Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis

Tobias Wauer†, Kirby N Swatek†, Jane L Wagstaff†, Christina Gladkova, Jonathan N Pruneda, Martin A Michel, Malte Gersch, Christopher M Johnson, Stefan MV Freund & David Komander‡

Abstract

The protein kinase PINK1 was recently shown to phosphorylate ubiquitin (Ub) on Ser65, and phosphoUb activates the E3 ligase Parkin allosterically. Here, we show that PINK1 can phosphorylate every Ub in Ub chains. Moreover, Ser65 phosphorylation alters Ub structure, generating two conformations in solution. A crystal structure of the major conformation resembles Ub but has altered surface properties. NMR reveals a second phosphoUb conformation in which β5-strand slippage retracts the C-terminal tail by two residues into the Ub core. We further show that phosphoUb has no effect on E1-mediated E2 charging but can affect discharging of E2 enzymes to form polyUb chains. Notably, UBE2R1- (CDC34), UBE2N/UBE2V1- (UBC13/UEV1A), TRAF6- and HOIP-mediated chain assembly is inhibited by phosphoUb. While Lys63-linked poly-phosphoUb is recognized by the TAB2 NZF Ub binding domain (UBD), 10 out of 12 deubiquitinases (DUBs), including USP8, USP15 and USP30, are impaired in hydrolyzing phosphoUb chains. Hence, Ub phosphorylation has repercussions for ubiquitination and deubiquitination cascades beyond Parkin activation and may provide an independent layer of regulation in the Ub system.

Keywords deubiquitinase; Parkin; phosphorylation; PINK1; ubiquitin

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

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Introduction

The covalent modification of Lys residues with the 76 amino acid protein ubiquitin (Ub) constitutes one of the most important cellular signals, most commonly targeting the substrate protein for proteasomal degradation (Hershko & Ciechanover, 1998). The last decade has revealed that ubiquitination is much more versatile and affects virtually all cellular processes, including transcription, translation, protein kinase, cytokine and DNA damage signaling, intracellular trafficking and most forms of protein degradation, such as ERAD, autophagy and mitophagy (Chen & Sun, 2009; Komander & Rape, 2012; Shaid et al., 2013; Randow & Youle, 2014). With such wide-ranging roles, it is not surprising that imbalances in the Ub system lead to disease, and many proteins involved in assembly, binding or disassembly of Ub conjugates are mutated in human disorders. A prime example is Parkinson’s disease, a neurodegenerative disorder characterized by loss of dopaminergic neurons, which affects 1–2% of the human population especially at an older age. Mutations in the Ub E3 ligase Parkin predispose individuals to autosomal recessive juvenile Parkinsonism (AR-JP), a form of the disease where neurological symptoms show at an early age. Heterozygous mutations of Parkin have also been implicated in the more common, late-onset form of the disease (Sun et al., 2006; Wang et al., 2008; Corti et al., 2011). Parkin belongs to the family of RBR E3 ligases (Wenzel & Klevit, 2012), and recent structural data revealed that Parkin is autoinhibited and requires activation (Riley et al., 2013; Trempe et al., 2013; Wauer & Komander, 2013). Parkin is activated by the protein kinase PINK1, which is stabilized on depolarized mitochondria upon mitochondrial damage (Youle & Narendra, 2011). Once activated, Parkin ubiquitinates numerous mitochondrial and cytosolic proteins (Chan et al., 2011; Sarraf et al., 2013) including mitofusins and Miro, eventually triggering mitophagy (Youle & Narendra, 2011).

Many substrates of PINK1 and mechanisms for PINK1-mediated Parkin activation have been postulated (Wang et al., 2011; Okatsu et al., 2012; Chen & Dorn, 2013). Most recently, based on mass spectrometry and following from earlier findings that PINK1 phosphorylates the Parkin Ub-like (Ubl) domain (Kondapalli et al., 2012), three groups showed that Ub itself is a substrate for PINK1, which phosphorylates Ub Ser65 exclusively, in vitro and in cells (Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014). Moreover, purified phosphoUb directly activated Parkin in the absence of PINK1, suggesting a new model of Parkin activation (Sauvé & Gehring, 2014; Zheng & Hunter, 2014) which is still being refined (Ordureau et al., 2014).

Aside from this gain-of-function role of phosphoUb in Parkin activation, it is unclear whether phosphorylation has structural consequences for Ub and whether phosphorylation affects other Ub-mediated processes. These fundamental questions have not been addressed to date.
We here show that PINK1 is a Ub and polyUb kinase and reveal wide-ranging consequences of Ub Ser65 phosphorylation. These include significant structural changes within phosphoUb wherein the protein interconverts between two conformations in solution, one of which has a retracted C-terminal tail; this Ub^Ser65 conforma-
tion has not been observed to date. While phosphoUb is still activ-
ated by E1 and charged onto E2 enzymes, discharging and polyUb
chain assembly is impaired in several E2 enzymes and E2/E3
complexes. Moreover, 10 out of 12 tested deubiquitinases (DUBs)
hydrolyze phosphoUb chains with significantly lower activity.

In summary, we show that PINK1-mediated phosphorylation of Ub generates a functionally altered Ub that would be incompetent in various Ub-regulated processes, consistent with a model that Ser65-
phosphorylated Ub is an independent signal with altered functions in cells.

Results

PINK1 is a Ub kinase

Like others (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014), we had also found that PINK1 is a Ub kinase while investigating the mechanism of Parkin activation (Fig 1A). In an attempt to reconstitute Parkin-mediated ubiquitination of a reported substrate, Miro (Wang et al., 2011; Birs et al., 2014), we performed a coupled kinase/ligase reaction with Tribolium castaneum (Tc)
PINK1 (Kondapalli et al., 2012), human Parkin lacking the Ubl domain (Wauer & Komander, 2013), GST-Miro (residues 1–580), E1, UBE2L3 (UBCH7) and wild-type (wt) Ub. Tandem mass spectrom-
etry on the reaction was searched for peptides containing
phos-tag gels of reactions using Lys63-linked triUb as substrate
resulted in multiple bands and diffuse signals, suggesting that more
than one Ub molecule can be targeted by PhPINK1 in this chain type
(Supplementary Fig S1B, C and E). For further analysis and quantifi-
cation, we used AQUA-based mass spectrometry (Kirkpatrick et al.,
2006; Orduere et al., 2014). Digestion of a ubiquitinated protein
in vitro

While this demonstrated that PINK1 phosphorylates monoUb, it was
not clear whether PINK1 could also phosphorylate polyUb chains.
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2006; Orduere et al., 2014). Digestion of a ubiquitinated protein
in vitro
Figure 1. Ser65-phosphorylated ubiquitin

A. Ser65 phosphorylation of ubiquitin (Ub) using hPINK1 or TcPINK1 or PhPINK1.

B. Relative abundance of Ub and Ser65 phospho-ubiquitin (phosphoUb) over time.

C. Mass spectrometry analysis of Ub and phosphoUb peaks.

D. PhPINK1 phosphorylation of K63 Ub.

E. PhPINK1 phosphorylation of Ub4.

F. wtUb, phosphoUb (20%) and phosphoUb (100%) over time.

G. Kinetics of Ub and phosphoUb with anti-Ub (FK2) antibody.
phosphoUb in the reaction. Increasing the phosphoUb ratio diminished Parkin activity (Fig 1G). This unexpected behavior has also been reported by the Harper lab (Ordoueu et al., 2014). It is known that the phosphoUb-mediated activation of Parkin proceeds with a Ub mutant that cannot proceed through E1/E2/E3 cascades, pointing toward an allosteric mechanism (Koyano et al., 2014). The fact that phosphoUb is seemingly unable to be assembled into polyUb by Parkin in vitro, suggests that phosphorylation affects Ub structure and/or affects its ability to be passed through the ubiquitination cascade.

Ser65 phosphorylation changes Ub structure

We first assessed biophysical and structural properties of phosphoUb. Ub is dynamic in solution yet generates excellent high-resolution NMR spectra with typically ~72 resonances (Lange et al., 2008; Vogeli et al., 2009). To understand whether and how Ser65 phosphorylation affects Ub structure, we performed a phosphorylation reaction in situ, by adding PhIPINK1/MgATP to 15N-labeled Ub in a time-dependent NMR experiment, which led to complete and specific Ser65 phosphorylation as confirmed by ESI-MS (see Supplementary Materials and Methods). To our astonishment, the 2D-NMR spectrum of phosphoUb showed 72 resonances that are similar to wt Ub and in addition 58 new resonances (Fig 2A). This suggests that phosphorylation of Ub at Ser65 generates two non-identical Ub conformations. These results were corroborated using purified 13C, 15N-labeled phosphoUb and it was possible to assign all resonances (Supplementary Fig S2). PhosphoUb in solution exists as a major species (70% as determined by relative peak intensities) that resembles wt Ub with resonances perturbed in the vicinity of pSer65 (Fig 2B). In addition, we observed a minor species (30%) with significant chemical shift perturbations (CSPs) as compared to wt Ub (Fig 2C) or to the major phosphoUb species (Fig 2D). ZZ-exchange measurements revealed that the two species are in a slow equilibrium with a conversion rate of ~2 per second (Supplementary Figs S3 and S4).

The second, minor species of phosphoUb has to our knowledge not been observed before. Secondary structure calculations for the major and minor species using TALOS+ (Fig 2E, Supplementary Fig S5) revealed a similar secondary structure to wt Ub, indicating no global conformational changes or significant unfolding of the Ub molecule (Fig 2E) (see below).

To test whether the conformational change was restricted to monoUb, we characterized phosphorylated, 13C, 15N-labeled Lys63-linked polyUb, which due to its open conformation displays BEST-TROSY spectra that are unperturbed by interface formation and are almost identical to Ub (72 peaks; Thr9, Glu24 and Arg74 resonances are exchange-broadened, while Lys63 and Glu64 resonances are split due to proximal and distal differences for these residues, and one extra peak for the isopeptide bond between the distal Gly76 and the side chain of the proximal Lys63). We found that phosphorylation also generated a minor conformation in uniformly labeled Lys63-linked di-, tri- and tetraUb (Supplementary Fig S6 and data not shown) with 128 peaks in phosphorylated Lys63-linked diUb. This indicates that phosphorylation-induced conformational changes can take place in the context of polyUb. Future work will need to assess whether the conformational changes exist in distal, proximal or both Ub moieties, whether it occurs in all chain types, and whether this leads to changes to polyUb structure.

Phosphorylation affects Ub stability

Ub is highly stable and structurally unaffected by low pH, SDS or high temperatures [see e.g. (Ibarra-Moler et al., 1999)]. However, a novel conformation of Ub and the observed conformational transition could result in a less stable molecule. Indeed, we found that melting temperatures were decreased by 10°C in differential scanning calorimetry (DSC) experiments, from 93°C for Ub to 83°C for phosphoUb (Supplementary Fig S7). This is consistent with an alternative, less stable conformation being present, but also confirms that neither of these conformations is unfolded at the temperatures of NMR measurements. However, it does suggest that thermal denaturation of phosphoUb occurs earlier, and standard protocols of Ub purification should be used with care (Koyano et al., 2014).

Crystal structure of phosphoUb

The two conformations of Ub were analyzed in more detail. We obtained crystals of phosphoUb and determined a structure to 1.9 Å resolution (Table 1). The new Ub crystal form (space group P1) contains eight phosphoUb molecules in the asymmetric unit (Fig 3A). Six of the molecules interact similarly via their Ile44 patches, resembling structures of Lys48-linked diUb (pdb-id 1aar [Cook et al., 1992]) (Fig 3B). Unambiguous electron density is observed for phospho-Ser65 in all molecules (Fig 3C). The phosphate group forms a hydrogen bond with the backbone amide of Gln62, but no other contacts (Fig 3D). In wt Ub, the same hydrogen bond is formed via the hydroxyl group of Ser65 (Fig 3D). The small CSP for Gln62 in the major NMR species compared to wt Ub (Fig 2B) is consistent with the conservation of the hydrogen bond of this backbone amide. This and the fact that there are no significant conformational differences between the phosphoUb structures in the crystal and a Ub reference structure [pdb-id 1ubq, RMSDs 0.45–0.69 Å (Vijay-Kumar et al., 1987)] indicate that the major phosphoUb NMR species had crystallized. While there are no conformational changes, phosphorylation of Ser65 leads to significant changes in the electrostatic surface potential of Ub (Fig 3E), which may alter interactions with other proteins (see below and Discussion).

Characterization of the minor phosphoUb species

As crystallization of phosphoUb did not provide information on the more intriguing minor species of phosphoUb, this was further investigated using a variety of solution NMR techniques. While the significant CSPs of the minor species compared to wt Ub and major phosphoUb species suggest substantial changes, the secondary structure prediction showed only small changes (Fig 2E). It was unlikely that N- or C-terminal regions of Ub were unfolded, as this would lead to loss of β-sheet character in TALOS+ calculations (Supplementary Fig S5). Additionally, hetNOE measurements were collected to observe changes in local stability of the secondary structure (Supplementary Fig S8). Differences in motion on the picosecond timescale are usually seen at the termini of proteins, resulting in reduced or negative hetNOE values due to increased flexibility and motion. Resonances of the first β-strand (aa 2–6) have lower hetNOE values in the minor species as compared to the major species, indicating increased flexibility. Interestingly, Arg74 is significantly stabilized in the minor species. In wt Ub, this residue is part
Figure 2. NMR analysis of phosphoUb.

A  $^1$H, $^{15}$N-BEST-TROSY spectrum of phosphoUb (shades of blue) overlaid with wild-type (wt) Ub (orange). The phosphoUb spectrum contains 130 non-sidechain resonances that have been colored in dark blue for the major species and light blue for the minor species based on the assignment of each species in Supplementary Fig S2B–D.

B–D Weighted chemical shift perturbation (CSP) graphs for (B) wt Ub versus major phosphoUb species, (C) wt Ub versus minor phosphoUb species, (D) major versus minor phosphoUb species.

E Comparison of Ub secondary structure [derived from pdb-id 1ubq (Vijay-Kumar et al, 1987)] with the secondary structure predictions of major and minor phosphoUb forms as calculated from backbone chemical shifts using TALOS+ (Supplementary Fig S5).
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Table 1. Data collection and refinement statistics.

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*Values in parentheses are for highest resolution shell.

Ser65 phosphorylation induces β-strand slippage in phosphoUb

Closer inspection of Ub structure and sequence reveals that such a β-strand slippage is entirely feasible. The β-strand has four Leu residues (Leu67, Leu69, Leu71 and Leu73), and Leu67 and Leu69 are integral to the Ub core (Fig 4B and C). The intermittent residues His68, Val70 and Arg72 are solvent-exposed (Fig 4C). Modeling of a β-strand slippage of two residues, that is, replacing Leu67 with Leu69, Leu69 with Leu71, etc., reveals a model for the minor phospho-Ub species in which the C-terminal tail of Ub has retracted into the Ub core by two amino acids (Fig 4D). The generated model is consistent with all data: (i) The four measured long-range HNCO contacts are now satisfied (Fig 4A, B and D), (ii) the through space NOE contacts are reconciled (Supplementary Fig S9), (iii) retraction of the C-terminal tail would lead to core contacts and stabilization of Lee73 and Arg74 as observed in the hetNOE experiment (Supplementary Fig S8), (iv) the C-terminal tail would extend the β-sheet, as was predicted in the secondary structure analysis (Fig 2E, Supplementary Fig S5) and (v) all residues in β5, the preceding loop (aa 62–66), in β1 (aa 2–7) and in β3 (Ile44 and Phe45) have changed their environment significantly, explaining the large chemical shift perturbations (Fig 2A–D). The largest CSP in the minor species occurs for Gln62 (Fig 2C), indicating a drastically altered environment around the Gln62 amide, possibly due to loss of the conserved hydrogen bond of the backbone amide (see above).

Retracting the Ub C-terminus would extend the phospho-Ser65 containing loop (aa 62–66), which in the hetNOE data appears to be rigid, suggesting that pSer65 forms new interactions on the Ub surface. Likewise, retraction of the important Ub C-terminus would affect interactions with most enzymes in the Ub system. Clearly, this model for a new Ub conformation requires further structural validation, which could be obtained by identifying binding partners or mutations that stabilize this species (see Discussion).

Together, the data show that phosphorylation has dramatic effects on Ub structure. It changes the surface properties of the major species, and, more intriguingly, generates a previously unobserved Ub conformation (which we will refer to as the UbrefαCT conformation). Ser65 phosphorylation leads to a β-strand slippage by two residues, which is enabled by the sequence of the β5-strand. These changes in Ub structure may have many repercussions on the Ub system, which relies on highly conserved and regulated interactions of Ub with the many enzymes facilitating its attachment and removal.
Effects of phosphoUb on the Ub system

Ub is almost invariant in evolution, is identical in higher eukaryotes and differs only by one non-conservative and two conservative mutations between Saccharomyces cerevisiae and humans. PINK1 from all species tested to date phosphorylates the invariant Ub Ser65, suggesting that the gain-of-function of phosphoUb in Parkin activation and mitophagy is evolutionarily conserved. However, Ub plays many roles, and it is currently unclear whether phosphoUb has other, potentially detrimental effects on the Ub system. The structural data described above (Figs 2–4) show that phosphoUb is structurally distinct and could potentially adopt new functions. For example, we posit that the minor species could be recognized by as-yet unknown phosphoUb binding proteins. It is also clear that in the major phosphoUb species, the main Ub interactions sites, the Ile44 and the Ile36 patches, are structurally intact and extensively utilized in crystal lattice interfaces (Fig 3). Nonetheless, all surfaces of Ub are highly conserved, and most have been implicated in protein interactions during Ub chain assembly, binding and/or disassembly.

Figure 3. Crystal structure of Ser65-phosphoUb.

A Structure of phosphoUb in space group P2₁ with 8 molecules in the asymmetric unit (Table 1). Ub molecules are colored differently, and atoms for pSer65 are shown as spheres with red oxygen and purple phosphorous atoms.

B Six of eight phosphoUb molecules form similar dimer interactions, where phosphoUb molecules interact via their Ile44 patch. This resembles the crystal structure of Lys48-linked diUb [pdb-id 1aar (Cook et al., 1992)]. Atoms for pSer65 are shown as in (A), and the Ile44 side chain is shown as blue sticks.

C Representative 2Fo-Fc electron density, contoured at 1 σ, covering the phosphorylation site. pSer65 is shown in ball-and-stick representation, and neighboring side chains are shown in stick representation with red oxygen and blue nitrogen atoms.

D In wt Ub (left), Ser65 forms a hydrogen bond with the backbone amide of Gln62. Upon phosphorylation (right), an oxygen atom from the phosphate forms the same backbone hydrogen bond.

E Cartoon representation and electrostatic surface potential of Ub (left) and phosphoUb (right) in identical orientations. Electrostatics were calculated using CHARMM (www.charmm-gui.org) and are colored from red (negative potential) to blue (positive potential).
Figure 4. The EMBO Journal
Consequences of phosphoUb on E2 charging and discharging

The E1-mediated charging of E2 enzymes is a sophisticated multistep reaction in which Ub is engaged in a variety of ways (Schulman & Harper, 2009). We screened 13 of the 19 human E2 enzymes that are charged by the Ub E1 UBA1, to find that all tested enzymes were charged by Ub and phosphoUb similarly (Fig 5).

The E1-generated E2–Ub conjugate is relatively stable until discharges by a ligase. In NMR and SAXS ensembles of charged E2 enzymes, Ub is flexibly attached to the E2 and can take many conformations (Pruneda et al, 2011). Importantly, Ser65 is remote from the active site and does not interact with the enzyme (Fig 5B, Supplementary Fig S10A), suggesting that the E2–phosphoUb conjugate may behave similarly to an E2–Ub conjugate.

A subset of E2 enzymes ubiquitinated themselves on one or multiple sites in vitro, and this was in most cases independent of Ub phosphorylation (Fig 5A). We also noted considerable auto-polyubiquitination of UBE2E1 and UBE2T in our assays. UBE2S and UBE2R1 (cdc34) assemble free Ub chains linked via Lys11 and Lys48, respectively. Interestingly, while UBE2S assembled Lys11-linked chains regardless of whether Ub was phosphorylated or not, UBE2R1, UBE3E1 and UBE2T were significantly impaired in discharging and chain formation when phosphoUb was used in the reaction (Fig 5A and C, Supplementary Fig S10B–D). This was quantified by AQUA mass spectrometry indicating GlyGly-modified Ub peptides in E2 reactions with phosphoUb were significantly reduced (Fig 5C, Supplementary Fig S10B–D). Hence, while UBE2R1, UBE2T and UBE2E1 were charged with phosphoUb (Fig 5A), they were unable to generate significant levels of phosphoUb chains.

The most dramatic effects of phosphoUb on E2 chain elongation were observed with UBE2N (UBC13), which in complex with the inactive E2-fold proteins UBE2V1 (UBE1A) or UBE2V2 (MMS2) assembles free Lys63-linked polyUb. UBE2N was charged identically with Ub or phosphoUb as observed for the remaining E2s (Fig 5A and D). Addition of UBE2V1 led to robust Lys63 chain assembly with Ub, but the enzyme complex was inactive with phosphoUb (Fig 5D). This finding can be explained structurally from the complex of S. cerevisiae Ubc13–Ub with Mms2 (Eddins et al, 2006). The acceptor Ub is bound by Mms2, which positions it such that Lys63 points toward the active site. Ser65 of the acceptor Ub interacts directly toward the active site. Ser65 of the acceptor Ub is bound by Mms2, which positions it such that Lys63 points toward the active site. Ser65 of the acceptor Ub interacts directly with Ub and does not make any contacts, phosphorylation would clash with Gly18 of Mms2 (Fig 5E). All functionally important residues in Ubc13/Mms2 are invariant in human UBE2N/UBE2V1. A reaction with Ub K63R as a donor Ub and Ub lacking the C-terminus (Ub ΔGG) as an acceptor Ub assembled Lys63-linked diUb specifically (Fig 5F). Increasing amounts of phosphoUb ΔGG did not affect diUb generation (Fig 5F), suggesting that this species was unable to compete with Ub ΔGG for UBE2V1 binding. Hence, the defect in UBE2N/UBE2V1 chain assembly is due to blocked acceptor Ub binding, consistent with the structure.

In summary, E2 charging with phosphoUb is unaffected for the majority of E2 enzymes, showing that E1 does not differentiate between Ub and phosphoUb. Importantly, some E2 enzymes are impaired in polyUb chain formation with phosphoUb, and, for example, the UBE2N/UBE2V1-mediated assembly of Lys63-linked chains is switched off by phosphoUb.

Effects of phosphoUb on E3-mediated ubiquitination

E2 enzymes are discharged with the help of E3 ligases, which either induce a high-energy state of the E2–Ub complex (RING, U-box E3 ligases) or accept Ub to form a second thioester intermediate (HECT, RBR E3 ligases) (Berndsen & Wolberger, 2014). Recent structural work has revealed how RING E3 ligases generate a spring-loaded E2–Ub complex (Dou et al, 2012; Plechanovova et al, 2012; Pruneda et al, 2012), and Ser65 of Ub is not involved in E2 or E3 interactions in any of the studied trimeric complexes (Supplementary Fig S11A). Likewise, Ub Ser65 is not contacted in HECT and RBR Ub complex structures (Supplementary Fig S11B and C). However, as seen with E2 enzymes, a switch from Ub to phosphoUb could lead to differences in ligase activity due to effects on acceptor Ub binding or may change linkage composition during chain formation.

We tested GST-tagged RING domains of clAP1 (aa 363–614) and TRAF6 (aa 1–285 and 50–285) in autoubiquitination reactions with Ub– or phosphoUb-charged UBE2D1 and UBE2D3 (Yang et al, 2005). UBE2D1 and UBE2D3 work with most E3 ligases, are non-specific for substrate modification and assemble multiple linkage types in vitro (Kim et al, 2007; Dynek et al, 2010). Autoubiquitination of GST-clAP1 proceeded with Ub and phosphoUb similarly with either E2 enzyme, leading to a depletion of monoUb and to qualitatively similar chain laddering on GST-clAP1 (Fig 6A and B, Supplementary Fig S11D). AQUA mass spectrometry revealed that GST-clAP1/UBE2D1 assembled chains to a similar extent (Fig 6C) and with similar composition with Ub and phosphoUb, namely Lys63 (~50%), Lys48 (~25%), Lys11 (~18%), and Lys6 and Lys27 linkages in smaller amounts (Fig 6D). The high amounts of Lys63 linkages (> 50% in both samples) were interesting, as this contrasts the finding of UBE2N/UBE2V1 where this linkage type was no longer assembled (Fig 5D). Hence, phosphoUb has no impact on clAP1-mediated chain assembly.

Surprisingly, with TRAF6, the same E2 enzymes, UBE2D1 and UBE2D3, assembled chains processively with Ub but not with
Figure 5.
phosphoUb (Fig 6E and F). This was observed for several GST-TRAF6 constructs that include the RING domain as well as zinc finger modules (Fig 6E and F, Supplementary Fig S11E). AQUA mass spectrometry confirmed this result, showing that only a small fraction of phosphoUb is involved in chain linkages (Fig 6G and H). The low activity with phosphoUb was TRAF6 dependent (Supplementary Fig S11F). These results are unexpected as they suggest that TRAF6 is able to recognize the Ser65 phosphorylation status.

To understand whether phosphoUb affects other ligase classes, we tested NEDD4L, a Lys63-specific enzyme (Kamadurai et al, 2009) with the E2 enzyme UBE2L3 that specifically transfers Ub onto the catalytic Cys of HECT and RBR E3 ligases (Wenzel et al, 2011). NEDD4L/UBE2L3 assembled Ub chains with Ub and phosphoUb identically (Fig 6I). Also, the bacterial effector HECT-like enzyme NleL (Hospenthal et al, 2013) assembled chains similarly with Ub and phosphoUb (Fig 6J, Supplementary Fig S11G).

Finally, we tested another member of the RBR E3 ligase family, HOIP, which is part of the linear Ub chain assembly complex (LUBAC) (Kirisako et al, 2006). The HOIP RBR domain extended C-terminally by a Ub binding domain assembles Met1-linked chains processively in vitro (Smit et al, 2012; Stieglitz et al, 2012). Interestingly, this reaction proceeded with Ub, but was inhibited with phosphoUb (Fig 6K, Supplementary Fig S11H). The structural basis for this is unclear. A structure of a minimal HOIP construct sufficient for Met1-chain assembly bound to two Ub molecules (Stieglitz et al, 2013) showed that Ser65 is solvent-exposed in both donor and acceptor Ub (Supplementary Fig S11C). This suggests that the extended construct used in the assay forms additional Ub interactions involving Ser65 of donor or acceptor Ub that cannot be formed when Ser65 is phosphorylated. Alternatively, the phosphate affects either access to or the pK_a of the acceptor 3-amine of Met1.

In summary, although testing only a small subset of E3 ligases, we uncovered that phosphoUb can lead to marked defects in polyUb chain generation.

Attributing phosphoUb ligation defects to the major conformation

Known structures of the ubiquitination machinery are incompatible with the observed minor phosphoUb^extraCT conformation. An interesting finding with phosphomimetic Ub mutants confirmed that the surface charge changes of the major conformation in phosphoUb accounted for the defects in the ligation deficiency in TRAF6/UBE2D and UBE2N/UBE2V1.

Parkin activation in cells could be mimicked by phosphomimetic Ub mutants, where the phosphorylated Ser65 is replaced with Asp or Glu (Kane et al, 2014; Koyano et al, 2014). We tested whether phosphomimetic mutants displayed two conformations in NMR experiments, by analyzing spectra of ^15N-labeled Ub S65E and S65D (Supplementary Fig S12A). Both mutants did not show the additional peaks of the Ub^extraCT conformation, but revealed a spectrum with 69 resonances (Thr9, Glu24 and Ala46 were exchange-broadened as compared to Ub) resembling the major conformation of phosphoUb (Supplementary Fig S12B). This is important as it suggests that phosphomimetic Ub mutants may not reconcile all effects of Ub Ser65 phosphorylation, and results from cell biology studies should be interpreted with care.

Still, this result was useful as it enabled us to understand whether the phosphoUb^extraCT conformation or the major phosphoUb conformation accounted for the observed defects in chain assembly. Indeed, consistent with structural considerations (Fig 2K), Ub S65E is unable to perform ubiquitination with UBE2N/UBE2V1 (Supplementary Fig S13A). Similarly, for TRAF6/UBE2D, Ub S65E significantly inhibited chain assembly, although not to the same extent as phosphoUb (Supplementary Fig S13B). There could be multiple reasons for the difference, and it is possible that the conformational equilibrium of phosphoUb may exaggerate the observed loss of activity.

Lys63-linked phosphoUb chains are recognized by TAB2

Ubiquitin binding domains (UBDs) decode Ub signals, and some preferentially interact with Ub polymers (Husnjak & Dikic, 2012). As mentioned above, the principle UBD recognition site on Ub, the Ile44 patch, is not grossly affected in the major conformation of phosphoUb, and we would expect small UBDs to interact similarly with Ub and phosphoUb. The case may be different for chain interacting UBDs. The TAB2 NZF domain “bends” a Lys63-linked dUb to interact with its two Ile44 patches simultaneously (Kalathu et al, 2009; Sato et al, 2009), yet Ser65 of either molecule is solvent-exposed (Fig 7A).

To test whether TAB2 binds poly-phosphoUb, we performed pull-down experiments using GST-tagged TAB2 NZF domain

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Figure 6. E3-mediated autoubiquitination with Ub and phosphoUb.
A Time course for a ligase reaction with GST-tagged cIAP1 (aa 363–614), UBE2D1 and Ub or phosphoUb. See Supplementary Fig S11D for a reaction with UBE2D3.
B Anti-Ub Western blot of an identical reaction run for 3 h.
C Comparison of the number of Ub chain linkages in the reaction in (B).
D Ub chain linkage profile for the reaction in (B).
E Reaction of GST-tagged TRAF6 (aa 1–285) with UBE2D3 as in (A). See Supplementary Fig S11E and F for additional TRAF6 reactions.
F Anti-Ub Western blot of GST-TRAF6/UBE2D1 run for 3 h.
G Comparison of the number of Ub chain linkages in the reaction in (F).
H Linkage composition in GST-TRAF6-UBE2D1 reactions reveals 58% Lys63 chains with wt Ub and UBE2D1. Significantly smaller quantities of linkages are assembled with phosphoUb.
I–K Chain assembly as in (A) with UBE2L3 and (I) GST-tagged NEDD4L (aa 576–955) [Mund & Pelham, 2009], (J) NleL (aa 170–782) [Hospenthal et al, 2013] and (K) HOIP RBR-LDD (aa 699–1072) [Smit et al, 2012]. While the HECT E3 ligases NEDD4L and NleL are active with Ub and phosphoUb, HOIP is significantly less active with phosphoUb. See also Supplementary Fig S11G and H.
(Kulathu et al., 2009), revealing strong interactions with Lys63-linked but not Met1-linked tetraUb regardless of phosphorylation status (Fig 7B). TAB2 has previously been used to sense Lys63-linked chains on mitochondria after depolarization (van Wijk et al., 2012), and it can be assumed that the detected chains were partly phosphorylated.

**DUBs hydrolyze phosphoUb chains with lower activity**

Finally, we analyzed the ability of DUBs to hydrolyze phosphoUb chains. The human genome encodes ~80 active DUBs, >50 of which contain Ub-specific protease (USP) domains. The remaining enzymes belong to four structurally distinct families (Komander et al., 2009; Clague et al., 2013). USP enzymes such as USP2 (Fig 8A) engulf ~40% of the solvent accessible surface of a bound distal Ub (Komander et al., 2009). Importantly, Ser65 is involved in these interactions, and phosphorylation may affect DUB recognition (Fig 8A). Indeed, when we tested USP2 activity against ubiquitinated GST-cIAP1 (Fig 8A) as a substrate in DUB assays, we found that USP2 was markedly less active against poly-phosphoUb (Fig 8B) and only cleaved phosphoUb chains at later time points or at higher enzyme concentration (data not shown).

Identical behavior was found for USP8, USP15 and USP30 (Fig 8C-E). Also, the Josephin-family DUB Ataxin-3 showed less activity against poly-phosphoUb (Fig 8F). Interestingly, USP8, USP15, USP30 and Ataxin-3 have all been implicated in Parkin regulation (Durcan et al., 2012, 2014; Bingol et al., 2014; Cornelissen et al., 2014). In contrast, known non-specific enzymes USP21 and the small viral OTU domain DUB vOTU (Akutsu et al., 2011; Ye et al., 2011) were able to cleave polyUb and poly-phosphoUb similarly (Fig 8G and H).

USP30 was significantly less active toward cIAP polyUb chains as compared to other USP DUBs. To test whether this was due to a different chain preference, we performed a specificity analysis against a panel of differently linked diUb, revealing that USP30 preferred Lys6-linked chains (Fig 8I). This is interesting as Lys6-linkages are enriched in Parkin substrates (Durcan et al., 2014; Ordureau et al., 2014). USP30 was still less active toward phosphorylated Lys6-linked diUb (Fig 8J).

Finally, we tested the activity of linkage-specific DUBs against defined phosphorylated substrates. The JAMM-family DUB AMSH is Lys63-specific (McCullough et al., 2004), but is less active against phosphorylated chains (Fig 8K). Similarly, Lys29/Lys33-specific TRABID, Lys48-specific OTUB1 and Met1-specific OTULIN are impaired in cleaving phosphorylated chains (Fig 8L-N).

Structurally, some of these results are surprising. While USP Ub binding may explain the weak activity of this enzyme class against poly-phosphoUb, the outlier USP21 shows that subtle differences in the binding site apparently enable a USP domain to target phosphorylated chains (Supplementary Fig S14A). Ser65 in distal or proximal Ub is solvent-exposed in known structures of OTU DUBs, such as OTUB1 (Juang et al., 2012; Wiener et al., 2012) (Supplementary Fig S14B) and OTUB1 and Met1-specific OTULIN are impaired in cleaving phosphorylated chains (Fig 8L-N).
Figure 8. Defects in hydrolyzing phosphoUb chains.

A Structure of USP2 (red) bound to Ub (cyan) [pdb-id 2hd5 (Renatus et al, 2006)], with a close-up on Ub Ser65 in the distal Ub. A negative charge at the USP2 Asp345 position is conserved in most USP enzymes (Ye et al, 2009).

B–H Ubiquitination reactions from Fig 6A were used as input for deubiquitinase (DUB) analysis. Silver-stained time-course gels are shown. All DUBs tested (USP2, USP8, USP15, USP30, Ataxin-3, USP21) hydrolyze Ub chains but have significantly lower activity against phosphoUb chains, with the exception of vOTU and USP21.

IU S P30 activity was significantly lower for cIAP polyUb as compared to other USP DUBs. Cleavage assays against the diUb panel reveals preference for Lys6-linked chains.

J USP30 activity is decreased against phosphorylated Lys6 diUb.

K–N Deubiquitinase assays against phosphorylated tetraUb. The activity of AMSH (K), TRABID (L), OTUB1 (M) and OTULIN (N) are inhibited with Lys63, Lys33, Lys48 and Met1 phosphorylated tetraUb, respectively.
The fact that 10 out of 12 tested DUBs have notably weaker activity against chains that contain phosphoUb suggests that phosphorylated chains are more stable as compared to unphosphorylated chains. Importantly, chains can be phosphorylated by PINK1 (Fig 1), and poly-phosphoUb exists in cells (Ordureau et al., 2014). This together with the inhibition of ligases could change the dynamics of modified substrates, their degradation rates or signaling capacity. Hence, Ub phosphorylation may have consequences on the dynamics of the entire Ub system.

Discussion

PINK1-mediated phosphorylation of Ub at Ser65 has dramatic consequences for Ub structure, and key processes in the Ub system, namely Ub attachment and removal.

It could be expected that phosphorylation of Ub would change its surface properties due to the addition of a negative charge. The obtained high-resolution crystal structure and solution studies agree that the majority of phosphoUb is structurally similar to wt Ub. To our amazement, NMR studies showed a second, minor conformation of phosphoUb, which is in slow exchange with the major conformation. Strikingly, the minor conformation shows distinct hydrogen bonding patterns and long-range NOEs for its C-terminal β5-strand, which can only be structurally satisfied when this strand is shifted by two residues. Our phosphoUb*retract model explains numerous observations and is structurally feasible due to the existence of four Leu-Xaa repeats in the β5-strand that would allow a shift of two residues without significantly disturbing the Ub core.

β-strand slippage is a known phenomenon occurring in, for example, Arf family GTPases (Pasqualato et al., 2002) and Serpins (Zeraik et al., 2014). In these proteins, strand slippage is induced by changes of nucleotide (GDP/GTP) in the binding pocket. While conceptually similar, we are not aware of a phosphorylation-induced β-strand slippage as reported here, and to our knowledge, the Ub*retract species has not been observed previously.

Structurally, β5-strand slippage would have significant implications: Firstly, shifts of His68 and Val70 would disrupt the important Ile44 hydrophobic patch which is involved in most Ub interactions described to date (Husnjak & Dikic, 2012; Komander & Rape, 2012). Secondly, the Ub*retract conformation can presumably not be recognized by enzymes of the assembly cascade (E1, E2, E3) as well as DUBs since these proteins rely on interactions with the extended Ub C-terminus. Thirdly, retraction of the C-terminus may change the dynamics and structure of polyUb chains, adding further complexity to the already vast structural landscape adopted by Ub polymers (Kulathu & Komander, 2012).

The relevance and potential roles of the Ub*retract conformation remain unclear. Its existence raises fundamental questions for Ub and Ub conformation, dynamics and folding. Biologically, the Ub*retract conformation could be recognized as a distinct signal. One could speculate that a mitophagy adaptor or other phosphoUb binding proteins may preferentially recognize the Ub*retract conformation of phosphoUb, and it will be interesting to identify such proteins.

Biochemically, it was not clear whether phosphoUb can be assembled into chains and whether chains incorporating phosphoUb are recognized by UBDs and hydrolyzed by DUBs. Using representative members from all branches of the Ub assembly and disassembly

![Diagram](image_url)

**Figure 9. New roles for Ser65-phosphoUb.**

Phosphorylation of Ub by PINK1 has multiple consequences. Structurally, it alters the electrostatic potential of Ub, but also generates a new Ub*retract* conformation with a retracted C-terminus. The Ub*retract* conformation is in slow exchange with the common Ub conformation. Functionally, Ub phosphorylation leads to a gain-of-function of Ub, as it becomes an allosteric activator of Parkin. We here show that phosphoUb also has loss-of-function effects, as it inhibits some assembly and disassembly systems. Additionally, it is possible that phosphoUb is recognized specifically by Ub receptors.
cascades, we demonstrated that, in principle, E1 activation, E2 charging and E3-mediated discharging can progress with phospho-Ub, although we made several surprising observations. Most strikingly, the Lys63-specific assembly complex UBE2N/UBE2V1 was inactivated by phosphoUb, which can be explained structurally. PhosphoUb also affected discharging in other E2 enzymes; the reasons for this are less clear.

We also investigated two RBR, two HECT and two RING E3 ligases, uncovering surprising differences for the latter. With identical E2 enzymes, the well-studied RINGs of cIAP1 and TRAF6 processed Ub and phosphoUb differently, and our results suggest that TRAF6 recognizes Ub and is unable to bind phosphoUb. The two tested HECT E3 ligases worked similarly with Ub and phosphoUb, while the RBR enzyme HOIP and Parkin are less active with phosphoUb.

Ub phosphorylation severely affects DUB activity, and 5 out of 6 USP enzymes, 4 out of 5 OTU enzymes and the tested JAMM and Josephin DUBs were significantly less active against phosphoUb. We were surprised that all DUBs implicated in Parkin biology and mitophagy were less active with phosphoUb; we wonder whether a phosphoUb-specific DUB exists. Since phosphoUb chains are more resistant to DUB activity, they likely constitute a more stable Ub signal.

Our work also revealed some insights for the PINK1/Parkin system. We show that, at least in vitro, Ub tetramers comprising six of the eight linkage types can be phosphorylated at every Ub in the chain. Harper and colleagues showed that this likely holds true in vivo, where PINK1 phosphorylates polyUb of various chain linkages on mitochondria (Ordureau et al., 2014). This is interesting from a kinase perspective since, apparently, PINK1 is able to access each Ub molecule within chains to phosphorylate Ser65. Moreover, Ser65 is in a folded and structurally stable environment in Ub, not exposing any stretch of primary sequence. This indicates that PINK1 likely recognizes a three-dimensional motif present in Ub and the Parkin Ub domain, rather than, as more common for kinases, a peptide motif.

According to proteomic analysis, PINK1 phosphorylates 2.5–3% of total Ub and 10–20% of mitochondrial Ub upon mitochondrial depolarization (Koyano et al., 2014; Ordureau et al., 2014). Different models have been proposed for phosphoUb-mediated Parkin activation, and the sequence of events is still debated (Zheng & Hunter, 2013; Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014; Ordureau et al., 2014). We here show that for phosphoUb to activate Parkin, unphosphorylated Ub must be present. This was also shown by Harper and colleagues, although in their case, the effects of pure phosphoUb appear less dramatic (50% reduction in chain formation) (Ordureau et al., 2014). The mechanistic basis for this is not clear and will need to be revealed in structural studies.

In summary, we show that phosphoUb, beyond the described gain-of-function in Parkin activation, has primarily detrimental effects on the Ub system, partly due to significant structural perturbations (Fig 9). Many issues remain, in particular regarding specific recognition of phosphoUb by (novel?) UBDs, specific reversal of phosphoUb signals by DUBs, and regarding functional consequences from inactivating E2/E3 ligase systems. Structurally, it is of prime importance to understand how phosphoUb is recognized by Parkin, how PINK1 can phosphorylate Ub specifically, and whether the Ub\textsuperscript{extra\textregistered} conformation has additional binding partners.

It is also clear that the identification of Ub phosphorylation by PINK1 is the tip of the iceberg with regard to Ub modifications. Several Ser/Thr/Tyr residues of Ub, including Thr7, Thr12, Ser57 and Tyr59, are known to be phosphorylated by as-yet unknown kinases (http://www.phosphosite.org), and all may have dedicated functions and pathways. This vastly increases the complexity in the Ub system and requires careful systematic future studies.

**Materials and Methods**

Please find complete Materials and Methods in the Supplementary Information online.

**Data deposition**

Crystallographic data for Ser65-phosphoUb have been deposited with the protein data bank, accession number 4wzp.

**Supplementary information** for this article is available online: http://emboj.embopress.org

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**Author contributions**

DK, KNS, TW, CG, MAM and MG performed biochemical experiments, and TW and DK solved and refined the phosphoUb crystal structure. KNS established mass spectrometry including AQUA and PRM analysis in the laboratory, performed all mass spectrometry and analyzed the data. JNP generated reagents and provided expertise and protocols. CMJ performed stability measurements; JW and SMVF performed NMR analysis. KNS, TW and CG discovered ubiquitin phosphorylation by PINK1 in vitro. DK wrote the manuscript with input from all authors.

**Conflict of interest**

DK is part of the DUB Alliance that includes Cancer Research Technology and FORMA Therapeutics, and is a consultant for FORMA Therapeutics.

**References**


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Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis

Supplementary Material

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# These authors contributed equally to this work

Running title: Ser65-phosphorylated ubiquitin
Supplementary Figure 1

A

B

C

D

E

F

G
Supplementary Figure 1. Phosphorylation of Ub and polyUb.

A) Phos-tag gel as in Figure 1B, but in this case active (left) and kinase inactive D359A (right) forms of GST-tagged TcPINK1 were used in a time course. B) Phosphorylation of Lys63-linked triUb with GST-tagged PhPINK1, followed by Phos-tag gel in a time course. Distinct mono-phosphorylated (monoP) and di-phosphorylated (diP) forms are labeled while presumably tri-phosphorylated (triP) chains appear as a smear. C) Phosphorylation time course of Lys63-linked hexaUb (K63 Ub6) with GST-tagged PhPINK1 on Phos-tag gel. D) Reaction from C analyzed by AQUA mass spectrometry as in Figure 1D. E) Phosphorylation of Lys11-linked tetraUb (K11 Ub4) on Phos-tag gel. F) AQUA mass spectrometry analysis on reactions from Figure 1E. F) Anti-Ub (FK2) antibody recognizes phosphoUb chains in a chain assembly reaction with cIAP as performed in Supplementary Figure 11D. See Figure 6 below for more details.
Supplementary Figure 2. Assignment of phosphoUb species.

Complete chemical shift assignment based on 3D triple resonance experiments with $^{13}$C, $^{15}$N-labeled phosphoUb. Chemical shift positions of the major (m) (A) and of the minor (n) (B) form of phosphoUb are labeled. Where the two forms share the same chemical shift only the residue type and number is given.
Supplementary Figure 3. ZZ exchange spectroscopy.

ZZ exchange experiment of phosphoUb with a mixing time of 92 ms illustrating the occurrence of exchange (cross) peaks between the (auto) resonances for the major and minor forms.
Supplementary Figure 4. ZZ exchange data.

Peak intensities of (auto) signals of the major form (blue) and minor form (lightblue) (left column) are fitted simultaneously to cross peaks as result of major to minor exchange (blue) and minor to major exchange (lightblue) (right column) for residues Ile23, Phe45, Ser57 and Leu73 using the methods described in (Latham et al, 2009). This compensates for the loss of signal intensity due to longitudinal $T_1$ relaxation (apparent from the decaying auto peaks). The exchange rate was calculated to be $1.76 \pm 0.09 \text{ s}^{-1}$. 
Supplementary Figure 5. PhosphoUb secondary structure prediction.
Backbone chemical shifts (HN, N, CA, CB and HA) were submitted to TALOS+ (Shen et al, 2009) for secondary structure prediction. Confidence in the prediction of α-helix (blue) or β-sheet (red) is given for the major (A) and minor (B) phosphoUb species. See Figure 2E for annotation.
Supplementary Figure 6

A

B

phosphoUb major vs phosphoUb Lys63 diUb major

C

phosphoUb minor vs phosphoUb Lys63 diUb minor
Supplementary Figure 6. Phosphorylation of Lys63 diUb.

A) A comparison of the BEST-TROSY spectrum of wild-type Lys63 diUb (black, 72 peaks) with phosphorylated wild-type Lys63 diUb (blue, 128 peaks). B) Weighted chemical shift perturbations of the major species of phosphoUb (Figure 2A) against the major species of phosphorylated wild-type Lys63 diUb. C) Weighted chemical shift perturbations of the minor species of phosphoUb (Figure 2A) against the minor species of phosphorylated wild-type Lys63 diUb.
Supplementary Figure 7

Excess Heat Capacity (kcal/mole\(^o\)C) vs. Temperature (\(^o\)C)

- phospoUb data
- phospoUb fitted curve
- wt Ub data
- wt Ub fitted curve
**Supplementary Figure 7. Stability measurements of Ub and phosphoUb.**

DSC endotherms for Ub and phosphoUb (dark cyan / blue lines, respectively) with fits to the data (light cyan / blue lines) for Ub; T\textsubscript{m} 93.6 °C, \(\Delta H\) calorimetric 88 kcal/mol and \(\Delta H\) van’t Hoff 87 kcal/mol, and for phosphoUb T\textsubscript{m} 83.7 °C, \(\Delta H\) calorimetric 65 kcal/mol and \(\Delta H\) van’t Hoff 61 kcal/mol.
Supplementary Figure 8. HetNOE of phosphoUb.

$^{15}\text{N}(^1\text{H})\text{hetNOE}$ values for the major (dark blue) and minor (light blue) species of phosphoUb. The value for the major form of pSer65 is omitted as it is artificially suppressed in this experiment due to the proximity of its cross peak to the highly flexible and therefore negative peak of Gly76 (see Supplementary figure 2A).
Supplementary Figure 9
Supplementary Figure 9. Long distance NOE contacts of major and minor species of phosphoUb.

The long distance NOE contacts of A) the major species of phosphoUb and B) the minor species of phosphoUb. The direction of the arrow indicates the assignment of a NOE cross peak from the HN proton to either an HN, HA or HB for a given residue. As a rule of thumb, NOE cross peaks are observed for resonances up to 5 Å apart. The observed contacts confirm the altered hydrogen-bonding pattern seen for the minor species of phosphoUb (Figure 4).
Supplementary Figure 10. Ubiquitin chain composition in E2 autoubiquitination reactions.

A) SAXS-derived ensemble of UBE2N, where the E2 is shown under a blue surface and the Ub is shown as a green ribbon. Ser65 is shown in sphere representation. Ser65 does not contact the E2 enzyme in any orientation of Ub. PDB files were downloaded from the Klevit-lab website (http://depts.washington.edu/klvtlab/). B-D) Coomassie gels of a reaction with UBE2T (B), UBE2E1 (C) and UBE2R1 (D) comparing Ub and phosphoUb, and subsequent AQUA-based chain composition analysis. Quantification of linkages is shown in Figure 5C.
Supplementary Figure 11. E3 ligase mediated assembly of phosphoUb chains

A) Structure of BIRC7 (red) bound to Ub~UBE2D2 (cyan and blue) (pdb-id 4auq, (Dou et al, 2012)). Ser65 is not contacted by the E2 or E3 enzyme. B) Composite model that combines the crystal structure of NEDD4 bound to Ub~UBE2D (pdb-id 3jw0, (Kamadurai et al, 2009), UBE2D is omitted for clarity) and the crystal structure of NEDD4L with Ub bound to HECT N-lobe (2xbb, (Maspero et al, 2011)). In either position of Ub, Ser65 is not contacting the E3 ligase. C) Structure of the catalytic core of HOIP, comprising the catalytic IBR (CBR) domain (also known as RING2) and the C-terminal LDD domain (4ljo, (Stieglitz et al, 2013)), shown with acceptor and donor Ub (cyan) bound. Ser65 does not contact the CBR-LDD in either Ub. D) Ubiquitination reaction as in Figure 6A with GST-cIAP1 and UBE2D3. E) Ubiquitination reaction with UBE2D3 and GST-TRAF6 50-285. F) Ubiquitination reaction as in E with or without TRAF6, demonstrating that chain formation is TRAF6-dependent also with phosphoUb. G) Ubiquitination reaction with NleL and UBE2L3. This combination makes free Lys6/Lys48-linked polyUb (Hospenthal et al, 2013). A longer time point as compared to Figure 6J is shown. H) Longer time course of the reaction of HOIP RBR-LDD / UBE2L3 as shown in Figure 6K, showing that HOIP generates short linear chains at later time points.
Supplementary Figure 12. Comparison of Ub S65E with wild-type Ub.

A) The BEST-TROSY spectrum of wild-type Ub (red) overlaid with the BEST-TROSY spectrum of Ub S65E (blue) and Ub S65D (green). The phosphomimetic Ub mutants shows no signs of the Ub\textsuperscript{retract} conformation as observed for phosphoUb. B) A minimal map of the weighted chemical shift perturbations (CSPs) between the peaks of S65E Ub and wild-type Ub. Resonances for Thr9, Glu24 and Ala46 are presumed to be missing due to line broadening.
Supplementary Figure 13A

E1, ATP
E2: UBE2D1
E3: GST-TRAF6 1-285

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Coomassie

Supplementary Figure 13B

E1, ATP
E2: UBE2N, UBE2V1

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Coomassie
Supplementary Figure 13. Comparison of Ub, phosphoUb, and Ub S65E in chain assembly.

A) Time course for a ligase reaction using GST-TRAF6 (1-285) and UBE2D1 combined with Ub, phosphoUb, or S65E Ub. Proteins were detected with Coomassie and anti-Ub Westerns. B) Reactions with UBE2N/UBE2V1 combined with Ub, phosphoUb, or S65E Ub. Proteins were detected as in A.
Supplementary Figure 14. PhosphoUb chain disassembly by deubiquitinases.
Structures of DUBs (red under a semi-transparent surface) bound to one or where available two Ub molecules (cyan). Ub Ser65 atoms are shown as spheres and indicated with a blue circle. **A)** Structure of USP21 bound to linear diUb-aldehyde (2yb5, (Ye et al, 2011)). The insert shows the distal binding site with USP21 Glu435 contacting the backbone of Ub. **B)** Structure of OTUB1 bound to E2 (blue, here UBE2D) with distal and proximal Ub bound (pdb-id 4ddg, (Juang et al, 2012; Wiener et al, 2012)). **C)** Structure of inactive OTULIN bound to Met1-linked diUb (3znz, (Keusekotten et al, 2013)). **D)** Crystal structure of the MJD-family protein Ataxin-3L with Ub bound in the distal site (3o65, (Weeks et al, 2011)). **E)** Crystal structure of the JAMM family protein AMSH-LP with Lys63-linked diUb bound across the active site (2znv, (Sato et al, 2008)).
**Table S1.** Peptides used for parallel reaction monitoring quantitation. Internal standards were isotopically labelled (\(^{13}\text{C},^{15}\text{N}\)) and the corresponding residue(s) is underlined. Modified residues with ubiquitination and phosphorylation are indicated by (GG) or p, respectively. Fragment ions used for quantitation are listed.

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SUPPLEMENTARY MATERIAL AND METHODS

PINK1 purification
Constructs of GST-tagged PhPINK1 (aa 115-575) and TcPINK1 (aa 128-570) codon-optimized for bacterial expression were expressed in Rosetta2 pLacI cells by inducing with 150 µM IPTG (OD600 ~1.0) at 37°C. The cells were grown at 18°C for 12 h and lysed by sonication in 270 mM sucrose, 10 mM glycerol 2-phosphate disodium, 50 mM sodium fluoride, 14 mM β-mercaptoethanol, 50 mM Tris (pH 8.0) with added DNase, Lysozyme and EDTA-free protease inhibitor tablets (Roche). After centrifugation (45000 x g, 30 min, 4°C) the supernatant was applied to Glutathione Sepharose 4B beads (GE Healthcare), agitated for 1 h at 4°C and subsequently washed in high salt buffer (500 mM NaCl, 10 mM DTT, 25 mM Tris pH 8.5) and equilibrated in low salt buffer (200 mM NaCl, 10 mM DTT, 25 mM Tris pH 8.5). The GST-tagged protein was eluted in low salt buffer containing 40 mM glutathione and applied to gel filtration (Superdex 200, GE Life Sciences) in low salt buffer. Untagged protein was cleaved for 12 h at 4°C with GST-3C protease and further purified by gel filtration (Superdex 75, GE Life Sciences) in low salt buffer. Protein-containing fractions were pooled, concentrated using a 30-kDa MWCO spin concentrator (Sartorius) and flash frozen in liquid nitrogen.

Generation of phosphoUb
10 mg of bovine Ub (Sigma-Aldrich, U6253) were incubated with glutathione S-transferase (GST)-tagged PhPINK1 (aa 115-575) in reaction buffer (40 mM Tris pH 7.5, 10 mM MgCl2, 0.6 mM DTT) for 3 h at room temperature. Reaction progress was monitored by ESI-MS. Reactions were stopped with apyrase, PINK1 was removed using glutathione sepharose 4B resin (GE Healthcare), and the reaction mixture was buffer exchanged to 20 mM Tris, pH 8.7. PhosphoUb was purified by anion exchange using a pH gradient from 20 mM Tris pH 8.7 to 50 mM Tris pH 7.4. Phosphorylated but not unphosphorylated Ub binds to MonoQ anion exchange resin at pH 8.7 and elutes at lower pH. Fractions containing phosphoUb were concentrated using
VivaSpin 3.5K concentrators and frozen. For NMR analysis, samples were generated as described below.

**Phosphorylation analysis of Ub**
Phosphorylation assays were performed by mixing 5 µM GST-PINK1 (species as indicated), 0.2 mg/ml Ub or Ub chains as indicated, 10 mM ATP in reaction buffer (40 mM Tris pH 7.5, 10 mM MgCl₂, 0.6 mM DTT) and incubated at room temperature for the specified time. The reaction was quenched in 4x LDS sample buffer and samples were applied on a 12% polyacrylamide gel containing 50 µM Phos-tag acrylamide (Wako Chemicals) and 0.77 mM ZnCl₂. MS analysis was performed as described below.

**Intact protein MS analysis**
Ub and phosphoUb protein stocks were diluted to 1 µM (50% ACN, 0.1% FA) prior to MS analysis. Samples were directly injected into the Q-Exactive (Thermo Fisher Scientific) mass spectrometer at a flow rate of 10 µl min⁻¹. Protein ionization was achieved using Heated Electrospray Ionization (HESI-II probe, Thermo Fisher Scientific). Ionization settings included the following: spray voltage, 6.0 kV; capillary temperature, 320°C; sheath gas, 10; S-lens RF level, 50. Raw spectra were deconvoluted using the Xtract node of Thermo Xcalibur Qual Browser version 2.2. Extract node settings included the following: generated mass mode, MH⁺; resolution, 140,000; S/N threshold, 3; mass range, 800-2000 m/z; fit factor, 44%; remainder, 25%.

**Parkin activity assays**
For Parkin activation assays a mixture of 0.1 µM E1, 9 µM Parkin, 10 mM ATP and ligation buffer (40 mM Tris pH 7.5, 10 mM magnesium chloride, 0.6 mM dithiothreitol) was pre-incubated for 1 h at 30°C. The total Ub concentration was kept at 0.5 mg/ml with the indicated mass percentages of phosphoUb supplemented. The ubiquitination reaction was started by adding 0.1 µM E1 and 3 µM UBE2L3 and samples were taken at the indicated time points by quenching 5 µl of the reaction with 5 µl LDS loading buffer (Invitrogen). Disulfide bridges were reduced by adding 2 µl of 200 mM DTT and prevented from reforming by acetylation with 1 µl of 0.5 M iodoacetamide.
Proteins were separated on NuPAGE 4-12% gradient Bis-Tris gels and Western blotting was performed by transfer on a nitrocellulose membrane and detection using a monoclonal anti-Ub FK2 antibody (Millipore).

**PhosphoUb preparation for NMR analysis**

Isotope labeled Ub was expressed and purified as described previously (Hospenthal et al., 2013). The phosphorylation of double labeled $^{15}$N, $^{13}$C-Ub was monitored in the spectrometer by $^1$H,$^{15}$N 2D BEST-TROSY at room temperature containing 100 µM $^{15}$N, $^{13}$C-Ub, 2.5 µM PhPINK1, 10 mM ATP, 25 µl 20 x reaction buffer (400 mM Tris pH 7.5, 100 mM MgCl$_2$, 6 mM DTT) which was adjusted to 500 µl with NMR buffer (18 mM Na$_2$HPO$_4$, 7 mM NaH$_2$PO$_4$ pH 7.2, 150 mM NaCl). 25 µl D$_2$O was added as an internal reference. After disappearance of the wt Ser65 signal of $^{15}$N, $^{13}$C-Ub, 21 µl of a 500 mM EDTA solution was added to quench the reaction.

For the long range HNCO and $^{15}$N-edited NOESY experiments, 1.7 mM of $^{15}$N, $^{13}$C-Ub was phosphorylated with 17 µM GST-PhPINK1 in a 6 h reaction containing 10 mM ATP, 10 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.6 mM DTT. After 6 h at room temperature the sample was applied to 600 µl equilibrated Glutathione Sepharose 4B beads (GE Healthcare). After 1 h agitation the beads were removed by gravity flow filtration and the flow through was applied to gel filtration (Superdex 75, GE Life Sciences) equilibrated in NMR buffer. Protein containing fractions were pooled and concentrated to 1 mM in a 3-kDa MWCO spin concentrator (Sartorius).

For Lys63 $^{15}$N, $^{13}$C-diUb generation the $^{15}$N, $^{13}$C-Ub was purified on an gel filtration column (Superdex 75, GE Life Sciences) and Lys63 chain assembly initiated in a volume of 1 ml by incubating 1.4 mM $^{15}$N, $^{13}$C-Ub with 2 µM E1, 16 µM UBE2N and 16 µM UBE2V1 in the presence of 10 mM ATP and ligation buffer (40 mM Tris pH 7.5, 10 mM magnesium chloride, 0.6 mM dithiothreitol). After 3 h at 37 °C the reaction was diluted with 50 mM sodium acetate pH 4.5, applied on a MonoS cation exchange column (GE Life Sciences) and eluted with a linear gradient to 50 mM sodium acetate pH 4.5, 1000 mM NaCl. The fractions containing Lys63 $^{15}$N, $^{13}$C-diUb were pooled, concentrated and flash frozen. The phosphorylation of 80 µM Lys63 $^{15}$N, $^{13}$C-diUb was performed with 2.5 µM PhPINK1 as described above and monitored
in $^1$H, $^{15}$N-2D BEST-TROSY experiments. Upon disappearance of the wt Ser65 signal the reaction was quenched by adding 20 µM EDTA. Longer chains (triUb, tetraUb) were purified and phosphorylated identically.

**Solution studies of phosphoUb**

All NMR experiments were performed in phosphate buffered saline (18 mM Na$_2$HPO$_4$, 7 mM NaH$_2$PO$_4$ pH 7.2, 150 mM NaCl).

NMR acquisition was carried out at 298 K on Bruker Avance III 600 MHz and Avance2+ 700 MHz spectrometers equipped with cryogenic triple resonance TCI probes. Topspin (Bruker) and Sparky (Goddard & Kneller, UCSF; [http://www.cgl.ucsf.edu/ home/sparky/](http://www.cgl.ucsf.edu/ home/sparky/)) software packages were used for data processing and analysis, respectively. $^1$H, $^{15}$N 2D BEST-TROSY experiments (Favier & Brutscher, 2011) were acquired with in-house optimized Bruker pulse sequences incorporating a recycling delay of 400 ms and 512*64 complex points in the $^1$H, $^{15}$N dimension, respectively. High quality 2D data sets were acquired in ~8 min.

Backbone chemical shift assignments were completed using Bruker triple resonance pulse sequences. CBCACONH and HNCACB spectra were collected with 1024*32*55 complex points in the $^1$H, $^{15}$N and $^{13}$C dimensions. HNCO and HNCACO experiments were collected with Non Uniform Sampling (NUS) at a rate of 25% of 1024*50*47 complex points in the $^1$H, $^{15}$N and $^{13}$C dimensions, respectively. HA and HB proton shifts were obtained from an HBHACONH spectrum collected with 50% NUS and 512*40*80 in the $^1$H, $^{15}$N and indirect $^1$H dimensions, respectively. These data sets were processed with Multi-Dimensional Decomposition or Compressed Sensing using the MddNMR software package (Orekhover & Jaravine, 2011; Kazimierczuk & Orekhov, 2011).

Weighted chemical shift perturbation calculations were completed using the equation $\sqrt{(\Delta^1H)^2+((\Delta^{15}N)^2)/5}$.

Secondary structure calculations were completed using TALOS+ (Shen *et al*, 2009) incorporating HN, N, CA, CB and HA shifts.

$^{15}$N($^1$H)-heteronuclear NOE (hetNOE) measurements were carried out using a Bruker pseudo 3D pulse program, applying a 120° $^1$H pulse train with a 5 ms interpulse delay for a total of 5 s interleaved on- or off-resonance saturation.
The hetNOE values were calculated from peak intensities according the equation $I_{on}/I_{off}$.

The rate of exchange between the major and minor forms of phosphoUb was established using ZZ exchange spectroscopy. Mixing times of 6, 18, 30, 48, 66, 96, 132, 192, 258, 324, 372, 426 and 492 ms were used in the pseudo 3D data set. Peak intensities of the major and minor forms (auto) and their exchange peaks (cross) of Ile23, Phe45, Ser57 and Leu73 were fitted in Mathematica 9 (Wolfram) using the methods described in (Latham et al, 2009).

Differences in the hydrogen bonding network were established using the long range TROSY-based HNCO (trHNCO) experiment described by (Cordier et al, 2008). A 1 mM sample of $^{15}$N,$^{13}$C phosphoUb was used to collect a 3D version of the long range trHNCO (133 ms N-C' magnetization transfer time) with 128 scans, 25% NUS and 1024*32*55 complex points in the $^1$H, $^{15}$N and $^{13}$C dimensions (~6 days). Cross peaks were assigned with reference to a standard trHNCO data set, with 8 scans and 33 ms N-C' magnetization transfer time, and processed as above.

The altered hydrogen bonding network was further confirmed by analysis of a $^{15}$N-edited 3D NOESY, collected on the 1 mM sample with 30% NUS and 1024*48*128 complex points in the direct $^1$H, $^{15}$N and indirect $^1$H dimensions respectively.

Minimal maps of chemical shift perturbations (CSP) were created to compare the unassigned BEST-TROSY spectra of phosphomimetic $^{15}$N-labelled Ub S65E and wild type Ub. The weighted chemical shift difference was calculated for each assigned peak of wild type Ub and all peaks in the Ub S65E spectrum, using the equation $\sqrt{(\Delta \text{H})^2+((\Delta^{15}\text{N})^2/5)}$. The smallest value was then reported.

**Stability measurements of Ub and phosphoUb**

Differential scanning calorimetry (DSC) was performed using a Microcal Capillary DSC instrument. Samples of Ub and phosphoUb were dialyzed into standard NMR buffer (25 mM phosphate, 150 mM NaCl, pH 7.2) and scanned at a heating rate of 90 °C / hour in mid feedback mode. Sample rescans
indicated significant levels of reversibility for thermal denaturation with > 50% of signal recovered despite heating initial runs to 115 °C. Data were corrected for instrumental baseline using buffer scans recorded immediately before Ub runs. After concentration normalization 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 fit to a non-two state model allowing $T_m$, $\Delta H$ calorimetric and $\Delta H$ van’t Hoff to vary independently.

**Crystallographic analysis of phosphoUb**

PhosphoUb for crystallization was generated by setting up a phosphorylation reaction at room temperature with 10 mg/ml recombinant Ub and 23.3 $\mu$M GST-PhPINK1 in 10 mM ATP, 40 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.6 mM DTT (total volume 250 $\mu$l). After 4 h the reaction was applied to 400 $\mu$l Glutathione Sepharose 4B beads (GE Healthcare) and agitated for 1 h. The flow through was collected and buffer exchanged into water with a PD-10 desalting column (GE Healthcare) and applied on a MonoQ anion exchange column (GE Life Sciences). Pure phosphoUb eluted in 50 mM Tris pH 7.4 and, was concentrated in a 3-kDa MWCO spin concentrator (Sartorius), and crystallized at 3 mg/ml using the vapor diffusion method in sitting drop experiments. Crystals grew in 30% (w/v) PEG 8000, 0.2 M ammonium sulfate, and were vitrified in mother liquor containing 25.5% (w/v) PEG 8000, 0.17 M ammonium sulfate, 15% (v/v) glycerol. Data were collected at the Diamond Light Source (Harwell, UK) beamline I-03. The structure was solved by molecular replacement in Phaser (McCoy et al, 2007), using truncated Ub (pdb-id 1UBQ, residues 1-71) as a search model. Subsequent rounds of model building in coot (Emsley et al, 2010) and refinement in Phenix (Adams et al, 2011) generated a final model with statistics shown in **Supplementary Table 1**. All structure figures were done in PyMol (www.pymol.org) and electrostatics were calculated with CHARMM (http://www.charmm-gui.org).
Ubiquitin chain composition mass spectrometry analysis

Chain assembly reactions were resolved on NuPAGE 4-12% gradient Bis-Tris gel prior to in-gel digestion and the addition of 400 fmoles AQUA peptide standards according to (Kirkpatrick et al, 2006) and (Ordureau et al, 2014). Supplementary Table 1 contains a list of all AQUA peptide standards. Isotopically labeled AQUA peptide standards were synthesized and purchased from Cell Signalling Technology®. Extracted peptides were lyophilized and stored at -80°C. Prior to MS analysis, peptides were resuspended in 30 µl of reconstitution buffer (7.5% ACN, 0.5% TFA, 0.01% H2O2). Oxidation of methionine-containing peptides was performed according to (Phu et al, 2010). 10 µl was directly loaded onto an EASY-Spray reverse-phase column via partial loop injection (C18, 3µm, 100Å, 75µm x 15µm) using a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific). Peptides were eluted using a 25 min ACN gradient (2.5-35%) at a flow rate of 1.4 µl min⁻¹ and flowmeter pressure of ~6,500 psi. Peptides were analyzed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) using parallel reaction monitoring (PRM), similar to (Tsuchiya et al, 2013). For PRM assays, monoisotopic precursor masses were isolated (2 m/z window) and fragmented at predetermined chromatographic retention times. Precursor masses were fragmented using the following settings: resolution, 17,500; AGC target, 1E5; maximum injection time, 120 ms; normalized collision energy, 28. Raw files were searched and fragment ions quantified using Skyline version 2.5.0.6157© (MacLean et al, 2010). The fragment ions used for quantitation are listed in Supplementary Table1. Data generated from Skyline was exported into a Microsoft Excel spread sheet for further analysis according to (Kirkpatrick et al, 2006).

Ubiquitin chain assembly studies

For E2 charging assays, 250 nM E1 were mixed with 4 µM E2 enzymes and 15 µM Ub or phosphoUb in ligation buffer (40 mM Tris pH 7.5, 10 mM magnesium chloride, 0.6 mM dithiothreitol) and incubated at 37 ºC. At indicated timepoints, 9 µl samples were mixed with 9 µl 4xLDS loading buffer (Invitrogen) without reducing agent, and resolved on 4-12% gradient SDS
PAGE gels in MES buffer (Invitrogen). Gels were Coomassie-stained with InstantBlue (Expedeon).

For ligase reactions, 5-10 µM of respective E3 ligases were added to the E2 mixture. Western blotting was performed using rabbit polyclonal anti-Ub antibody (Millipore).

**UBD pull-down assay**

Pull-down assays were essentially performed as previously described (Kulathu *et al.*, 2009). 30 µg of GST-tagged TAB2 NZF was immobilized on 25 µl of Glutathione Sepharose 4B (GE Life Sciences) and washed three times with pull-down assay buffer (PDAB: 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.1 % NP-40). Then, 1.5 µg of the indicated tetraUb species (see section above; Generation of phosphoUb) was incubated with the immobilized TAB2 NZF overnight at 4 ºC in a total volume of 450 µl pull-down assay buffer containing 0.2 mg/ml BSA. The beads were then washed five times with PDAB prior to separation by SDS-PAGE. Proteins were transferred to PVDF and blotted using a polyclonal rabbit anti-Ub antibody (Millipore).

**Disassembly of phosphorylated polyubiquitin**

DUBs were either kind gifts from Marc Pittmann, purchased, or purified according to published procedures (Mevissen *et al.*, 2013). Polyubiquitinated cIAP substrate was generated from a ligase reaction with GST-tagged cIAP1 and UBE2D1, which was stopped with 0.1 U apyrase. 10 µl of this reaction were used in a 30 µl DUB reaction, that contained 3 µl 10 x DUB buffer (500 mM sodium chloride, 500 mM Tris pH 7.5, 50 mM dithiothreitol) and DUBs at indicated concentrations. During incubation at 37 ºC, aliquots of 6 µl of the reaction were taken at the time points indicated and mixed with 6 µl 4 x LDS loading buffer (Invitrogen) to stop the reaction. Samples (10 µl) were resolved by SDS-PAGE as above and silver stained using the Bio-Rad Silver Stain Plus kit according to the manufacturer’s protocol.

DiUb and tetraUb chains were phosphorylated with GST-PhPINK1 and Ser65 phosphorylation efficiency was assessed by either AQUA MS (tetraUbs) or ESI-MS (K6 diUb). GST-PhPINK1 was removed by Glutathione 4B Sepharose beads. Ub chains were diluted in PBS + 5 mM DTT at 1 µM (1.5x, tetraUb) or
3 µM (1.5x, diUb), DUBs were diluted to their respective 3x concentrations in PBS + 5 mM DTT and both solutions were incubated at 37 °C for 10 min prior to start of the reaction. Samples were taken after the indicated time points and quenched by addition 4xLDS loading buffer (Invitrogen), and analyzed by SDS-PAGE and silver staining as described above.

SUPPLEMENTARY REFERENCES


