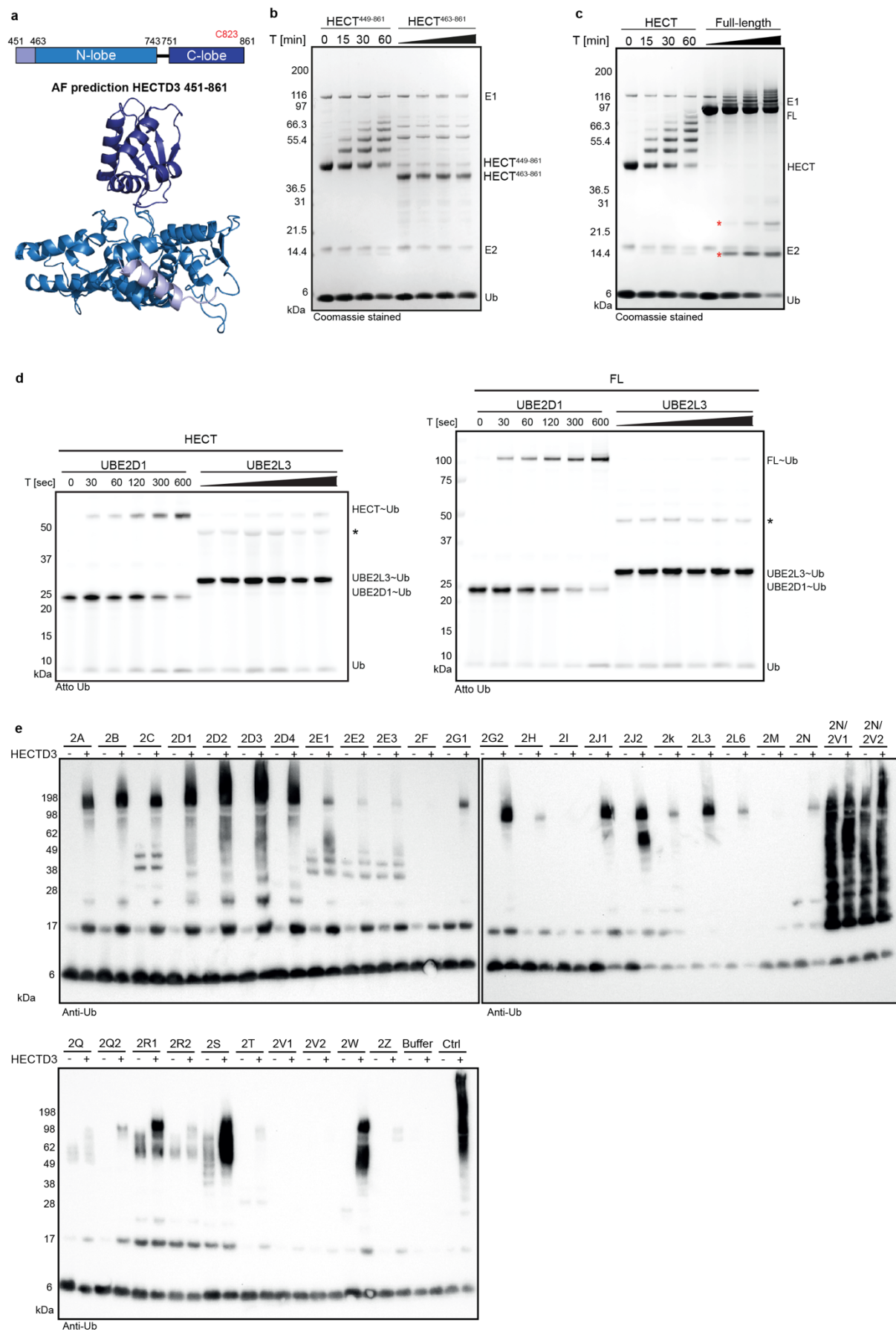
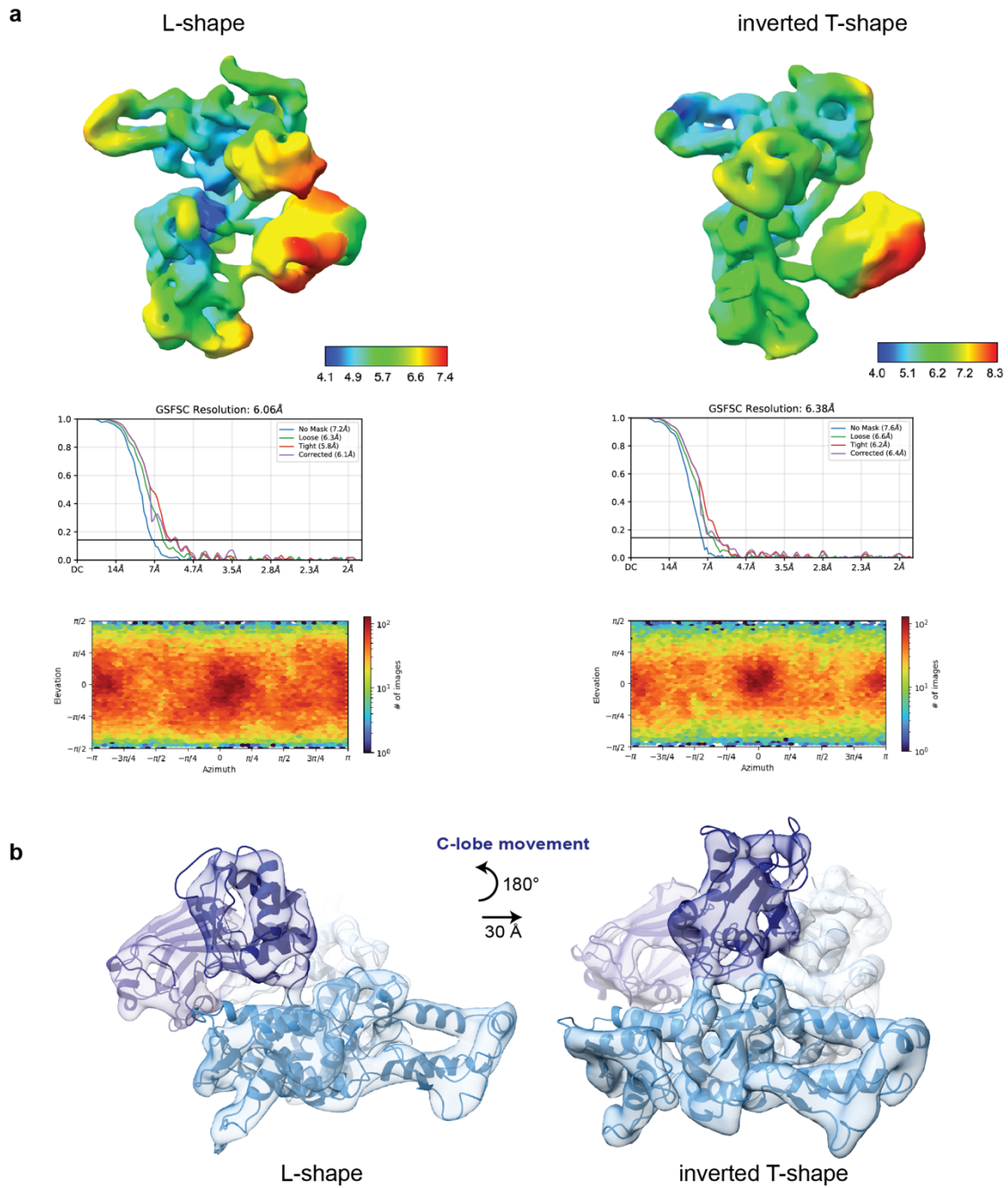




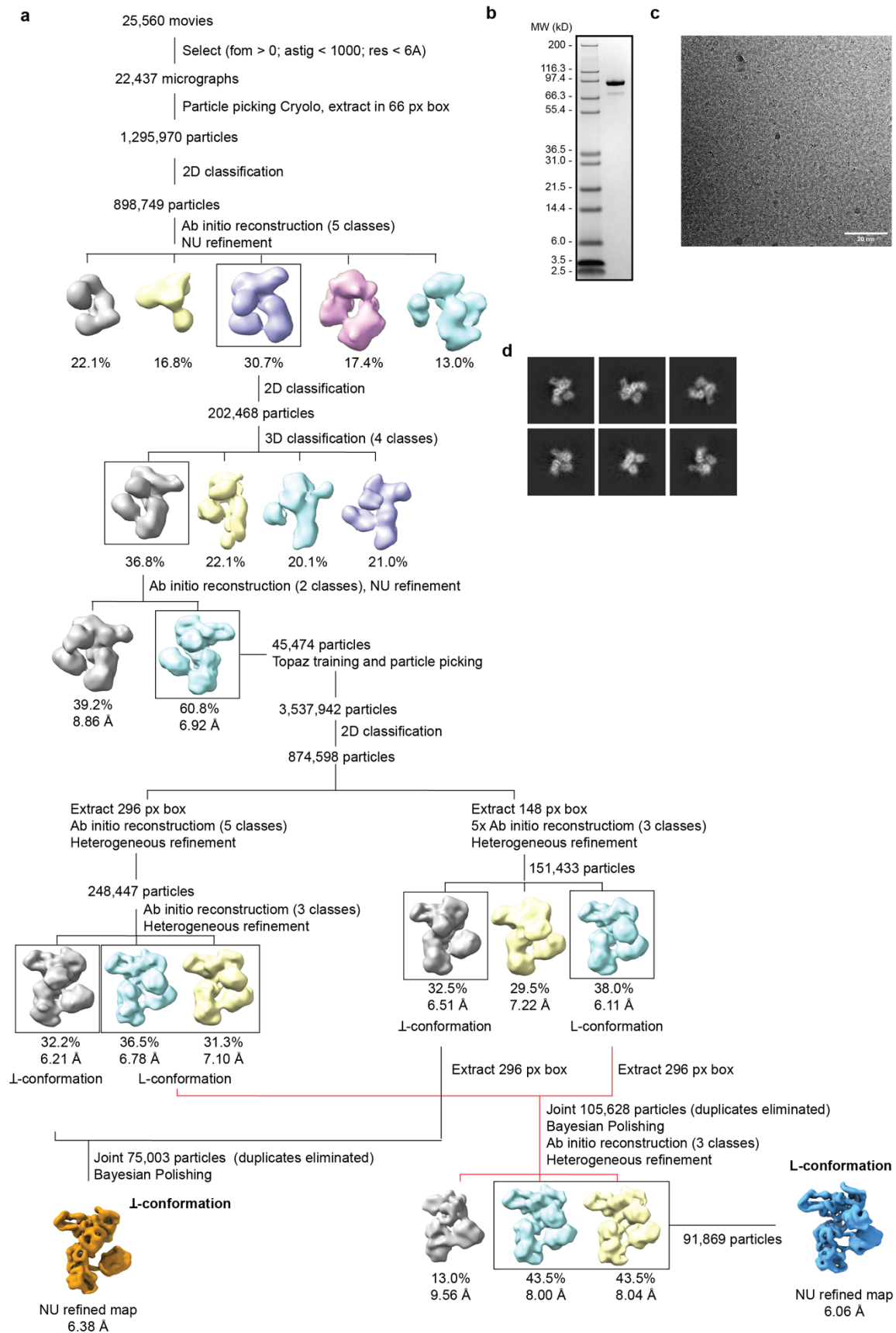
Supplementary Figure 2: Ubiquitination activity of HECTD3

a, Cartoon representation and AlphaFold3 prediction of HECTD3 HECT domain. The HECT domain harbours an N-terminal helical extension (light purple) that interacts with the N-lobe (blue). The C-lobe (dark blue) harbours the catalytic cysteine C823. **b**, Auto-ubiquitination activity of HECTD3 HECT with (449-861) and without (463-861) the helical extension. **c**, Auto-ubiquitination activity of HECTD3 HECT domain compared to HECTD3 full-length. Asterix highlights unanchored di- and tri-ubiquitin. Replicates of this assay are present as controls in multiple figures of the main manuscript and Supplementary Information. **d**, Trans-thiolation activity of HECTD3 HECT (left) and full-length (right) with either UBE2D1 or UBE2L3. Transfer of the labelled ubiquitin from the E2 to the catalytic cysteine was observed in a time-dependent manner (n=2). **e**, E2 screen for HECTD3. HECTD3 auto-ubiquitination reactions were set-up using the E2scanTM Kit version 2 and incubated for 60 min. Source Data are provided as a Source Data file.



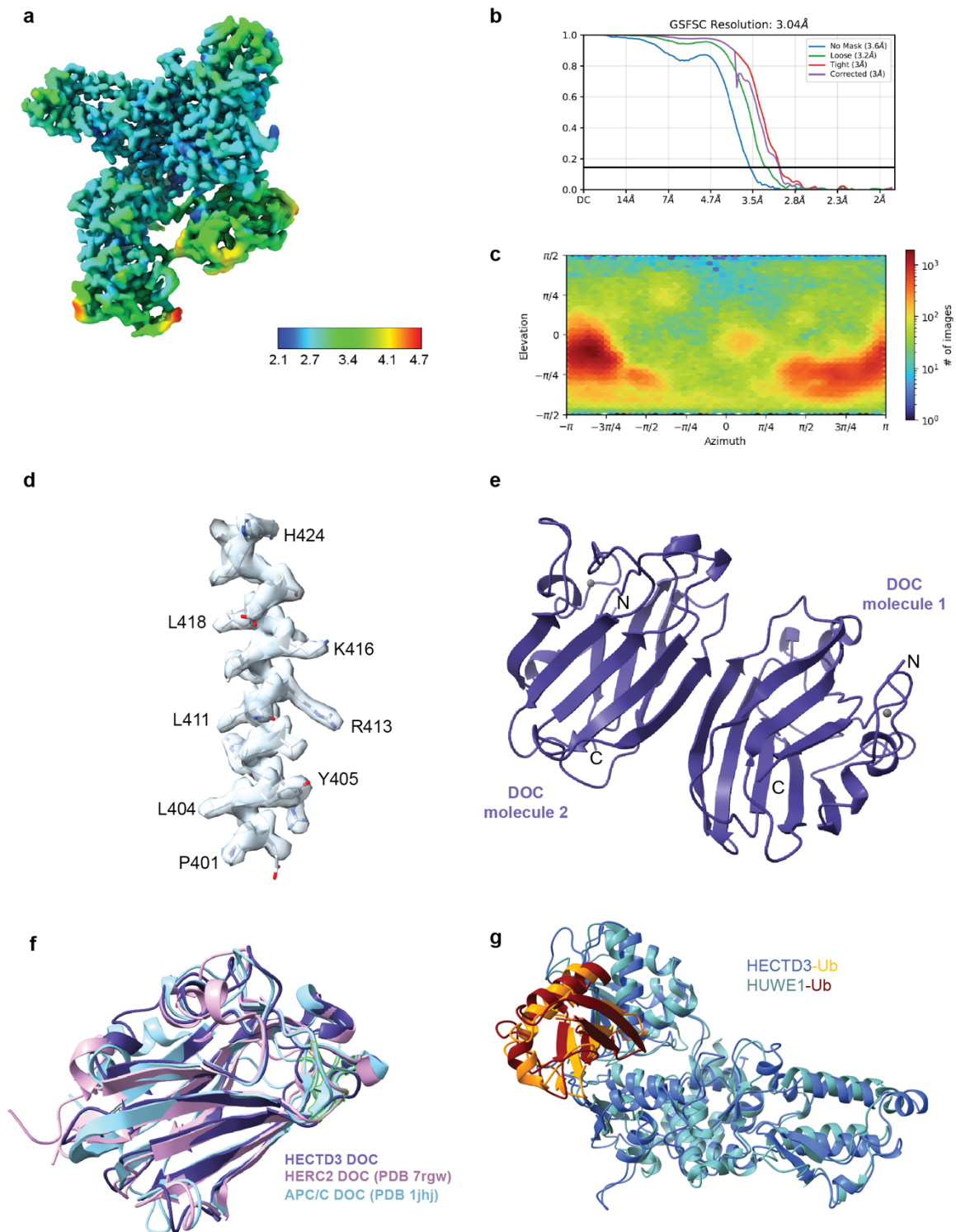
Supplementary Figure 3: Local resolution, particle distribution and C-lobe conformational transition in HECTD3.

a, Density maps colored by local resolution for the HECTD3 L- and “inverted-T” conformations, gold-standard Fourier Shell Correlation (FSC) curves of the 3D reconstructions indicating the resolution at FSC=0.143, and viewing direction distribution plots for the datasets for the two conformations. **b**, Cartoon representation of the two HECTD3 conformations showing the transition of the C-lobe from the HECTD3 L-shape to the “inverted-T” one. The C-lobe rotates 180 ° and shifts by ~30 Å.



Supplementary Figure 4: Cryo-EM data collection and processing workflow for HECTD3.

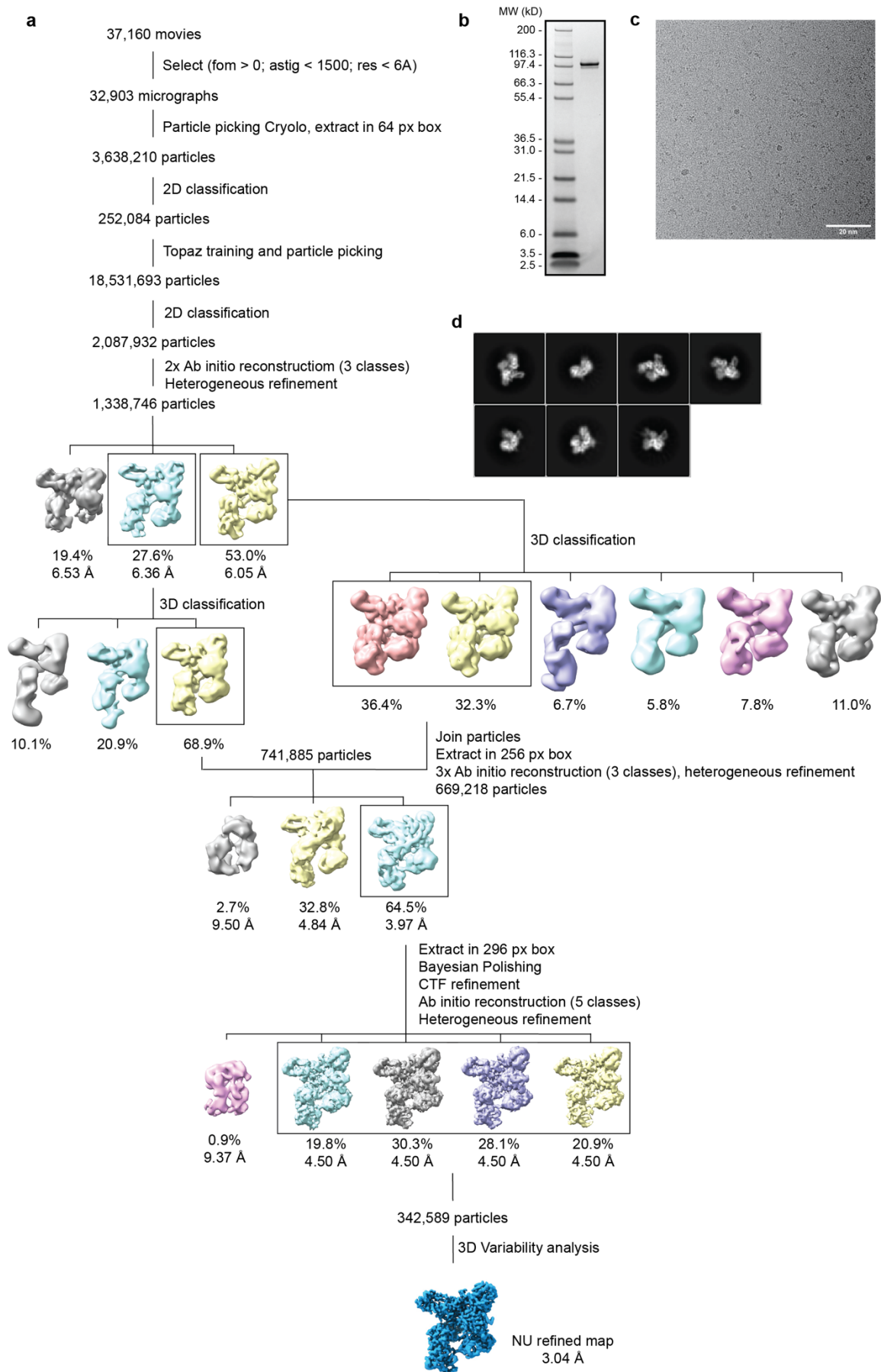
a, Flowchart of the image processing steps of the cryo-EM data. **b**, SDS-PAGE analysis of the cryo-EM HECTD3 sample. **c**, Representative micrograph from 22,437 selected for further processing. **d**, Representative 2D-class averages from the initial 2D classification.



Supplementary Figure 5: Local resolution, particle distribution in HECTD3-Ub and analysis of the DOC domain

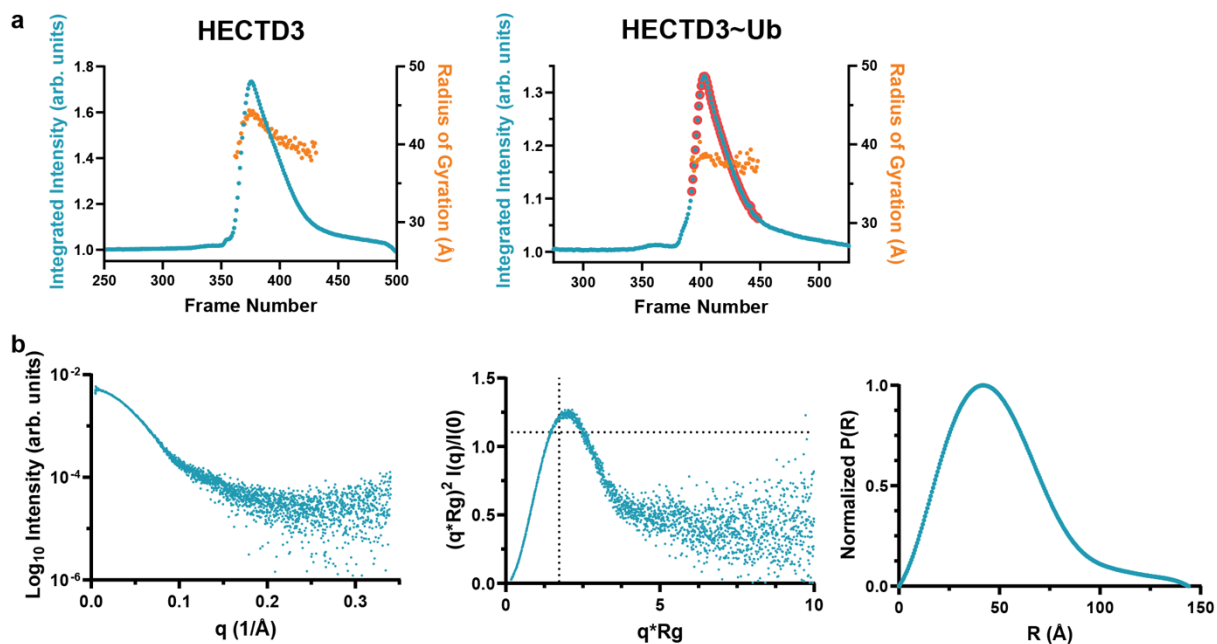
a, HECTD3-Ub density map colored by local resolution. **b**, Gold-standard Fourier Shell Correlation (FSC) curves of the 3D reconstructions indicating the resolution at FSC=0.143. **c**, Viewing direction distribution plot for the HECTD3-Ub dataset. **d**,

Example of the EMReady-sharpened density map surrounding one of the long N-terminal helices (residues 401–425), permitting model building of the HECTD3–Ub structure, with colours consistent with those used in the full-length model. **e**, HECTD3 DOC domain dimer in the crystallographic asymmetric unit. **f**, Alignment of the crystal structure of the HECTD3 DOC domain (dark purple) with that of HERC2 (PDB 7rgw, pink) and the APC/C complex (PDB 1jhl, cyan). **g**, Structural alignment between the ubiquitin-conjugated HECT domains of HECTD3 (blue) and HUWE1 (cyan, PDB 6zy1).



Supplementary Figure 6: Cryo-EM data collection and processing workflow for HECTD3-Ub.

a, Flowchart of the image processing steps of the cryo-EM data. **b**, SDS-PAGE analysis of the cryo-EM sample. **c**, Representative micrograph from 32,903 selected for further processing. **d**, Representative 2D-class averages from the initial 2D classification.



Supplementary Figure 7: SEC-SAXS analysis of HECTD3 and HECTD3-Ub.

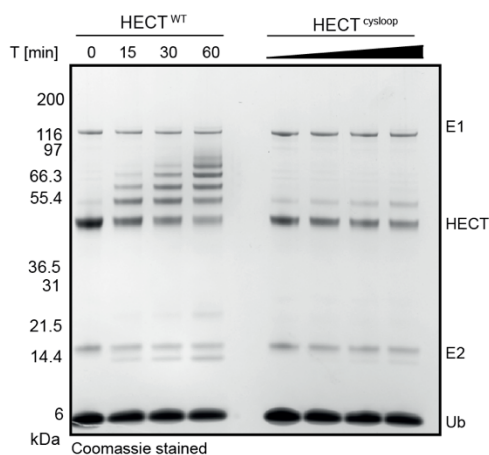
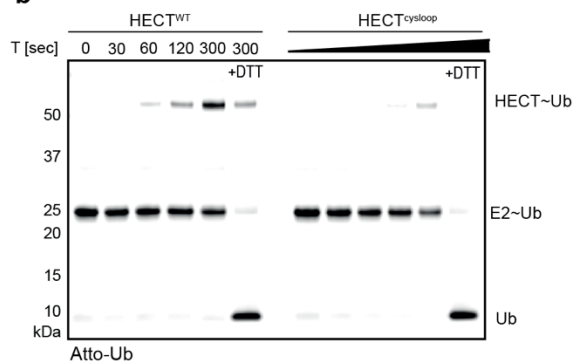
a, Integrated intensity of recorded small angle X-ray scattering as a function of frames recorded off a Superdex 200 3.2 x 300 column at 75 $\mu\text{l}/\text{min}$ flow rate. Plotted as orange dots are the values of the derived radii of gyration for the background subtracted recorded profiles. Highlighted are the frames averaged and used for the analysis of HECTD3-Ub. **b**, HECTD3-Ub X-ray scattering profile, Kratky plot and normalized pair-distribution function $P(R)$. The dotted lines in the Kratky plot are drawn at $qR_g = \sqrt{3}$ and $(qR_g)^2 I(q)/I(0) = 1.104$. Folded and spherical proteins have a maximum where the two lines intersect. Source Data are provided as a Source Data file.

a

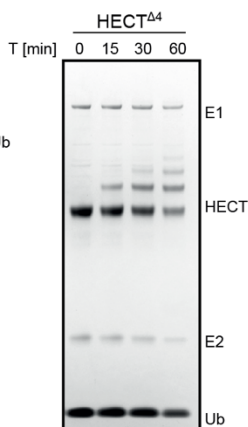
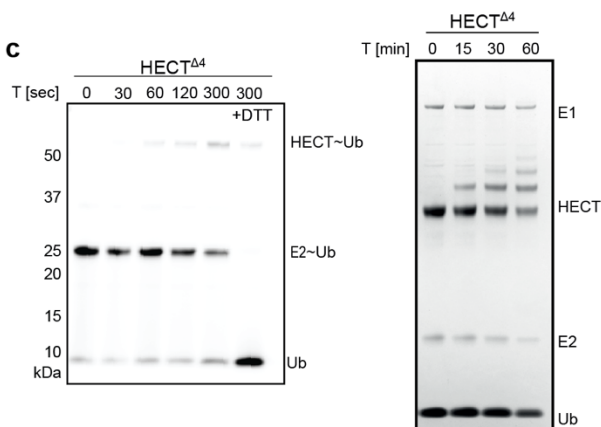
HECTD3 S S T C S S T L F L P H Y A S A K V C E E K L R Y A A Y N C V A I D - T D M S L W E E
 NEDD4 A H T C F N R L D L P P Y E S F E E L W D K L Q M A I E N T Q G F D G V - - - - - D
 NEDD4L A H T C F N R L D L P P Y E T F E D L R E K L L M A V E N A Q G F E G V - - - - - D
 SMURF1 A H T C F N R I D I P P Y E S Y E K L Y E K L L T A V E E T C G F - A V - - - - - E
 SMURF2 A H T C F N R I D I P P Y E S Y E K L Y E K L L T A I E E T C G F - A V - - - - - E
 ITCH S H T C F N R L D L P P Y K S Y E Q L K E K L L F A I E E T E G F - G Q - - - - - E
 WWP1 S H T C F N R L D L P P Y K S Y E Q L K E K L L F A I E E T E G F - G Q - - - - - E
 WWP2 S H T C F N R L D L P P Y K S Y E Q L R E K L L Y A I E E T E G F - G Q - - - - - E
 HECW1 A H T C F N R L D L P P Y P S Y S M L Y E K L L T A V E E T S T F - G L - - - - - E
 HECW2 A H T C F N R L D L P P Y P S F S M L Y E K L L T A V E E T S T F - G L - - - - - E

cys-loop

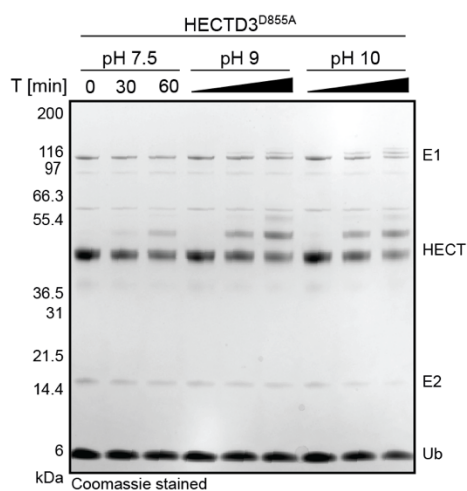
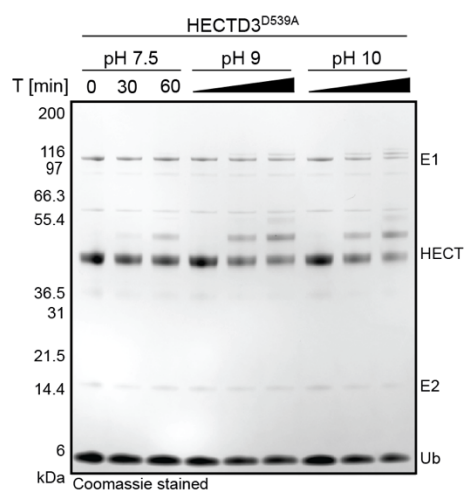
b



c

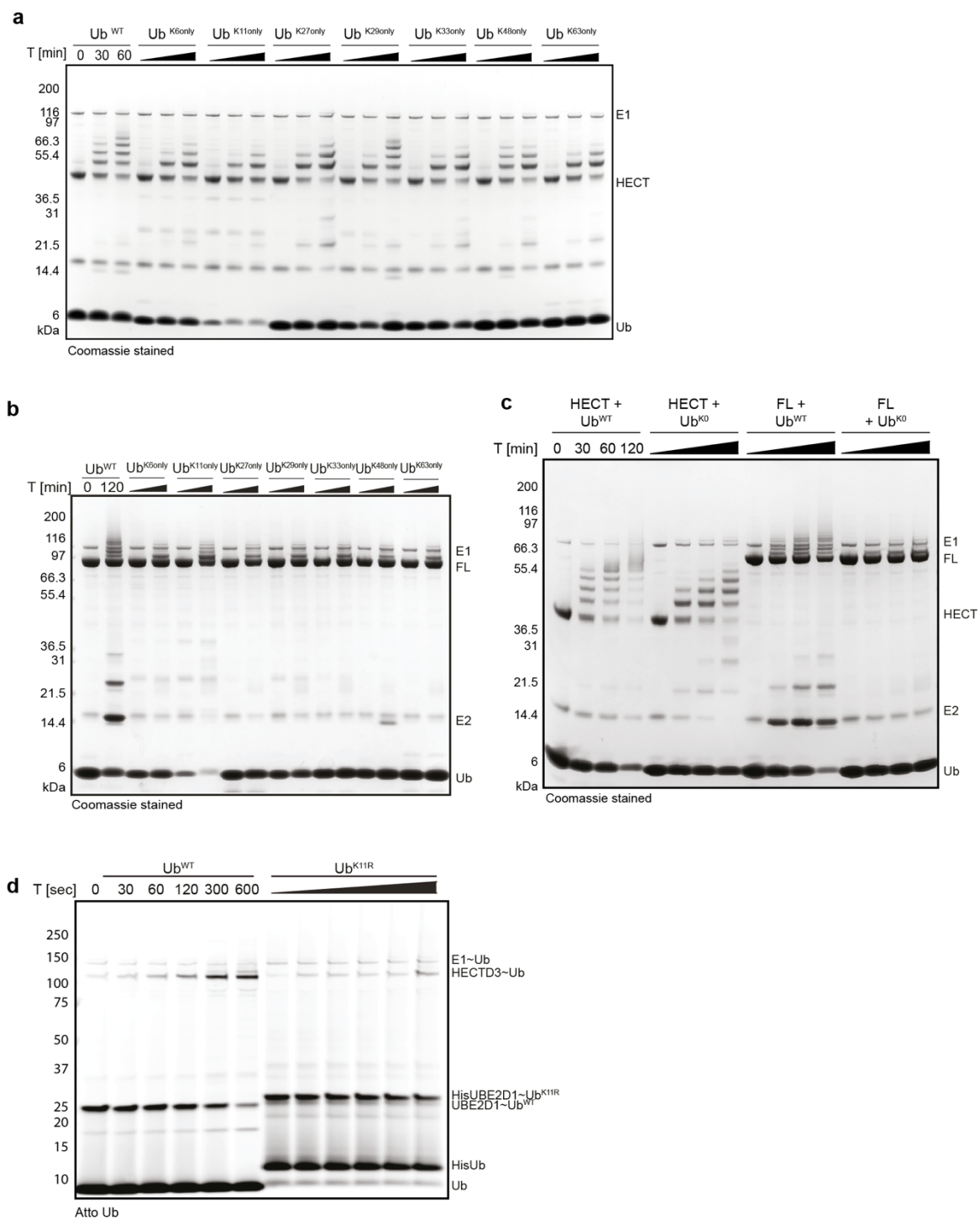


d



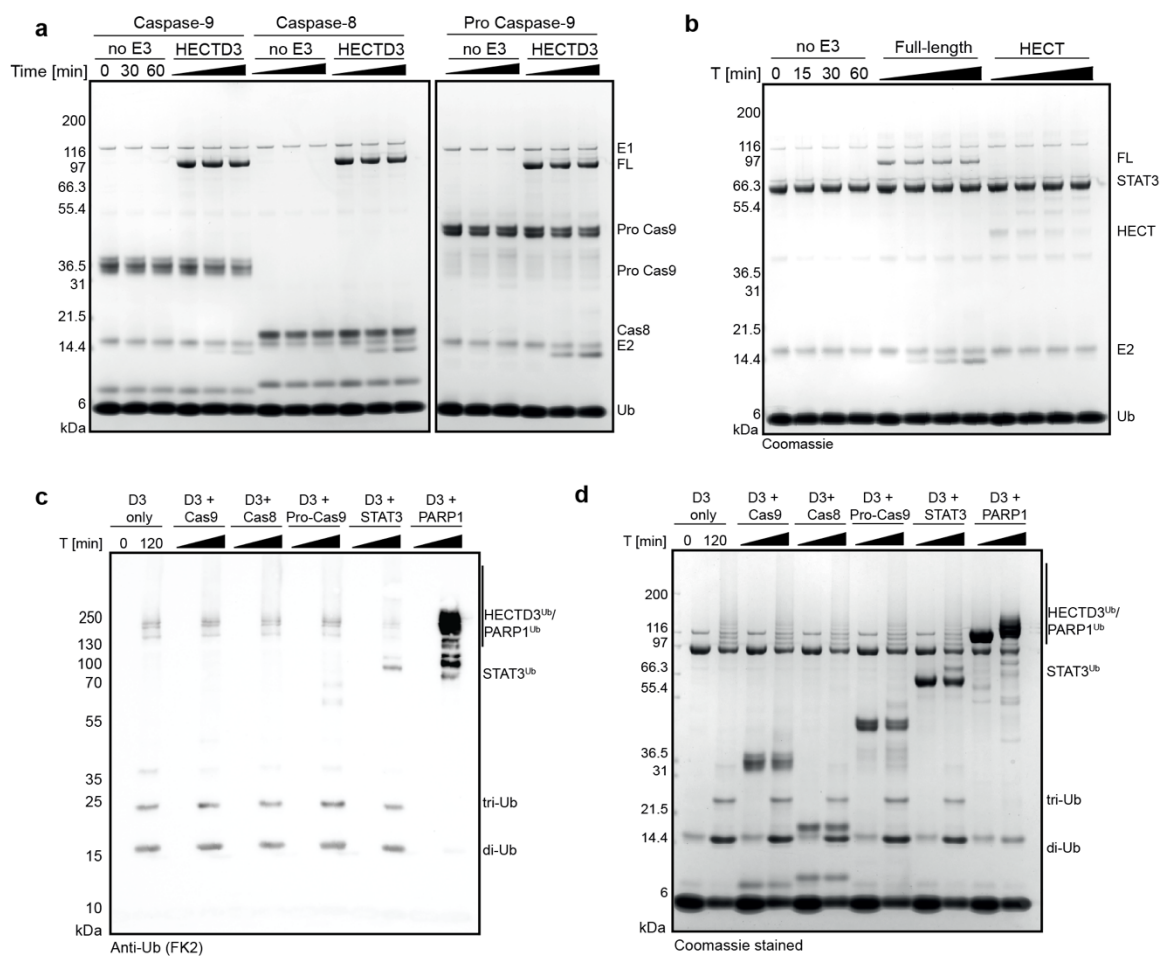
Supplementary Figure 8: Catalytic mechanism and interfaces between HECTD3 and ubiquitin

a, Sequence alignment of the C-terminal region of HECTD3 with members of the NEDD4 HECT family. The loop harbouring the catalytic cysteine is highlighted (cys-loop). Residues are coloured based on the Clustal Colour scheme. **b**, Left: Trans-thiolation activity of HECTD3 HECT wild-type (WT) and a HECT variant that has the catalytic loop exchanged to that of NEDD4 HECTs (STCS to HTCF). Right: Auto-ubiquitination reaction of the same proteins as in **b** (n=2). **c**, Left: Trans-thiolation activity of HECTD3 HECT that has the last four amino-acids deleted ($\Delta 4$). Right: Auto-ubiquitination reaction of HECTD3 HECT $\Delta 4$ (n=2). **d**, Auto-ubiquitination reaction of HECTD3 HECT D539A (left) and D855A (right) performed at different pH (pH 7.5, pH 9 and pH 10). Reactions were analysed after 0, 30 and 60 min and analysed by SDS-PAGE and Coomassie staining (n=1). Source Data are provided as a Source Data file.



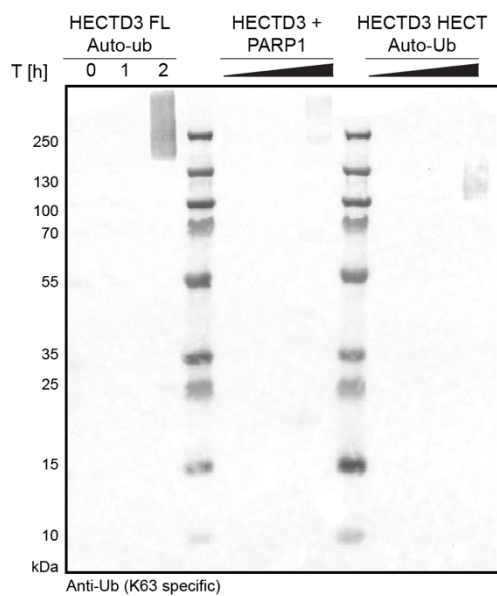
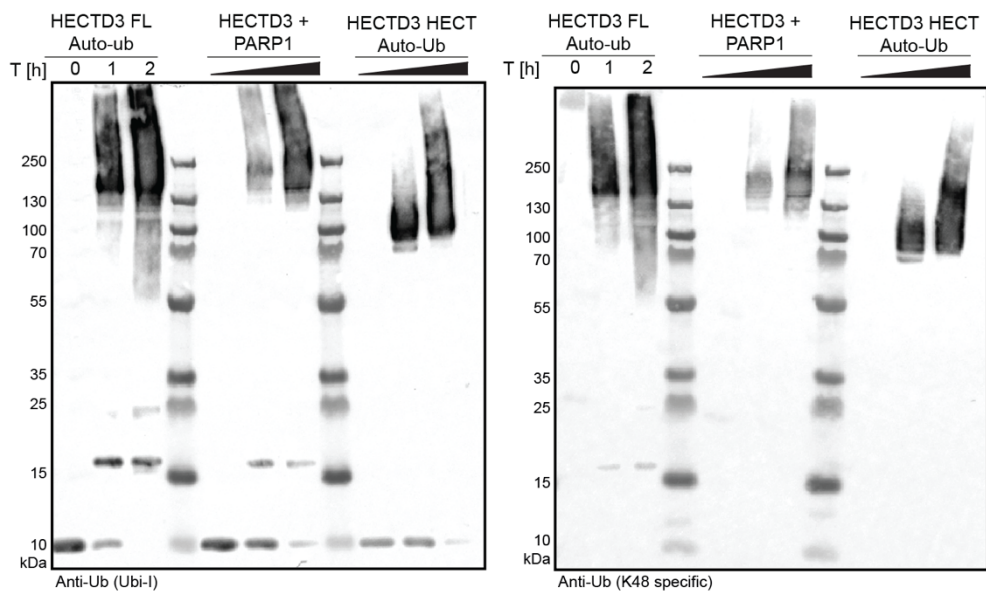
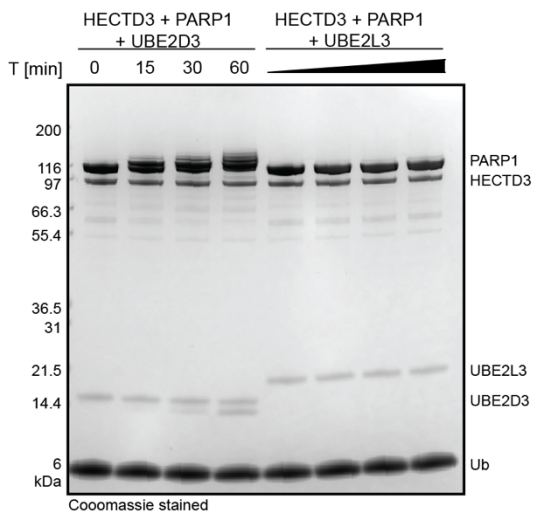
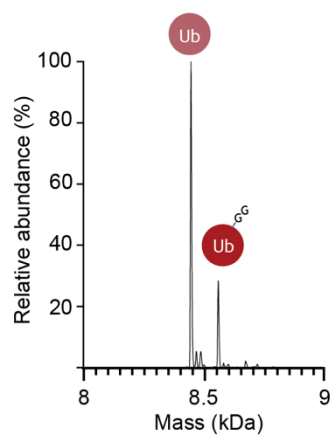
Supplementary Figure 9: Chain linkage selectivity of HECTD3

Auto-ubiquitination activity of **a**, HECTD3 HECT domain (n=1) and **b**, HECTD3 full-length with wild-type (WT) ubiquitin and ubiquitin that harbours a lysine to arginine mutation for all seven lysine residues (K0)(n=2). Samples were analysed using Coomassie staining **c**, Auto-ubiquitination activity of HECTD3 HECT domain with either wild-type (WT) ubiquitin or a ubiquitin variant that has all seven lysine residues mutated (K0) over 120 min (n=3). **d**, Trans-thiolation activity of HECTD3 full-length with wild-type ubiquitin (Ub^{WT}) or ubiquitin that harbours a K11R mutation (Ub^{K11R})(n=2). Transfer of fluorescently labelled ubiquitin from the E2 to the catalytic cysteine of HECTD3 was observed in a time-dependent manner for both ubiquitin variants. Please note the K11R ubiquitin contains a N-terminal His₆-tag and therefore runs higher on the SDS Coomassie-stained gel than WT ubiquitin. Source Data are provided as a Source Data file.



Supplementary Figure 10: Substrate ubiquitination by HECTD3

a, Substrate ubiquitination assays with Caspase-9, Caspase-8 and pro Caspase-9 monitored in absence of E3 (no E3) and in presence of HECTD3 full-length (FL). **b**, Substrate ubiquitination assays with STAT3. Ubiquitination of STAT3 was monitored in absence of E3 (no E3) and in presence of HECTD3 HECTD domain and HECTD3 full-length (FL). **c**, Substrate ubiquitination assays with higher E3 concentration to highlight auto-ubiquitination analysed by western blot. Samples contained either HECTD3 only or were supplemented with Caspase 9 (Cas9), Caspase 8 (Cas8), pro-Caspase 9 (Pro-Cas9), STAT3 or PARP1. **d**, as **c**, but with Coomassie staining. Source Data are provided as a Source Data file.

a**b****c**

Supplementary Figure 11: Analysis of auto- and substrate ubiquitination with chain-specific antibodies

a, The auto-ubiquitination activity of HECTD3 HECT domain, HECTD3 full-length and PARP1 ubiquitination was analysed by a general anti-Ub and linkage specific anti K48 and K63 antibodies. **b**, PARP1 ubiquitination assays with two different E2s (UBE2D3 and UBE2L3 (n=2)). **c**, Ub-clipping analysis of PARP1 ubiquitination by HECTD3. Substrate-ubiquitination assays were performed and subsequently treated with Lb^{pro} protease. Composition of ubiquitin moieties was analysed by intact-mass spectrometry. Source Data are provided as a Source Data file.

Supplementary Figure 12: DOC domain binding and potential E2 binding interfaces

a, Consurf analysis of HECTD3 DOC domain sequence. Residues are based on conservation as shown and annotated based on whether they are surface exposed, buried, functional or structural residues. **b**, PARP1 ubiquitination assays with HECTD3 wild-type (WT) and two DOC domain mutants (Y252A and F256A) analysed by Coomassie-staining (left) or western-blot (right)(n=2) **c**, Auto-ubiquitination assays with HECTD3 full-length wild-type (WT) or full-length HECTD3 that harbours mutations in the DOC domain (Y252A and F256A)(n=1). **d**, Trans-thiolation activity of HECTD3 full-length type (WT) or full-length HECTD3 that harbours mutations in the DOC domain (Y252A and F256A). Transfer of fluorescently labelled ubiquitin from the E2 to the catalytic cysteine of HECTD3 was observed in a time-dependent manner (n=2). **e**, Structural alignment of apo HECTD3 in the inverted T-shape with NEDD4L (green) in complex with UBE2D2-Ubiquitin (PDB 3jw0, orange and pink, respectively). Both structures of the HECT align well (left). However, in the context of the full-length protein, the position of ubiquitin sterically clashes with the N-terminal region (right) as seen in the zoomed-in panel. Source Data are provided as a Source Data file.

Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics.

	HECTD3-Ub EMD-53804/9R85	HECTD3-L EMD-53852/9R94	HECTD3-T EMD-53852/9R94
Data collection and processing			
Magnification	x130,000	x130,000	x130,000
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å)	40.99 (31 movies)	39.76 (31 movies)	39.76 (31 movies)
Defocus range (μm)	-0.6 to -3.0	-1.2 to -3.0	-1.2 to -3.0
Pixel size (Å)	0.95	0.95	0.95
Symmetry imposed	C1	C1	C1
Initial particles images (no.)	2,087,932	3,537,942	3,537,942
Final particles images (no.)	342,589	91,869	75,003
Map resolution (Å)	3.0	6.1	6.4
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.0 - 5.3	4.1 - 7.5	4.5 - 8.9
Refinement			
Initial model	AF2	HECTD3-Ub	HECTD3-Ub
Model resolution (Å)	2.9 3.1	4.9 5.7	5.6 6.4
FSC threshold	0.143 0.5	0.143 0.5	0.143 0.5
Map sharpening B factor (Å ²)	-145.5	-489.0	-588.8
Model composition			
Non-hydrogen atoms	14072	6441	6581
Protein residues	886	807	812
Bfactors (Å ²)	4.73/182.76/74.37	48.40/411.36/204.03	55.73/433.67/224.34
min/max/mean			
RMS deviations			
Bond length (Å)	0.002	0.002	0.002
Bond angles (°)	0.419	0.440	0.474
Validation			
MolProbity score	0.96	1.67	1.62
Clashscore	1.91	14.4	12.77
Rotamers outliers (%)	0.26	0.86	0.85
Ramachandran plot			
Favoured (%)	98.63	98.38	98.14
Allowed (%)	1.37	1.62	1.86
Disallowed (%)	0	0	0
Map-model correlation coefficients			
CCmask	0.80	0.82	0.79
CCbox	0.67	0.84	0.82

Supplementary Table 2. Data collection and refinement statistics for HECTD3**DOC domain**

	HECTD3 DOC (PDB 9R6V)
Beamline	MASSIF-3 (ESRF)
Wavelength (Å)	0.96770
Resolution range (Å)	38.85 - 1.61 (1.65 - 1.61)
Space group	<i>P</i> 21 21 21
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	47.82, 52.64, 115.16
α , β , γ (°)	90, 90, 90
Total reflections	271730 (19916)
Unique reflections	40411 (2852)
Multiplicity	6.7 (7.0)
Completeness (%)	99.2 (99.6)
Mean <i>I</i> / σ (<i>I</i>)	11.7 (1.6)
Wilson B-factor	19.54
R-meas	0.0947 (1.36)
R-pim	0.0364 (0.512)
CC1/2	0.998 (0.455)
CC*	0.999 (0.791)
Reflections used in refinement	38155 (2721)
Reflections used for R-free	1930 (117)
R-work	0.195 (0.300)
R-free	0.212 (0.326)
Number of non-hydrogen atoms	2339
macromolecules	2162
ligands	2
solvent	175
Protein residues	278
RMS (bonds) (Å)	0.004
RMS (angles) (°)	0.69
Ramachandran favored (%)	98.2
Ramachandran allowed (%)	1.8
Ramachandran outliers (%)	0.0
Rotamer outliers (%)	0.0
Clashscore	1.39
Average B-factor (Å ²)	25.07
macromolecules (Å ²)	24.52
ligands (Å ²)	21.31
solvent (Å ²)	31.91

Statistics for the highest-resolution shell are shown in parentheses.

Supplementary Table 3: SAXS acquisition and processing parameters for HECTD3-Ub

Data collection

Beamline	B21 at Diamond
Wavelength	0.9464 Å
q range (Å ⁻¹)	0.0045 – 0.34
Detector	EigerX 4M (Dectris)
Beamsize	< 75 µm
Energy	13.1 keV
Column	Superdex 200 3.2 x 300 (total volume = 2.4 ml)
Flow rate (ml/min)	0.075
Temperature (°C)	15

Samples details

Sample volume (µl)	50
Sample concentration (mg/ml)	5.5 (50 µM)

Structural parameters

Reciprocal Space

Rg (Å) Guinier	39.3 ± 0.09
I(0) (cm ⁻¹)	0.005213 ± 0.000006
qRg limit	1.26

Real Space

Rg (Å) P(R)	39.2 (34.1 ¹)
I(0) (cm ⁻¹)	0.005213
Rc (Å)	23.2
Dmax (Å)	144 (118 ¹)
Porod volume (Å ³)	192562

Molecular mass determination

Theoretical MW (kDa)	106
DATPOROD MW (kDa) (Vp/1.7)	113
SAXS MoW2 (q = 0.25 Å ⁻¹)	109

Fit of cryo-EM structure

χ ² (crysol)	3.8
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¹ Values calculated for the cryo-EM structure of HECTD3-Ub with Crysol