Supplementary Information

A cascading activity-based probe sequentially targets E1-E2-E3 ubiquitin enzymes

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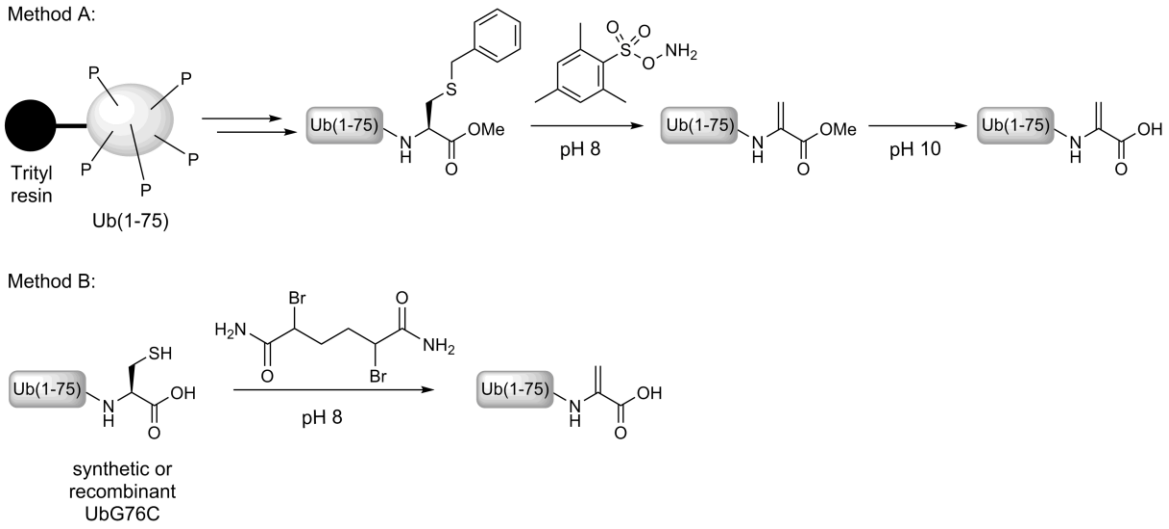
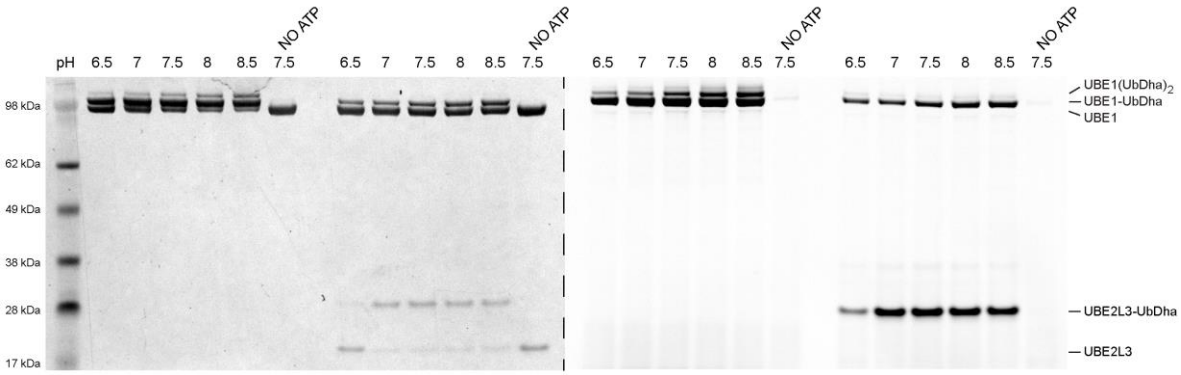
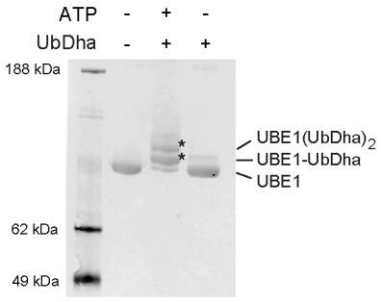
Supplementary Results



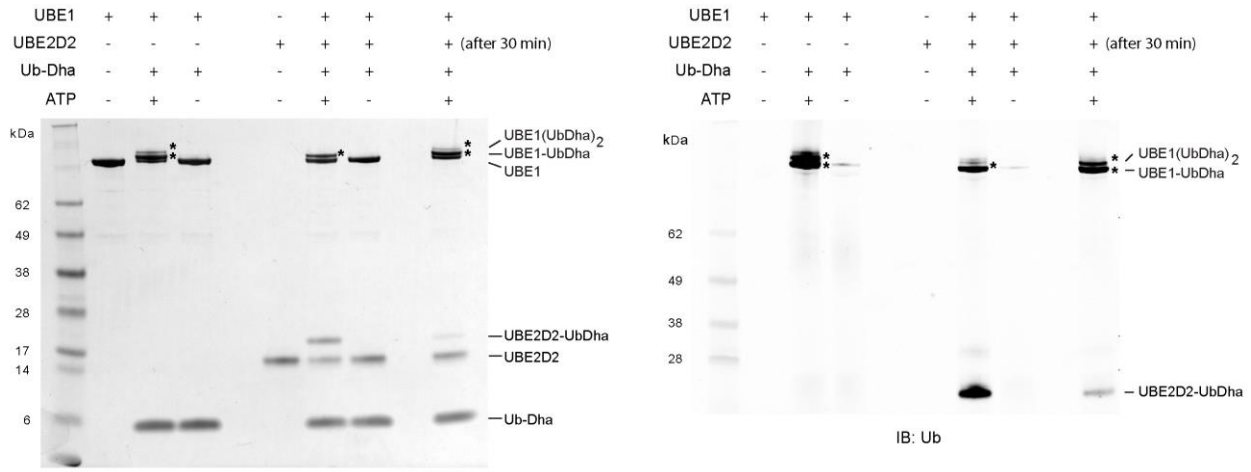
Supplementary Figure 1. Synthesis of Ub-Dha starting from Ub(1-75)-Cys(Bn)-OMe (method A) or UbG76C (method B).



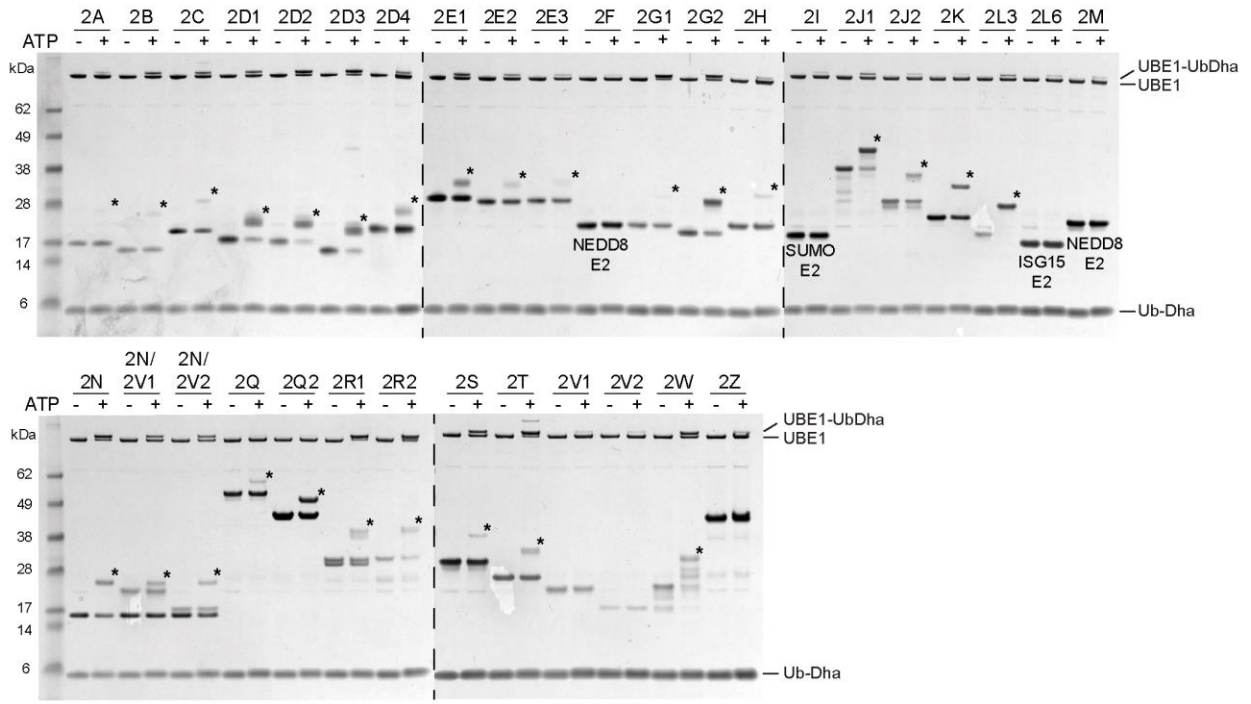
Supplementary Figure 2. pH effect on labeling of Cy5-UbDha with UBE1 and UBE2L3. Left: silver stain, right: fluorescent scan.



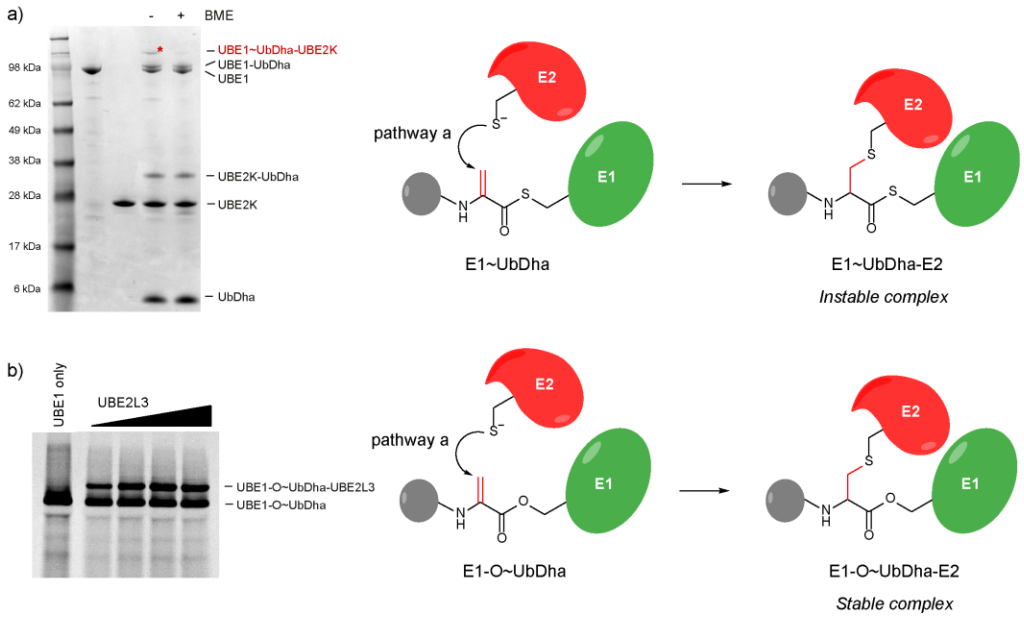
Supplementary Figure 3. Western Blot of UBE1 labeling reaction with UbDha (blotted against His-tagged UBE1) showing the UBE1-UbDha and UBE1(UbDha)$_2$ adducts (indicated by asterisks).



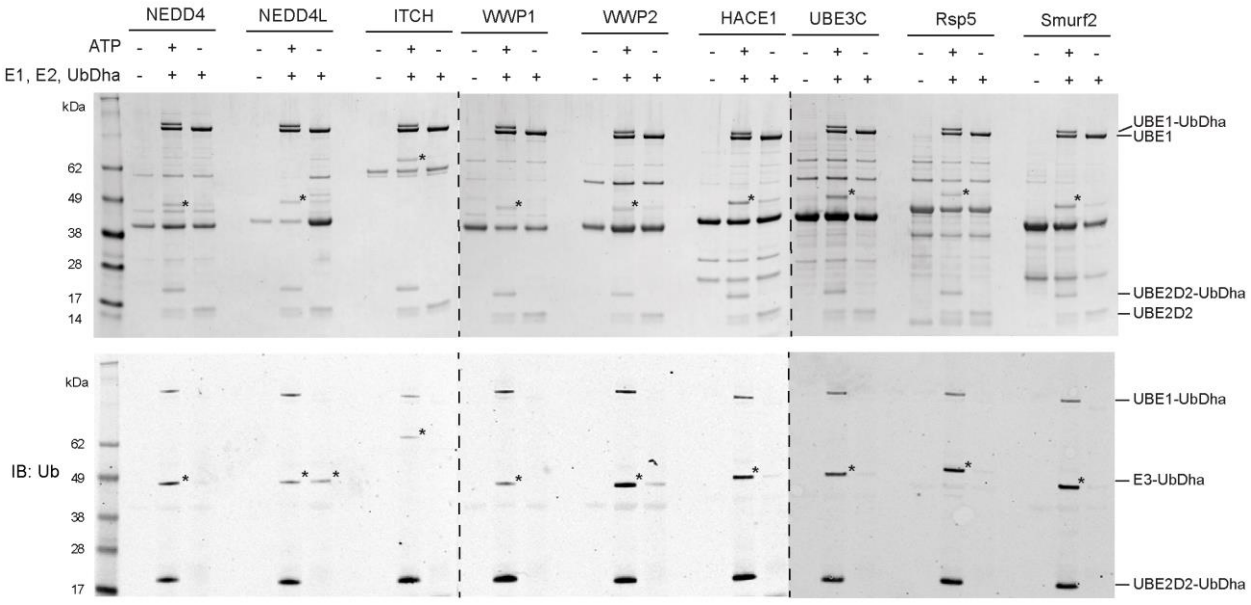
Supplementary Figure 4. Doubly loaded UBE1 intermediate is not formed in the presence of UBE2D2. Silver stain (left) and western blot against Ub (right). The asterisks indicate the thioether-linked UBE1-UbDha and UBE1(UbDha)$_2$ adducts.



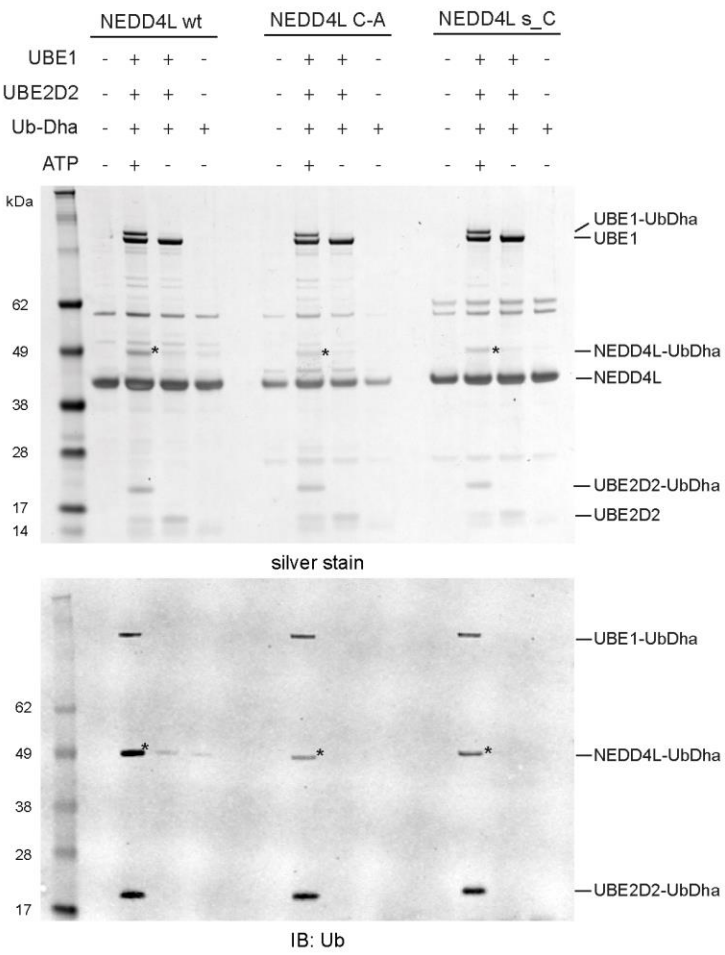
Supplementary Figure 5. UbDha labels 27 E2s specific for Ub transfer but not E2s employing Ubls. The asterisks indicate the thioether-linked E2-UbDha adducts. (E2-scan kit, Ubiquigent).Visualized by silver stain.



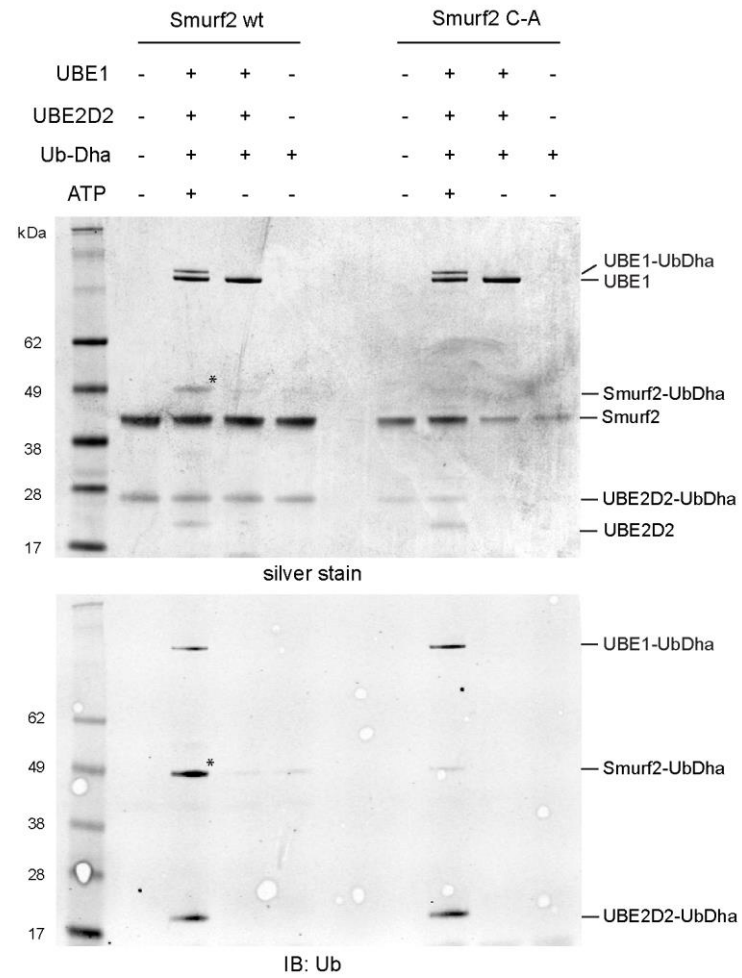
Supplementary Figure 6. A ternary complex is formed via a third pathway. The acceptor enzyme directly reacts with the Michael acceptor on the probe donating enzyme thioester adduct. a) SDS-PAGE analysis of UbDha reaction with UBE1 and UBE2K. Under non-reducing conditions the ternary complex is visible on gel, while under reducing conditions this instable complex is not (visualized by coomassie). b) In gel fluorescence analysis of Cy5-UbDha reaction with UBE1 active site Cys-Ser mutant and UBE2L3. Under reducing conditions the more stable ternary UBE1-O~UbDha-UBE2L3 is still visible.



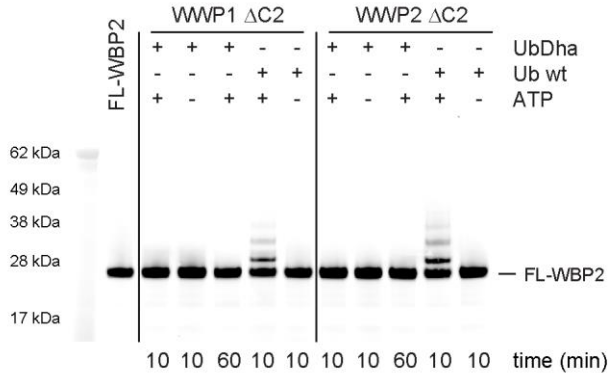
Supplementary Figure 7. UbDha shows reactivity towards E3 HECT enzymes under ATP dependent conditions. The asterisks indicate the E3-UbDha thioether-linked adduct. Visualized by silver staining (upper panel) and western blot against Ub (lower panel).



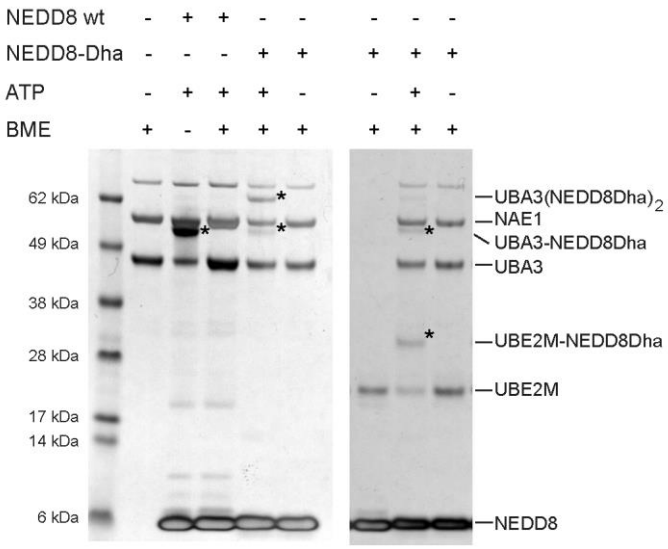
Supplementary Figure 8. Labeling of NEDD4L wt, Cys-to-Ala mutant and single Cys mutant with UbDha visualized by silver staining (upper panel) and western blot against Ub (lower panel). The asterisks indicate the thioether-linked NEDD4L-UbDha adduct.



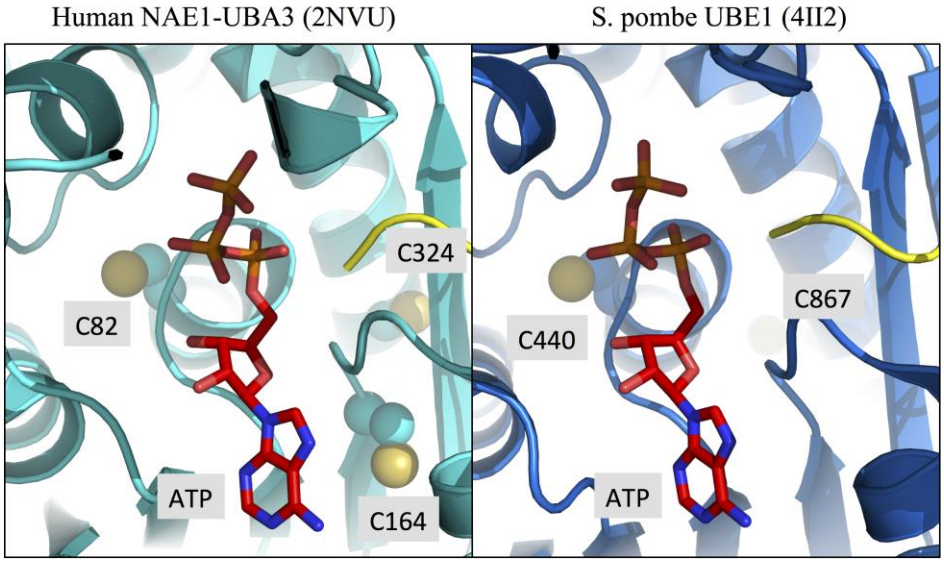
Supplementary Figure 9. Labeling of Smurf2 wt and Cys-to-Ala mutant with UbDha, visualized by silver staining (upper panel) and western blot against Ub (lower panel). The asterisks indicate the thioether-linked Smurf2-UbDha adduct.



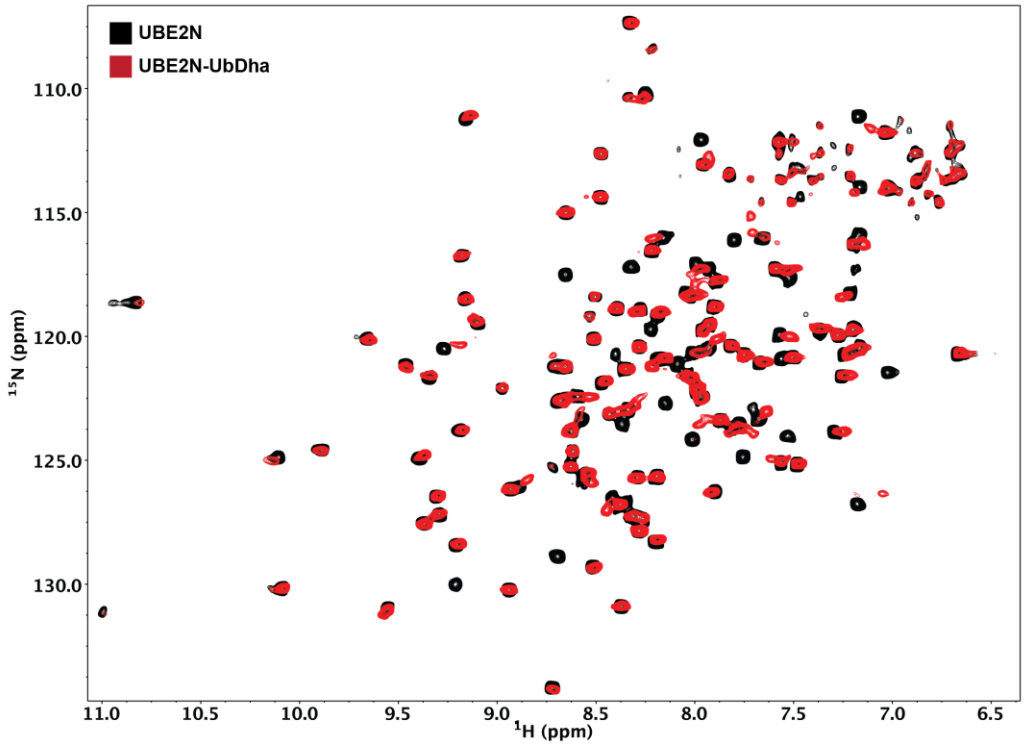
Supplementary Figure 10. Multiple turnover ubiquitination on substrate WBP2 does not occur with UbDha.



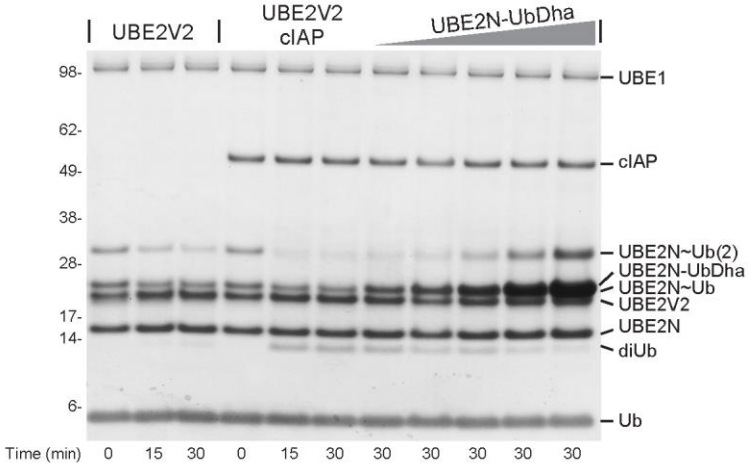
Supplementary Figure 11. NEDD8Dha shows covalent bond formation with the E1 UBA3 and E2 UBE2M (visualized by silver stain). Additionally a higher-running E1 band was detected, presumably corresponding to one NEDD8Dha marking the active site Cys and the other bound to the adenylation domain mimicking an E1 double-loaded intermediate (Supplementary Figure 12). The asterisks indicate the labeled enzyme adducts.



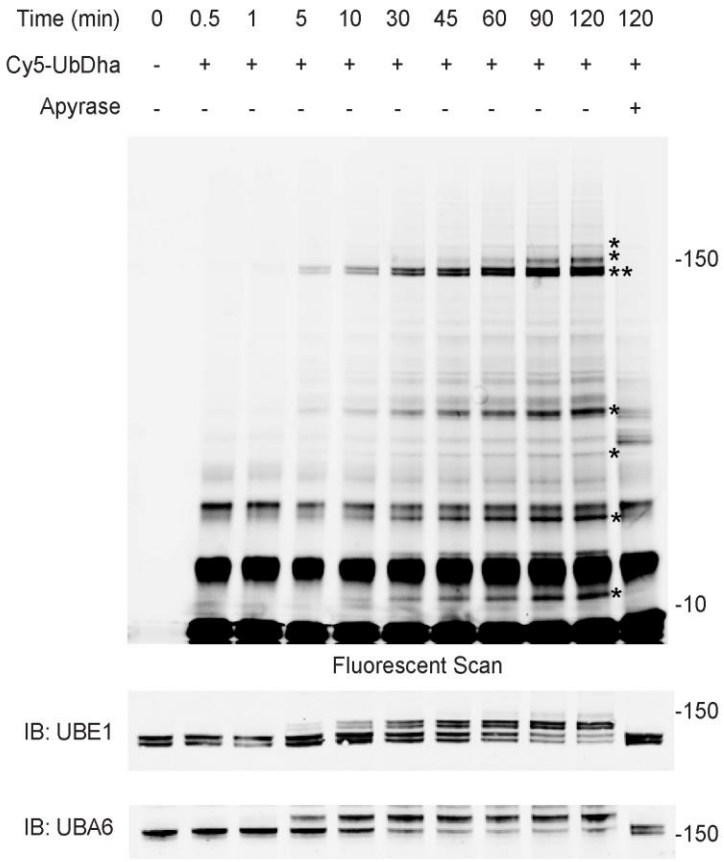
Supplementary Figure 12. Aligned E1 structures of the ATP binding site. The ATP binding site of NAE1 is flanked by three cysteines (Cys82, Cys164 and Cys324)[1], one of these (Cys82) is conserved in human UBE1 (Cys481). Note: There is no human E1 crystal structure available. Therefore we used a yeast UBE1 structure[2], as a homology model of human UBE1, to present the location of conserved cysteine near ATP (C481 of human UBE1 and C440 of Sp UBE1, human UBE1 and S. pombe share 53.8% sequence identity).



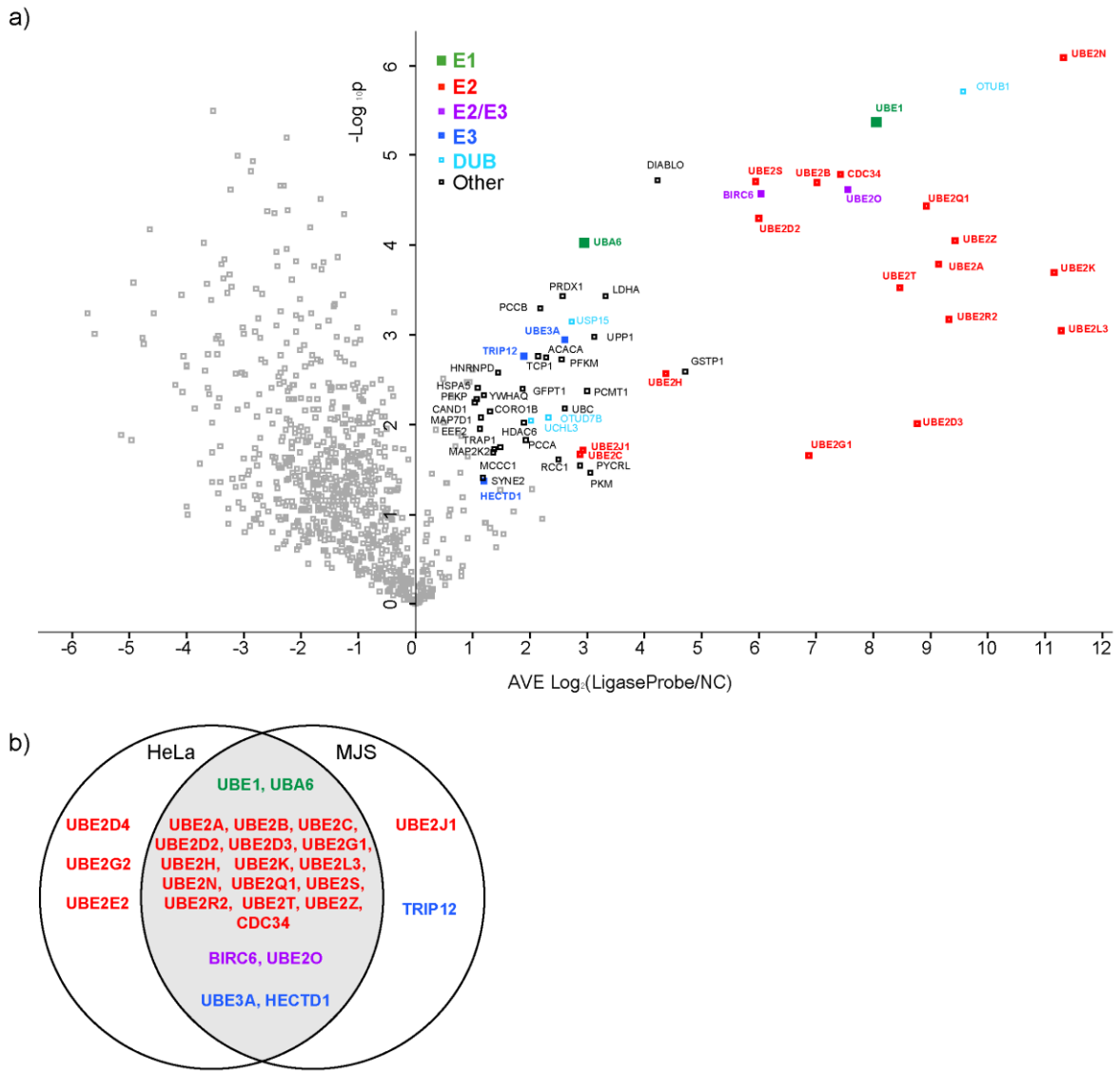
Supplementary Figure 13: Full ^{1}H, ^{15}N HSQC spectra of UBE2N (black) and thioether-linked UBE2N-UbDha (red).



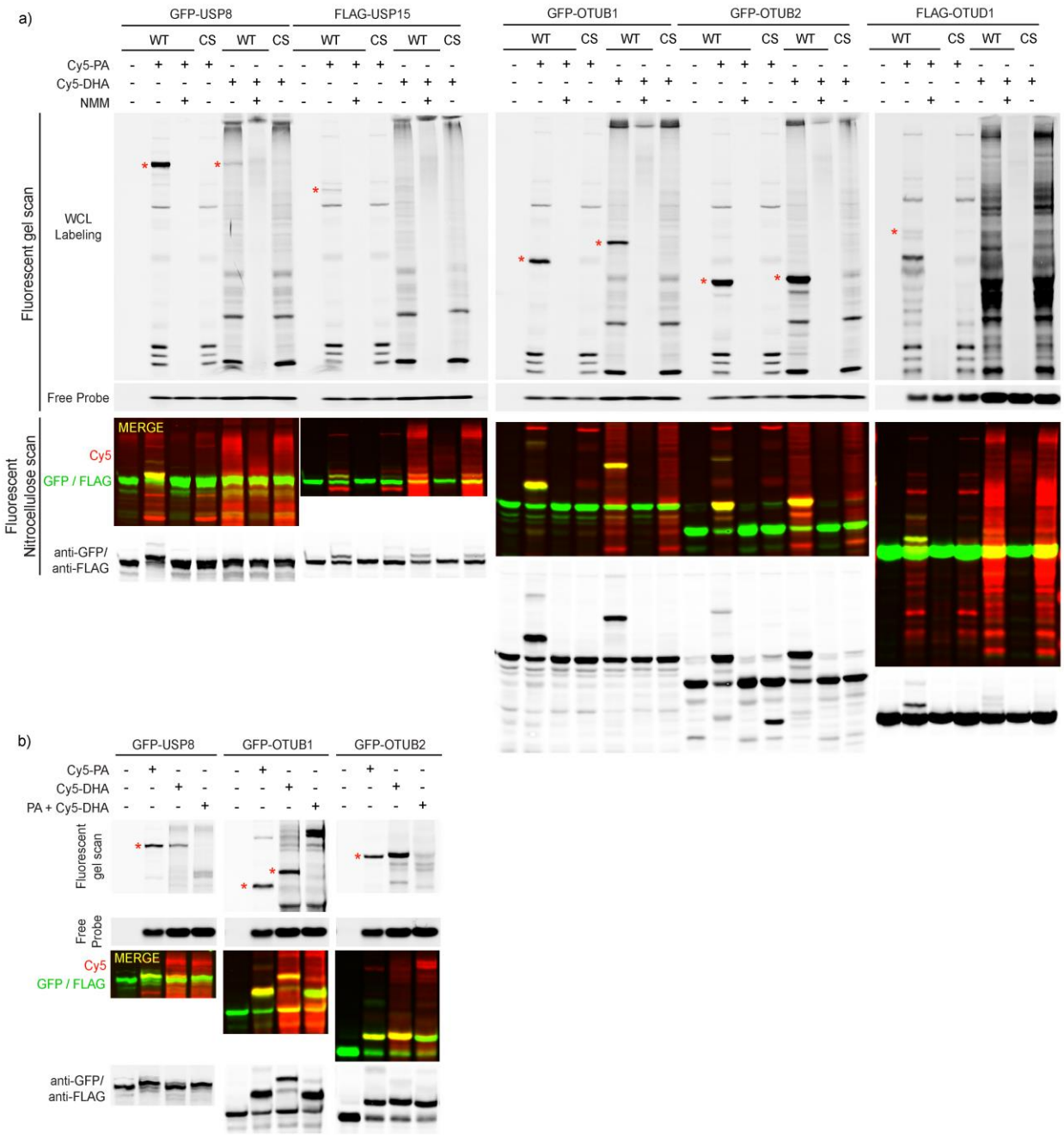
Supplementary Figure 14. Thioether-linked UBE2N-UbDha adduct can compete with downstream ubiquitination enzymes. Single-turnover ubiquitination assay monitoring the formation of diUb from thioester-linked UBE2N~Ub. Titration of the stable thioether-linked UBE2N-UbDha into the reaction results in diminished diUb production.



Supplementary Figure 15. Activation of UbDha in cell extracts. Time-course of HeLa cell lysates labeling with Cy5UbDha in the absence (-) or presence (+) of ATP scavenger apyrase. The asterisks indicate ATP-dependent bands.

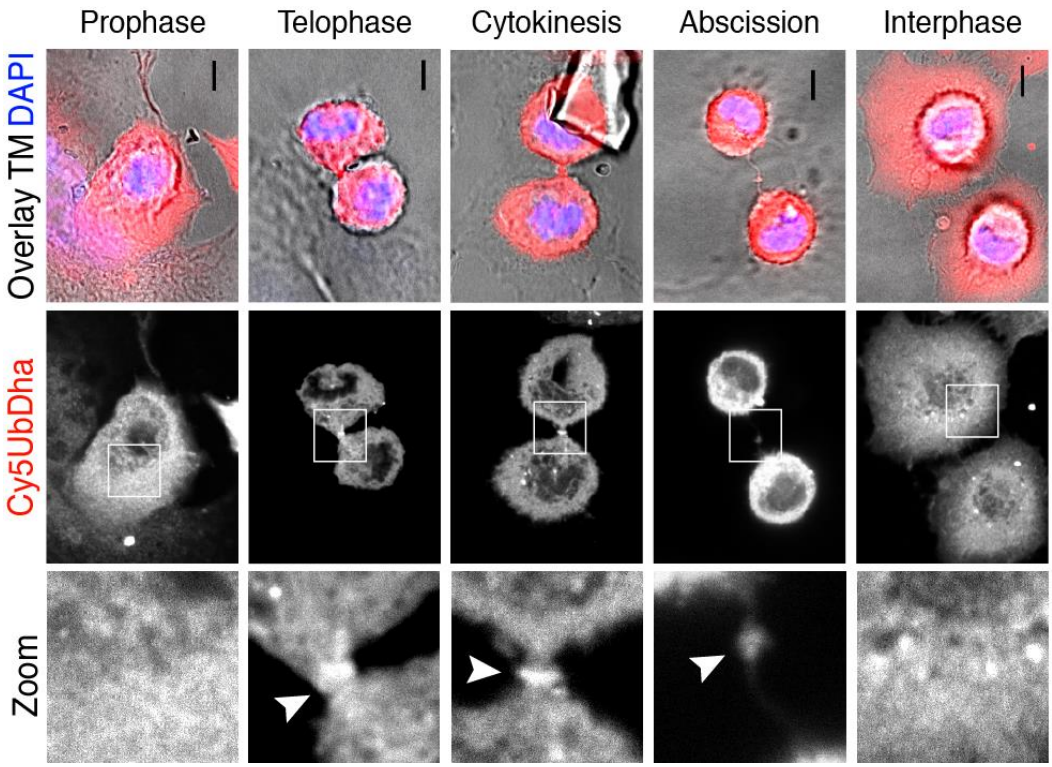


Supplementary Figure 16. Proteome-wide activity profiling of the Ub conjugation machinery in MelJuSo cells. **a**) Volcano plot of pairwise comparison of proteins bound to the Biotin-UbDha probe relative to the apyrase-treated negative control (negative $\log_{10}$ p-value, y-axis) as a function of average $\log_2$ fold enrichment (x-axis). Colored dots represent confidently identified ubiquitin machinery components as follows: E1 (green), E2 (red), HECT E3 (blue), hybrid E2/E3 (purple) and DUBs (light blue), with an average $\log_2$ ratio greater than 1 and with $p < 0.05$. Hits unrelated to the Ub cycle are marked in black, and proteins falling below the threshold are shown in gray. **b**) Venn diagram of shared Ub-conjugation enzymes recovered from HeLa and MelJuSo cells.

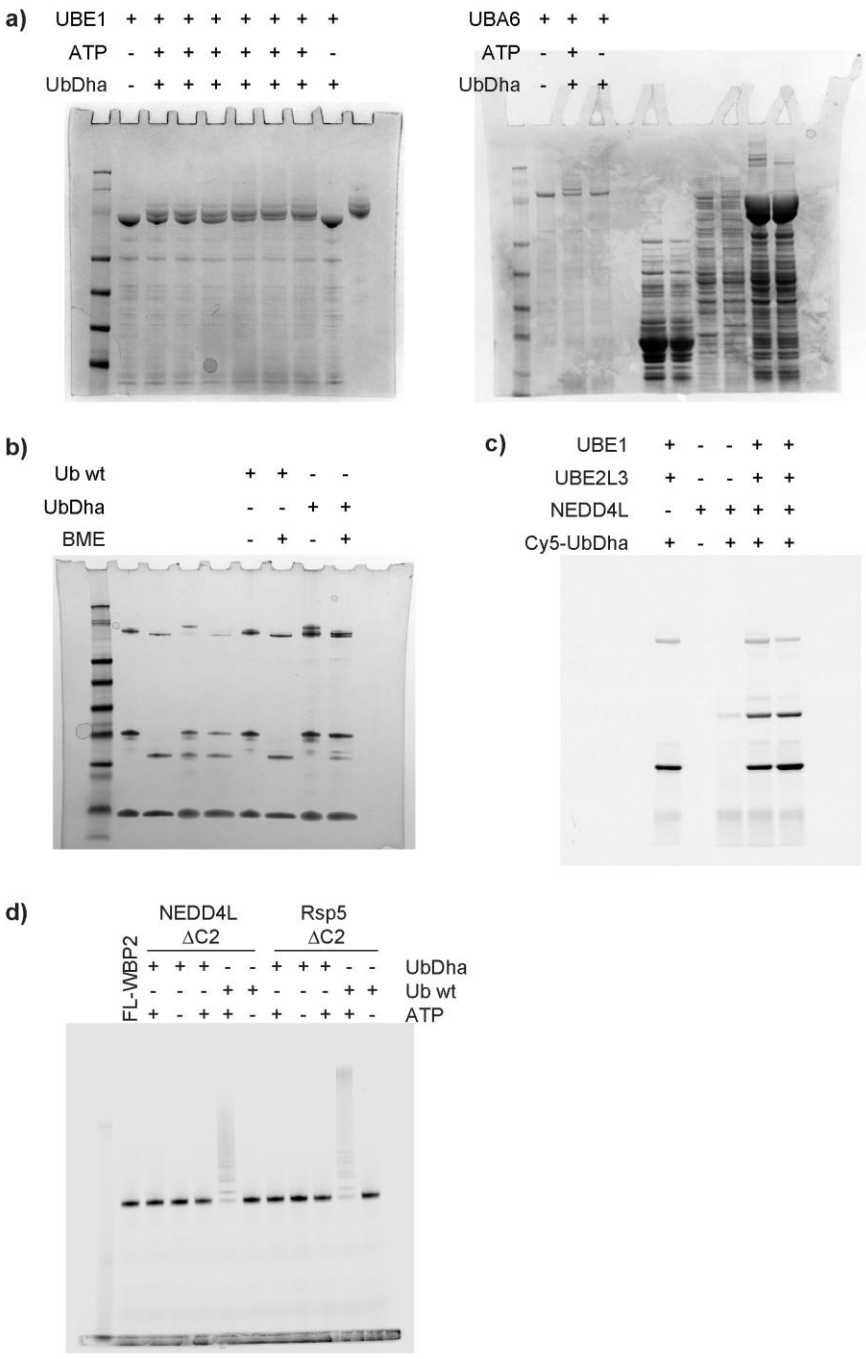


Supplementary Figure 17. DUB labeling: comparison of UbPA and UbDha. Labeling of DUBs in lysates of HEK293T cells transfected as indicated with Cy5-UbPA or Cy5-UbDha, visualized by fluorescence gel scan and immunoblotting against GFP or FLAG. **a**) Red asterisks indicate labeling of active DUBs. OTUB1 is doubly modified with UbDha, a known characteristic for OTUB1.[3] UbPA readily modifies all catalytically competent DUBs tested, while UbDha does not exhibit the same degree

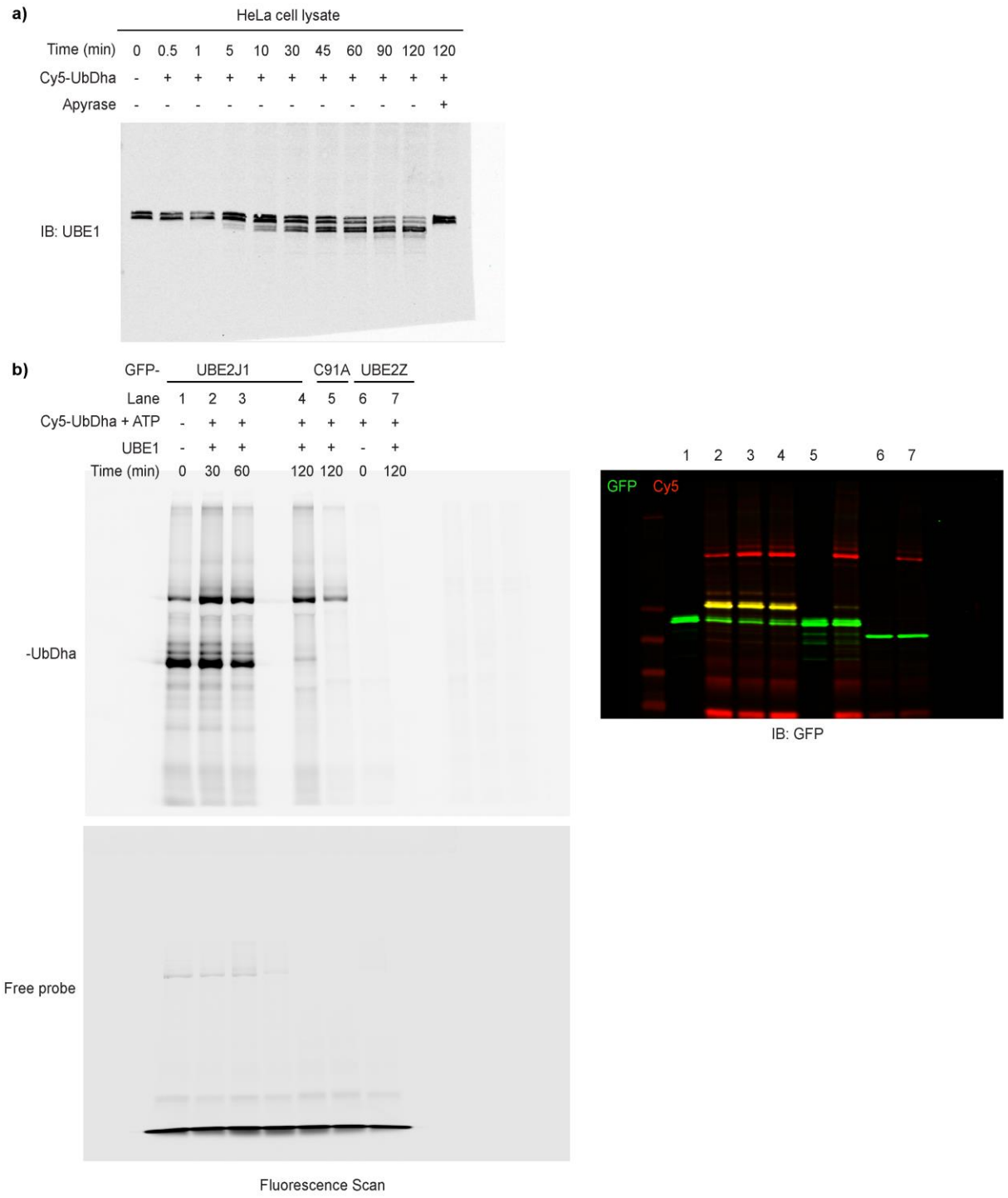
of reactivity. Mutations of active-site cysteine residues to serines abolished DUB labeling. **b**) Labeling of DUBs with Cy5-UbDha was completely abolished upon pretreatment with UbPA.



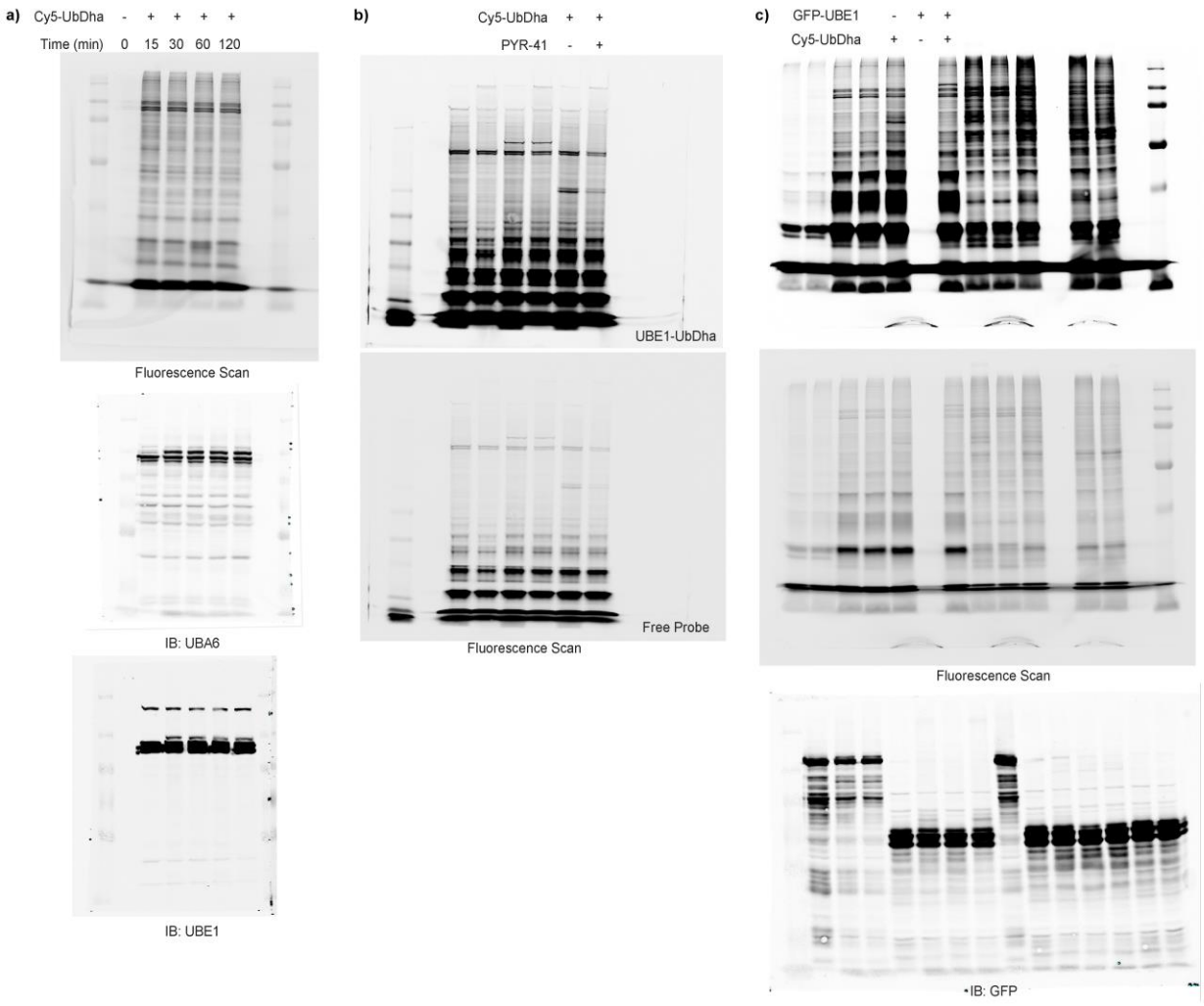
Supplementary Figure 18. Accumulation of Cy5-UbDha probe at the abscission site in diving cells. Cy5-UbDha probe (white, bottom panels) was introduced into HeLa cells by electroporation. Following 1 hr recovery period, cells were fixed and visualized by confocal microscopy. Representative images of cells harboring Cy5-UbDha undergoing various mitotic phases are shown; overlays correspond to Cy5 (red), nuclear DAPI (blue) and the transmission images; scale bars = 5 µm. Arrows point towards the site of abscission, where applicable.



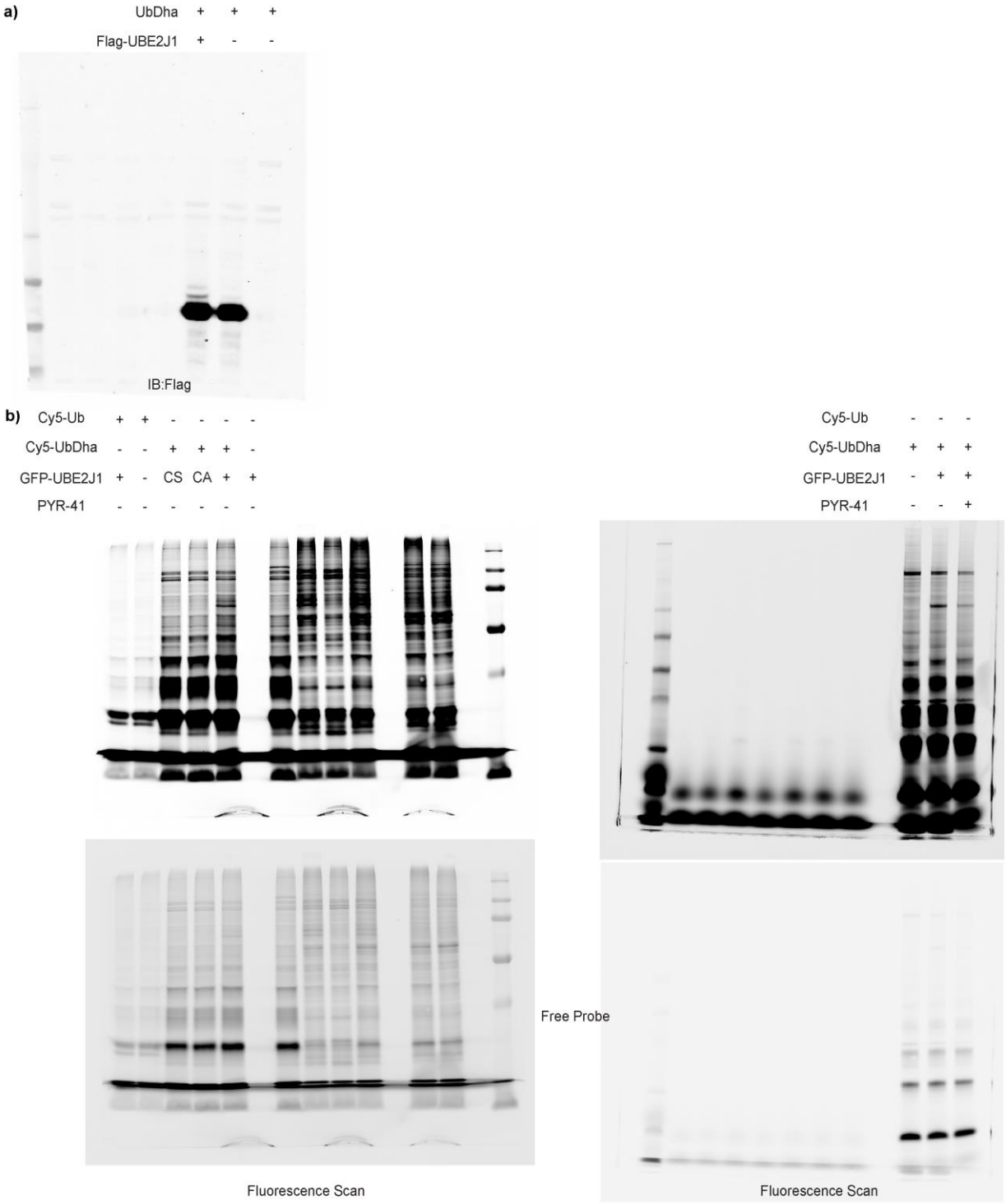
Supplementary Figure 19. Uncut gel images of Figure 2.



Supplementary Figure 20. Uncut gel images of Figure 4. **a**) Corresponds to Figure 4a, Full Fluorescence scan shown in Supplementary Figure 15; **b**) Corresponds to Figure 4c.



Supplementary Figure 21. Uncut gel images of Figure 5. **a**) Corresponds to Figure 5a; **b**) Corresponds to Figure 5b; **c**) Corresponds to Figure 5e.



Supplementary Figure 22. Uncut gel images of Figure 6.

Supplementary Table 1. Data collection and refinement statistics

	UBE2D3-UbDha
Data collection	
Space group	P 1 21 1
Cell dimensions	
a, *b*, *c* (Å)	42.13, 52.27, 53.62
α, β, γ (°)	90, 100.78, 90
Resolution (Å)	52.68 – 2.20 (2.28-2.20) *
R_{merge}	0.077 (0.76)
$I / \sigma I$	8.8 (2.0)
Completeness (%)	98.6 (99.6)
Redundancy	3.5 (3.6)
Refinement	
Resolution (Å)	52.68 – 2.20
No. reflections	40492
R_{work} / R_{free}	19.1 / 24.4
No. atoms	
Protein	1771
Ligand/ion	6
Water	77
B-factors	
Protein	49.4
Ligand/ion	67.9
Water	46.9
R.m.s. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.58

*Highest-resolution shell is shown in parentheses.

References

1. Huang, D.T. *et al.* Basis for a ubiquitin-like protein thioester switch toggling E1-E2 affinity. *Nature.* **445**, 394-398 (2007).
2. Olsen, S.K. & Lima, C.D. Structure of a ubiquitin E1-E2 complex: insights to E1-E2 thioester transfer. *Mol Cell.* 49, 884-896 (2013).
3. Wang, T. *et al.* Evidence for bidentate substrate binding as the basis for the K48 linkage specificity of otubain 1. *J Mol Biol.* **386**, 1011-1023 (2009).