Expanded View Figures

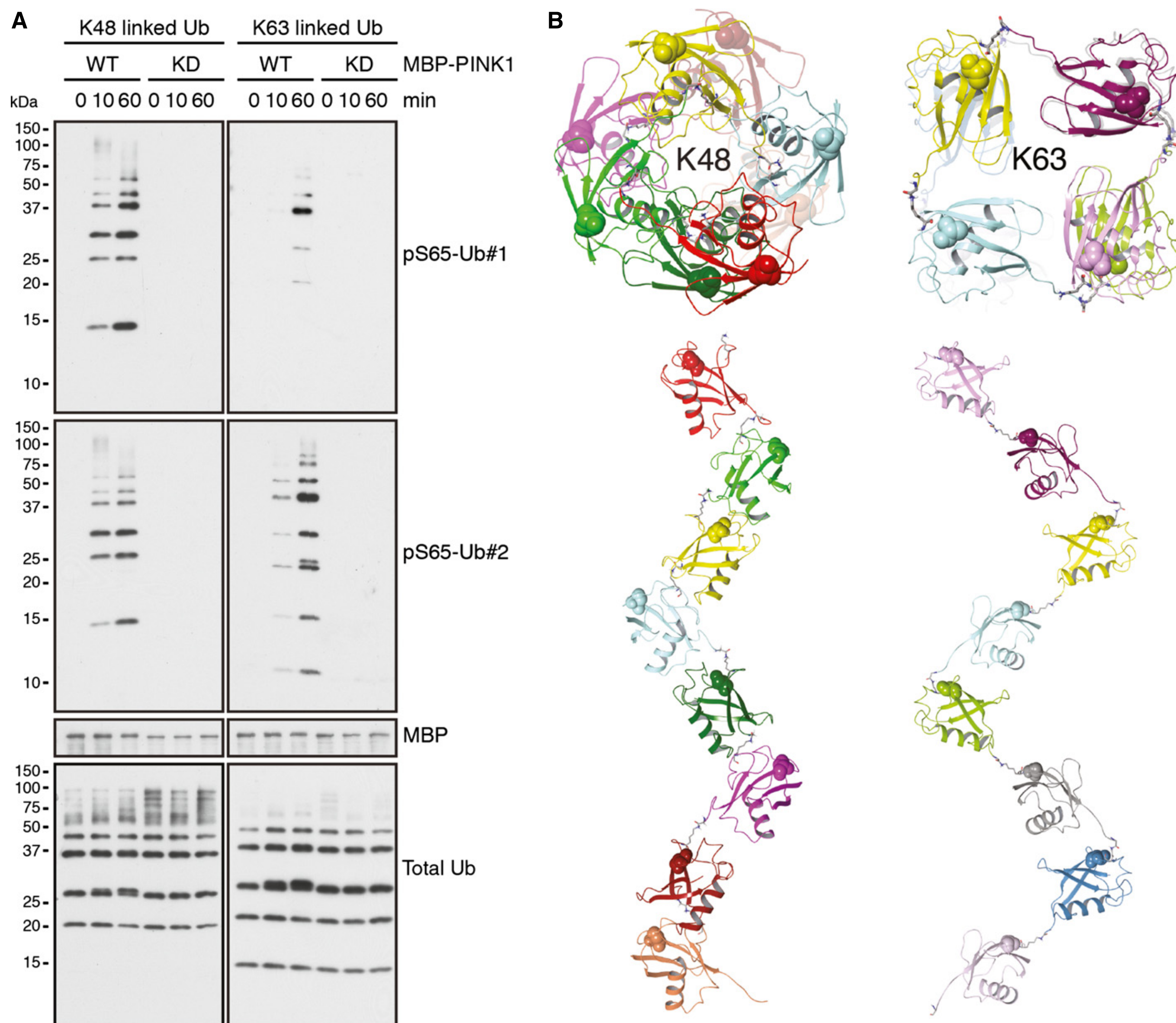

Figure EV1. anti-pS65-Ub antibodies detect PINK1-phosphorylated poly-Ub chains.

A Untagged K48- and K63-linked poly-Ub chains (n = 2–7) were incubated with recombinant MBP-PINK1 WT or KD for the indicated times *in vitro* prior to WB. Both, pS65-Ub#1 and #2 similarly recognized phosphorylated K48-linked poly-Ub chains, whereas pS65-Ub#2 appeared to react stronger with phosphorylated K63-linked Ub conjugates. Blots were probed with MBP and total Ub antibodies to show equal loading.

B Axial and lateral views of a K48-linked or K63-linked (8-mers) poly-Ub chains show the different topologies. In the more closed and compacted structure of K48-linked poly-Ub chains, S65 residues of the individual Ub moieties are faced outwards. However, in the rather extended conformation of K63-linked chains with larger spacing between Ub moieties, S65 residues are positioned more toward the inside of the chain. Each Ub moiety and corresponding S65 is shown in a different color. pS65 is shown in VdW representation. K48-Gly76 and K63-Gly76 linkages are shown in licorice rendering colored by atom type.

 © 2015 The Authors

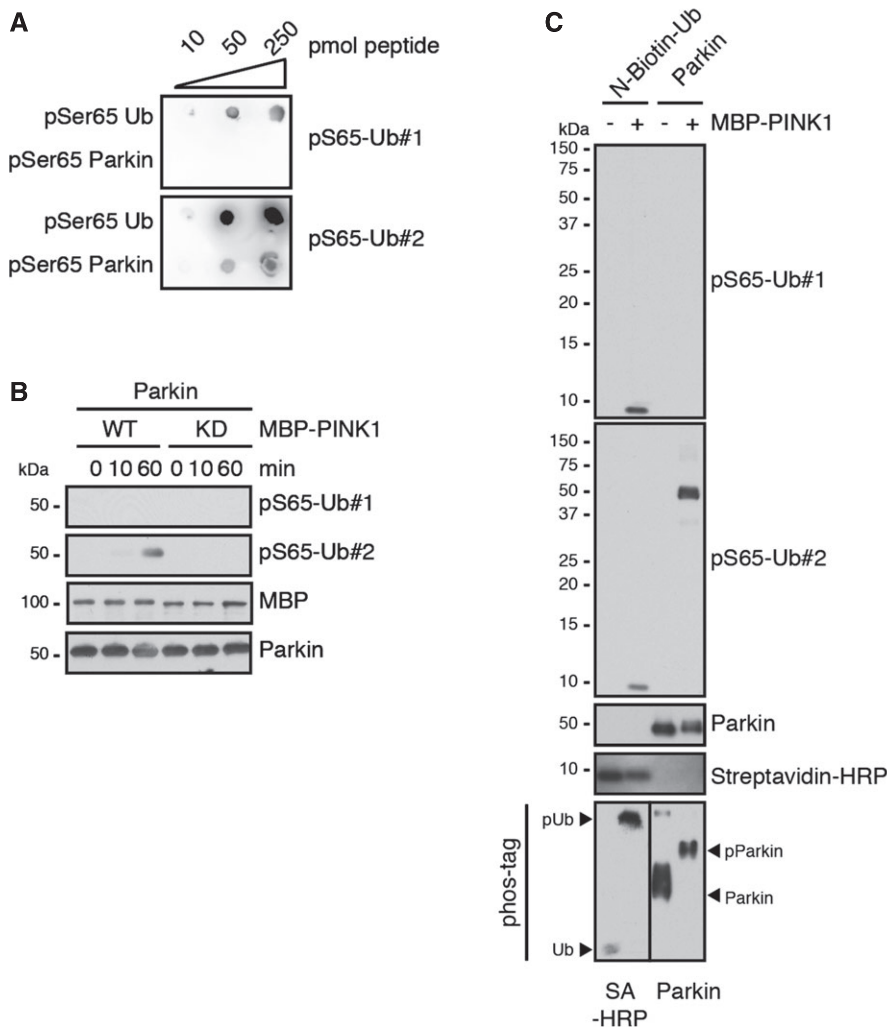

Figure EV2. pS65-Ub#1 is highly specific, whereas pS65-Ub#2 recognizes both PINK1-modified substrates, pS65-Ub and pS65-Parkin.

A Dot blots were performed with the immunogen (pSer65-Ub, amino acids 59–71 of Ub: YNIQKE[pS]TLHLVL) and a phosphorylated Parkin peptide (pSer65-Parkin, amino acids 60–71 of Parkin: DLDQQ[pS]IVHIVQ) and probed with affinity-purified antibodies pS65-Ub#1 and #2. While pS65-Ub#1 was specific to the phosphorylated Ub peptide, pS65-Ub#2 showed reactivity toward Ub and Parkin phospho-peptides.

B Recombinant untagged Parkin was incubated with MBP-tagged PINK1 WT or KD for the indicated times *in vitro*. PINK1-phosphorylated Parkin is detected by pS65-Ub#2, but not #1, although at much lower level than pS65-Ub (compared to Fig 1C).

C Equimolar amounts of recombinant N-biotin-Ub or untagged Parkin were incubated with MBP-PINK1 for 2 days to achieve complete phosphorylation as shown by phos-tag gel electrophoresis. Under these conditions, pS65-Ub#1 specifically detected pS65-Ub, while pS65-Ub#2 cross-reacted somewhat stronger with pS65-Parkin. Both antibodies did not recognize unphosphorylated Ub or Parkin, respectively.

© 2015 The Authors

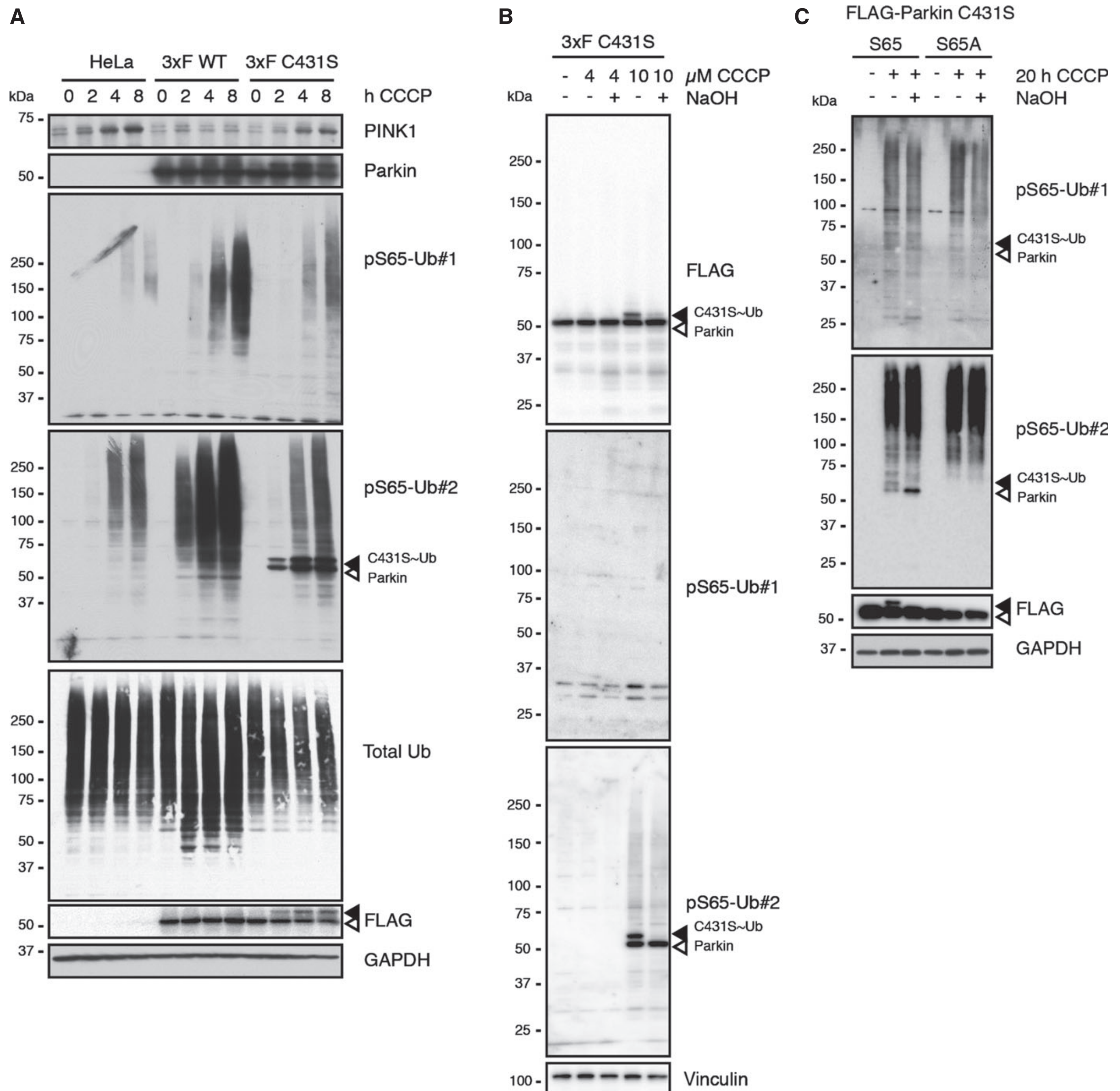

Figure EV3. Cross-reactivity of the pS65-Ub#2 antibody with cellular pS65-Parkin.

A Parental HeLa cells and cells stably expressing 3× FLAG-tagged Parkin WT or C431S mutants were treated with CCCP for the indicated times, lysed and analyzed by WB. In comparison with parental HeLa cells and with cells overexpressing the ligase-dead Parkin mutant C431S, cells overexpressing functional Parkin show strongly enhanced pS65-Ub signal. Similarly, enhanced overall ubiquitination is present in WT overexpressing cells as reflected by total Ub signal. In addition to pS65-Ub, pS65-Ub#2 also recognizes Parkin as observed with the inactive Parkin mutant C431S, where it labels two discrete bands, which likely correspond to pS65-Parkin (open arrowhead) and pS65-Parkin charged with Ub or pS65-Ub (filled arrowhead).

B HeLa cells stably expressing 3× FLAG-tagged Parkin C431S were incubated with 4 or 10 μM CCCP for 2 h, lysed and left untreated or treated with NaOH to strip off Ub from "Ub-charged" Parkin. Chemical cleavage of the Ub moiety from C431S by NaOH results in the collapse of the upper band (Ub-charged pS65-Parkin, filled arrowhead) into the lower band (pS65-Parkin, open arrowhead) as shown by FLAG and pS65-Ub#2 antibodies.

C HeLa cells were transiently transfected with FLAG-Parkin C431S or a C431S+S65A double mutant as an additional specificity control. Cells were challenged with CCCP, lysed, and left untreated or treated with NaOH. Of note, pS65-Ub#2 does only recognize a discrete band where Parkin WT but not the phospho-dead variant Parkin S65A was expressed.

 © 2015 The Authors

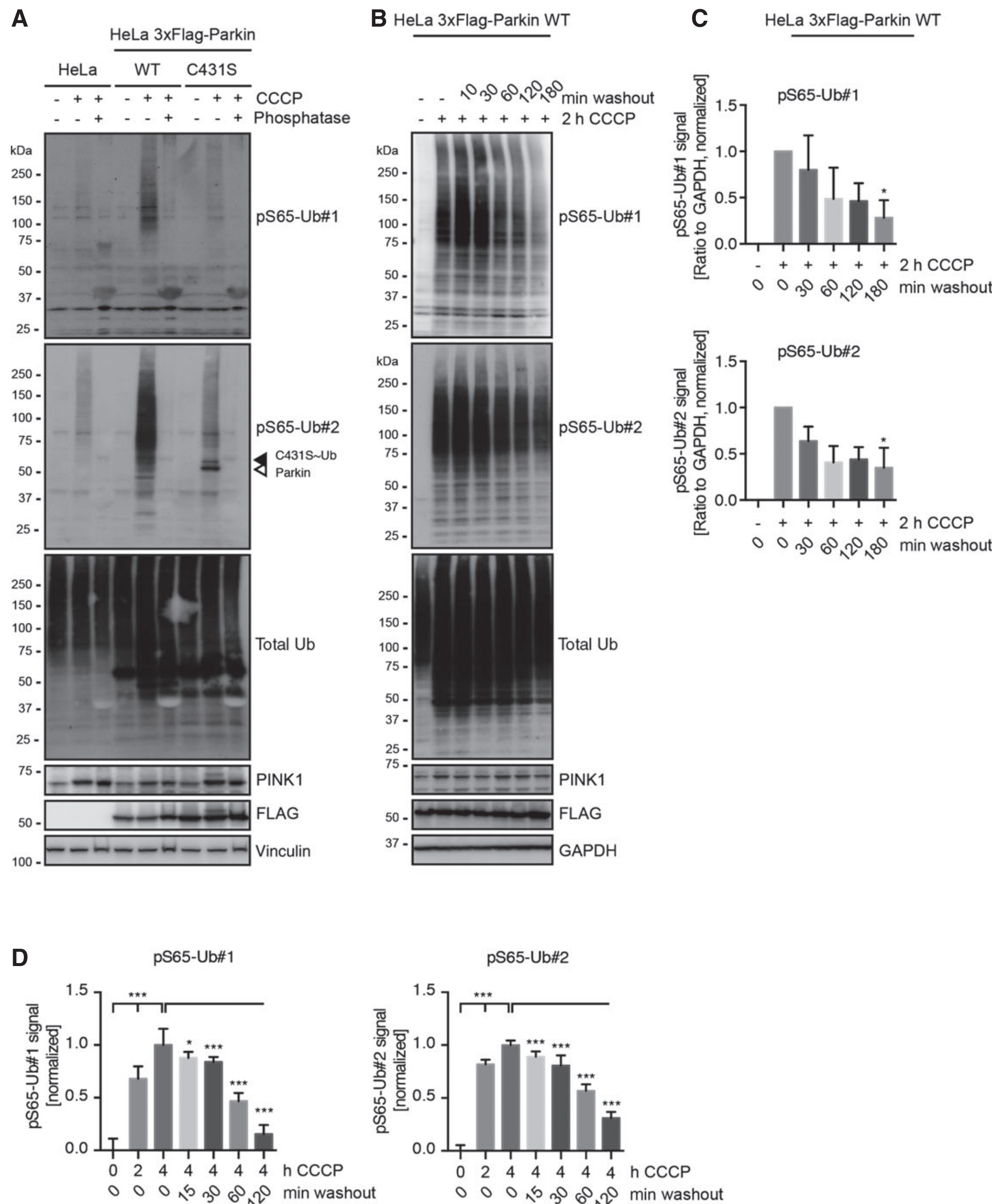

Figure EV4. pS65-Ub is sensitive to phosphatase and is quickly de-phosphorylated during recovery from mitochondrial stress.

A Parental HeLa cells or HeLa cells stably expressing 3× FLAG-tagged Parkin WT or C431S were treated for 2.5 h with CCCP, lysed and incubated with or without alkaline phosphatase. Treatment with phosphatase results in removal of phospho-moieties from poly-Ub chains and Parkin, which are no longer recognized by the anti-pS65-Ub antibodies.

B HeLa cells stably expressing 3× FLAG-tagged Parkin WT were treated with CCCP for 2 h. CCCP was washed out for different times in medium lacking the uncoupler. Over time of washout, pS65-Ub levels dropped considerably, as did total poly-Ub levels that were initially induced by CCCP.

C Quantification of pS65-Ub de-phosphorylation from WB experiments shown in (B). $n = 5$ independent experiments; shown are mean values ± SD (one-way ANOVA with Tukey *post hoc*; $*P < 0.05$) for each pS65-Ub antibody.

D (De-)phosphorylation of pS65-Ub signal upon CCCP treatment and subsequent recovery was quantified by high content imaging (HCI) [7,9]. HeLa Parkin WT cells were incubated with CCCP for 0, 2, or 4 h. Then, CCCP was washed out for the indicated times prior to fixation and staining with pS65-Ub antibodies and a nuclear counterstain. Mean intensity of pS65-Ub in a cytoplasmic ring around the nucleus was measured and normalized to untreated cells and cells that had been treated with CCCP for 4 h. $n = 4$ experiments; shown are mean values ± SEM (one-way ANOVA with Tukey *post hoc*; $*P < 0.05$; $***P < 0.0005$).

© 2015 The Authors

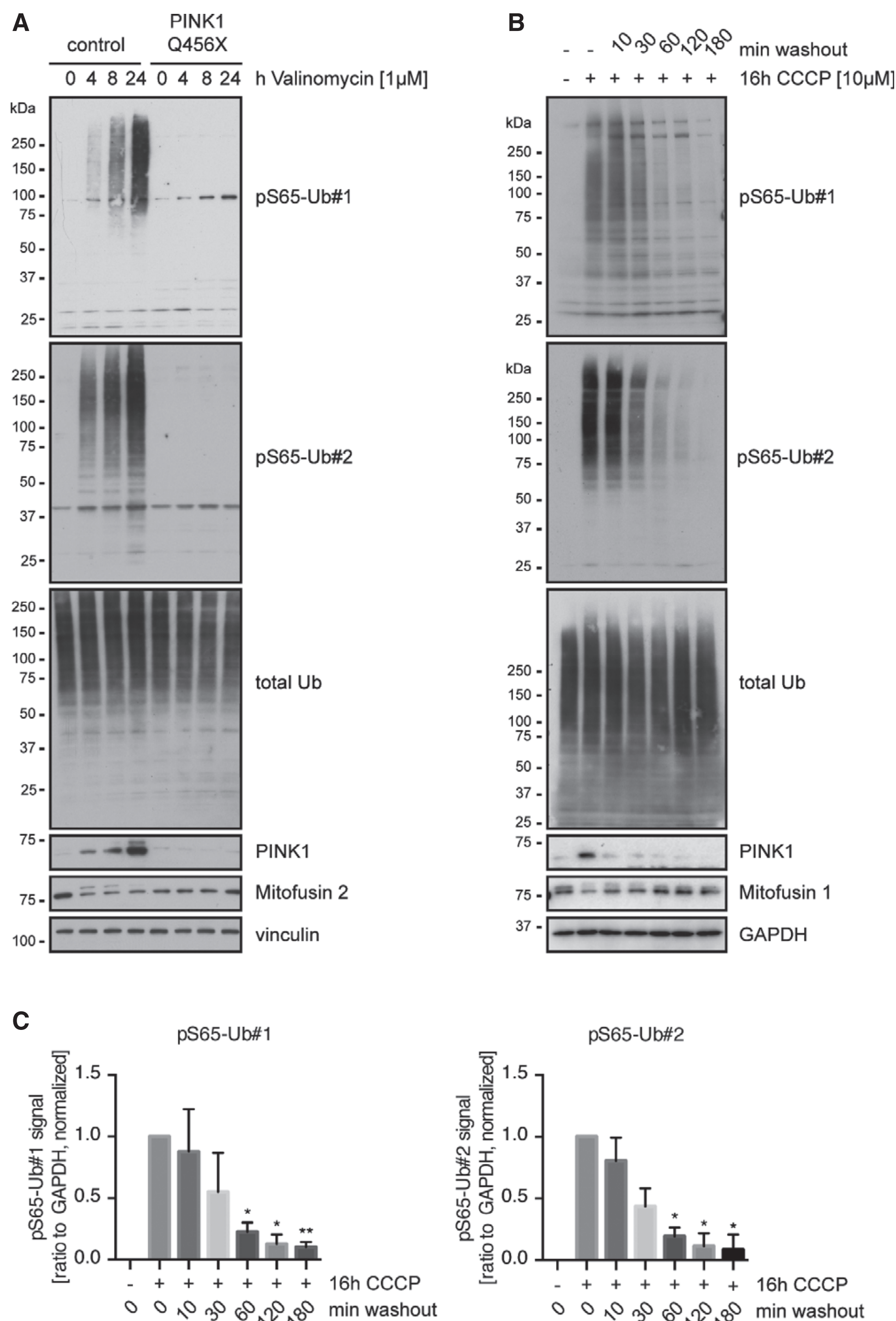

Figure EV5. pS65-Ub is PINK1 dependent and reversible in human primary fibroblasts

A Primary human fibroblasts derived from healthy controls or PD patients harboring a PINK1 Q456X mutation were incubated with the alternative mitochondrial damaging agent valinomycin as indicated. Cells were lysed and analyzed by WB with antibodies against pS65-Ub and total Ub.

B Human control fibroblasts were left untreated or were treated with CCCP for 16 h and then subjected to washout using medium lacking CCCP for the times indicated. Within 30 min after medium change, pS65-Ub signal was drastically diminished.

C Quantification of pS65-Ub de-phosphorylation from WB experiments shown in (B). $n = 3$ independent experiments; shown are mean values ± SD (one-way ANOVA with Tukey *post hoc*; $*P < 0.05$; $**P < 0.005$) for each pS65-Ub antibody.

 © 2015 The Authors