

WITHDRAWN ARTICLE

## WITHDRAWN: Repurposing FDA Approved Drugs as FXR Agonists: A Structure Based *in silico* Pharmacological Study

*Sandra Jose; Sreevidya S. Devi; Anjana Sajeev; Sosmitha Girisa; Mohammed Alqahtani; Mohamed Abbas; Abdulrahman Alshammari; Gautam Sethi; Ajaikumar Kunnumakkara*

*Biosci Rep* (2022) BSR20212791; <https://doi.org/10.1042/BSR20212791>

The Accepted Manuscript version of this article (published on 29 March 2022) was withdrawn on 07 June 2022 due to concerns over data validity.

# 1        **The retracted conformation of ubiquitin Ser65 phosphorylation** 2        **inhibits the formation of K48-linked ubiquitin chains**

3        **Chang-Li Zhang<sup>1,2,\*</sup>, Xian-Mo Wang<sup>1,2,\*</sup>, Ju Yang<sup>3</sup>, Chen-Qi Xin<sup>1,2</sup>, Wen Fan<sup>1,2</sup> and Hua-Wei Yi<sup>1,2</sup>**

4        <sup>1</sup>The First Affiliated Hospital of Yangtze University, Jingzhou, Hubei Province 434000, China; <sup>2</sup>The First People's  
5        Hospital of Jingzhou, Jingzhou, Hubei Province 434000, China; <sup>3</sup>Innovation Academy for Precision Measurement  
6        Science and Technology of the Chinese Academy of Sciences, Wuhan, Hubei Province 430071, China

7        \*These authors contributed equally to this work.

8        **Correspondence:** Wen Fan (931412421@qq.com), Hua-Wei Yi (202071848@yangtzeu.edu.cn)

## 9        **Abstract**

10        PINK1, as the first reported ubiquitin kinase, can phosphorylate ubiquitin (Ub) at  
11        Ser65 site, which regulates the structure and function of Ub monomer. However, the  
12        levels of PINK1 and phosphorylated Ub (pUb) are very low in normal cells. Here we  
13        show that when proteasome activity is inhibited, the levels of soluble PINK1 (sPINK1)  
14        and pUb will increase significantly. Further we show that ubiquitin phosphorylation can  
15        inhibit the formation of K48-linked ubiquitin chains in vivo and in vitro, and the  
16        retracted state of pUb plays a leading role in the inhibition process. Ubiquitination is a  
17        necessary process for substrates degradation. Thus, phosphorylation can regulate  
18        proteasomal degradation of substrates.

## 19        **Introduction**

20        PINK1 is the earliest discovered and most widely studied ubiquitin kinase. It  
21        anchors to the mitochondrial outer membrane through the N-terminal transmembrane  
22        region [1, 2]. PINK1 can phosphorylate ubiquitin (Ub) at Ser65 site [1]. The resulting  
23        phosphorylated ubiquitin (pUb) can be recognized by Parkin, an E3 ubiquitin ligase.  
24        The pUb will regulate the conformation of Parkin, thus activates its E3 ubiquitin ligase  
25        activity and ubiquitinates mitochondrial outer membrane (MOM) proteins. Further  
26        recruits mitophagy adaptors and initiates clearance of the damaged mitochondria [3-5].  
27        PINK1 is the substrate of 26 S proteasome and can be degraded by ubiquitin proteasome  
28        system (UPS) through N-end rule pathway, so the levels of PINK1 and pUb are very

low in cells [6].

Phosphorylated ubiquitin at Ser65 site has two conformations: relaxed and retracted in solution, and the relaxed state is similar to wild-type Ub [2]. Compared with the relaxed state, the 5th  $\beta$ -sheet of the retracted state moves two amino acid residues to the N-terminus [7, 8]. Gladkova C et al found that point mutations, such as the L67S, can disrupt the original hydrophobic interaction and shift the position of the 5th  $\beta$ -sheet, thus converting the conformation of Ub to the retracted state [9]. Interestingly, the ratio of the relaxed to retracted state of pUb can be regulated by solution pH [10].

Phosphorylation can regulate the tertiary structure of ubiquitin monomer, which will undoubtedly increase the complexity of ubiquitin system and remodel the function of ubiquitin monomer and ubiquitin chains [11]. Lys48-linked ubiquitin chains that mainly mediating substrates degradation can also be affected by ubiquitin Ser65 phosphorylation. Previous studies have shown that ubiquitin phosphorylation can inhibit the substrates degradation in cells [2]. Deubiquitinating enzymes (DUBs) can eliminate the ubiquitination of substrates and regulate the proteasomal degradation. Wauer T et al found that phosphorylation could affect the activities of a variety of DUBs [12]. Ubiquitination is a necessary condition for proteasomal degradation of substrates. However, there is a lack of detailed research on the regulation of ubiquitination and formation of ubiquitin chains by phosphorylation.

PINK1 and pUb has been shown to play a cytoprotective role in the mitochondrial autophagy pathway [3, 13, 14]. However, there are few reports on the role of PINK1 and pUb in other aspects. Here, we focus on regulation of proteins proteasomal degradation by Ser65 phosphorylation from the aspect of ubiquitin chains formation. We want to characterize how phosphorylation regulates the formation of K48-linked ubiquitin chains and the role of the newly generated retracted conformation.

## Material and methods

### Protein sample preparation

The sequence of human Ub was cloned into pET11a vector, and the relevant Ub mutants were obtained by quick-change method. PINK1 derived from body Louse (*Pediculus humanus corporis*) has been reported to phosphorylate ubiquitin at Ser65 site [14]. The *Ph*PINK1 (residues 115-575) gene sequence was cloned into pGEX-4T-1 vector, with a GST tag and TEV restriction site at the N-terminal. Human E1 (Uba1) was cloned into pGEX-4T-1 vector, with a GST tag and Thrombin restriction site at the N-terminal. Ubiquitin binding enzyme E2 (Ube2K) was cloned into pET28a vector, with a 6xHis tag and Thrombin restriction site at the N-terminal.

All proteins were expressed in the *E. coli* BL21 strain. Ubiquitin and its mutants were purified on Sepharose SP and Sephacryl S100. Uba1 was purified from GST column and Sephacryl S100, and Ube2K was purified from His-affinity column and Superdex-75. For the preparation of *Ph*PINK1 and pUb, the reported methods can be directly referred to [14].

### Cell culture and treatments

HEK293 cells were cultured in DMEM supplemented with 10% FBS and at 37°C in humidified conditions with 5% CO<sub>2</sub>. Cell transfections were performed by using Hieff Trans™ Liposomal Transfection Reagent (Yeasten Biotech Co., Ltd., Shanghai, China, 40802ES02) according to manufacturer's protocol and MG132 (Topsience, Shanghai, China, T2154) was used at concentration of 5 μM for 24 h.

### Antibodies

Primary antibodies used were against PINK1 (6946, Cell Signaling Technology, 1:800), pUb (ABS1513-I, Millipore, 1:1000), GAPDH (60004-1-Ig, Wuhan San-Ying Proteintech Group, 1:3000), Flag (80010-1-RR, Wuhan San-Ying Proteintech Group, 1:500).

### Western blot assays

The HEK293 cell lysates were prepared with RIPA buffer (Beyotime Biotechnology, Shanghai, China, catalog number P0013B). The supernatant samples

83 (~50 µg) were used for western blotting, the assays have been performed as described  
84 earlier [15]. The antibody information was as described above.

### 85 **Flag pull-down assay**

86 HEK293 cells were harvested after co-transfected with PRK5/flag-Ub48K (all Lys  
87 on Ubiquitin were mutated to Arg except Lys48) and pcDNA3.1/sPINK1, or after  
88 transfected with PRK5 or with pcDNA3.1 null vector. The supernatant samples after  
89 lysed with sonication were incubated with anti-flag M2 agarose beads (Millipore,  
90 M8823) for 1 h, the beads were then washed and the bound proteins were eluted by  
91 buffer containing flag peptides followed by western blot.

### 92 **In vitro ubiquitin-chain synthesis assay**

93 In vitro ubiquitin-chain synthesis assay was carried out as previously described  
94 [15]. To synthesize Lys48-linked ubiquitin chains, add 2.5 µM human E1 (Uba1), 20  
95 µM ubiquitin binding enzyme E2 (Ube2K), 2.5 mM ATP, 5 mM MgCl<sub>2</sub>, 0.5 mM Ub (or  
96 pUb, Ub mutant), and ATP regeneration system into the reaction buffer (20 mM Tris,  
97 1mM DTT, pH8.0). The ATP regeneration system includes 10 mM creatine phosphate  
98 (Sigma-Aldrich, 27920), 2 U/ml inorganic pyrophosphatase (Sigma-Aldrich, I1643),  
99 and 1U/ml creatine phosphokinase (Sigma-Aldrich, C3755). The reaction was  
100 incubated at 30 °C for ~10 h, the prepared Lys48-linked ubiquitin chains can be detected  
101 by SDS-PAGE.

## 102 **Results**

### 103 **The impairment of proteasomal activity promotes sPINK1 and pUb level**

104 PINK1 is anchored to the outer membrane of mitochondria through the N-terminal  
105 transmembrane region. The PARL, a protease on the inner mitochondrial membrane,  
106 can cleave PINK1 to generate soluble PINK1 (sPINK1). sPINK1 is a proteasomal  
107 substrate, and it can be degraded by the ubiquitin-proteasome system through the N-  
108 terminal degradation pathway (Figure 1A). Therefore, the levels of PINK1 and pUb are  
109 very low in normal cells [6].

110 We treated HEK293 cells with proteasome inhibitor MG132 [16], and then  
111 detected the levels of PINK1 (Figure 1B) and pUb (Figure 1C) in soluble components  
112 by western blot. The sPINK1 and pUb levels were hardly detected in the control group.  
113 After treated with 5  $\mu$ M MG132, the levels of sPINK1 and pUb increased significantly.  
114 Meanwhile, the content of full-length PINK1 was almost unchanged. To directly  
115 investigate the relationship between sPINK1 and pUb levels, we transfected sPINK1 to  
116 HEK293 cells and monitored the PINK1 and pUb levels by western blot (Figure 2A),  
117 we found that overexpression of sPINK1 could significantly increase the levels of pUb.

### 118 **Phosphorylation impedes the formation of K48-linked ubiquitin chains**

119 To detect the levels of K48-linked ubiquitin chains in cells, flag-Ub48K was  
120 overexpressed in wild-type (WT) and PINK1 knock out (KO) HEK293 cells. The  
121 Ub48K means all Lys were mutated to Arg except Lys48. Thus, only K48-linked  
122 ubiquitin chains could be formed. All the HEK293 cells were treated with MG132 to  
123 inhibit the degradation of K48-linked ubiquitin chains by 26S Proteasome. The levels  
124 of K48-linked ubiquitin chains were detected by western blot (anti-flag). As shown in  
125 Figure 2B on the left, large amounts of K48-linked ubiquitin chains could be detected  
126 in both wild-type and PINK1 KO HEK293 cells. There is no significant difference in  
127 amounts of ubiquitin chains in the two lanes, possibly because the levels of PINK1 and  
128 pUb are extremely low in HEK293 cells. Further, we also co-transfected flag-Ub48K  
129 and full-length PINK1 or sPINK1 into HEK293 cells. After overexpression of PINK1  
130 or sPINK1, the levels of K48-linked ubiquitin chains in both lanes decreased  
131 significantly as shown in Figure 2B on the right. It should be noted that the Ser65 site  
132 of flag-Ub48K could be phosphorylated in cells, as shown in the pull-down assay  
133 (Figure 2C).

134 We further studied the relationship between pUb levels and the formation of K48-  
135 linked ubiquitin chains in vitro. Using *Ph*PINK1 kinase, we successfully prepared  
136 Ser65 phosphorylated Ub (pUb) in vitro. The Mass Spectrometry results were shown  
137 in figure 2D. In addition, we also detected the pUb by Western blot with phospho-

Ubiquitin (Ser65) antibody (data not shown). Ubiquitin activating enzyme E1 (Uba1) and ubiquitin binding enzyme E2 (Ube2K) were used as the main enzymes for ubiquitin chains synthesis [17], and wild-type Ub or pUb was added as raw materials to synthesize K48-linked ubiquitin chains, and the synthesis process can be detected by SDS-PAGE, as shown in Figure 2E. When Ub was added into the ubiquitin chain synthesis system, a large number of di-Ub and poly-Ub could be detected. For pUb, however, only a small fraction di-Ub could be detected, and most of them still existed in the form of pUb monomer. The above results have shown that phosphorylation at ubiquitin Ser65 site inhibits the formation of K48-linked ubiquitin chains.

### **The retracted state plays a leading role in the inhibition process**

Phosphorylation at the Ser65 site makes ubiquitin to have two stable conformations in solution, namely the relaxed and the retracted state [10]. Gladkova C et al found that L67S mutation could destroy the original hydrophobic interaction and transfer the structure almost entirely into the retracted state [9]. Thus, the Ub/S65E was used to simulate the relaxed state of pUb, while Ub/L67S and Ub/L67S/S65E were used to simulate the retracted state of pUb. In addition, Ub/S65A mutant was used as control. Then, we investigated the effects of these ubiquitin mutants on the formation of K48-linked ubiquitin chains, as shown in Figure 3A. Ub/S65A and Ub/S65E, like wild-type Ub, could efficiently synthesize ubiquitin chains of different lengths, and the S65E mutation cannot simulate pUb as previously reported [7]. However, for Ub/L67S and Ub/L67S/S65E, their SDS-PAGE bands were similar to that of pUb. Meanwhile, we have achieved the same results in synthesis of Lys48-linked diubiquitin (K48-diUb) as shown in Figure 3B. These results have shown that the retracted conformation of pUb is the main reason for inhibiting the formation of K48-linked ubiquitin chains.

Next, we resort to protein complex structures. Lv Z et al have analyzed the complex structure of Uba1/Ub [19], as shown in Figure 4A. The retracted state of pUb (pdb: 5xk4, marked in yellow cartoon) is superimposed onto the Ub (marked in green cartoon) in the complex. The key residues interacting with Ub are shown as purple sticks, and the 5th  $\beta$ -sheet (marked in red) is just on the interface. The 5th  $\beta$ -sheet on

167 the retracted conformation of pUb moves two amino acid residues to the N-terminus,  
168 and the amino acid residues on the  $\beta$ -sheet are different from Ub in the complex, which  
169 could destroy the interaction between Ub and Uba1. In addition, the C terminal of Ub  
170 (indicated by a purple arrow) is close to the active site of Uba1[20], and the retracted  
171 state could change its position to inhibit Uba1 activation. The complex structure of  
172 Ube2K/K48-diUb [19] is shown in Figure 4B. The 5th  $\beta$ -sheet of proximal subunit is  
173 also on the interface, and the Ser65 site of the two subunits are far away from the  
174 interface. To sum up, the retracted state shifts the 5th  $\beta$ -sheet to the N terminal,  
175 destroying the interface between Ub and Uba1 or Ube2K, thereby inhibiting the  
176 synthesis of the K48-linked ubiquitin chains.

### 177 **3.4 Phosphorylation inhibits proteasomal degradation of substrates**

178 Ubiquitination is the prerequisite for proteasomal degradation of substrates. The  
179 substrates to be degraded need to be connected with ubiquitin chains (mainly K48-  
180 linked ubiquitin chains) of different lengths through the E1~E2~E3 enzymatic cascade.  
181 The ubiquitin receptors on 26 S proteasome can recruit the substrates through the K48-  
182 linked ubiquitin chains, thereby degrading the substrates through the ubiquitin-  
183 proteasome system [20-22], as shown in Figure 5. Under the action of ubiquitin kinase  
184 PINK1, the level of pUb will increase in cells [6], and proportion of the retracted state  
185 is about 50% under physiological pH condition [10]. The retracted state could inhibit  
186 ubiquitination of substrates and the formation of K48-linked ubiquitin chains, so as to  
187 inhibit the degradation of substrates by 26 S proteasome.

### 188 **Discussion**

189 PINK1, as a widely reported ubiquitin kinase, can phosphorylate ubiquitin  
190 monomer and poly-Ub chains at Ser65 site [12]. Phosphorylation regulates the structure  
191 of the Ub monomer and affects the deubiquitinating enzymes (DUBs) activity have  
192 been well characterized [12, 23]. However, there is little research on how  
193 phosphorylation regulates the formation of poly-Ub chains and the ubiquitin dependent  
194 proteasomal degradation of substrates. Here, we found that ubiquitin phosphorylation  
195



196 inhibits the formation of K48-linked ubiquitin chains, thus hindering the proteasomal  
197 degradation of substrates. Moreover, we further found that the retracted state of pUb  
198 played a leading role in inhibiting the formation of K48-linked ubiquitin chains.

199 In normal cells, the levels of PINK1 and pUb are very low. However, when cells  
200 aging or under certain physiological conditions, the levels of pUb will increase  
201 significantly [24-26]. The phosphorylation of ubiquitin at Ser65 site can regulate the  
202 structure of Ub monomer to have two stable conformations in the solution, namely the  
203 relaxed and retracted state. The regulation of tertiary structure will further impacts the  
204 quaternary arrangements of ubiquitin subunits in poly-Ub chains [10]. Besides,  
205 ubiquitin phosphorylation inhibits the activities of many enzymes responsible for  
206 attaching and removing poly-Ub chains, thus remodeling the ubiquitin system [11]. A  
207 recent study has demonstrated that pUb can affect discharging of E2 enzymes to form  
208 poly-Ub chains [12, 23]. In this study, we found that phosphorylation could inhibit the  
209 formation of K48-linked ubiquitin chains *in vivo* and *in vitro*. Furthermore, we  
210 simulated the retracted state by L67S/S65E mutant and found that the retracted state  
211 played a leading role in inhibiting the formation of K48-linked ubiquitin chains.

212 At present, the reports on the biological function of pUb mainly focus on the  
213 activation of parkin by pUb and the initiation of mitochondrial autophagy pathway [13,  
214 27-29]. However, Swaney DL et al reported that ubiquitin phosphorylation at Ser65 site  
215 could cause a dramatic accumulation of ubiquitylated proteins in cells [2]. In this study,  
216 we found that phosphorylation could inhibit the synthesis of K48-linked ubiquitin  
217 chains, thus hindering the proteasomal degradation of substrates. The retracted  
218 conformation of pUb plays a leading role in inhibiting the formation of K48-linked  
219 ubiquitin chains. Phosphorylation can regulate proteasomal degradation in many  
220 aspects, such as regulate the structure of poly-Ub chains, affect the degradation activity  
221 of DUBs, and regulate the recognition of ubiquitin chains by shuttle factors or ubiquitin  
222 receptors, which need to be further studied.

## 223 Conclusion

Our study elucidates the retracted conformation of pUb as one of the mediators responsible for inhibiting the formation of K48-linked ubiquitin chains, which opens a new window for modulating proteasomal activity.

## Data Availability

All data are available upon request

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Funding

This work was supported by Natural Science Foundation of Hubei Province of China [grant number 2021CFB261 (to Hua-Wei Yi)] and the Yangtze University Medical Innovation Fund [grant number 2022MIF08 (to Hua-Wei Yi)].

## CRedit Author Contribution

**Chang-Li Zhang:** Resources, Supervision, Validation, Methodology. **Xian-Mo Wang:** Data curation, Project administration, Writing-original draft. **Ju Yang:** Formal analysis, Visualization. **Chen-Qi Xin:** Writing-review and editing. **Wen Fan:** Conceptualization, Resources, Supervision. **Hua-Wei Yi:** Data curation, Funding acquisition, Methodology, Project administration, Writing-review & editing.

## Acknowledgments

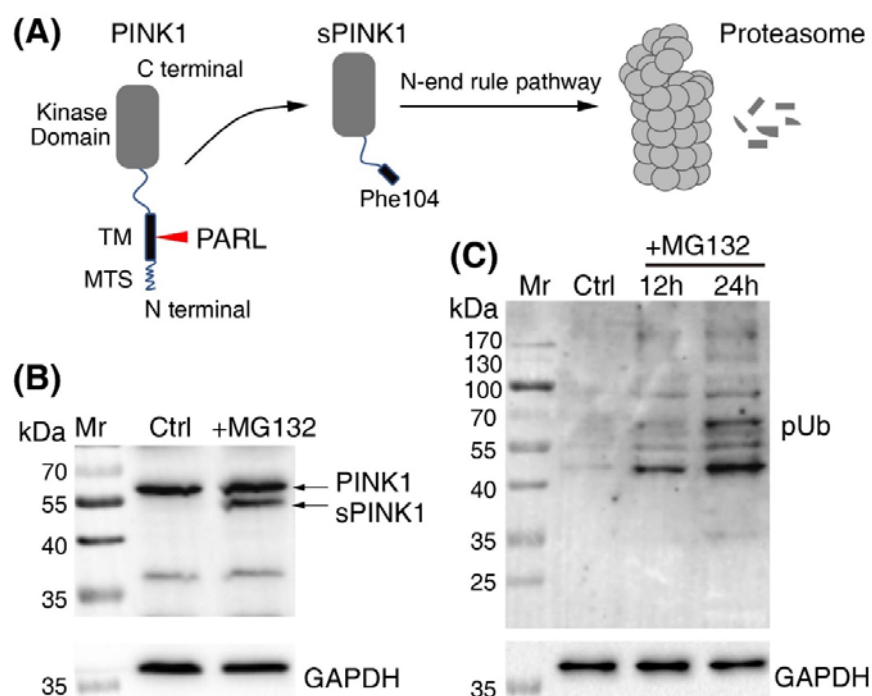
Our sincere thanks to Prof. Chun Tang, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China, for his help with the original experimental design.

## References

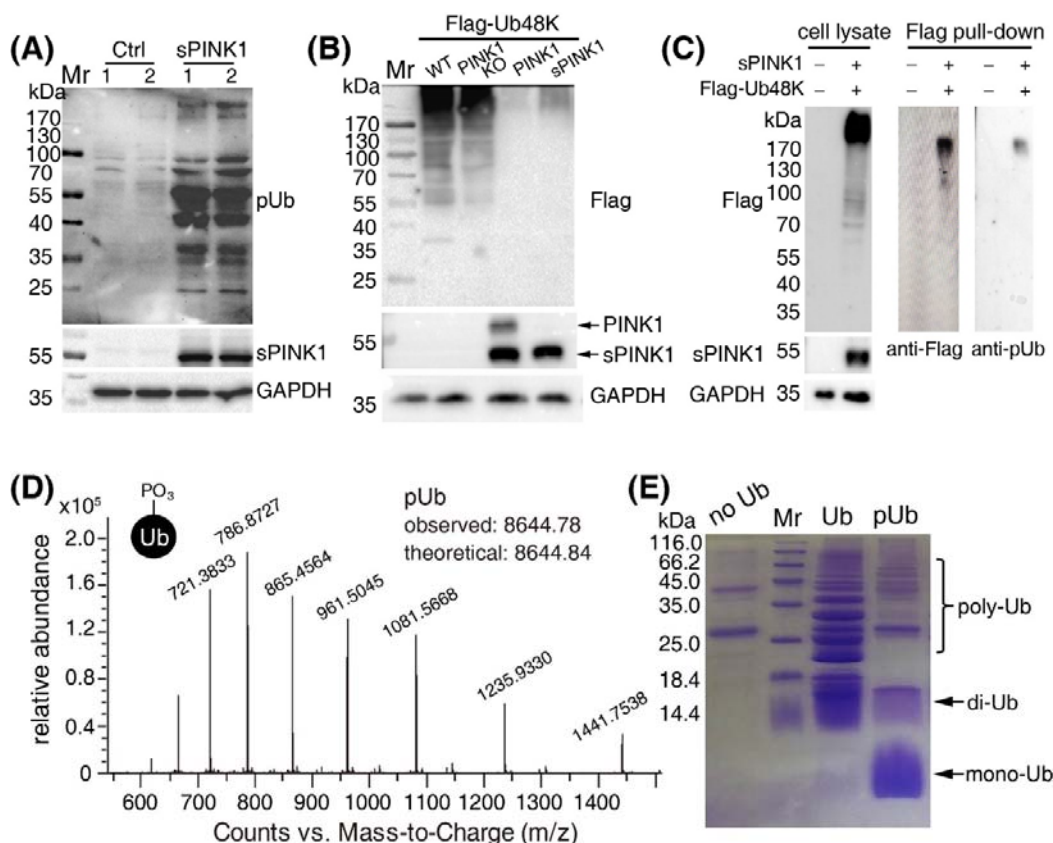
- Schubert, A.F., Gladkova, C., Pardon, E., Wagstaff, J.L., Freund, S., Steyaert, J. et al. (2017) Structure of PINK1 in complex with its substrate ubiquitin. *Nature*. **552**, 51-56, <https://doi.org/10.1038/nature24645>
- Swaney, D.L., Rodríguez-Mias, R.A. and Villén, J. (2015) Phosphorylation of ubiquitin at Ser65 affects its polymerization, targets, and proteome-wide turnover. *Embo Rep*. **16**, 1131-1144,

- <https://doi.org/10.15252/embr.201540298>
- 3 Kumar, A., Tamjar, J., Waddell, A.D., Woodroof, H.I. and Aalten, D.M.V. (2017) Structure of PINK1 and mechanisms of parkinson's disease-associated mutations. *eLife*. **6**, e29985, <https://doi.org/10.7554/eLife.29985>
  - 4 Aguirre, J.D., Dunkerley, K.M., Mercier, P. and Shaw, G.S. (2017) Structure of phosphorylated Ubl domain and insights into PINK1-orchestrated parkin activation. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 298-303, <https://doi.org/10.1073/pnas.1613040114>
  - 5 Trempe, J.F., Sauvé, V., Grenier, K., Seirafi, M., Tang, M.Y., Ménade, M. et al. (2013) Structure of parkin reveals mechanisms for ubiquitin ligase activation. *Science*. **340**, 1451-1455, <https://doi.org/10.1126/science.1237908>
  - 6 Eldeeb, M.A. and Ragheb, M.A. (2020) N-degron-mediated degradation and regulation of mitochondrial PINK1 kinase. *Curr Genet*. **6**, 693-701, <https://doi.org/10.1007/s00294-020-01062-2>
  - 7 Kazansky, Y., Lai, M.Y., Singh, R.K. and Fushman, D. (2018) Impact of different ionization states of phosphorylated serine-65 on ubiquitin structure and interactions. *Sci Rep*. **8**, 2651-2662, <https://doi.org/10.1038/s41598-018-20860-w>
  - 8 Ye, S.X., Gong, Z., Yang, J., An, Y.X., Liu, Z., Zhao, Q. et al. (2021) Ubiquitin is double-phosphorylated by PINK1 for enhanced pH-sensitivity of conformational switch. *Protein & cell*. **10**, 908-913, <https://doi.org/10.1007/s13238-019-0644-x>
  - 9 Gladkova, C., Schubert, A.F., Wagstaff, J.L., Pruneda, J.N., Freund, S. and Komander, D. (2017) An invisible ubiquitin conformation is required for efficient phosphorylation by PINK1. *Embo J*. **36**, 3555-3572, <https://doi.org/10.15252/emboj.201797876>
  - 10 Dong, X., Gong, Z., Lu, Y.B., Liu, K., Qin, L.Y., Ran, M.L., Zhang, C.L., Liu, Z., Zhang, W.P. and Tang, C. (2017) Ubiquitin S65 phosphorylation engenders a pH-sensitive conformational switch. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 6770-6775, <https://doi.org/10.1073/pnas.1705718114>
  - 11 Tang, C. and Zhang, W.P. (2019) How phosphorylation by PINK1 remodels the ubiquitin system: A perspective from structure and dynamics. *Biochemistry*. **59**, 26-33, <https://doi.org/10.1021/acs.biochem.9b00715>
  - 12 Wauer, T., Swatek, K.N., Wagstaff, J.L., Gladkova, C., Pruneda, J.N., Michel, M.A., Gersch, M., Johnson, C.M. et al. (2015) Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis. *Embo J*. **34**, 307-325, <https://doi.org/10.15252/emboj.201489847>
  - 13 Wauer, T., Simicek, M., Schubert, A., Komander, D. (2015) Mechanism of phospho-ubiquitin-induced PARKIN activation. *Nature*. **524**, 370-374, <https://doi.org/10.1038/nature14879>
  - 14 Schubert, A., Gladkova, C., Pardon, E. et al. (2017) Structure of PINK1 in complex with its substrate ubiquitin. *Nature*. **552**, 51-56, <https://doi.org/10.1038/nature24645>
  - 15 Liu, Z., Dong, X., Yi, H.W., Yang, J., Gong, Z., Wang, Y., Liu, K., Zhang, W.P. and Tang, C. (2019) Structural basis for the recognition of K48-linked Ub chain by proteasomal receptor Rpn13. *Cell Discov*. **5**, 19-33, <https://doi.org/10.1038/s41421-019-0089-7>
  - 16 Yamano, K. and Youle, R.J. (2013) PINK1 is degraded through the N-end rule pathway. *Autophagy*. **9**, 1758-1769, <https://doi.org/10.4161/auto.24633>
  - 17 Pickart, C.M. and Raasi, S. (2005) Controlled synthesis of polyubiquitin chains. *Method Enzymol*. **399**, 21-36, [https://doi.org/10.1016/S0076-6879\(05\)99002-2](https://doi.org/10.1016/S0076-6879(05)99002-2)
  - 18 Dikic, I., Wakatsuki, S., Walters, K.J. (2009) Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol*. **10**, 659-671, <https://doi.org/10.1038/nrm2767>
  - 19 Middleton, A.J. and Day, C.L. (2015) The molecular basis of lysine 48 ubiquitin chain synthesis by

- 295 Ube2K. *Sci Rep.* **5**, 16793-16806, <https://doi.org/10.1038/srep16793>
- 296 20 Lu, Y., Lee, B.H., King, R.W., Finley, D., Kirschner, M.W. (2015) Substrate degradation by the  
 297 proteasome: a single-molecule kinetic analysis. *Science*. **348**, 1250834-1250853,  
 298 <https://doi.org/10.1126/science.1250834>
- 299 21 Bard, J.A.M., Bashore, C., Dong, K.C. and Martin, A. (2019) The 26S proteasome utilizes a kinetic  
 300 gateway to prioritize substrate degradation. *Cell*. **177**, 286-298,  
 301 <https://doi.org/10.1016/j.cell.2019.02.031>
- 302 22 Dong, Y., Zhang, S., Wu, Z., Li, X., Wang, W.L., Zhu, Y. et al. (2019) Cryo-EM structures and  
 303 dynamics of substrate-engaged human 26S proteasome. *Nature*. **565**, 49-55,  
 304 <https://doi.org/10.1038/s41586-018-0736-4>
- 305 23 Huguenin-Dezot, N., Cesare, V.D., Peltier, J., Knebel, A., Kristaryianto, Y.A., Rogerson, D.T. et al.  
 306 (2016) Synthesis of isomeric phosphoubiquitin chains reveals that phosphorylation controls  
 307 deubiquitinase activity and specificity. *Cell Rep.* **16**, 1180-1193,  
 308 <https://doi.org/10.1016/j.celrep.2016.06.064>
- 309 24 Xu, H., Fiesel, F.C., Dominika, T., Monica, C.C., Wen-Lang, L., Soto, A.I. et al. (2018) Age- and  
 310 disease-dependent increase of the mitophagy marker phospho-ubiquitin in normal aging and Lewy  
 311 body disease. *Autophagy*. **14**, 1404-1418, <https://doi.org/10.1080/15548627.2018.1461294>
- 312 25 Fiesel, F.C., Ando, M., Hudec, R., Hill, A.R., Castanedes-Casey, M., Caulfield, T.R. et al. (2015)  
 313 (Patho-)physiological relevance of PINK1-dependent ubiquitin phosphorylation. *Embo Rep.* **16**,  
 314 1114-1130, <https://doi.org/10.15252/embr.201540514>
- 315 26 Ordureau, A., Sarraf, S., Duda, D., Heo, J.M., Jedrychowski, M., Sviderskiy, V. et al. (2014)  
 316 Quantitative proteomics reveal a feedforward mechanism for mitochondrial parkin translocation  
 317 and ubiquitin chain synthesis. *Mol Cell*. **56**, 462-462, <https://doi.org/10.1016/j.molcel.2014.10.006>
- 318 27 Walden, H. and Muqit, M. (2017) Ubiquitin and parkinson's disease through the looking glass of  
 319 genetics. *Biochem J.* **474**, 1439-1451, <https://doi.org/10.1042/BCJ20160498>
- 320 28 Kumar, A., Aguirre, J.D., Condos, T.E., Martinez-Torres, R.J., Chaugule, V.K., Toth, R. et al. (2015)  
 321 Disruption of the autoinhibited state primes the E3 ligase parkin for activation and catalysis. *Embo*  
 322 *J.* **34**, 2506-2521, <https://doi.org/10.15252/emboj.201592337>
- 323 29 Gladkova, C., Maslen, S.L., Skehel, J.M. and Komander, D. (2018) Mechanism of Parkin activation  
 324 by PINK1. *Nature*. **559**, 410-414, <https://doi.org/10.1038/s41586-018-0224-x>



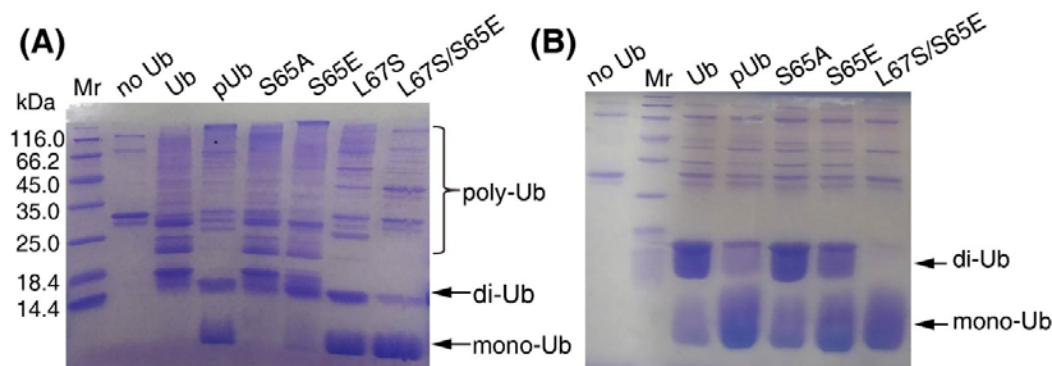
**Figure 1. Inhibition activity of proteasome leads to increase of sPINK1 and pUb levels**  
**(A)** A schematic for PINK1 degradation by 26S proteasome through N-end rule pathway. **(B)** MG132 treatment increases the level of sPINK1. Images of western blot analysis with anti-PINK1 and anti-GAPDH antibodies for control and MG132 treatment (5 μM for 24 h) of HEK293 cells, and the GAPDH was used as an internal reference. **(C)** MG132 treatment increases the level of pUb. Images of western blot analysis with anti-pUb and anti-GAPDH antibodies for control and MG132 treatment (5 μM for 12-24 h) of HEK293 cells.



**Figure 2. Ubiquitin phosphorylation impedes the formation of K48-linked ubiquitin chains**

(A) Representative images of western blot analysis with anti-pUb and anti-PINK1 antibodies for HEK293 supernatant samples. The HEK293 cells were transfected with pcDNA3.1/sPINK1 or pcDNA3.1 null vector. There are two experimental groups in the figure. (B) Representative images of western blot analysis with anti-flag and anti-PINK1 antibodies for HEK293 cells. The HEK293 or PINK1 knock out (KO) cells were co-transfected with PRK5/flag-Ub48K (all Lys on Ubiquitin were mutated to Arg except Lys48) and pcDNA3.1/PINK1, pcDNA3.1/sPINK1, or pcDNA3.1 null vector, and the cells were treated with 5  $\mu$ M proteasome inhibitor MG132 for 24 h. (C) Representative images of western blot analysis with anti-flag and anti-pUb antibodies for HEK293 cell lysate. The HEK293 cells were co-transfected with PRK5/flag-Ub48K and pcDNA3.1/sPINK1, or transfected with PRK5 or pcDNA3.1 null vector. The anti-flag M2 agarose beads were used for pull-down assay. (D) Representative Mass Spectrum of pUb. The observed molecular weight was the same as theoretical results. (E) Representative SDS-PAGE images for detecting the formation of K48-linked ubiquitin chains. Wild-type Ub and pUb were added into the synthesis system of K48-linked ubiquitin chains.

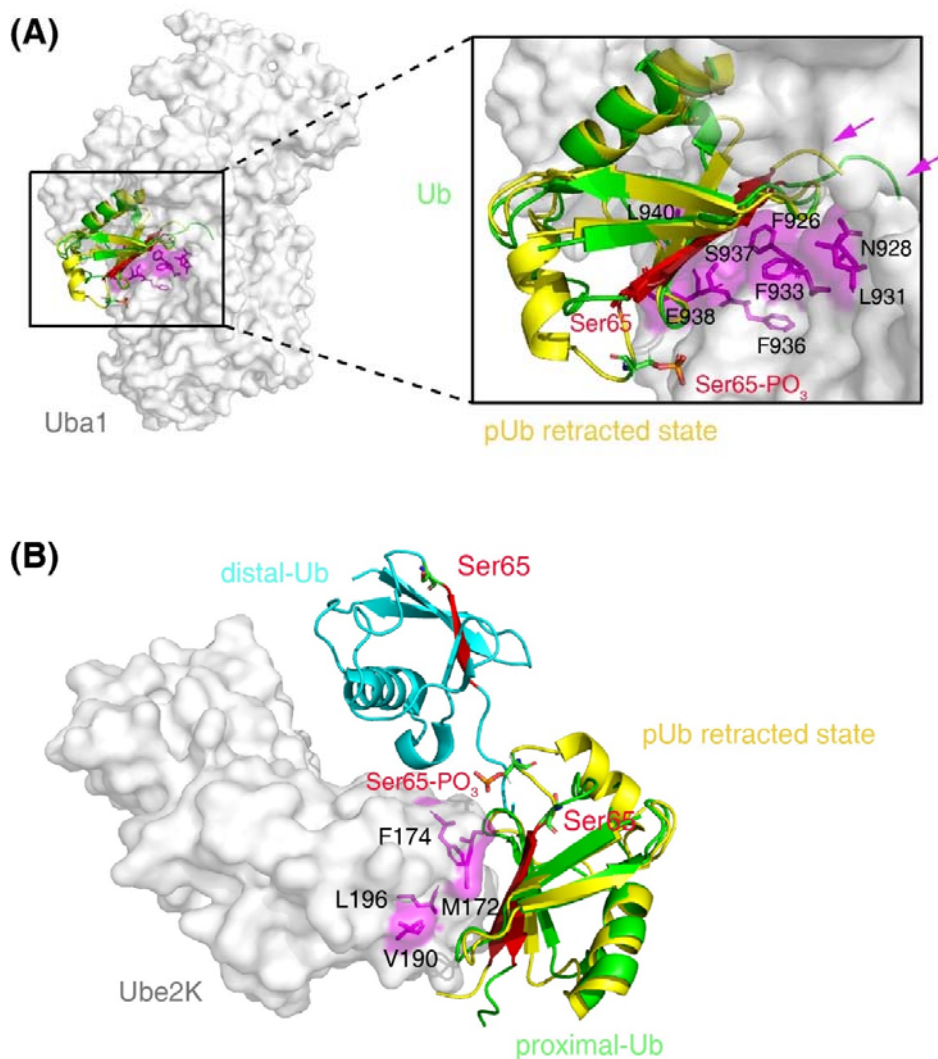




**Figure 3. The retracted conformation inhibits the formation of ubiquitin chains**

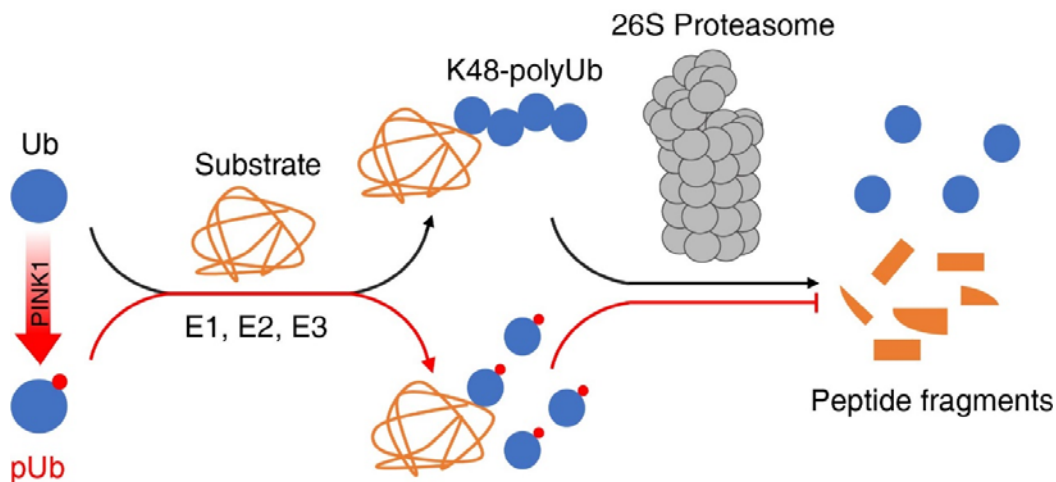
(A) Representative SDS-PAGE images for ubiquitin chains synthesis ability of Ub, Ub mutant and pUb. The wild-type Ub, Ub mutant and pUb were added into the synthesis system of K48-linked ubiquitin chains, and there were no Ub or Ub mutant in the first lane. (B) Representative SDS-PAGE images for K48-diUb synthesis ability. To ensure a single final product of diubiquitin, K48R mutation was introduced to the distal Ub and 77D was introduced to the proximal Ub.

WITHDRAWN



**Figure 4. The retracted conformation destroys the binding interface between Ub and E1/E2**  
(A) Complex structure of Ub monomer and Uba1. pdb: 6DC6. The Ser65 of Ub is represented as sticks, and the 5th  $\beta$ -sheet is marked in red. Residuals located on the interface and closer to the 5th  $\beta$ -sheet are represented by purple sticks. (B) Complex structure of K48-diUb and Ube2K. pdb: 6IF1. The distal and proximal subunit of K48-diUb are represented in cyan and green, respectively. The 5th  $\beta$ -sheet of each subunit is marked in red. Residuals located on the interface and closer to the 5th  $\beta$ -sheet are represented by purple sticks. In addition, the retracted state of pUb (yellow, pdb: 5xk4) is superimposed onto the Ub in the complex (green)





**Figure 5. A schematic for phosphorylation inhibits proteasomal degradation of substrates**

Under the sequential action of E1, E2 and E3, the substrates can be modified by ubiquitination and degraded through ubiquitin proteasome system. Phosphorylation inhibits the formation of K48-linked ubiquitin chains and hinders the ubiquitination of substrates, thus hindering the proteasomal degradation of substrates.

WITHDRAWN