APPENDIX

Appendix Figure 1: Validation of pS65-Ub antibodies in cells expressing GFP-Parkin WT or C431S by IF. HeLa cells stably expressing GFP-tagged Parkin WT (left) or Parkin C431S mutation (right) were left untreated or treated with CCCP for 8h, fixed and stained. Cells were analyzed for GFP-Parkin epifluorescence (green), and stained with pS65-Ub antibodies (red) together with Hoechst (blue, nuclei) and either **(A)** anti-total Ub (cyan) or **(B)** anti-TOM20 (cyan, mitochondria) antibody. Both pUb antibodies detect pS65-Ub signals on highly clustered, Ub-labeled mitochondria that were also labeled by GFP in cells expressing functional WT Parkin after 8h CCCP treatment. In cells expressing ligase-dead Parkin C431S signals are increased as well, but remained rather diffuse. In contrast to the clear cytoplasmic staining of GFP-Parkin C431S, pS65-Ub signals distinctively co-localized with the dispersed mitochondrial network as detected with both antibodies albeit weaker with S65-Ub#1. Scale bars correspond to 10µm.

Appendix Figure 2: (Co-)immunoprecipitation of pS65-Ub. (A) HeLa cells stably expressing 3xFLAG-tagged Parkin were incubated with CCCP for the indicated times, lysed under denaturing conditions and subjected to immunoprecipitation (IP) with anti-pS65-Ub antibodies. Immunoprecipitated proteins were analyzed with total Ub and pS65-Ub antibodies to confirm their specificity. Asterisks denote bands derived from the IgG heavy chain. **(B)** HeLa cells stably overexpressing 3xFLAG-tagged Parkin WT or C431S were treated with CCCP as indicated, lysed under stringent conditions in RIPA buffer and subjected to IP with an anti-FLAG antibody. Parkin WT appeared to strongly bind to poly-Ub chains. pS65-Parkin (open arrowhead) and ‘Ub-charged’ phospho-

Parkin (closed arrowhead) are only detectable for C431S Parkin, but not WT. Asterisks denote likely cleaved Parkin species.

Appendix Figure 3: pS65-Ub signals are specific to mitochondrial stress in various cells. (A) HeLa cells stably overexpressing untagged Parkin were treated with CCCP, tunicamycin (to induce ER stress), or etoposide (to induce DNA damage) as indicated. Phosphorylation of poly-Ub is observed only upon mitochondrial stress. Antibody against cleaved PARP served as a control for effectively induced ER stress and DNA damage. **(B)** Non-neuronal Hek293E or HeLa cells as well as H4 glioblastoma or M17-BE neuroblastoma cells were treated with CCCP as indicated. Cells were lysed and analyzed by Western blot to monitor pS65-Ub signal over time.

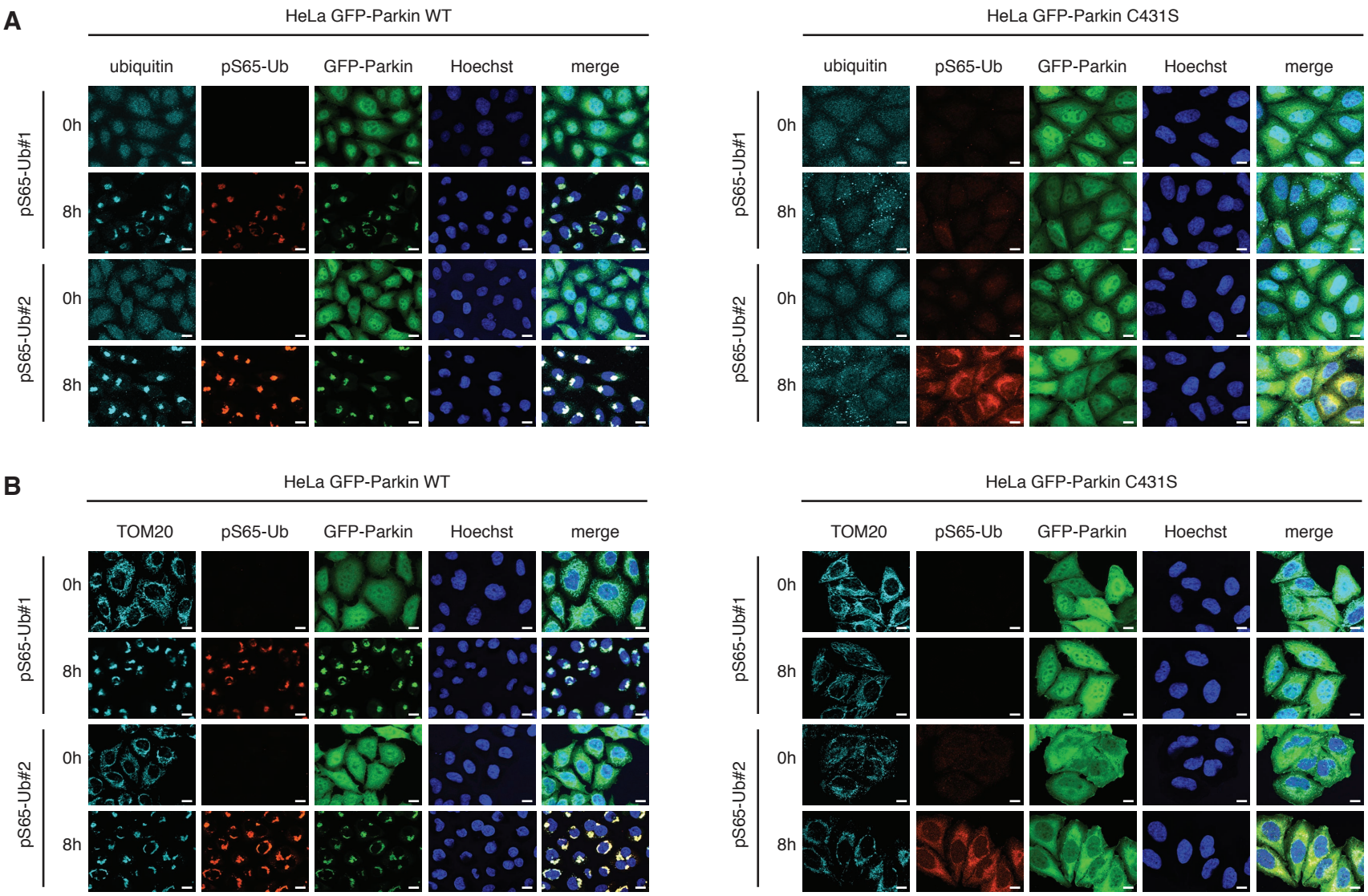
Appendix Figure 4: pS65-Parkin C431S is de-phosphorylated with similar kinetics compared to pS65-Ub. HeLa cells stably expressing 3xFLAG-tagged Parkin C431S were treated with CCCP for 4h. CCCP was washed out for different times in medium lacking the uncoupler. Over time washout, pS65-Parkin and pS65-Ub levels dropped considerably.

Appendix Table 1: Details on human post-mortem brains analyzed in this study.

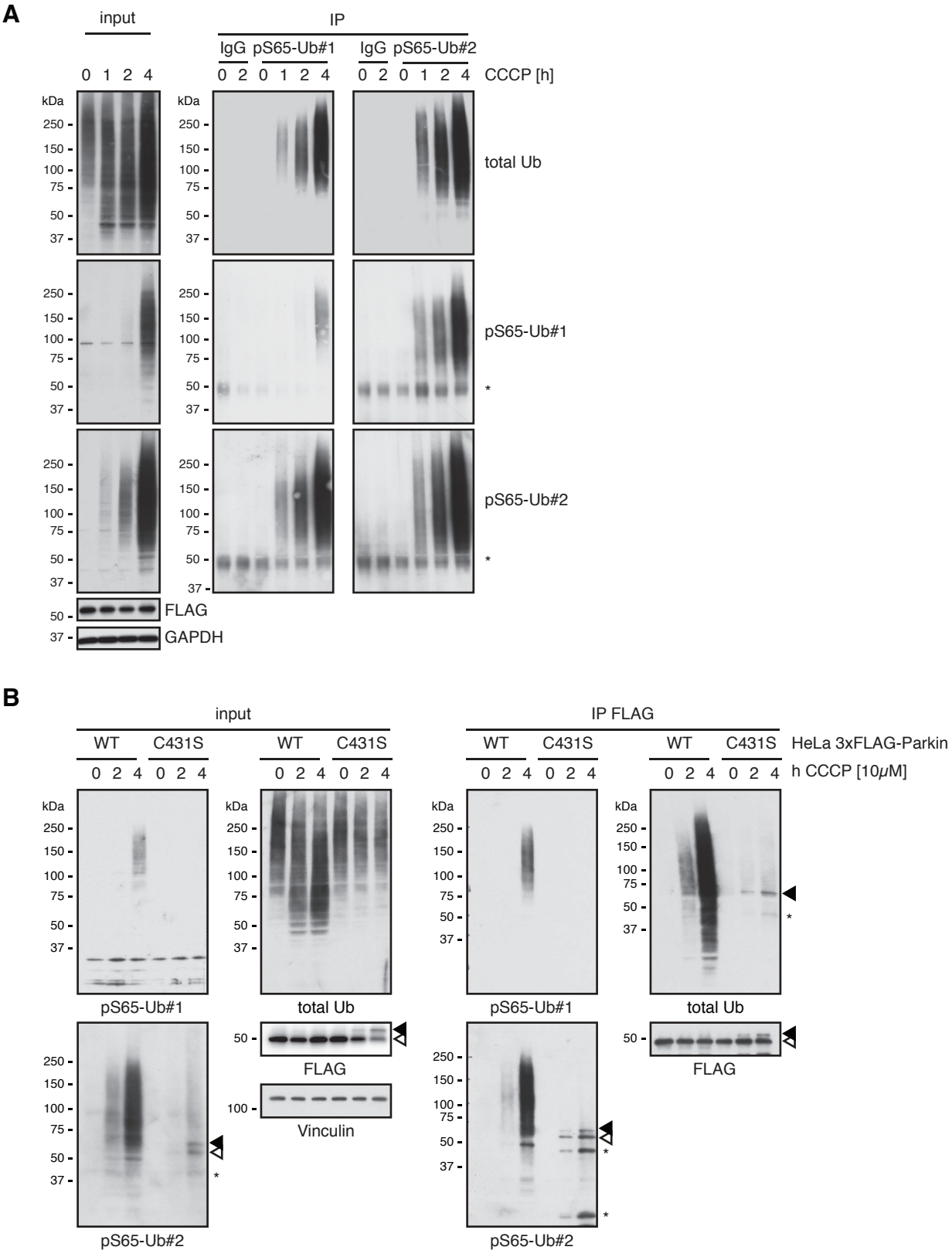
Table lists all 15 human post-mortem brains analyzed for pS65-Ub signals from rabbit sera to affinity-purified and immuno-depleted antibodies. Table shows brain details including sex, age, neurologic and neuropathologic diagnoses (Dx), Braak neurofibrillary tangles (NFT) stage, Thal amyloid phase, brain weight [gram], and fixation [days]. pS65-Ub antibodies were tested over an age-range from 23 to 102 years from cognitively normal controls with or without age related tauopathy as well as from PD patients (brain

stem LB disease and limbic LB disease). Our observations are consistent with an increase in pS65-Ub with age and disease.

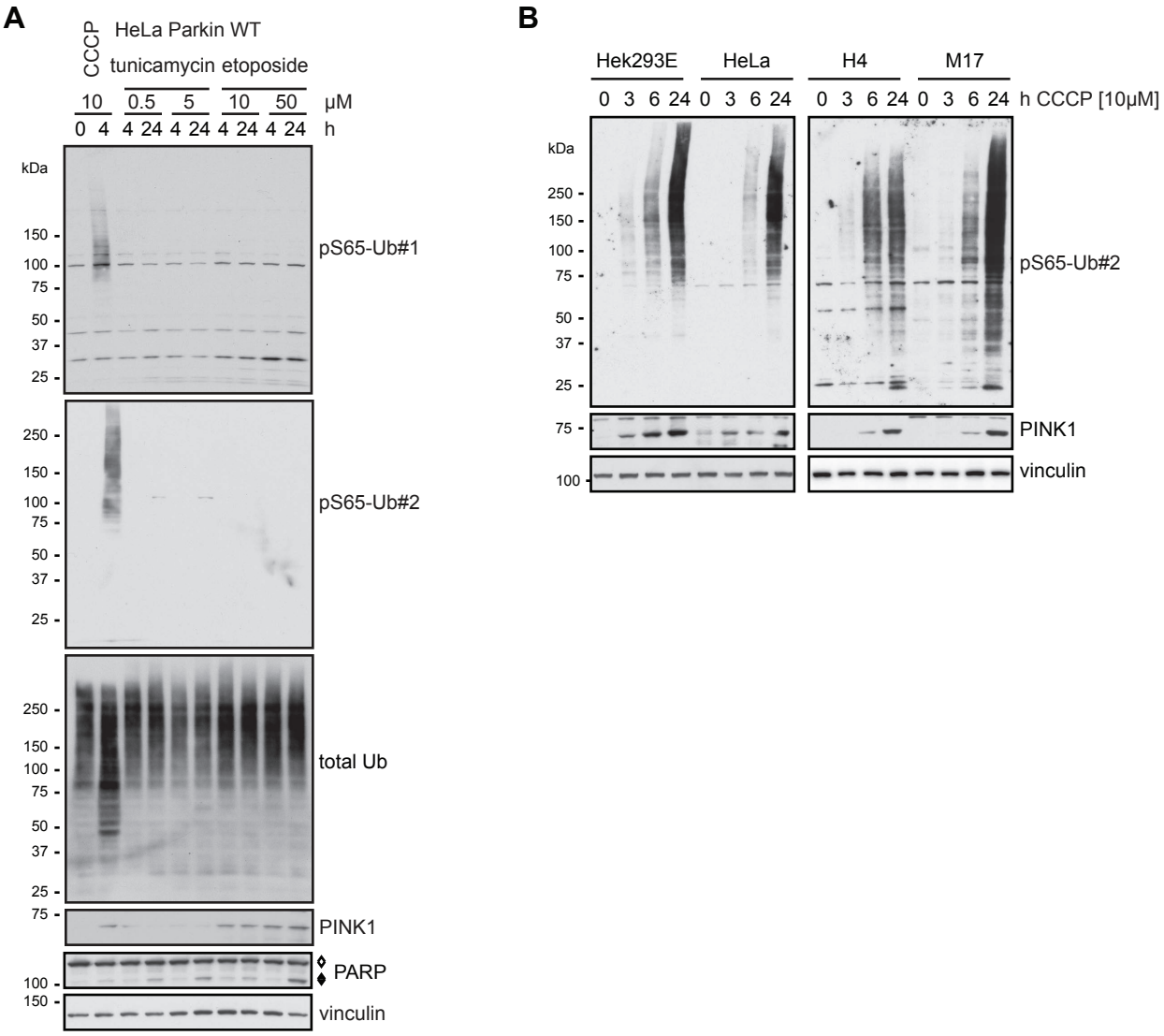
Appendix Figure 1



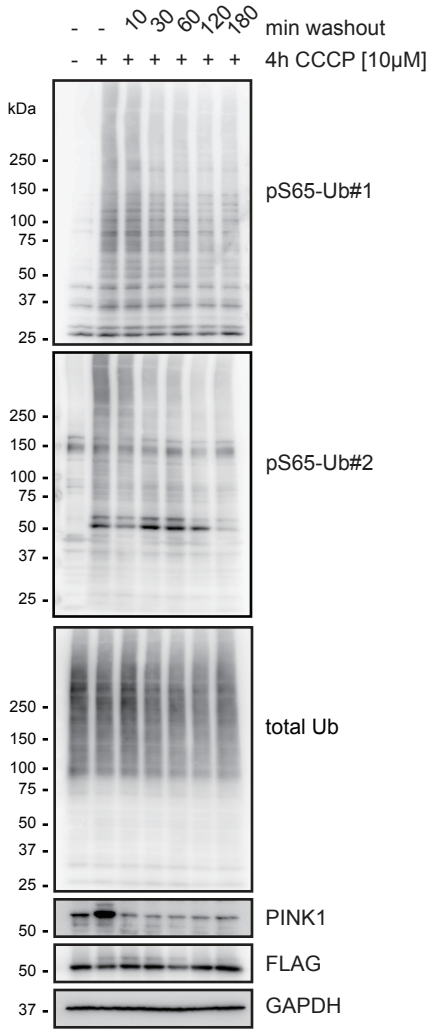
Appendix Figure 2



Appendix Figure 3



Appendix Figure 4



Appendix Table 1:

Category	Sex	Age	Neurologic Dx	Neuropath-ologic Dx	Braak NFT stage	Thal amyloid phase	Brain weight (g)	Fix (days)
young	F	23	No cognitive impairment	Normal	0	0	1280	14
young	F	26	No cognitive impairment	Normal	0	0	1430	38
young	M	28	No cognitive impairment	Normal	0	0	1160	16
young	F	37	No cognitive impairment	Normal	0	0	1120	13
older	M	63	No cognitive impairment	Age related tauopathy	III	0	1440	11
older	F	66	No cognitive impairment	Normal	0	0	1180	23
older	M	85	No cognitive impairment	Age related tauopathy	I	0	1270	41
older	M	89	No cognitive impairment	Normal	0	0	1200	na
older	F	91	No cognitive impairment	Age related tauopathy	III	0	1200	29
older	M	93	No cognitive impairment	Normal	I	1	1070	16
PD	F	58	Parkinson disease	Brainstem Lewy body disease	0	1	1320	73
PD	M	67	Parkinson disease	Brainstem Lewy body disease	III	3	1000	274
PD	F	69	Parkinson disease	Brainstem Lewy body disease	0-I	0	1160	258
PD	M	71	Parkinson disease	Limbic Lewy body disease	0	0	1200	169
PD	F	78	Parkinson disease	Limbic Lewy body disease	I-II	1	1620	17
PD	M	84	Parkinson disease	Brainstem Lewy body disease	I	0	1080	71