Appendix Figures

Structural basis for feedforward control in the PINK1/parkin pathway

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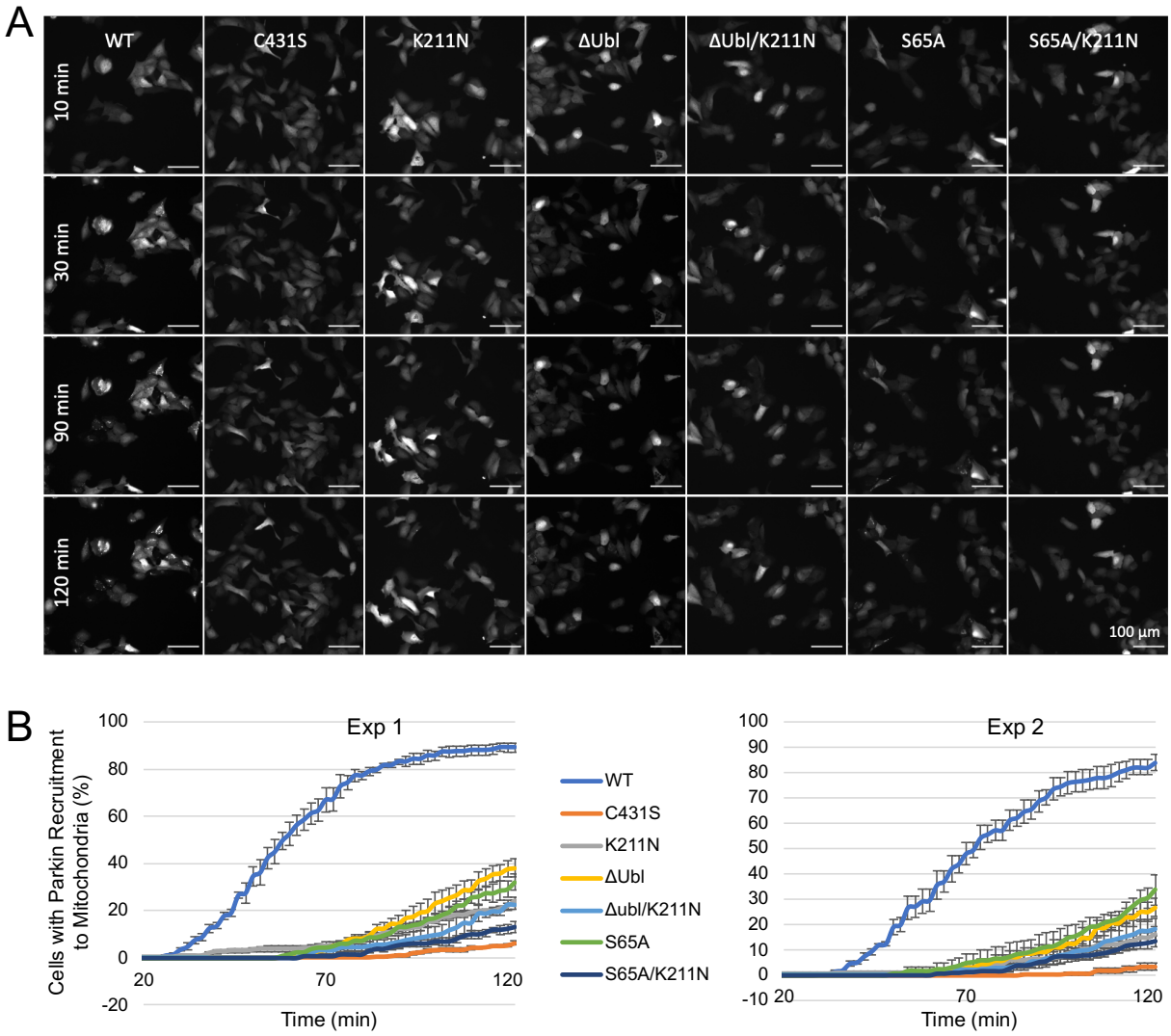
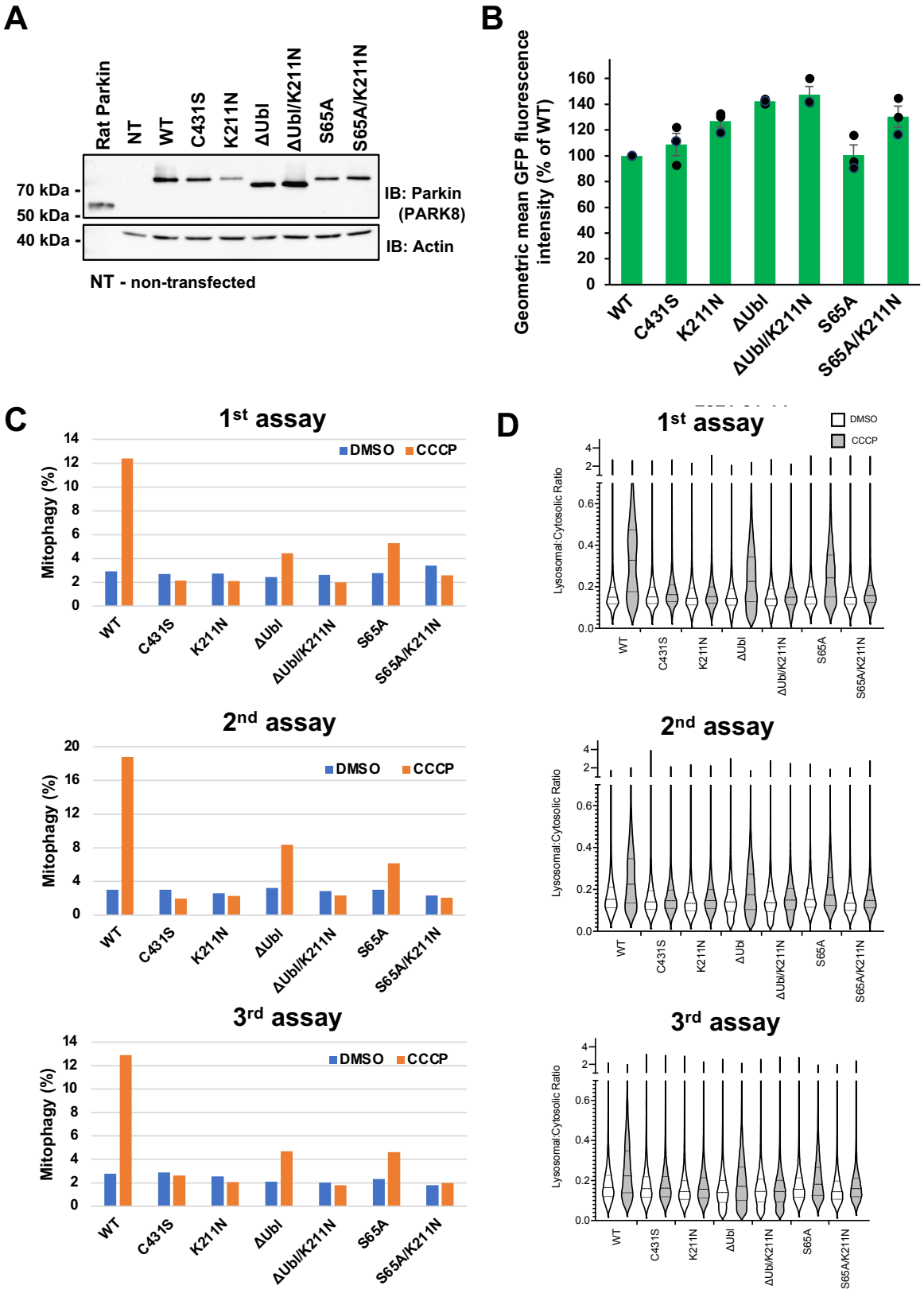
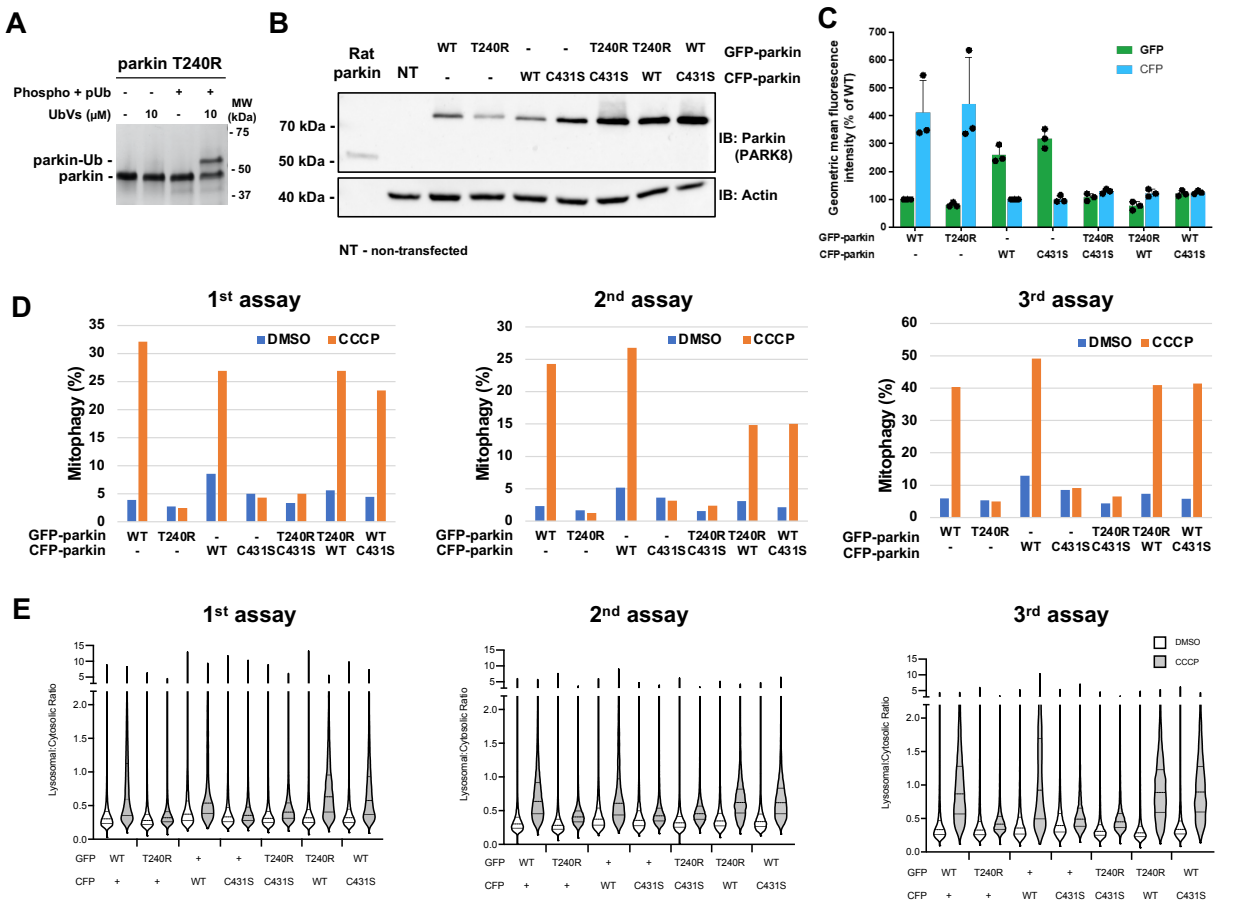
Table of contents



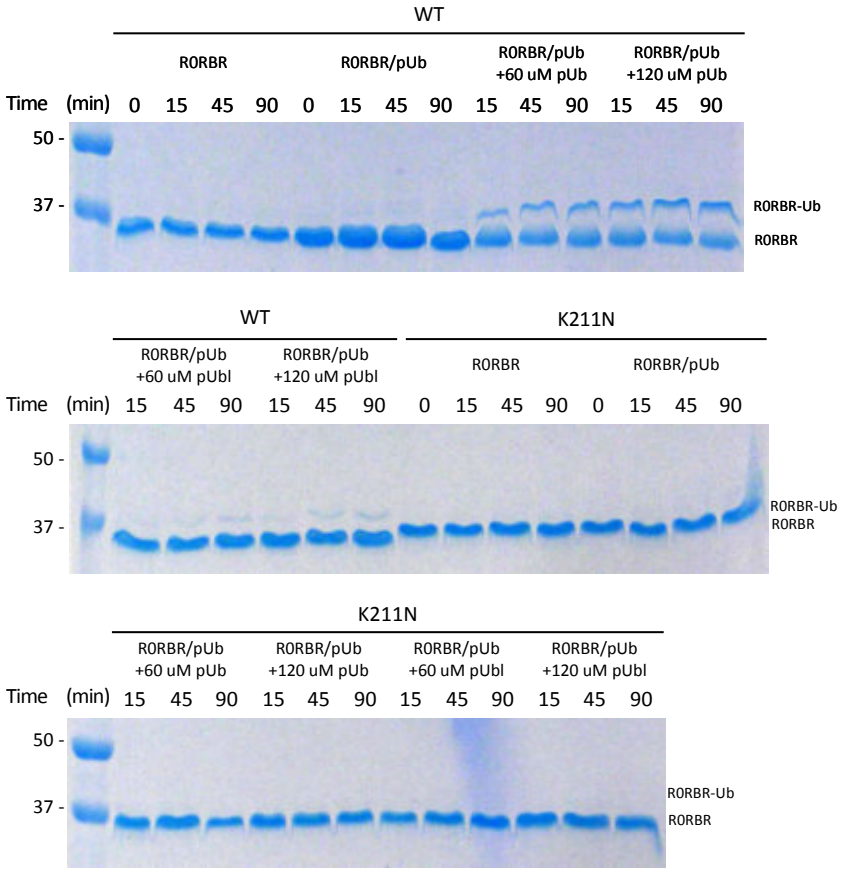
Appendix Figure S1. Parkin recruitment without Ubl phosphorylation. A. Time-lapse imaging of parkin recruitment to mitochondria upon treatment with 20 µM CCCP in U2OS cells stably expressing WT, K211N, C431S, ΔUbl (residues 1-76 deleted), ΔUbl K211N, S65A, S65A K211N GFP-parkin. Recruitment can be visualized by the appearance of punctate GFP fluorescence. Scale bar: 100 µm. **B**. Percentage of cells showing parkin puncta determined every 2 min for 120 min after addition of CCCP. Error bars are s.e.m. from four images per time point.


A
Rat Parkin
NT
WT
C431S
K211N
ΔUbl
ΔUbl/K211N
S65A
S65A/K211N
70 kDa -
50 kDa -
40 kDa -
IB: Parkin (PARK8)
IB: Actin
NT - non-transfected
B
Geometric mean GFP fluorescence intensity (% of WT)
WT
C431S
K211N
ΔUbl
ΔUbl/K211N
S65A
S65A/K211N
C
1st assay
2nd assay
3rd assay
Mitophagy (%)
DMSO
CCCP
D
Lysosomal:Cytosolic Ratio

Appendix Figure S2. Effects of the loss of the pUbl-phosphorylation site on mitophagy. A. Western blotting detection of the expression levels of GFP-parkin variants in U2OS cells used for the mt-Keima assay. **B.** Average GFP fluorescence intensities of parkin fusion proteins used in three replicates of mt-Keima assay. **C.** Mitophagy detected for three replicates of FACS-based analysis of mt-Keima acidification of U2OS cells transfected with GFP-parkin variants and treated with 20 μM CCCP for 4 hrs. **D.** Violin plots of ratio/gating of the three replicated of mt-Keima assay.



Appendix Figure S3. Absence of parkin mutant complementation in mitophagy. A. UbVS assay of the activation of parkin mutant T240R upon phosphorylation and in the presence of pUb. **B.** Western blotting detection of the expression levels of GFP- and CFP-parkin proteins in U2OS cells used for the mt-Keima assay. **C.** Average GFP and CFP fluorescence intensities of parkin fusion proteins used in three replicates of complementation mt-Keima assay. **D.** Mitophagy detected for three replicates of FACS-based analysis of mt-Keima acidification of U2OS cells transfected with GFP, CFP, and GFP/CFP- and treated with 20 μM CCCP for 12 hrs. **E.** Violin plots of ratio/gating of the three replicated of mt-Keima assay.



Appendix Figure S4. UbVS assays of parkin activation by pUb or pUbl. Replicates of UbVS assays performed with R0RBR/pUb (WT or K211N). In this case, 10 µM UbVS was added to 5 µM R0RBR and incubated at 37 °C for a total time of 90 minutes in 25 mM Tris, pH 8.5, 200 mM NaCl, 5 mM TCEP. Reactions were stopped with SDS-PAGE loading buffer and analyzed on 10% Tris-glycine gels stained with Coomassie blue.