic Cys of the RING2 domain remained inaccessible if phospho-Ubl was unable to interact with its UPD binding site, and explained how AR-JP-causing K211N or K161N mutations produce parkin variants that cannot be activated by Ser65 phosphorylation.

The position of the Ubl on the UPD overlaps only marginally with the position of RING2 in autoinhibited states of parkin, and while binding of both would lead to steric clashes (Fig. 3d, Extended Data Fig. 7a), the hydrophobic RING2 binding site (Extended Data Fig. 1b) would remain unusually exposed upon opening of the RING2-UPD interface. In our structure, clear electron density for a stretch of residues was apparent at the RING2-binding site of the UPD (Extended Data Fig. 6c), and we could unambiguously assign this density to the sequence corresponding to the first conserved region of the Ubl-UPD linker (Fig. 4, Extended Data Figs. 4, 6). In particular, residues Leu102, Val105 and Leu107 occupy pockets previously bound by RING2 residues Met458, Trp462 and Phe463 (Fig. 4a, b). Hence the Ubl-UPD linker shields the hydrophobic patch on the UPD that was opened by release of RING2. Indeed, similar to the K211N mutation, phosphoparkin with deletion of the first set of conserved linker residues  $(\Delta 101-109)$  was unable to be charged by Ub-VS (Fig. 4c).

The linker provides additional contact points for the phospho-Ubl interface. Arg104 is located between two key hydrophobic residues, and contacts with its side chain the Ser65 loop in phospho-Ubl. Notably, parkin(R104W) is a mutation found in patients with AR-JP<sup>2.30</sup>, and we would predict that this mutation would disrupt or misalign the observed hydrophobic interactions. A phospho-parkin(R104A) mutant was charged less efficiently by Ub-VS (Fig. 4d), showed slower E2–Ub discharge activity (Extended Data Fig. 8a, b) and reduced in vitro poly-ubiquitination activity (Fig. 4e), whereas its thermal stability remained unperturbed (Extended Data Fig. 8c).

Together, structural, biochemical and patient data confirm the crucial importance of the first conserved stretch of the Ubl–UPD linker for parkin activity, and define a new activating element, which we term ACT, in this understudied regulatory region of parkin, which also contains several phosphorylation sites (see further discussion in Extended Data Fig. 8d).

Our work resolves the activation mechanism of parkin, finally visualizing large-scale domain rearrangements and showing that the parkin Ubl switches between an inhibitory position in the unphosphorylated molecule to an activating position in phosphorylated parkin. Our data are consistent with a model in which the phosphorylated Ubl and the ACT element in the Ubl-UPD linker dislodge RING2 from its autoinhibited position, enabling it to be charged by E2–Ub, and ubiquitinate substrates in its vicinity independently of the parkin core (Fig. 4f). Notably, our model does not require parkin dimerization<sup>6,26</sup>.

Our structure of an activated parkin core will inform drug discovery efforts that have set out to identify parkin activators. With the realization that the RING2–UPD interface opens and exposes a hydrophobic pocket, small molecules could be directed towards this interface. Such molecules may become particularly useful to restart mitophagy in patients with AR-IP who carry parkin variants that are not activated by PINK1-mediated Ubl phosphorylation.



Fig. 4 | An activating element (ACT) in parkin. a, Structural detail of the ordered ACT within the parkin-phospho-Ubl-UPD linker. Three hydrophobic ACT residues bind the hydrophobic UPD groove, and polar ACT residues contact phospho-Ubl. **b**, Superposition of the ACT with RING2 (PDB 5N2W<sup>6</sup>, semi-transparent) in the same orientation as in **a**. Hydrophobic ACT residues mimic RING2 interactions. c, Ub-VS charging assay of phospho-parkin, and phospho-parkin variants lacking the ACT ( $\Delta$ 101–109) or the second conserved hydrophobic linker sequence ( $\Delta$ 116– (A101-109) of the second conserved hydrophobic linker sequence (A116 123). Experiments were performed in duplicate with identical results; for gel source data, see Supplementary Fig. 1. **d**, Ub-VS charging assay as in c for wild-type (WT) phospho-parkin or the R104A mutant. Patients with parkin(R104W) suffer from AR-JP<sup>2,30</sup>. Experiments were performed in duplicate with identical results; for gel source data, see Supplementary Fig. 1.

### **Online content**

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586 018-0224-x

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e, Activity of wild-type phospho-parkin and the R104A mutant in vitro, with UBE2L3 as the E2 enzyme. The reaction was resolved by SDS-PAGE and western blotted for ubiquitin. A representative gel of three independent experiments is shown. For source data, see Supplementary Fig. 1. f, Model of the sequential domain rearrangements required for full parkin activation, extended from a previous model<sup>3</sup> (also see Fig. 1a). In autoinhibited parkin, the Ubl, REP and RING2 assume inhibitory positions. Binding of phospho-ubiquitin induces localization of parkin to the MOM, repositioning of the IBR domain and release of the Ubl domain. Phosphorylation of parkin allows the phospho-Ubl domain and ACT element to bind to the UPD, to replace and release the RING2 and REP, enabling ubiquitination of MOM substrates.

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### METHODS

**Molecular biology.** cDNA of *Thamnophis sirtalis* (*Ts*) parkin was obtained from GeneArt (Invitrogen) with codon-optimization for bacterial expression and cloned into a pOPIN-K vector<sup>31</sup>, using the In-Fusion HD Cloning Kit (Takara Clonetech). Human (*Hs*)parkin and *Pediculus humanus corporis* (*Ph*)PINK1 constructs were also expressed from a pOPIN-K vector, while UBE2L3 was expressed from a pGEX6 vector. *Hs*UBE1/PET21d was a gift from C. Wolberger (Addgene plasmid # 34965<sup>32</sup>).

Site-directed mutagenesis was carried out using the QuikChange protocol with Phusion polymerase. A TEV cleavage site was introduced into the parkin constructs using the NEB Q5 Site-Directed Mutagenesis Kit (NEB). In *Ts*parkin, residues 368–374 (KSPGATA) were replaced by the ENLYFQS TEV cleavage sequence, while in *Hs*parkin residues 382–378 (EASGTTT) were replaced by the TEV cleavage sequence to yield cleavable constructs.

**Protein purification.** For parkin expression, *Escherichia coli* Rosetta2 pLacI cells (Novagen) were grown in 2×TY medium at 37°C. At OD<sub>600</sub> = 0.6 the temperature was reduced to 18°C; expression was induced at OD<sub>600</sub> = 0.8–1.0 with 30 µM IPTG and the medium supplemented with 200 µM ZnCl<sub>2</sub>. Cells were harvested after overnight growth at 18°C and frozen at  $-20^{\circ}$ C.

For parkin purification, cells were resuspended in lysis buffer (300 mM NaCl, 10% (w(v) glycerol, 25 mM Tris (pH 8.5), 14.3 mM  $\beta$ -mercaptoethanol) supplemented with 2 mg/ml lysozyme, 0.2 mg/ml DNaseI and 80 µg/ml PMSE. The suspension was homogenized using an EmulsiFlex-C3 (Avestin) for two passes at ~15,000 p.s.i. and cleared by centrifugation at 46,000g for 35 min at 4 °C. The clarified lysate was applied to Amintra glutathione resin (Expedeon), resin washed with high salt buffer (25 mM Tris (pH 8.5), 500 mM NaCl, 10 mM DTT) and GST-fusion parkin cleaved from the resin overnight at 4 °C with GST-3C protease.

Samples were eluted and resin washed with no-salt buffer (25 mM Tris (pH 8.5), 10 mM DTT). All following purification steps were carried out on an Åkta Pure system (GE Healthcare). Pooled fractions were subjected to anion-exchange chromatography on a 6-ml Resource Q column (GE Healthcare) with a 0–25% linear gradient from buffer A (25 mM Tris (pH 8.5), 10 mM DTT, 50 mM NaCl) to buffer B (25 mM Tris (pH 8.5), 10 mM DTT, 1,000 mM NaCl) over 15 column volumes. For phosphorylated parkin, the resulting sample was phosphorylated using a 1:100 molar ratio of GST–*Ph*PINK1 in phosphorylation buffer (10 mM ATP, 10 mM MgCl<sub>2</sub>, 200 mM NaCl, 50 mM Tris (pH 8.5), 10 mM DTT). P1NK1 was subsequently removed by incubation with Amintra glutathione resin (Expedeon) and phosphorylated parkin purified using anion exchange chromatography as above. Finally, samples were subjected to size-exclusion chromatography (HiLoad 16/600 Superdex 75 pg, GE Healthcare) into buffer C (25 mM Tris (pH 8.5), 10 mM DTT, 200 mM NaCl).

In short, *Hs*UBE1 was purified as follows. An N-terminal GST–Ub fusion protein was expressed and lysed in β-mercaptoethanol-free lysis buffer and applied to Amintra glutathione resin (Expedeon). Upon washing, the resin was equilibrated with 50 mM Tris (pH 8.5) and 2 mM ATP. *Hs*UBE1 β-mercaptoethanol-free clarified lysate was generated as above, supplemented with 10 mM ATP and 10 MgCl<sub>2</sub> and incubated with the GST–Ub fusion-bound glutathione resin at room temperature for 30 min. The resin was then washed with DTT-free high salt buffer supplemented with 5 mM MgCl<sub>2</sub>. *Hs*UBE1 was eluted in DTT-containing buffer and protein-containing fractions were applied to anion-exchange and size-exclusion chromatography as above. UBE2L3, UBE2D3 and GST–*Ph*PINK1 were purified as described previously<sup>33</sup>.

Generation of non-dischargeable E2-Ub complex. UBE2L3 (C86K) and ubiquitin were stored in charging buffer (25mM CAPSO (pH 9.5), 20 mM MgCl<sub>2</sub>, 150 mM NaCl). UBE2L3 (C86K) (450 µM) was incubated with Ub (900 µM) and HsUBE1 (2.5 µM) in charging buffer supplemented with 10 mM ATP at 37 °C overnight. The resulting mixture was applied to size-exclusion chromatography as above in buffer C. Fractions containing UBE2L3-Ub were pooled, concentrated and again applied to size-exclusion chromatography to remove free UBE2L3. Ub-VS generation and parkin coupling. Ub(1-75)-MesNa was prepared as described previously<sup>34</sup>, H-Gly-VS hydrochloride was a kind gift from H. Ovaa

Ub-VS generation and parkin coupling. Ub(1–75)–MesNa was prepared as described previously<sup>34</sup>. H-Gly-VS hydrochloride was a kind gift from H. Ovaa and B.-T. Xin (Leiden University). Ub–MesNa, stored in buffer D (20 mM HEPES, 50 mM sodium acetate (pH 6.5), 75 mM NaCl) at ~20 mg/ml, was used to dissolve ~50 mg H-Gly-VS hydrochloride together with ~30 mg of N-hydroxysucinamide (Fluka), acting as a catalyst. The pH was raised to 8.5 by addition  $\sigma$ -60 µl of 4 M NaOH and reaction incubated at 37 °C. Reaction progress was monitored by LC–MS analysis. When the ratio of Ub(1–75)-VS to hydrolysed Ub–MesNa product was ~1:1, with a minimum formation of the doubly coupled, Ub(1–75)–VS-VS species, the reaction was diluted in 50 mM sodium acetate (pH 4.5) and applied to cation-exchange chromatography on a 1-ml MonoS column (GE Healthcare) with a 10–35% linear gradient between 50 mM sodium acetate (pH 4.5) containing 0 M and 1 M NaCl, respectively. The resulting fractions were analysed by LS–MS

and Ub(1–75)–VS containing fractions were pooled and applied to size-exclusion chromatography as above in buffer D.

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For quantitative parkin–Ub-VS coupling, phospho-parkin was purified as above where 10 mM DTT in buffer C was replaced with 5 mM TCEP. Phospho-parkin and Ub-VS were mixed at a 1.3 molar ratio and incubated at room temperature. Reaction progress was monitored by LC–MS analysis and, upon completion, the reaction was quenched by addition of DTT (~30 min). The resulting sample was purified using size-exclusion chromatography (buffer C).

Mass-spectrometry analysis. LC–MS analysis was carried out on an Agilent 1200 Series chromatography system coupled to an Agilent 6130 Quadrupole mass spectrometer. Samples were eluted from a Phenomenex Jupiter column (5 ml, 300 Å, C4 column, 150  $\times$  2.0 mm) using an acetonitrile gradient + 0.2% (v/v) formic acid. Protein was ionized using an ESI source (3 kV ionization voltage), and spectra were analysed in positive ion mode with a mass range between 400 and 2,000 m/z. Averaged spectra were deconvoluted using Promass (Novatia, LLC) and plotted using GraphPad Prism (version 7).

Limited proteolysis. Tsparkin, phospho-Tsparkin, phospho-Tsparkin-phosphoubiquitin, and phospho-Tsparkin-phospho-ubiquitin charged with Ub-VS were purified as described above. A 1 mg/ml protein solution was mixed with 5 µg/ml solution of elastase from the Proti-Acc Kit (Hampton research) and incubated for 1 h at room temperature. The reactions were quenched by addition of DTT- and iodoacetamide-containing LDS buffer and resolved on a 4–12% SDS NuPAGE gradient gels (Invitrogen) and stained with Instant Blue SafeStain (Expedeon).

Hydrogen–deuterium exchange mass spectrometry (HDX-MS). Complexes were formed on ice and incubated for 30 min to give a final parkin concentration of 10 µM. Deuterium-exchange reactions of parkin and the different complexes were initiated by diluting the protein in D<sub>2</sub>O (99.8% (v/v) D<sub>2</sub>O ACROS, Sigma) in 25 mM Tris (pH 8.5), 200 mM NaCl, 1 mM TCEP to give a final D<sub>2</sub>O percentage of ~95%. For all experiments, deuterium labelling was carried out at 23 °C (unless otherwise stated) at five time points: 0.3 s (3 s on ice), 3 s, 30 s, 300 s and 3,000 s, in technical triplicate. The labelling reaction was quenched by the addition of chilled 2.4% (v/v) formic acid in 2 M guanidinium hydrochloride and immediately frozen in liquid nitrogen. Samples were stored at -80 °C before analysis.

The quenched protein samples were rapidly thawed and subjected to proteolytic cleavage with pepsin followed by reversed phase HPLC separation. In brief, the protein was passed through an Enzymate BEH immobilized pepsin column,  $2.1 \times 30$  mm,  $5 \,\mu$ m (Waters, UK) at 200 µl/min for 2 min, the peptic peptides were trapped and desalted on a  $2.1 \times 5$  mm C18 trap column (Acquity BEH C18 Vanguard pre-column,  $1.7 \,\mu$ m, Waters). Trapped peptides were subsequently eluted over 11 min using a 3-43% gradient of acetonitrile in 0.1% (v/v) formic acid at 40 µl/min. Peptides were separated on a reverse phase column (Acquity UPLC BEH C18 Column  $1.7 \,\mu$ m, 100 mm  $\times 1$  mm; Waters) and detected on a SYNAPT G2-Si HDMS mass spectrometer (Waters) over an *m*/z of 300 to 2,000, with the standard electrospray ionization (ESI) source with lock mass calibration using [Glu1]-fibrino peptide B (50 fmol/µl). The mass spectrometer was operated at a source temperature of 80 °C and a spray voltage of 2.6 kV. Spectra were collected in positive ion mode.

<sup>1</sup> Peptide identification was performed by  $MS^{e35}$  using an identical gradient of increasing acetonitrile in 0.1% (v/v) formic acid over 11 min. The resulting  $MS^e$  data were analysed using Protein Lynx Global Server software (Waters, UK) with an MS tolerance of 5 ppm.

Mass analysis of the peptide centroids was performed using DynamX software (Waters). Only peptides with a score >6.4 were considered. The first round of analysis and identification was performed automatically by the DynamX software, however, all peptides (deuterated and non-deuterated) were manually verified at every time point for the correct charge state, presence of overlapping peptides, and correct retention time. Deuterium incorporation was not corrected for backexchange and represents relative, rather than absolute changes in deuterium levels. Changes in H/D amide exchange in any peptide may be due to a single amide or a number of amides within that peptide.

**Protein preparation for crystallization.** TEV-cleavable parkin was purified as described above. An anion-exchange purified parkin sample (step 1) was incubated with Ub-C3Br in a 1:4 molar ratio and GST-PhPINK1 in a 9:1 molar ratio in phosphorylation buffer (10 mM ATP, 10 mM MgCl<sub>2</sub>, 200 mM NaCl, 50 mM Tris (pH 8.5), 10 mM DTT) at a final parkin concentration of ~70 µM, yielding a phosphorylated, phospho-ubiquitin conjugated, TEV-cleavable parkin sample (step 2). GST-PhPINK1 was subjected to His<sub>6</sub>-TEV cleavage overnight at 4°C (step 3). His<sub>6</sub>-TEV was removed using Ni-NTA agarose (Qiagen), sample diluted in buffer A and applied to anion-exchange and size-exclusion chromatography as described above.

To generate Ub-C3Br, Ub(1–75)–MesNa was prepared as described previously<sup>34</sup>. Ub-MesNa, stored in buffer D was incubated with 0.2 g/ml 3-bromopropylamine hydrobromide (Fluka) dissolved in PBS (pH 4.8) at 2:1 molar ratio

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with final Ub(1-75)-MesNa concentration of 445 µM. The coupling was carried out on ice for 30 min following addition of 50 µl 4 M NaOH to raise the pH to 10.5. The reaction was quenched by addition of 12  $\mu l$  of 12 M HCl and sample buffer exchanged using a disposable PD-10 desalting column (GE Healthcare) into buffer C.

Crystallization. Initial crystals were found from crystallization experiments carried out at 18 °C in a 96-well sitting drop vapour diffusion plates in the MRC format (Molecular Dimensions) by mixing 100 nl of 4 mg/ml protein solution with 100 nl reservoir solution. The crystallization condition of 12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, 12.5% (v/v) MPD, 0.03 M of each sodium nitrate, disodium hydrogen phosphate, ammonium sulfate, 0.1 M MOPS/ HEPES-Na (pH 7.5) was found from the MORPHEUS screen (Molecular Dimensions). Seeds were obtained from a fine screen and streak seeding was carried out in a hanging drop format from an 8 mg/ml protein solution. Larger crystals were obtained after 6 days in the original crystallization condition. Crystals were soaked in mother-liquor supplemented with 10% (v/v) glycerol before vitrification in liquid nitrogen.

Data collection, phasing and refinement. Diffraction data were collected at the Diamond Light Source, beamline I-24 (0.9686 Å, 100 K), and processed using DIALS<sup>36</sup>. The crystal structure was determined by molecular replacement in Phaser37 using the structure of the human parkin core (PDB 5N2W6) truncated after the IBR, as well as a human parkin Ubl structure (PDB 5C1Z<sup>19</sup>). The structure was built at 1.80 Å, in multiple rounds of model building in Coot<sup>38</sup> and refine-ment in PHENIX<sup>39</sup>. Phenix ReadySet-derived geometry restraints for the 3CN warhead were used, with external restraints defining the linkage points. Final Ramachandran statistics: 98.9% favoured, 1.1% allowed, and 0% outliers. Structural figures were generated using PyMol (http://www.pymol.org). Data collection and refinement statistics can be found in Extended Data Table 1.

Parkin activity assays. Ub-VS conjugation assays. Indicated parkin variants stored in either DTT- or TCEP-containing buffer were incubated with Ub-VS that was prepared as described above. The reactions were quenched at indicated time points by addition of DTT- and iodoacetamide-containing LDS buffer and resolved on a 4–12% SDS NuPAGE gradient gels (Invitrogen) and stained with Instant Blue SafeStain (Expedeon).

Parkin assembly assays. Wild-type or R104A phospho-parkin (5  $\mu$ M) were incubated in ubiquitination buffer (30 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM ATP, 10 mM MgCl<sub>2</sub>) with HsUBE1 (0.2 μM), UBE2L3 (2 μM) and Ub (20 μM). The reactions were quenched at the indicated time points by addition of DTT- and iodoacetamide-containing LDS buffer and resolved on a 4-12% SDS NuPAGE gradient gels (Invitrogen ) and transferred to a PVDF membrane (BioRad). Membranes were blocked in a 5% (w/v) milk solution in PBS-T (PBS + 0.1% (v/v) Tween-20) for 30 min and incubated overnight at 4°C with a ubiquitinrecognizing antibody (Ubi-1, NB300-130, Novus Biologicals) in 5% (w/v) BSA in PBS-T and 0.1% (w/v) sodium azide. The membrane was then washed with PBS-T, incubated for 1 h at room temperature with anti-mouse IgG-HRP (NXA931, GE Healthcare) in 5% (w/v) milk in PBS-T, washed in PBS-T and visualized using the Amersham Western Blotting Detection Reagent (GE Healthcare) and a ChemiDoc Touch Imaging System (BioRad).

E2-Ub discharge assays. The UBE2D3-Ub conjugate was generated by incubating UBE2D3 (20  $\mu$ M) with HsUBE1 (20 nM) and Ub (80  $\mu$ M) in ubiquitination buffer supplemented with 5 µM CaCl2 at 37 °C for 10 min. To remove remaining

ATP, 0.5 U of Apyrase (NEB) was added and the reaction incubated at 30 °C for 30 min

The discharge reaction was studied by addition of 1 µM wild-type or R104A phospho-parkin to a diluted charging reaction mixture (final UBE2D3 concen tration was 9  $\mu$ M). The reactions were quenched at indicated time points by addition of DTT-free LDS buffer, while a final sample was collected at 11 min in UDTT-containing LDS buffer to assess the extent of isopeptide-linked UBE2D3– Ub species formation. Samples were resolved on 4–12% SDS NuPAGE gradient gels (Invitrogen) and stained with Instant Blue SafeStain (Expedeon). The gel band intensity was quantified in ImageJ by isolating the specific intensity of the UBE2D3~Ub thioester band as indicated, subtracting the background of the final reduced sample and normalized within each reaction

Thermal denaturation assays. Protein melting curves were recorded on a Corbett RG-6000 real time PCR cycler (30 °C to 85 °C with 7 s per 0.5 °C). Samples contained 4  $\mu$ M parkin protein and 4×SYPRO orange in ubiquitination buffer + 5 mM TCEP. Melting curves were obtained as the maxima of dF/dT versus T plots. All data were recorded in triplicate.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper

Data availability. Coordinates and structure factors have been deposited with the Protein Data Bank under accession code 6GLC. Uncropped versions of all gels are displayed in Supplementary Fig. 1. All reagents and data are available upon reasonable request from the corresponding author.

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Extended Data Fig. 1 | Mechanisms of parkin autoinhibition. a, Structure of autoinhibited, full-length human parkin (PDB 5C1Z<sup>19</sup>) shown schematically (top, as in Fig. 1a) and in cartoon representation in the same colours. Two insets show the UPD–RING2 interface (with Cys431 shown in ball-and-stick representation), and the blocked E2 binding site (with the E2 position, modelled according to PDB 5EDV<sup>28</sup>, shown as grey surface). Zn ions are shown as grey spheres. **b**, An 'openbook' view of the UPD–RING2 interface, with hydrophobic residues

coloured white on each surface. **c**, Structure of phospho-ubiquitin bound to full-length parkin (PDB 5N2W<sup>6</sup>) as in **a**. Phospho-ubiquitin binding leads to helix straightening, and IBR domain repositioning, which releases the Ubl domain for phosphorylation<sup>5,6</sup>. In the shown structures of unphosphorylated parkin, the Ubl and REP (red) inhibit E2 binding, and the RING2–UPD interface is intact, with Cys431 being inaccessible. The Ubl–UPD linker was removed from crystallized constructs in **a** and **c**<sup>6,19</sup>.



Extended Data Fig. 2 | Sample preparation for HDX-MS and selected raw data. a, Representative LC–MS spectrum of the prepared Ub-VS probe (see Methods). Experiment was performed in duplicate. b, Representative LC–MS spectrum of Ub-VS-reacted phospho-parkin. Experiment was performed in duplicate. c, Samples used in HDX-MS analysis. In HDX-MS, non-covalent complexes with phospho-ubiquitin were used. Covalent complexes are indicated with a dash and non-covalent

complexes by a colon. This is representative of at least three independent experiments; for gel source data, see Supplementary Fig. 1. **d**, Relative deuterium uptake (in Da) is shown for exemplary selected peptides across the parkin molecule, over the timecourse of the experiment. Each point for the technical replicate experiments is shown. Data points were taken at identical time points, but are offset on the *x* axis for clarity.



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Extended Data Fig. 3 | Graphical representation of HDX-MS data. Data from HDX-MS experiments (Fig. 1b–e) were plotted onto a stylized 'open domain' model of parkin, with identical colouring (blue, more protected from solvent exchange compared to previous state; red, less protected from solvent exchange compared to previous state). Grey regions correspond to peptides that were not covered or could not be analysed owing to modification. Schematic domain representations indicate an average change of the corresponding interfaces across all time points. White regions indicate no change. a, Parkin compared to parkin–phosphoubiquitin. b, Parkin–phospho-ubiquitin compared to phospho-parkin– phospho-ubiquitin. c, Phospho-ubiquitin in complex with an isopeptide UBE21.3–Ub thioester mimetic (see Methods). This experiment confirmed a previously reported binding site for the E2-conjugated ubiquitin on the RBR<sup>6,28</sup> (8). **d**, Phospho-parkin-phospho-ubiquitin compared to Ub-VSreacted phospho-parkin-phospho-ubiquitin. Reaction with Ub-VS leads to modification of the catalytic Cys431-containing-peptide, generating non-identical peptides precluding comparison by HDX-MS. Low coverage of the RING2 domain can be explained by ubiquitin resistance to pepsin cleavage, leading to protection of the linked RING2 domain and subsequent peptide loss. To allow comparison, these peptides were also omitted from analysis of the UBE2L3-Ub-bound sample. In c and d, the structure representation is deceiving because REP and RING2 are highly mobile and are no longer bound to the parkin core. Indeed, the high hydrogen-deuterium exchange in the REP sequence in active parkin (Fig. 1d, e; peptide (4) in Extended Data Fig. 2d) indicates an additional loss of secondary structure in this helical element when REP and RING2



**Extended Data Fig. 4** | A **conserved linker between Ubl and UPD.** Sequence alignment of parkin, with domains coloured corresponding to 5N2W<sup>6</sup> as in Extended Data Fig. 1. Phosphate binding pockets are labelled. The linker region between Ubl and UPD (amino acids 76–143) contains two strings of highly conserved residues. Residues upstream and downstream of the conserved region are unconserved both in sequence and linker length. Tsparkin shows the smallest number of residues in the linker (upstream, 25 amino acids in human parkin, 18 amino acids in Tsparkin; downstream, 18 amino acids in human parkin, 11 amino acids in Tsparkin; See also Extended Data Fig. 8d.



Extended Data Fig. 5 | See next page for caption.

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Extended Data Fig. 5 | Tsparkin and pre-crystallization biochemistry for human parkin. a, HDX-MS experiment comparing phospho-Tsparkin reacted with phospho-ubiquitin-C3Br and Ub-VS with identical colouring (blue, more protected from solvent exchange; red, less protected from solvent exchange; grey, not covered in all of the compared states, see Fig. 1). The experiment was performed in technical triplicate. The Tsparkin profile is highly similar to the profile of human parkin in an analogous state (Fig. 1e). Higher peptide resolution in this sample reveals protection of the RING2 interface by reacted Ub-VS, but the C terminus of RING2 that binds to the UPD interface is surface exposed. Both phospho-Ubl and the Ubl-UPD linker are protected in activated parkin. **b**, Limited proteolysis of Tsparkin with elastase, in different stages of activation. In unphosphorylated, autoinhibited Tsparkin, the Ubl is cleaved off in the Ubl-UPD linker. In activated forms of Tsparkin, Phospho-Tsparkin, phospho-Tsparkin preacted with phospho-ubiquitin-C3Br and Ub-VS), the RING2 is readily cleaved off, while the Ubl is not efficiently removed. This suggests that the Ubl-UPD linker is not accessible in activated forms of Tsparkin. A representative gel from three independent experiments is shown. For gel source data, see Supplementary Fig. 1. **c**, A TEV cleavage site was introduced after the IBR domain, so that after activation by phospho-ubiquitin and UbI-phosphorylation, the released RING2 domain can be removed. Once removed, RING2 is no longer stably associated with the remaining parkin core. Shown is a gel filtration profile illustrating this point. A representative profile from three independent experiments is shown. **d**, SDS-PAGE analysis of sample preparation process (see Methods). Asterisk denotes ubiquitin probe (Ub-C3Br)reacted material that modifies the RING2 catalytic Cys, which explains the cleaved, probe-reacted RING2 band (asterisk in step 3). A representative gel from three independent experiments is shown. For gel source data, see Supplementary Fig. 1. **e**, HDX-MS experiment on Tsparkin, comparing phospho-Tsparkin reacted with phospho-ubiquitin-C3Br with phospho-Tsparkin reacted with phospho-Usiquitin-C3Br with phosphotorsparkin reacted with phospho-Tsparkin reacted with phosphotorsparkin reacted sin a. Identical profiles were obtained, showing that RING2 removal has no effect on the activated core of parkin. This further indicates that RING2 acts independently of the parkin core upon full activation. Notably, in both comparisons, we observed concomitant protection of phospho-Ubl and the UbI-UPD linker. The experiment was performed in technical triplicate.



Extended Data Fig. 6 [Quality control and electron density maps for human phospho-parkin-phospho-ubiquitin. a, LC-MS spectrum of crystallized human phospho-parkin (amino acids 1–382) bound to phospho-ubiquitin. This is representative of two independent experiments. **b**, Composite omit map (generated with simulated annealing) shown for the single complex in the asymmetric unit.  $2|F_0|-|F_c|$  electron density is shown at  $1\sigma$ . **c**, Electron density as in **b** for

the Ubl-UPD linker, **d**, Electron density as in **b** for the Ser65 phospho-Ubl binding site on the UPD linker, **e**, Electron density as in **b** for the Ser65 phospho-Ub binding site. As we are missing electron density for disordered regions in the Ubl-ACT and ACT-UPD linkers, we cannot exclude the possibility that phospho-Ubl may interact *in trans* with a neighbouring parkin molecule. Also see Extended Data Table 1.





**Extended Data Fig.** 7 | **The phospho-Ubl binding site on the UPD.** a, Side-by-side view of phospho-parkin–phospho-ubiquitin (left) and parkin–phospho-ubiquitin (PDB 5N2W<sup>6</sup>, right), and superposition of both (below). The green Ubl domain changes position by >50 Å. b, E2–Ub from the structure of the HOIP RBR domain in complex with UBE2D2–Ub<sup>28</sup> was modelled onto phospho-parkin–phospho-ubiquitin, by superposition of the RING1 domains of each complex. The E2conjugated ubiquitin molecule in the 'open' conformation binds to the previously recognized cryptic ubiquitin binding interface on RING1– IBR<sup>6</sup>. The contact points correlate with HDX-MS data (Fig. 1d, Extended Data Figs. 2, 3c). **c**, HDX-MS data from Fig. 1e were plotted onto the phospho-parkin-phospho-ubiquitin structure with identical colouring (blue, more protected from solvent exchange; red, less protected from solvent exchange; grey, not covered in all of the compared states, compare with Fig. 1). Protected regions on UPD match the observed phospho-Ubl interface. **d**, HDX-MS experiments comparing parkin with a mutation in the phospho-acceptor binding site on the UPD (phospho-parkin(K211N)-phospho-ubiquitin) compared with phospho-parkin-phospho-ubiquitin, coloured as in **c**. The mutant is unable to protect the Ubl, and to release RING2 and REP. Experiments were done as technical triplicate.



heterozygous mutation (2 families)

Extended Data Fig. 8 | A regulatory role of the parkin Ubl–UPD linker. a, b, E2 discharge assay resolved on a Coomassie stained SDS–PAGE gel (a) and quantified from band intensities (b) for phospho-parkin and phospho-parkin(R104A). This is representative of at least two independent experiments; for gel source data, see Supplementary Fig. 1. The mutation in the ACT element leads to a reduction in discharge activity, suggesting that the residue is required to dislodge RING2 from the parkin core. c, Parkin(R104A) is equally stable as wild-type parkin, in the unphosphorylated or phosphorylated form. Thermal denaturation experiments were performed as technical triplicate. d, Sequence detail of the Ubl–UPD linker, which contains the ACT element described here. In the ACT element as bound to phospho-parkin-phosphoubiquitin, the positions of two annotated (in PhosphoSitePlus) parkin phosphorylation preventing phospho-Ubl and/or linker binding to the UPD. It is hence highly likely that phosphorylation of parkin on these residues provides additional layers of parkin peosphorylation by PKA was recently reported to be a mechanism of parkin inhibition in beigeto-white adipocyte transition, although phosphorylation sites remained unclear<sup>41</sup>. Residues before the ACT element (amino acids 73–99) and after the ACT element (amino acids 109–142) are disordered in our structure. The last ordered residue, Ser108, is tantalizingly close to the REP binding site as well as to the phospho-ubiquitin binding pocket, but disorder suggests that clear binding sites for other conserved linker residues, in particular for the parkin GLAVIL motif, are not present. HDX-MS also does not reveal additional protection of the linker, even when the E2–Ub conjugate is bound, suggesting that the GLAVIL motif may not bind the E2 (Fig. 1d, Extended Data Figs. 2, 3c). On the other hand, there are at least three additional annotated phosphorylation sites, Ser116, Ser131 and Ser136<sup>15,40,42,43</sup>, suggesting that the second part of the linker may also be regulated. Phosphorylation on these residues could change the ability of the disordered parts of the linker to interact with parkin *in cis*. For example, we would speculate that a phosphorylated Ser116 could for example, reach the phosphate binding pocket occupied by phospho-Ser65 of ubiquitin. Alternatively, the remaining UbI–UPD linker may be important for substrate recruitment, or involved in other, PINK1independent mechanisms of parkin activation.

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Extended Data Table 1 | Data collection and refinement statistics

|                             | phospho-parkin (1-382) - phospho-Ub |
|-----------------------------|-------------------------------------|
| Data collection             |                                     |
| Space group                 | <i>P</i> 3 <sub>2</sub> 2 1         |
| Cell dimensions             |                                     |
| a, b, c (Å)                 | 83.93, 83.93, 105.12                |
| $\alpha, \beta, \gamma$ (°) | 90, 90, 120                         |
| Resolution (Å)              | 72.69 –1.80 (1.84 – 1.80)           |
| R <sub>merge</sub>          | 0.065 (0.773)                       |
| // σ/                       | 13.80 (2.40)                        |
| Completeness (%)            | 100.00 (99.30)                      |
| Redundancy                  | 6.7 (6.7)                           |
| Ş                           |                                     |
| Refinement                  |                                     |
| Resolution (Å)              | 59.79 –1.80                         |
| No. reflections / test set  | 40229 / 2020                        |
| Rwork / Rfree               | 0.180 / 0.205                       |
| No. atoms                   |                                     |
| Protein                     | 3039 (398 aa)                       |
| Ligand/ion                  | 41                                  |
| Water                       | 165                                 |
| <i>B</i> -factors           |                                     |
| Protein                     | 45.05                               |
| Ligand/ion                  | 66.19                               |
| Water                       | 46.94                               |
| R.m.s. deviations           |                                     |
| Bond lengths (Å)            | 0.008                               |
| Bond angles (°)             | 1.19                                |
| <b>U</b> ()                 |                                     |

\*Values in parentheses are for highest-resolution shell.

Appendix F.

# An invisible ubiquitin conformation is required for efficient phosphorylation by PINK1

### An invisible ubiquitin conformation is required for efficient phosphorylation 302 by PINK1



Article



# An invisible ubiquitin conformation is required for efficient phosphorylation by PINK1

Christina Gladkova<sup>†</sup>, Alexander F Schubert<sup>†</sup>, Jane L Wagstaff, Jonathan N Pruneda<sup>(D)</sup>, Stefan MV Freund & David Komander<sup>\*</sup>

### Abstract

The Ser/Thr protein kinase PINK1 phosphorylates the well-folded, globular protein ubiquitin (Ub) at a relatively protected site. Ser65. We previously showed that Ser65 phosphorylation results in a conformational change in which Ub adopts a dynamic equilibrium between the known, common Ub conformation and a distinct, second conformation wherein the last  $\beta$ -strand is retracted to extend the Ser65 loop and shorten the C-terminal tail. We show using chemical exchange saturation transfer (CEST) nuclear magnetic resonance experiments that a similar, C-terminally retracted (Ub-CR) conformation also exists at low population in wild-type Ub. Point mutations in the moving  $\beta 5$  and neighbouring B-strands shift the Ub/Ub-CR equilibrium. This enabled functional studies of the two states, and we show that while the Ub-CR conformation is defective for conjugation, it demonstrates improved binding to PINK1 through its extended Ser65 loop, and is a superior PINK1 substrate. Together our data suggest that PINK1 utilises a lowly populated yet more suitable Ub-CR conformation of Ub for efficient phosphorylation. Our findings could be relevant for many kinases that phosphorylate residues in folded protein domains.

Keywords nuclear magnetic resonance; Parkin; Parkinson's disease; PINK1; ubiquitin phosphorylation

Subject Categories Post-translational Modifications, Proteolysis & Proteomics; Structural Biology

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### Introduction

Protein ubiquitination and protein phosphorylation are the two main regulatory post-translational modifications of proteins (Hunter, 2007). While phosphorylation provides a binary signal, the ubiquitin (Ub) signal is highly tuneable and exists in many variations. For example, polyUb chains of many architectures exist and encode distinct biological outcomes (Komander & Rape, 2012); moreover, Ub itself can be phosphorylated or acetylated, expanding its functional versatility (Swatek & Komander, 2016; Yau & Rape, 2016). Mass spectrometry has enabled the discovery and quantitation of the plethora of Ub modifications, including ubiquitin phosphorylation (Ordureau *et al*, 2015), yet proteins regulating and responding to these have remained by-and-large unclear, with one exception. Ub phosphorylation at Ser65 has been linked to mitophagy, the process by which damaged parts of mitochondria are isolated and targeted for autophagic clearance (Pickrell & Youle, 2015; Nguyen *et al*, 2016).

Ser65-phosphorylated ubiquitin (hereafter phosphoUb) is generated on mitochondria by the Ser/Thr protein kinase PINK1 (Kane et al, 2014; Kazlauskaite et al, 2014; Koyano et al, 2014; Ordureau et al, 2014; Wauer et al, 2015a), which is stabilised on the cytosolic face of mitochondria upon membrane depolarisation (Narendra et al, 2010). PINK1 phosphorylates Ub attached to outer mitochondrial membrane proteins, and this recruits and allosterically activates the E3 ligase Parkin (Kazlauskaite et al, 2015; Kumar et al, 2015; Sauvé et al, 2015; Wauer et al, 2015b). PINK1 also phosphorylates Parkin in its Ub-like (Ubl) domain, which is required for full Parkin activation and leads to strong, localised mitochondrial ubiquitination (Kondapalli et al, 2012; Ordureau et al, 2014; Wauer et al, 2015b). PINK1/Parkin action attracts adaptor proteins and recruits the mitophagy machinery, leading to clearance of the damaged organelle (Heo et al, 2015; Lazarou et al, 2015). The pathophysiological importance of PINK1/Parkin-mediated mitophagy is underlined by the fact that mutations in PINK1 and Parkin are linked to autosomal recessive juvenile Parkinson's disease (AR-JP). a neurodegenerative condition arising from loss of dopaminergic neurons in the substantia nigra (Corti et al, 2011; Pickrell & Youle, 2015).

The generation of phosphoUb by PINK1 is mechanistically poorly understood. PINK1 is an unusual Ser/Thr kinase, highly divergent from other kinases in the kinome (Manning *et al*, 2002). In part, this is due to several large insertions in the kinase N-lobe, which complicate structural modelling (Trempe & Fon, 2013). Also its substrate, Ub, is a non-classical kinase target since its 76 amino acids form a globular, highly robust and stable  $\beta$ -grasp fold, in which Ser65 is markedly protected. Ub Ser65 resides in the loop preceding the  $\beta$ 5 strand, and its side chain hydroxyl group engages in two backbone

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hydrogen bonds with Gln62. In addition, nearby side chains of Phe4 and Phe45 further stabilise the Ser65-containing loop (Fig EV1A). Ub Ser65 is structurally identical to Ser65 in the Parkin Ubl domain, but the two substrates lack similarity at the sequence level and a PINK1 phosphorylation consensus motif is not apparent (Kazlauskaite *et al*, 2014). The Ser65 position and interactions within a well-folded, globular domain make this residue an unlikely phosphorylation site for PINK1 or indeed any kinase.

Ub is highly similar in the > 300 Ub crystal structures in the protein data bank (Perica & Chothia, 2010; Harrison *et al*, 2016), and its biophysical properties and availability have made it a popular model system for protein folding studies (Jackson, 2006) and nuclear magnetic resonance (NMR) method development (Fushman *et al*, 2004; Lange *et al*, 2008; Torchia, 2015). NMR studies in particular have shown that despite its compact fold and high intrinsic stability, Ub is dynamic and contains several regions of local conformational flexibility (Lange *et al*, 2008). These include a mobile four-residue C-terminal tail, as well as a flexible  $\beta$ -hairpin structure, the  $\beta 1/\beta 2$ -loop, that alters the interaction profile of Ub (Lange *et al*, 2008; Hospenthal *et al*, 2013; Phillips & Corn, 2015). Importantly, we previously discovered that Ser65 phosphorylation resulted in a further, dramatic conformational change in Ub (Wauer *et al*, 2015a).

The Ser65 loop and the last ß5-strand were previously not known to be conformationally dynamic, yet phosphorylation led to an equilibrium between two phosphoUb conformations (Fig 1A). The first state resembles the common Ub conformation observed in all reported crystal structures to date. This phosphoUb conformation was confirmed in a crystal structure (Wauer et al, 2015a) and more recently by an NMR structure (Dong et al, 2017). More striking was a second conformation, in which the entire last β-strand slipped by two amino acids, extending the Ser65 loop, and simultaneously shortening the Ub C-terminal tail (hereafter referred to as the Ub-CR conformation for C-terminally retracted) (Wauer et al, 2015a; Dong et al, 2017). This change is facilitated by a Leu-repeat pattern in the ß5-strand: Leu67, Leu69 and Leu71 occupy complementary Leu pockets in the Ub core, whereas Leu73 is mostly solvent exposed. In the Ub-CR conformation observed in phosphoUb, Leu73 occupies the Leu71 pocket, Leu71 occupies the Leu69 pocket, and Leu69 occupies the Leu67 pocket, resulting in Leu67 residing in a more exposed position that was formerly occupied by Ser65 (Fig 1A). Experimentally, the phosphoUb-CR conformation was supported by large (> 1.5 ppm) chemical shift perturbations and by determination of the hydrogen bonding patterns for the  $\beta$ -sheet, using long-range HNCO-based NMR analysis (Wauer et al. 2015a). A recent NMR structure of the phosphoUb-CR conformation confirmed our findings (Dong et al, 2017).

We here show that the Ub-CR conformation can indeed be detected in unphosphorylated Ub, when analysing "invisible" populations accessible by chemical exchange saturation transfer (CEST) experiments (Fig 1B). This previously unrecognised equilibrium between a common Ub and a Ub-CR conformation in wild-type (wt) Ub can be shifted in either direction through point mutations in unphosphorylated Ub. Crystal structures as well as biophysical and NMR measurements enable in-depth characterisation of the Ub-CR conformation, and biochemical analyses reveal its functional relevance during Ser65

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phosphorylation. The Ub-CR conformation of Ub, with its mobile Ser65 loop, forms a more stable complex with PINK1 as assessed by NMR binding studies. More importantly, the Ub-CR conformation is required for efficient PINK1 phosphorylation. Together, we provide evidence that the preferred PINK1 substrate is a lowly populated form of Ub that is invisible to conventional biophysical techniques.

### Results

### Identification of a Ub-CR conformation in wild-type Ub

Dynamic aspects of Ub have been under intense scrutiny, in particular by NMR, and numerous studies have collectively covered most motional timescales from fast ps internal motions up to  $\mu$ s-ms conformational exchange processes using RDC analysis (Lange *et al*, 2008; Torchia, 2015). Our initial detection of the phosphoUb/phosphoUb-CR transition was enabled by a near-equal population of both states, and ZZ-exchange experiments indicated a slow exchange (~2 s<sup>-1</sup>) between these conformations (Fig 1A and B).

Given the timescales of motion probed in previous Ub studies, we hypothesised that a very lowly populated, transient Ub-CR conformation of wt Ub could have been systematically missed. Furthermore, we assumed that an increase in temperature would lower the energy barrier between the two conformers and potentially increase the population of the Ub-CR species. The detection of lowly populated, "dark" or "invisible" conformational states can be enabled by CEST experiments (Vallurupalli et al, 2012; Kay, 2016). In CEST, protein resonances are observed in the presence of a frequency-swept weak B1 saturation field where a series of experiments is acquired and the offset of the B1 field is varied systematically. If the B1 saturation field offset coincides with the lowly populated conformer, saturation transfer occurs during a fixed exchange period leading to an attenuation of the dominant species. This enables the indirect observation of an enhanced signal for the otherwise invisible state. Indeed, optimised 15N-CEST experiments (see Materials and Methods) revealed the existence of a second set of peaks in the 15N dimension for wt Ub in phosphatebuffered saline (25 mM NaPi (pH 7.2), 150 mM NaCl) at 37 or  $45^\circ\text{C}$ (Figs 1C, and EV1B and C). The chemical shift positions of this second, lowly occupied population correlated well with previously recorded phosphoUb-CR resonances (Figs 1C, and EV1B and D, Appendix Fig S1).

Pseudo two-dimensional CEST data with multiple B<sub>1</sub> fields were globally fitted for several resonances (see Materials and Methods) and allowed us to determine the occupancy of the Ub-CR conformation to be 0.68% in wt ubiquitin at 45°C, with an exchange rate to the common conformation of 63 s<sup>-1</sup> (Fig 1D). Together, CEST experiments revealed the existence of a previously undetected Ub conformation in wt Ub, which by chemical shift analysis resembles the phosphoUb-CR conformation reported earlier.

### Stabilisation of the Ub-CR conformation

With the occurrence of the wt Ub-CR conformation confirmed, we set out to stabilise it for further study. Following retraction of the  $\beta$ 5-strand, Leu67 occupies a position previously held by Ser65.

### An invisible ubiquitin conformation is required for efficient phosphorylation 304 by PINK1



### Figure 1. Ub adopts the C-terminally retracted (Ub-CR) conformation.

- A Centre: Schematic of the Ub surface, showing the position of the β5-strand (arrow) on the Ub core, and the position of phosphorylated Ser65. Cartoons to the left and right show a slice along the β5 strand, depicting the β5 residues and their positions in the respective common Ub conformation Leu pockets.
   B Timescales of NMR experiments to study the Ub/Ub-CR conformation in this and previous work (Wauer *et al*, 2015a).
- C CEST experiment on <sup>15</sup>N-labelled wt Ub (1.5 mM) in phosphate buffered saline (25 mM NaPi (pH 7.2), 150 mM NaCl) at 45°C. For a subset of resonances in the HSQC spectrum of Ub, a cross section taken at their <sup>15</sup>N frequency displays an additional resonance in this frequency-swept <sup>26 12</sup>N dimension (CEST profile) corresponding to the lowly populated Ub-CR conformation. The main peak in the CEST profile closely correlates to the corresponding HSQC resonance in the phospholbb conformation (grey), while the amplified smaller peak matches the resonance position of the phospholb-CR conformation (red). Note that the observed chemical shift positions in the wt Ub CEST data do not perfectly match phospholb resonances due to the chemical shift contribution of the phosphate group. Additional peaks can be found in Fig EVIB, and full spectra in Appendix Fig S1. A temperature profile for a selected resonance as well as a plot of all absolute <sup>15</sup>N shift differences can be found in Fig EVIC and D.
- D Schematic of the common/Ub-CR equilibrium for wt Ub. Occupancies and the rate of exchange generated from CEST at 45°C are reported (seven peaks fitted). A representative example of fit quality is shown in Appendix Fig S8.

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Therefore, we mutated Leu67 to Ser with the prediction that it would encourage  $\beta$ 5-strand slippage to place residue 67 in the Ser65 pocket, and fill the Leu67 hydrophobic pocket with Leu69 instead (Fig 2A). Indeed, <sup>1</sup>H-<sup>15</sup>N BEST-TROSY 2D spectra (bTROSY) of <sup>15</sup>N labelled Ub L67S showed 73 peaks implying a single Ub conformation (Appendix Fig S2). The chemical shift pattern did not match wt Ub, but more closely resembled the pattern seen for the phosphoUb-CR conformation. This can be assessed using well-dispersed reporter resonances, such as Lys11 (Fig 2B), while a global comparison of the full spectra can be drawn from chemical shift perturbation heat maps (Fig 2C). Hence, Ub L67S predominantly adopts the Ub-CR conformation despite lacking phosphorylated Ser65.

### Mimicking the Ub-CR conformer in Ser65 phosphoUb

We also wanted to study the phosphoUb-CR conformer in more detail, and hence, we phosphorylated Ub L67S with Pediculus humanus corporis (Ph)PINK1 (Woodroof et al, 2011; Wauer et al, 2015a). Strikingly, in situ phosphorylation transformed the simple Ub-CR bTROSY spectrum to a complicated spectrum with the occurrence of many additional peaks (data not shown). Phos-tag gels and mass spectrometry (MS) showed that PhPINK1 phosphorylates Ub L67S at multiple sites, on Ser65, and on Thr66 or on the introduced Ser67 (Fig EV2A and B). A mixture of phosphorylated species explains the complexity of the observed NMR spectrum. PINK1 shows exquisite preference for Ser65 in wt Ub and only phosphorylates Thr66 at very high enzyme concentrations and late time points (Wauer et al, 2015a). Hence, the doubly phosphorylated species are a result of the Ub-CR conformation induced by the L67S mutation. These data indicated that the Ub-CR conformation has profound effects on PINK1-mediated Ub phosphorylation, but suggested that this mutation was limited in its usefulness for the study of phosphoUb-CR.

To overcome this and to generate exclusively Ser65-phosphorylated Ub in the Ub-CR conformation, Leu67 was mutated to Asn, and Thr66 was mutated to Val (termed hereafter Ub TVLN mutant) (Fig 2D). Ub TVLN was phosphorylated only once, on Ser65 (Fig 2E), showed a clean, single-species bTROSY spectrum highly similar to Ub L67S in the unphosphorylated form, and when phosphorylated was highly similar to the phosphoUb-CR conformation (Fig 2F–H, Appendix Fig S3A and B) (Wauer *et al*, 2015a). Together, this showed that Ub TVLN is an excellent mimic for the Ub-CR conformer.

### Crystal structures of Ub in the Ub-CR conformation

The identification of Ub mutants stably in the Ub-CR conformation allowed us to obtain high-resolution crystal structures of Ub L67S (1.63 Å) and phosphoUb TVLN (1.6 Å) (Table 1, Figs 3 and EV3). Both structures confirmed that the  $\beta$ 5-strand is retracted by two amino acids, and Ser/Asn67, Leu69, Leu71 and Leu73 adopt near identical conformations as compared to Ser65, Leu67, Leu69 and Leu71, respectively, seen in previous Ub structures (Fig 3A–D). Hydrogen bonding patterns observed in the crystal structures matched the experimentally determined hydrogen bonding pattern for the phosphoUb-CR conformation (Wauer *et al*, 2015a), and the phosphoUb TVLN structure is similar to a recently reported NMR structure of phosphoUb-CR (Fig EV3E and F).

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The structures highlight important consequences of the Ub-CR conformation. The Ser65-containing loop (aa 62-66) protrudes from and lacks defined contacts with the Ub core, is flexible judging by B-factor analysis and in Ub L67S adopts distinct conformations in the two molecules in the asymmetric unit (Figs 3A and EV3D). Likewise, in phosphoUb TVLN, the Ser65-containing loop is extended and seemingly mobile, with the phosphate group exposed making no contacts to the Ub core. A further important feature of the Ub-CR conformation is the disruption of Ub interaction interfaces, the most important being the Ile44 hydrophobic patch, which also utilises Leu8 in the flexible  $\beta 1/\beta 2$ -hairpin, and Val70 and His68 of Ub ß5-strand (Komander & Rape, 2012). In the Ub-CR conformation, the Ile44 hydrophobic patch is disrupted due to dislocation of  $\beta 5$  residues Val70 and His68 (Fig 3E). In contrast, a second interaction site, the Ile36 hydrophobic patch (Hospenthal et al, 2013), is only altered, as Leu71 is now facing the protein core (Figs 3F and EV3C). Finally, retraction of the  $\beta$ 5-strand by two residues reduces the reach and conformational flexibility of the important Ub C-terminal tail.

### Affecting the Ub/Ub-CR conformational equilibrium

Mutating the first hydrophobic residue of the  $\beta$ 5-strand, Leu67, favours the Ub-CR conformation, since Leu69 and Leu71 can occupy alternative positions easily. We reasoned that mutating Leu71 to a larger residue, which cannot occupy the Leu69 position, might stabilise it in the common Ub conformation, and disfavour the Ub-CR conformation after phosphorylation (Fig 4A). Indeed, this was the case; Ub L71Y displays a common Ub spectrum without phosphorylation, and a spectrum highly similar to the common phosphoUb species after phosphorylation (Fig 4B–D, Appendix Fig S4A and B). Hence, Ub L71Y is a mutation in which the Ub-CR conformation is disfavoured.

Thus far, the introduced mutations change residues on the moving §5-strand. We wondered whether residues in the vicinity, for example, from the neighbouring  $\beta$ 1-strand, could also shift the observed equilibrium. A good candidate was Phe4 with its solvent exposed side chain (Fig 4E), which would be anticipated to have only subtle effects on Ub conformation per se. Indeed, Ub F4A displayed a wt-like bTROSY spectrum (Fig 4F-H, Appendix Fig S5A). However, strikingly, phosphorylation of Ub F4A resulted in a spectrum where the most intense peaks are in positions associated with the phosphoUb-CR conformation and peaks from a minor species (~12% by peak intensity) match the common Ub conformation (Fig 4G and H, Appendix Fig S5B), a reversal of that observed in the wt phosphoUb spectrum. This demonstrates that while the mutant resides in the common Ub conformation without phosphorylation, it almost completely shifts to a Ub-CR conformation upon phosphorylation. Hence, residues contacting and stabilising the slipping  $\beta 5\text{-strand}$  are able to affect the conformational equilibrium.

### Comparative stability studies of Ub mutants

The fascinating and unexpected conformational plasticity of Ub with regard to  $\beta$ 5-strand slippage was further confirmed in comparative studies. We had previously shown decreased thermal stability of phosphoUb, which we speculated was due to the

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Figure 2. Stabilising the Ub-CR conformation with point mutations.

- A Schematic of the L67S mutation, which places a Ser in the Leu67 pocket of the common conformation.
- В

Selected resonances of Ub L67S, compared to phosphoUb/phosphoUb-CR spectra. Ub L67S adopts the Ub-CR conformation. For full spectra, see Appendix Fig S2. Weighted chemical shift perturbation heat maps, comparing Ub L67S to indicated Ub spectra, revealing the similarity with phosphoUb-CR. For chemical shift values, c see Source Data. Schematic of Ub TVLN, introducing non-phosphorylatable residues at Thr66 and Leu67.

- D
- Phos-tag analysis of Ub TVLN phosphorylation by PhPINK1. Like Ub, Ub TVLN is phosphorylated only on Ser65. Data shown are representative of experiments Е performed in triplicate.
- Lys11 resonance of Ub TVLN in unphosphorylated and phosphorylated states. Ub TVLN adopts only the Ub-CR conformation regardless of phosphorylation status. For full spectra, see Appendix Fig S3A and B. Weighted chemical shift perturbation heat maps of Ub TVLN in comparison with indicated Ub species. For chemical shift values, see Source Data.
- G

H Weighted chemical shift perturbation heat maps of phosphoUb TVLN in comparison with indicated Ub species. For chemical shift values, see Source Data Source data are available online for this figure.

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| Table 1. Data collection and refinement statistics.    |          |                                   |                         |  |
|--|----------|-----------------------------------|-------------------------|--|
|  |          | Ub L67S                           | pUb T66V/L67N           |  |
| Data collection  |          |                                   |                         |  |
| Space grou   | ıp       | P 2 2 <sub>1</sub> 2 <sub>1</sub> | P 3 <sub>2</sub> 2 1    |  |
| Cell dimensions  |          |                                   |                         |  |
| a, b, c (Å)  |          | 41.06, 48.81, 74.40               | 49.77, 49.77, 89.27     |  |
| α, β, γ (°)  |          | 90, 90, 90                        | 90, 90, 120             |  |
| Resolution   | (Å)      | 35.95–1.63 (1.69–1.63)            | 24.49–1.601 (1.66–1.60) |  |
| R <sub>merge</sub>                                     |          | 0.059 (0.487)                     | 0.56 (0.227)            |  |
| Ι/σΙ   |          | 10.3 (2.0)                        | 22.7 (6.3)              |  |
| Completer  | iess (%) | 99.14 (99.58)                     | 98.47 (96.49)           |  |
| Redundan   | cy       | 3.6 (3.6)                         | 7.4 (7.5)               |  |
| Refinemen  | t        |                                   |                         |  |
| Resolution   | (Å)      | 35.95–1.63                        | 24.49–1.601             |  |
| No. reflect<br>test set                                | ions/    | 19,138/1,883                      | 17,194/1,675            |  |
| $R_{\rm work}/R_{\rm free}$                            |          | 0.193/0.237                       | 0.192/0.223             |  |
| No. atoms  |          |                                   |                         |  |
| Protein  |          | 1,189 (151 aa)                    | 608 (76 aa)             |  |
| Ligand/ion   |          | 30                                | 40                      |  |
| Water  |          | 134                               | 170                     |  |
| B-factors  |          |                                   |                         |  |
| Protein  |          | 21.1                              | 20.3                    |  |
| Ligand/ion   |          | 50.8                              | 51.3                    |  |
| Water  |          | 30.9                              | 35.0                    |  |
| R.m.s. devi  | ations   |                                   |                         |  |
| Bond lengt   | ths (Å)  | 0.010                             | 0.010                   |  |
| Bond angle   | es (°)   | 1.18                              | 1.08                    |  |
| Values in parentheses are fer highest resolution shell |          |                                   |                         |  |

Values in parentheses are for highest resolution shell.

Ub/Ub-CR equilibrium (Wauer *et al*, 2015a). Indeed, differential scanning calorimetry (DSC) experiments revealed that Ub-CR mutants Ub L67S and Ub TVLN display a  $T_m$  of ~83°C, with or without phosphorylation (Fig EV4A) [compared to 97°C for wt Ub and 87°C for phosphoUb (Wauer *et al*, 2015a)]. In comparison, Ub F4A displays an intermediate stability ( $T_m$  89°C) consistent with NMR findings. Importantly, Ub L71Y is as stable as wt Ub ( $T_m$  96°C), indicating that the mutation does not induce unfolding, but merely stabilises the common Ub conformation. Hence, the Ub-CR conformation is less thermostable as compared to the common Ub conformation, and this explains lower stability of phosphoUb.

We also previously used  $^{15}N\{^1H\}$  heteronuclear NOE (hetNOE) experiments to show stabilisation of Arg74 upon retraction of the  $\beta5$ -strand (Wauer *et al*, 2015a). Consistent with our analysis, the hetNOE for Arg74 in Ub TVLN resembles that of phosphoUb-CR, regardless of its phosphorylation status (Fig EV4B, Appendix Fig S6). Arg74 of Ub F4A behaved like the common conformation of wt Ub, but following phosphorylation was stabilised as in the Ub-CR conformation. Lastly, Arg74 of Ub L71Y was more dynamic irrespective of its phosphorylation and resembled the common conformation formation of wt Ub.

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### Additional NMR evidence for a common/Ub-CR equilibrium

Further evidence of the described conformational equilibrium was obtained either directly by CEST on equilibrium-perturbing mutants, or using solvent exchange experiments based on clean chemical exchange transfer (CLEANEX).

CLEANEX experiments measure the ability of backbone amide protons to exchange with the solvent, thus reporting on the relative solvent exposure of each residue, and is able to report on changes to ubiquitin dynamics, such as repercussions of C-terminal retraction or, for example, exposure of the Leu8-loop. Each Ub variant revealed a similar set of solvent accessible residues for wt Ub, Ub L71Y and Ub F4A, but considerably more solvent exchange was observed especially in the Ser65-loop region in Ub TVLN (Appendix Fig S7A and B). This is consistent with the structural data. Interestingly, residues of the nearby Leu8-loop report on the conformational preferences of each Ub mutant through their population averaged rates of solvent exchange (Fig 5A). In the TVLN mutant, the Leu8-loop demonstrates the greatest degree of solvent accessibility, with the F4A mutant and wt Ub rates being greater than the L71Y Ub-CR-inhibited mutant. This correlates with the overall stability seen in the  $T_m$  measurements (Fig EV4A) and the crystal structures (Fig EV3C).

As discussed above, we used CEST analysis to determine the Ub-CR occupancy in wt Ub to be 0.68% at 45°C with an exchange rate of 63 s<sup>-1</sup> (Fig 1D). We performed a similar analysis for the Ub variants to determine how the introduced mutations perturb the conformational equilibrium (Fig 5B, Appendix Fig S8). For Ub TVLN, we observe ~99% occupancy in the Ub-CR conformation at 45°C, with an exchange rate of 120 s<sup>-1</sup>. As indicated by our previous analyses, the Ub F4A mutant falls between Ub TVLN and wt, with a Ub-CR occupancy of 4.5% at 45°C, and a similar exchange rate of 83 s<sup>-1</sup>. Lastly, the Ub L71Y mutant is stabilised in the Ub-CR state under the conditions of our experiment.

To extend our analysis of the common/Ub-CR conformational equilibrium to room temperature (25°C), where the vast majority of Ub NMR experiments are performed, we chose to repeat the CEST experiment for the Ub F4A mutant which had sufficient populations of the two species for accurate fitting. At room temperature, we observed a Ub-CR occupancy of 1.3% and an exchange rate of 46 s<sup>-1</sup> for the Ub F4A mutant (Fig 5B). Extrapolating a similar temperature dependence on wt Ub would estimate a Ub-CR occupancy to be even lower than 0.68%, further explaining why the Ub-CR conformation is invisible to conventional biophysical methods.

### The Ub-CR conformation affects ubiquitination reactions

Our identification of Ub mutants adopting the Ub-CR conformation facilitated experiments to test the biochemical impact of this species, which has a shortened C-terminal tail and disrupted lle44 hydrophobic patch (see Fig 3E), on Ub assembly reactions.

We found that Ub TVLN, which adopts the Ub-CR conformation in solution, was readily charged by E1 onto E2 enzymes, including UBE2D3, UBE2L3, UBE2S, UBE2N and UBE2R1 (Fig 6A), which is perhaps surprising in the light of recent findings that that the hydrophobic patch is important for E1-mediated E2 charging

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A Ub L67S structure at 1.63 Å resolution. The two molecules of the asymmetric unit are superimposed. For electron density, see Fig EV3A.

- B PhosphoUb TVLN structure at 1.6 Å resolution. For electron density, see Fig EV3B.
   C Structure of wt Ub (1UBQ; Vijay-Kumar et al, 1987).
- D Superposition of structures from (A–C), showing residues of the β5-strand. RMSD values comparing to wt Ub (1UBQ) are reported.
   Position of residues making up the IIe44 hydrophobic patch in Ub or Ub-CR conformations.
- F As in (E), showing residues of the Ile36 hydrophobic patch.

(Singh et al, 2017). However, the E1 reaction is known to be relatively permissive and can also accommodate conformation-changing C-terminal Ub mutations such as Ub L73P (Békés et al, 2013).

While charging appeared unaffected, Ub TVLN demonstrated impaired (UBE2S) or abrogated (UBE2R1, UBE2N/UBE2V1) E2mediated chain assembly (Fig 6B), and also impaired or abrogated

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Figure 4. Mutations to modulate the Ub/Ub-CR equilibrium.

- Schematic of the Ub L71Y mutation. A large Tyr residue may not easily fit into the Leu69 pocket. А

Weighted chemical shift perturbation heat maps of Ub L/21Y in comparison with indicated Ub species. For chemical shift values, see Source Data. Weighted chemical shift perturbation heat maps of phosphoUb L/21Y in comparison with indicated Ub species. For chemical shift values, see Source Data. С

Lys11 resonance for Ub L71Y and phosphoUb L71Y in comparison with the split phosphoUb spectrum. For full spectra, see Appendix Fig S4A and B. Schematic of the Ub F4A mutation in which a residue from the neighbouring  $\beta$ 1-strand may modulate the Ub/Ub-CR equilibrium. D

Weighted chemical shift perturbation heat maps of Ub F4A in comparison with indicated Ub species. For chemical shift values, see Source Data. Weighted chemical shift perturbation heat maps of phosphoUb-CR F4A in comparison with indicated Ub species. For chemical shift values, see Source Data. G

н Lys11 resonance for Ub F4A and phosphoUb F4A in comparison with the split phosphoUb spectrum. For full spectra, see Appendix Fig S5A and B.

Source data are available online for this figure

chain assembly by RING E3 ligases (cIAP/UBE2D3, TRAF6/ UBE2D3) (Fig 6C), a HECT E3 ligase (HUWE1/UBE2L3), or RBR E3 ligases (Parkin/UBE2L3, HOIP/UBE2L3) (Fig 6D). This shows that the Ub-CR conformation severely affects the Ub system. Consistently, in a large-scale mutational study in Saccharomyces cerevisiae, the Ub L67S mutation was shown to have detrimental effects on

yeast growth (Roscoe et al, 2013; Roscoe & Bolon, 2014). While Ub contains many essential residues and interfaces, our data suggest that the reported lack-of-fitness can be attributed to the Ub-CR conformation.

To date, the only known role for phosphoUb in cells is to recruit and allosterically activate Parkin during mitophagy (Pickrell & Youle,

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Figure 5. Comparative CLEANEX and hetNOE studies of Ub/Ub-CR mutants.

- A CLEANEX experiments on Ub variants, comparing fitted solvent exchange rates (k) for selected residues of the Leu8 loop. See Appendix Fig S7A and B for complete Ub CLEANEX rates and a graphical representation of the dataset.
- B CEST analysis, as in Fig 1C, for Ub variants TVLN, F4A and L71Y. WT Ub data are overlaid for comparison. CEST data were fitted for TVLN (one peak) and F4A (eight peaks at 45°C, four peaks at 25°C) to determine occupancies and exchange rates. Representative fit quality is shown in Appendix Fig S8. For raw CEST data used for fitting, see Source Data.

Source data are available online for this figure.

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2015; Nguyen *et al*, 2016). Our previous structural analysis revealed that the common conformation of phosphoUb binds to Parkin (Wauer *et al*, 2015b), and this binding event leads to release of the Parkin Ubl domain and Ubl phosphorylation by PINK1. Phosphorylation of wt Ub increases the occupancy of the Ub-CR conformation (Wauer *et al*, 2015a), and we were now able to evaluate the impact of the phosphoUb CR conformation on Parkin activity. To test this, PINK1-dependent phosphorylation of the Parkin Ubl domain was monitored in response to either wt or TVLN phosphoUb (Fig 6E). As predicted, while addition of wt phosphoUb IVLN did not.

# The Ub-CR conformation stably binds PhPINK1

While a Ub-CR-inducing mutation had inhibitory effects on the ubiquitination cascade, we still wondered whether this conformation had physiological roles. A number of observations pointed towards potential importance in PINK1-mediated Ub phosphorylation. As discussed above, Ser65 in wt Ub is poorly accessible, but becomes more

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exposed in the Ub-CR conformation. Moreover, phosphorylation of Ub L67S and Ub TVLN mutants was markedly accelerated compared to wt Ub as shown by qualitative Phos-tag gels (Figs 2E and EV2A).

We hence tested how PINK1 interacted with its substrates and performed bTROSY experiments with unlabelled PhPINK1 (aa 115-575) and <sup>15</sup>N-labelled wt Ub, Ub mutants, or the Parkin Ubl domain (aa 1-76) (Figs 7 and EV5A, Appendix Figs S9-S11). In the presence of PhPINK1, all peaks were line-broadened to some extent due to the formation of a weakly-associated 62 kDa complex. A subset of Ub/ Ubl peaks, which were additionally exchange broadened, revealed the residues that interact with PhPINK1 (Fig 7, left column). Small chemical shift perturbations were also observed upon addition of PhPINK1, the most significant of which showed agreement with the differential line-broadening analysis (Fig EV5A). Addition of MgAMP-PNP had no apparent effect on the PhPINK1 interaction with Ub (Appendix Fig S12). PhPINK1 binding was also measured using CLEANEX experiments, whereby the binding to Ub or Ubl masks the interacting residues on the substrate from chemical exchange with the solvent (Fig 7, right column).

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[GST-PhPINK1] = 0.25 μM (22°C)

# Figure 6. Effects of Ub-CR on ubiquitination reactions.

Ubiquitination reactions were performed in parallel with wt Ub and the Ub-CR variant Ub TVLN at identical concentrations. Individual reactions were run for indicated times, resolved on 4–12% SDS-PAGE gradient gels and stained with Coomassie. Data shown are representative of experiments performed in at least duplicate.

- A E1 charging reactions with Ub and Ub TVLN on UBE2D3 (top) and UBE2L3 (bottom). B E2-based Ub chain assembly reaction using UBE2R1 (top), UBE2S (middle) and UBE2N/UBE2V1 (bottom). E2 charging proceeds identically but chain assembly is inhibited with Ub TVLN, indicated by the lack of free dibb assembly. C E3-based autoubiquitination reaction with GST-cIAP1 (aa 363–612) and GST-TRAF6 (aa 50–211) in conjunction with UBE2D3.
- HECT- and RBR-based chain assembly in conjunction with UBE2L3. Top: HUWE1 HECT domain (aa 3,993–4,374). Middle: phosphorylated full-length Parkin. Bottom:
- D RBR-LDD fragment of HOIP (aa 699-1,072).
- Е Parkin phosphorylation in the absence or presence of either wt or TVLN phosphoUb monitored by Phos-tag.

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Figure 7. PhPINK1 recognises the Ser65 loop in the Ub-CR conformation.

Left column: Complex formation between *Ph*PINK1 (without MgATP) and Ub variants or Parkin Ubl results in line broadening of NMR resonances due to formation of a 62 kDa complex. Relative line broadening of Ub resonances is determined by peak intensity and plotted with mean value indicated, and values decreasing by half or full standard deviation (stdv) from the mean are coloured orange and red, respectively. An analogous chemical shift perturbation analysis is shown in Fig EVSA. Full spectral overlays are shown in Appendix Figs S9–S11. Middle column: Line-broadened residues are plotted on the Ub surface. Right column: CLEANEX experiments showing solvent-exchanging residues (orange and light blue bars) on Ub variants and Parkin Ubl, and how these are affected by *Ph*PINK1 binding (red and dark blue bars). Orange/red bars highlight the Ser65-containing loop, which is exposed in Ub TVLN and highly protected after *Ph*PINK1 binding.

Remarkably, the footprint of *Ph*PINK1 on its substrates varied (Fig 7, middle column). In wt Ub, as well as Ub F4A and Ub L71Y, broadened residues correspond to the C-terminal tail and the lle44 patch, but strikingly did not include residues from the Ser65-containing loop. In these three samples, a similar degree of overall line broadening suggests similar (weak) binding. CLEANEX experiments of substrates without *Ph*PINK1 (light colours) and with *Ph*PINK1 (dark colours) reveal the lle44 patch interaction of these substrates and, although only Lys63 is sufficiently solvent exposed to be measured, some relative protection of the Ser65 loop following *Ph*PINK1 binding is also observed.

In contrast, the Ub TVLN mutant as well as the Parkin Ubl forms larger interfaces involving the entire ß5-strand, and importantly, all residues from the Ser65-containing loop. Moreover, overall line broadening was significantly stronger in Parkin Ubl and Ub TVLN samples as compared to wt Ub, suggesting that these substrates form a more stable complex. This was emphasised in the CLEANEX experiments collected for Ub TVLN, which in the apo state show the enhanced solvent accessibility of all resonances of the Ser65 loop. PhPINK1 interaction leads to almost complete protection of the entire Ser65 loop of Ub TVLN showing that in the Ub-CR conformation the phosphorylation site is part of the interface with PINK1. The stronger interaction between PhPINK1 and the Ub-CR conformation was confirmed by isothermal calorimetry (ITC), which provided a  $K_D$  of approximately 300  $\mu$ M for Ub TVLN and only very little binding for wt Ub that could not be quantified (Fig EV5B). As expected for the product of the phosphorylation reaction, phosphoUb TVLN showed a weaker interaction with PhPINK1, particularly in the Ser65 loop (Appendix Fig S13).

Together, these experiments indicate that the significantly faster rate of Ub phosphorylation seen in the Ub-CR mutants such as Ub TVLN can be explained with enhanced binding of PINK1 to Ub-CR, which can form additional interactions via the Ser65 loop.

# Ub conformations affect PINK1 activity

The fact that Ub mutations stabilise the Ub-CR conformation in the absence of phosphorylation (Figs 2–5), and the discovery that wt Ub dwells in a Ub/Ub-CR equilibrium (Figs 1 and 5), opened the fascinating possibility that the Ub-CR conformation is used or even required for PINK1-mediated Ub phosphorylation.

To test this, we compared phosphorylation rates by treating Ub, Ub mutants and Parkin Ubl samples with *PhPINK1/MgATP*, in qualitative experiments using Phos-tag gels (Fig EV6A), or in semiquantitative, real-time experiments using <sup>15</sup>N-labelled Ub/Ubl substrates in NMR experiments (Figs 8A and B, and EV6B). Direct assessment of individual peak disappearance/appearance over time from unphosphorylated to fully phosphorylated samples enabled generation of phosphorylation rate curves,

revealing strikingly different rates (Fig 8A and B). The fastest rates were observed for Ub TVLN and Parkin Ubl, and these were almost indistinguishable from each other, even at lower enzyme concentrations (Fig EV6B); 50% of the substrate was phosphorylated after ~2 min. The Ub F4A sample was also quite fast, being half-phosphorylated after ~5 min. In contrast, it took ~90 and ~275 min to phosphorylate 50% of wt Ub or Ub L71Y, respectively, under identical conditions. Hence, phosphorylation of the Ub-CR mutant Ub TVLN is ~45-140-fold faster as compared to variants where this conformation is much less populated (wt Ub) or disfavoured (Ub L71Y). The order of preferred PhPINK1 Ub substrates is in agreement with the occupancy of the Ub-CR conformation as determined by CEST analysis (Fig 5B). This suggested that PINK1 not only prefers the Ub-CR conformation, but that it requires it for efficient phosphorylation.

# Discussion

Ubiquitin is a most fascinating molecule. Despite being the focus of three decades of biochemical, biophysical and structural research, we here uncover a new conformation in which the C-terminal  $\beta5$ -strand of Ub is retracted by two residues. This extends the upstream Ser65-containing loop, perturbs the lle44 hydrophobic patch and shortens the otherwise extended Ub C-terminus. We show that Ub adopts the Ub-CR conformation and, although this conformation is lowly populated, our data suggest that it is functionally relevant.

Our previous work showed that the Ub-CR conformation was stabilised in Ser65 phosphoUb (Wauer et al. 2015a), which was recently confirmed by an NMR solution structure (Dong et al, 2017). We hence set out to identify stable versions of phosphoUb-CR for further study. We identified point mutations that readily adopted the Ub-CR conformation, even without phosphorylation, enabling us to shift the equilibrium. Still, the Ub-CR conformation in wt Ub evaded detection, despite a large number of published dynamics investigations probing timescales over multiple orders of magnitude (both experimental and computational) (e.g., Lange et al. 2008). We chose CEST experiments, which uniquely enhance the detection of otherwise invisible states, to study a potential lowly populated, transient Ub-CR conformer in wt Ub (Baldwin & Kay, 2009; Kay, 2016). Indeed, CEST experiments provided direct evidence for the existence of the Ub-CR conformation under nearphysiological conditions [25 mM NaPi (pH 7.2), 150 mM NaCl, at 37°C]. This is an exciting finding that adds new complexity to the Ub conformational landscape.

Our mutational analysis explains previous findings that mutations of seemingly non-functional Ub residues severely affected Ub as well as cellular fitness (Sloper-Mould *et al*, 2001; Roscoe *et al*, 2013; Roscoe & Bolon, 2014). Most Ub mutations have to date been

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Α time 2.2 5.0 7.7 16.0 27.0 65.7 0 (x10<sup>3</sup> s) Ub 1.00 0.67 0.50 0.38 0.15 0.05 0.00 pUb 0.00 0.24 0.40 0.63 0.87 0.94 1.00 119 4 **-**161 **-**161 -161 **-**161 mdd **-161** pl61 🗭 pl61 🔎 pl61 🔊 <u>pl</u>61 🔎 <u>pl</u>61 🔎 pl61 🔎 15N / 120.∠ 7.00 7.00 7.00 7.00 7.15 7.00 7.00 7.00 7.15 7.15 7.15 7.15 7.15 7.15 <sup>1</sup>H chemical shift / ppm Ub peak intensity 1.0 в pUb peak intensity Ub Ub TVLN normalised peak intensity Ub L71Y Ub F4A Ubl 0.5 50% Ub peak intensity pUb peak intensity 1x10<sup>4</sup> 0.0 1x10 1x10<sup>6</sup> 1x10 1x10 1x10<sup>3</sup> time (s)

#### Figure 8. Ub in the Ub-CR conformation is a superior PhPINK1 substrate.

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- A An in situ phosphorylation experiment was performed, in which suitable substrate signals were monitored for disappearance/appearance in an NMR time course as illustrated for IIe61.
- B A series of bTROSY spectra were acquired in ~4 (Ub TVLN, Ub F4A and Ubl) or ~8-min (wt Ub and Ub L71Y) increments following Ser65 phosphorylation by 350 nM PhPINKL. Peak intensities of unphosphorylated and phosphorylated Ub/Ubl as in (A) were normalised to the corresponding measurements in the initial (fully unphosphorylated) and final (fully phosphorylated) time points, respectively. Data from at least nine individual resonances were averaged with error bars indicating standard deviation from the mean.

explained with disruption of one of the various Ub binding interfaces (Komander & Rape, 2012). Whilst protein interactions are clearly a key function of Ub, we here reveal how some mutations may indirectly affect global Ub interaction capabilities by inducing a dysfunctional Ub-CR conformation.

Importantly, we also show a physiological role for a Ub-CR conformation. Ub is a well-folded, stable protein, and as such is an unlikely kinase substrate. Many protein kinases prefer or require disordered target sequences for phosphorylation. The well-ordered Ser65-containing loop in the common Ub conformer does not fit this criterion, but the more mobile loop provided in the Ub-CR conformation enables efficient binding and phosphorylation. Hence, Ub-CR mutants are superior PINK1 substrates. Considering wt Ub, it is tempting to speculate that PINK1 stabilises the Ub-CR conformation, or indeed, that a Ub conformational change may impose a rate-limiting step for phosphorylation. So far, we have not observed this with wt Ub, but the timescales of binding experiments (µs) vs. conformational change (ms) present a challenge to directly detect a precatalytic state with wt Ub. It is exciting that we may be able to mimic this precatalytic state with the Ub TVLN mutant, and this may be useful for future structural studies on PINK1.

Our findings likely have pathophysiological relevance. PINK1 mutations result in AR-JP, and our results reveal that one of its key substrates, Ub, needs to be in a particular conformation to enable efficient phosphorylation. It is easy to imagine that conditions or binding partners that stabilise Ub in a common conformation (e.g., Ile44-patch binding domains in mitochondrial associated proteins) may impede PINK1 activity and imbalance the system. In this context, it will also be interesting to test whether different chain contexts modulate the observed Ub/Ub-CR equilibrium and affect the rate at which chains can be phosphorylated. A further open question relates to the Parkin Ubl domain, for which there is no evidence at current of a similar, C-terminally retracted Ubl conformation.

We had previously shown that the Ub-CR conformation is present also in phosphoUb (Wauer *et al*, 2015a), but strikingly, the only known phosphoUb receptor, Parkin, recognises the common phosphoUb conformation and does not utilise the more distinctive phosphoUb-CR conformation. It is possible that alternative receptors for phosphoUb-CR exist, but it is also imaginable that Ub-CR exists predominantly to facilitate phosphorylation in the first place. The unique requirements for PINK1 phosphorylation, Parkin activation and Ub conjugation indicate that exchange between the common

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and Ub-CR conformations not only occurs but is required during the process of mitophagy.

While the Ub-CR conformation explains how Ser65 can be phosphorylated by PINK1, questions remain how other sites on Ub, such as well-ordered Thr12 and Thr14 on the  $\beta 2$ -strand, can be phosphorylated. More globally, our data explain how an inaccessible phosphorylation site in a folded protein can be targeted via exploitation of an invisible conformation. Hence, our work is likely relevant for other kinases that target folded protein domains.

# Materials and Methods

# Molecular biology

Ub constructs were cloned into pET17b vectors, and site-directed mutagenesis was carried out using the QuikChange protocol with Phusion polymerase (NEB). UBE2D3, UBE2S, UBE2L3, UBE2R1, UBE2N/UBE2V1 full-length proteins and GST-cIAP1 (aa 363–612), GST-TRAF6 (aa 50–211) were expressed from pGEX6 vectors. Full-length *Hs*Parkin, *Ph*PINK1 (aa 115–575) and HOIP RBR-LDD (aa 699–1,072) were expressed from a pOPIN-K vector, while *Hs*Parkin Ubl domain (aa 1–76) and HUWE1 catalytic domain (aa 3,993–4,374) were expressed from a pOPIN-S vector (Berrow *et al*, 2007). *Hs*Ube1/PET21d was a gift from Cynthia Wolberger [Addgene plasmid # 34965 (Berndsen & Wolberger, 2011)].

# Protein purification

All Ub mutants were expressed in Rosetta 2 (DE3) pLacl cells and purified following the protocol of Pickart and Raasi (2005). In short, unlabelled proteins were expressed in 2xTY medium, protein expression was induced at OD<sub>600</sub> of 0.6–0.8 with 200  $\mu$ M IPTG and cells were harvested after 4–5 h at 37°C. Singly <sup>15</sup>N-labelled or doubly <sup>15</sup>N- and <sup>13</sup>C-labelled proteins were expressed in minimal medium [M9 supplemented with 2 mM MgSO<sub>4</sub>, 50  $\mu$ M ZnCl<sub>2</sub>, 10  $\mu$ M CaCl<sub>2</sub>, trace elements, vitamins (BME vitamin solution, sterile-filtered, Sigma)], supplemented with 1 g <sup>15</sup>NH<sub>4</sub>Cl and 4 g glucose or <sup>13</sup>C<sub>6</sub> glucose where required. Protein expression was induced at OD<sub>600</sub> of 0.5–0.6 with 200  $\mu$ M IPTG, and cells were harvested after O/N growth at 18°C.

Labelled and unlabelled Parkin Ubl (aa 1–76) was expressed as a His-SUMO-fusion construct as described previously (Wauer *et al*, 2015a) and purified using HisPur<sup>TM</sup> Cobalt Resin (Thermo Fisher Scientific). The His-SUMO tag was cleaved using SENP1 during dialysis in cleavage buffer (25 mM Tris (pH 8.5), 300 mM NaCl, 2 mM  $\beta$ -mercaptoethanol) overnight at 4°C. The His-SUMO tag was captured on HisPur<sup>TM</sup> Cobalt Resin. GST-tagged *Ph*PINK1 (aa 115–575), E2 and E3 enzymes were purified and Parkin phosphorylated as described earlier (Wauer *et al*, 2015a). For NMR studies, the *Ph*PINK1 GST-tag was cleaved using PreScission protease.

As a final step, all proteins subjected to NMR analysis were purified by SEC (Superdex 75 or Superdex 200, GE Life Science) in NMR buffer (18 mM  $Na_2$ HPO<sub>4</sub>, 7 mM  $NaH_2$ PO<sub>4</sub>, 150 mM NaCl (pH 7.2) with 10 mM DTT added for *Ph*PINK1 and Parkin Ubl). Proteins for biochemistry were purified by SEC (Superdex 75 or Superdex 200, GE Life Science) in 25 mM Tris, 150 mM NaCl (pH 7.4). Ub-CR is a superior PINK1 substrate Christina Gladkova et al

### Phos-tag assays

Phosphorylation of Ub constructs and Parkin Ubl was performed by incubating 15  $\mu$ M substrate with indicated GST-*Ph*PINK1 concentrations in 25 mM Tris (pH 7.4), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM ATP, 1 mM DTT at 22°C or 37°C as indicated. Reactions were quenched at the given time points with EDTA-free LDS sample buffer.

Samples were analysed by  ${\rm Mn}^{2+}$  Phos-tag SDS–PAGE. A 17.5% (w/v) acrylamide gel was supplemented with 50  $\mu M$  Phos-tag AAL solution (Wako Chemicals) and 50  $\mu M$  MnCl<sub>2</sub> and stained with Instant Blue SafeStain (Expedeon). An EDTA-free Tris–glycine running buffer was used.

# Mass-spectrometry analysis

LC-MS analysis was carried out on an Agilent 1200 Series chromatography system coupled to an Agilent 6130 Quadrupole mass spectrometer. Samples were eluted from a phenomenex Jupiter column (5  $\mu$ m, 300 Å, C4 column, 150  $\times$  2.0 mm) using an acetonitrile gradient + 0.2% (v/v) formic acid. Protein was ionised using an ESI source (3 kV ionisation voltage), and spectra were analysed in positive ion mode with a mass range between 400 and 2,000 m/z. Averaged spectra were deconvoluted using the manufacturer's software and plotted using GraphPad Prism (version 7).

# Ub phosphorylation by PhPINK1

Purified Ub variants were incubated at a 100:1 ratio with *Ph*PINK1 in phosphorylation buffer (10 mM ATP, 20 mM Tris (pH 7.4), 10 mM MgCl<sub>4</sub>, 150 mM NaCl, 1 mM DTT). Reaction progress at  $25^{\circ}$ C was monitored using LC-MS, and once there were no changes in recorded spectra, the reaction mixture was dialysed against water, using a 3.5 kDa cut-off dialysis cassette (Thermo Scientific). The dialysate was applied to an anion exchange (MonoQ 5/50 GL, GE Life Sciences) column. PhosphoUb was eluted by 50 mM Tris (pH 7.4) and further purified by SEC (Superdex 75, GE Life Sciences) into NMR buffer. PhosphoUb TVLN for crystallography was purified by SEC in 25 mM Tris (pH 7.4).

### Crystallisation, data collection and structure determination

Ub L67S was crystallised at 12.5 mg/ml by sitting-drop vapour diffusion against 3 M  $(NH_4)_2SO_4$ , 0.1 M MES (pH 6.0) using a 2:1 protein-to-reservoir ratio at 18°C. A single crystal was harvested and vitrified in liquid nitrogen.

PhosphoUb TVLN was crystallised at 11.2 mg/ml by sitting-drop vapour diffusion against 3.2 M  $(NH_4)_2SO_4$ , 0.1 M bicine (pH 9.0), in a 1:1 protein-to-reservoir ratio at 18°C. A single crystal was harvested and vitrified in liquid nitrogen.

Ub L67S diffraction data were collected at the Diamond Light Source, beam line I-04, while phosphoUb TVLN was collected on an FR-E<sup>+</sup> SuperBright ultra-high-intensity microfocus rotating copper anode ( $\lambda = 1.5418A^\circ$ ) generator equipped with a MAR345 detector. Diffraction data were processed with iMosflm (Battye *et al*, 2011) and scaled with AIMLESS (Evans, 2006).

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Structures were determined by molecular replacement, using wt Ub [pdb-1UBQ, (Vijay-Kumar *et al.*, 1987)] aa 1–59 as a search model in Phaser (McCoy *et al.*, 2007). Iterative rounds of model building and refinement were performed with Coot (Emsley *et al.*, 2010) and PHENIX (Adams *et al.*, 2011), respectively. All structural figures were generated in Pymol (www.pymol.org).

Data collection and refinement statistics can be found in Table 1.

#### Stability measurements

Samples were dialysed into NMR buffer [18 mM Na<sub>2</sub>HPO<sub>4</sub>, 7 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl (pH = 7.2) using 3.5 kDa MW cut-off dialysis cassettes (Thermo Scientific)] and subsequently diluted to 50  $\mu$ M. DSC was performed using a VP-capillary DSC instrument (Malvern Instruments). Samples were scanned at a heating rate of 90°C/h in mid-feedback mode. Data were corrected for instrumental baseline using average buffer scans recorded immediately before and after Ub runs and plotted. After concentration normalisation, the intrinsic protein baseline between pre- and post-transitional levels was corrected using the progress function in the Origin software supplied with the instrument. Corrected endotherms were fitted to a non-two-state model allowing  $T_m$ ,  $\Delta H$  calorimetric and  $\Delta H$  van't Hoff to vary independently.

# Ubiquitination assays

Ubiquitination assays were essentially performed according to (Wauer *et al*, 2015a), with reactions performed in ubiquitination buffer (30 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM ATP, 10 mM MgCl<sub>2</sub>) at  $37^{\circ}$ C.

For E2 charging and E2-mediated assembly, *Hs*UBE1 was used at 0.2  $\mu$ M, Ub was used at 20  $\mu$ M and E2s were used a 4  $\mu$ M. For E3-mediated assembly, *Hs*UBE1 was used at 0.2  $\mu$ M, Ub was used at 20  $\mu$ M, E2s were used a 2  $\mu$ M and GST-cIAP1, GST-TRAF6, HUWE1, pParkin were used at 5  $\mu$ M, while HOIP RBR-LDD was used at 1  $\mu$ M. Samples were taken at indicated time points, the reactions quenched with LDS sample buffer with reducing agent unless otherwise indicated, resolved on 4–12% SDS gradient gels (NuPage) and stained with Instant Blue SafeStain. A representative example of an experiment done at least in duplicate is shown.

# Isothermal titration calorimetry

Experiments were performed using a MicroCal Auto-ITC200 (GE Healthcare) at 25°C. Samples of 1.5 mM wt or TVLN Ub were injected into the cell containing 250  $\mu$ M *Ph*PINK1 (aa 115–575), for a total of 20 injections of 2  $\mu$ l each, with 180-s spacing intervals. High salt buffer was used to stabilise the highly concentrated *Ph*PINK1 (25 mM Tris (pH 8.5), 400 mM NaCl, 2.5 mM TCEP). Binding curves were fitted to a one-site binding model using the MicroCal PEAQ-ITC Analysis Software (Malvern). Experiments were performed in duplicate.

#### Parkin phosphorylation assays

Phosphorylation of Parkin was performed by incubating 15  $\mu$ M substrate with 0.25  $\mu$ M GST-*Ph*PINK1 in the presence or absence of

15  $\mu M$  of specified ubiquitin variants at 22°C in phosphorylation buffer (25 mM Tris (pH 7.4), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM ATP, 10 mM DTT). Reactions were quenched at the given time points with EDTA-free LDS sample buffer.

Samples were analysed by  ${\rm Mn}^{2\,*}$  Phos-tag SDS–PAGE. A 12.0% (w/v) acrylamide gel was supplemented with 50  $\mu M$  Phos-tag AAL solution (Wako Chemicals) and 50  $\mu M$  MnCl<sub>2</sub> and stained with Instant Blue SafeStain (Expedeon). An EDTA-free Tris–glycine running buffer was used.

#### NMR

#### General acquisition parameters

Nuclear magnetic resonance acquisition was carried out at 25°C on either Bruker Avance III 600 MHz, Bruker Avance II+ 700 MHz or Bruker Avance III HD 800 MHz spectrometers equipped with a cryogenic triple-resonance TCI probes unless otherwise stated. Topspin (Bruker) and NMRpipe (Delaglio *et al.*, 1995) were used for data processing and Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, UCSF, https://www.cgl.ucsf.edu/home/sparky/) was used for data analysis. <sup>1</sup>H, <sup>15</sup>N 2D BEST-TROSY experiments (band-selective excitation short transients-transverse relaxation-optimised spectroscopy) were acquired with in-house optimised Bruker pulse sequences incorporating a recycling delay of 400 ms and 1,024\*64 complex points in the <sup>1</sup>H, <sup>15</sup>N dimension, respectively. High-quality data sets were collected in approximately 9 min.

# Backbone chemical shift assignments

De novo assignments or reassignments (L67S, TVLN, pTVLN, F4A, pF4A).

Nuclear magnetic resonance acquisition was carried out at 25°C on Bruker Avance III 600 MHz spectrometer equipped with a cryogenic triple-resonance TCI probe. Backbone chemical shift assignments were completed using Bruker triple-resonance pulse sequences. HNCACB spectra were collected with 512\*32\*55 complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions, respectively. CBCA (CO)NH, HNCO and HN(CA)CO spectra were collected with 512\*32\*48 complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions, respectively. All experiments were collected using non-uniform sampling (NUS) at a rate of 50% of complex points in the <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C dimensions, respectively, and reconstructed using compressed sensing (Kazimierczuk & Orekhov, 2011).

Assignment of the common conformation peaks seen in the pF4A <sup>15</sup>N-<sup>1</sup>H spectra was aided by analysis of ZZ-exchange experiments (Latham *et al*, 2009) collected with 50-, 75-, 150-, 200-, 400- and 800-ms delays using the Bruker 950 MHz Avance III HD spectrometer at the MRC Biomedical NMR centre for optimised sensitivity.

Due to the similarity of the L71Y and pL71Y HSQC spectra to wt Ub, cross-peak assignment was simply confirmed by analysis of a  $^{15}N$  NOESY-HSQC collected with a mixing time of 120 ms and 1,024\*32\*48 complex points in the  $^{1}H,$   $^{15}N$  and  $^{1}H$  dimensions.

Previously published assignments of peaks in the Parkin (1–76) Ubl and pUbl by (Aguirre *et al*, 2017) were downloaded from the BioMagResBank www.bmrb.wisc.edu (accession number 30197).

Weighted chemical shift perturbation calculations were performed using the following relationship:  $((\Delta^1 H)^2 + (\Delta^{15} N/5)^2)^{0.5}$  where the  $\Delta$  denotes the difference in ppm of the chemical shift

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between the peaks of phosphorylated and unphosphorylated peaks of the same ubiquitin or between different ubiquitin species. Data were plotted with GraphPad Prism (version 7).

# <sup>15</sup>N {<sup>1</sup>H}-heteronuclear NOE measurements

 $^{15}N$  {<sup>1</sup>H}-heteronuclear NOE (hetNOE) measurements were carried out using standard Bruker pulse programs, applying a 120° <sup>1</sup>H pulse train with a 5-ms inter-pulse delay for a total of 5-s interleaved on-or off-resonance saturation. The hetNOE values were calculated from peak intensities according to the equation  $I_{\rm on}/I_{\rm off}$ .

# CLEANEX experiment on Ub (Fitting)

All CLEANEX experiments were collected at 800 MHz with a 3-s acquisition delay and mixing times of 5.2, 10.4, 20.8, 41.6, 83.2 and 166.4 ms using standard Bruker pulse programs. Backbone amide protons that exchanged with the bulk solvent were fitted using established methods (Hwang *et al*, 1998), with exchange rates plotted using GraphPad Prism (version 7).

## CEST

Initial <sup>15</sup>N-pseudo-3D CEST experiments were collected at 700 MHz at 25, 37 and 45°C using established pulse sequences (Vallurupalli *et al*, 2012). At each temperature experiments were acquired with an exchange period of 400 ms and a weak B<sub>1</sub> saturation field of either 12.5 or 25 Hz, which was calibrated according to (Vallurupalli *et al*, 2012) and applied in a range between 102 and 134 ppm at 184 or 92 frequency points, respectively. <sup>15</sup>N CEST profiles were plotted as  $I/I_0$  against applied B<sub>1</sub> field, with the  $I_0$  value taken as first slice where the exchange period was omitted.

Higher resolution <sup>15</sup>N-pseudo-3D CEST experiments were then collected using Bruker 950 MHz Avance III HD spectrometer at the MRC Biomedical NMR centre. Here, experiments were collected at 45°C with an exchange period of 400 ms and weak frequency-swept  $B_1$  fields of 12.5, 25 and 50 Hz all at 12.5-Hz intervals for a total of 248 points. In order to optimise the experimental conditions and obtain exchange rates and invisible state populations, we modified the  ${\rm ^{15}N}\mbox{-}pseudo-3D$  CEST experiments with a mide proton to directly attached nitrogen-selective Hartmann-Hahn cross-polarisation periods to obtain highly selective pseudo-2D experiments (Pelupessy et al, 1999). Typically for each weak  $B_1$  saturation field, pseudo-2D CEST experiments were acquired with a relaxation delay of 5 s, 400ms exchange time, 184 frequency-swept points and eight scans in 2 h. To quantify the exchange rates and populations, we obtained  $^{15}\text{N-CEST}$  profiles at five weak B1 saturation fields of 12.5, 20, 25, 37.5 and 50 Hz for a subset of exchanging peaks, see Source Data. Experiments were processed in Topspin 3.2 and the peak intensities simultaneously fitted using ChemEx (https://github.com/gbouvig nies/chemex) as previously described (Vallurupalli et al, 2012).

# PhPINK1 – Ub/UbI binding experiments

Binding experiments were performed by recording BEST-TROSY and CLEANEX (with 166.4-ms mixing time) with 65 µM Ub/Parkin Ubl constructs with and without equimolar amounts of *Ph*PINK1. For the BEST-TROSY experiments, the peak heights of the datasets with *Ph*PINK1 were normalised against the respective peaks without *Ph*PINK1 for each Ub/Parkin Ubl construct and plotted accordingly. For the CLEANEX experiments, the absolute peak heights with and without *Ph*PINK1 were plotted side by side.

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# Phosphorylation rate measurements by NMR

Phosphorylation was performed by incubating 100 µM labelled substrate (Ub or Parkin Ubl) with 350 nM PhPINK1 in NMR buffer supplemented with 10 mM MgCl<sub>2</sub>/ATP at 25°C; 700 MHz BEST-TROSY experiments were carried out to monitor phosphorylation with eight scans and 128 increments for wt Ub and Ub L71Y (~8 min), and four scans and 100 increments for Ub F4A, Ub TVLN and Parkin Ubl (~3.5 min). To compare Ub TVLN and Parkin Ubl phosphorylation rates, 65  $\mu M$  Ub TVLN or Parkin Ubl were incubated with 20 nM PhPINK1 in NMR buffer and 10 mM MgCl<sub>2</sub>/ATP at 25°C. 600 MHz BEST-TROSY experiments were recorded with eight scans and 128 increments. Peak heights of each time point were normalised against the peak height of the first (no phosphorylation) and last (full phosphorylation) time point, respectively. A minimum set of nine peaks for each construct was used to plot phosphorylation rates (wt Ub: I3, F4, I13, T14, L15, E18, I23, V26, K29, I30, L43, I44, F45, G47, L50, E51, D52, S57, N60, I61, K63, E64, L67, H68; Ub TVLN: Q2, K6, T7, L15, I61, K63, E64, V66, N67, H68; Parkin Ubl: F4, R6, E16, S22, C59, D60, Q64, H68, V70; Ub F4A: K27, A28, K29, I30, D32, Q41, K48, L50, D52, L71; Ub L71Y: Q2, V5, K6, T14, L15, I23, K29, I44, F45, G47, L50, L56, S57, N60, I61, Q62, E64, S65, T66, L67, H68, V70, R72, L73) and a set of four peaks for the Ub TVLN and Parkin Ubl phosphorylation rate comparison (Ub TVLN: I3, K6, T7, Q62; Parkin Ubl: E16, Q25, K27, E28, F45, K48, E49, D60, Q64, V67). Data were plotted with GraphPad Prism (version 7).

# Data availability

Coordinates and structure factors have been deposited with the protein data bank accession codes 50XI (Ub L67S), 50XH (phosphoUb TLVN). NMR chemical shifts and raw CEST data used for fitting are provided as Source Data.

## Expanded View for this article is available online

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# Author contributions

Conceptualisation and experimental design, CG, AFS, JLW, JNP, SMVF, DK; investigation, CG, AFS, JLW, SMVF; writing, DK; funding acquisition, DK. All authors commented on and improved the text.