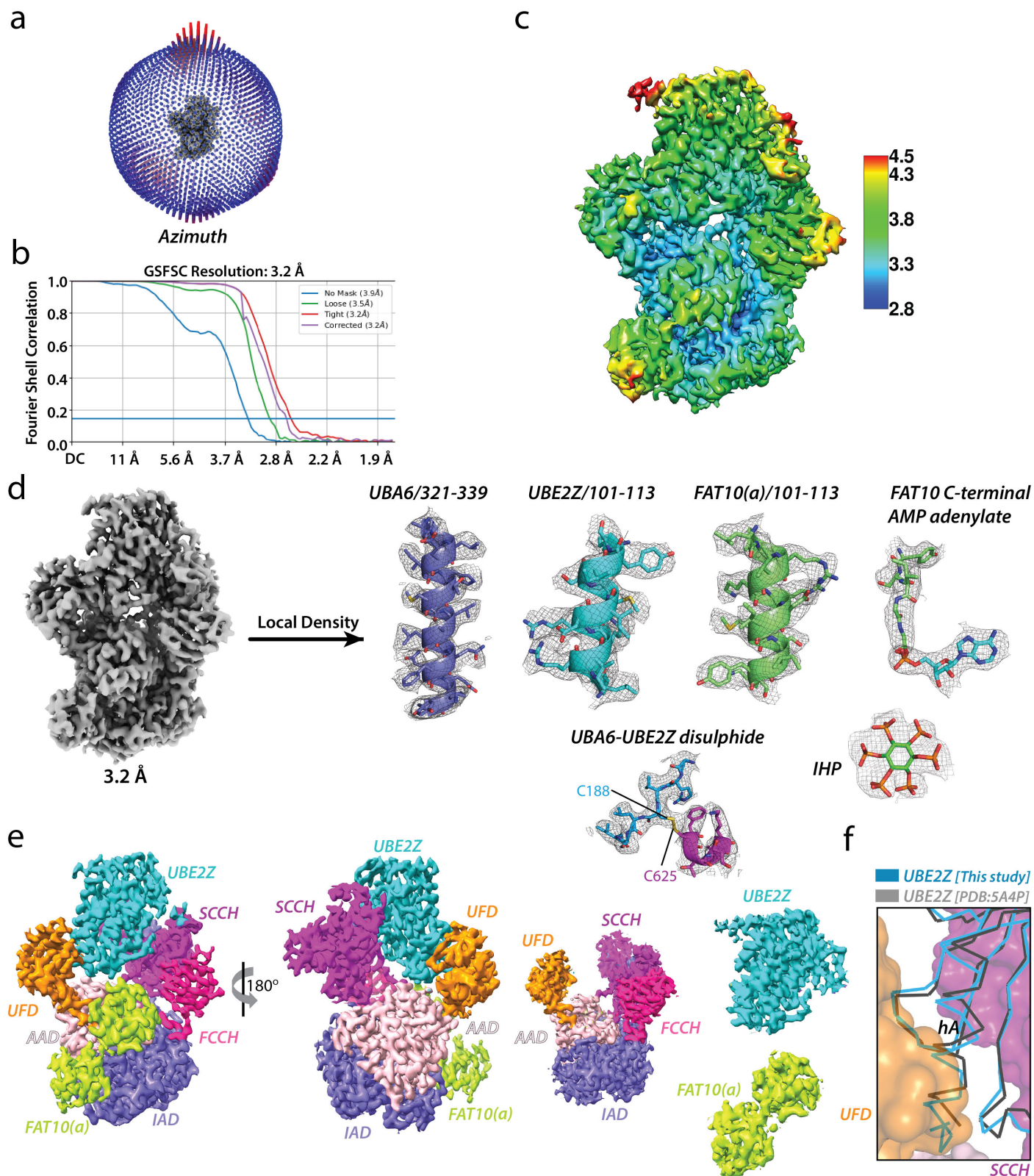
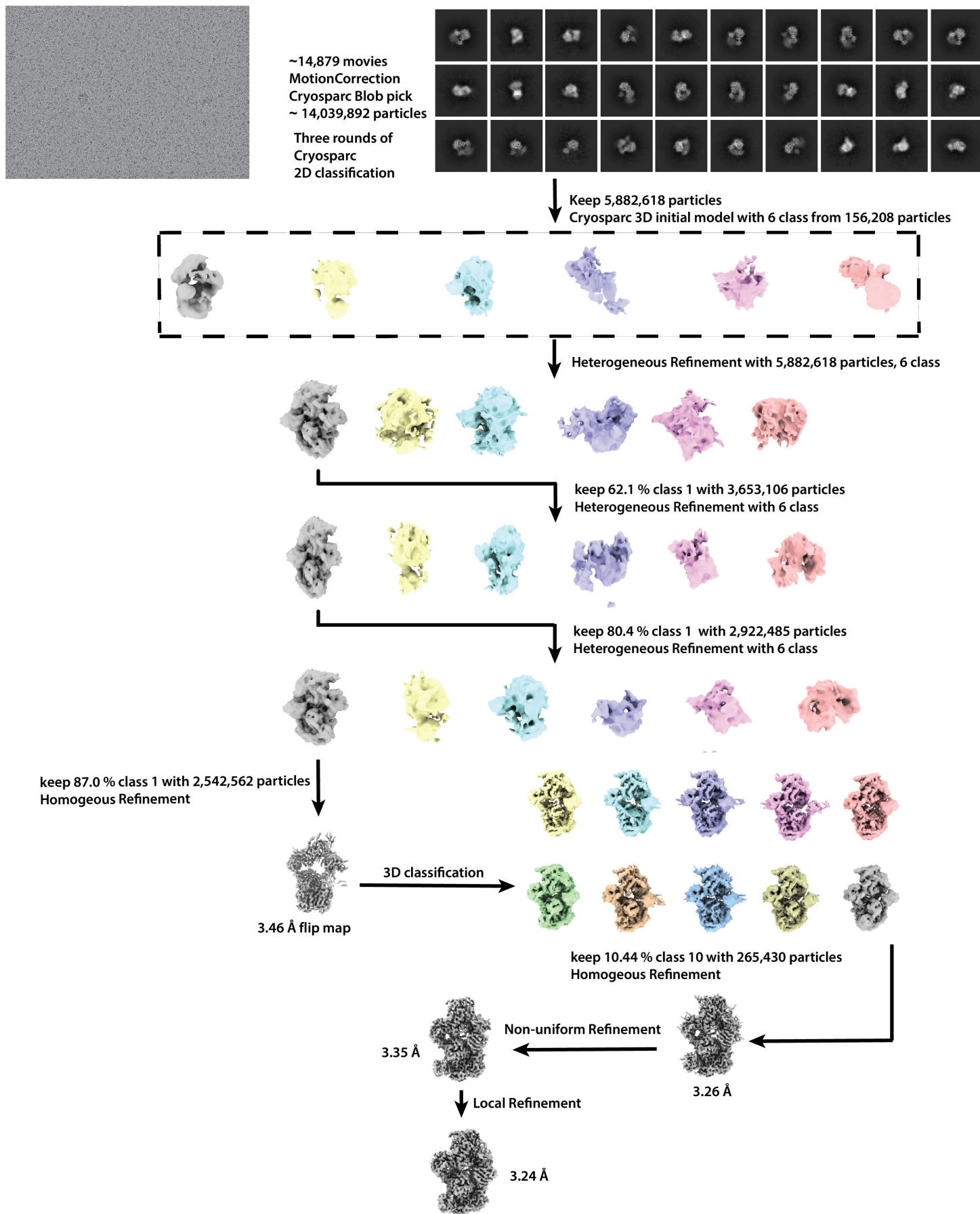


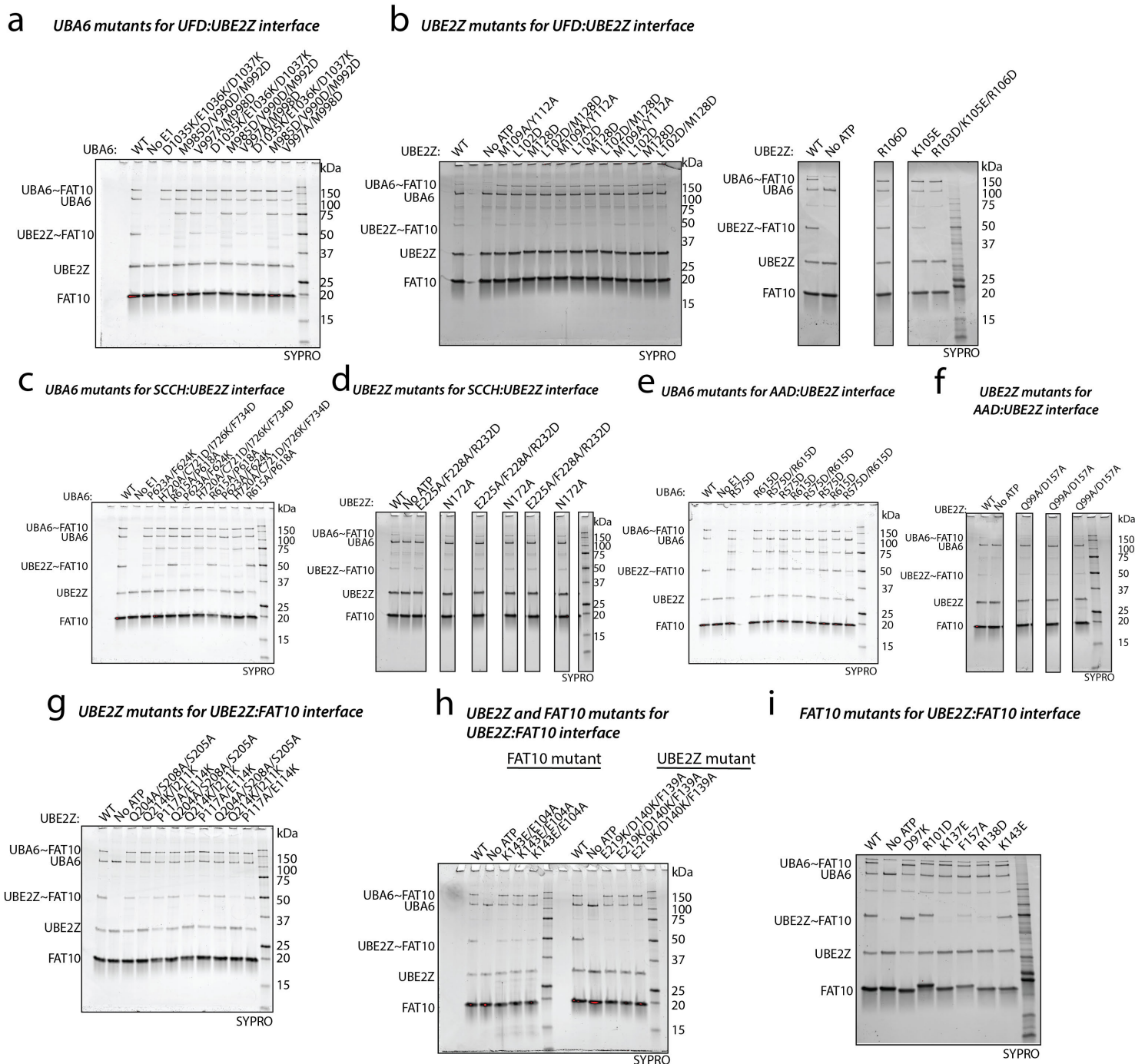


Supplementary Fig. 1 | Quality of the UBA6-UBE2Z/FAT10(a) complex cryo-EM reconstruction.

a, The final Euler angle distribution of particles used for 3D refinement. **b**, Fourier Shell Correlation (FSC) plot from two half maps, with an overall resolution of 3.2 Å as determined at 0.143 criterion. **c**, Local resolution map color-coded from lower (red) to higher resolution (blue). **d**, Overall map (left) and detailed views of local cryo-EM density for UBA6, UBE2Z, FAT10, FAT10 C-terminal AMP adenylate and Ins6 regions of the complex. **e**, Cryo-EM map of the UBA6-UBE2Z/FAT10(a) complex with two views related by a 180° rotation about the y-axis (left). Maps of the individual subunits are shown in the right with IAD (slate), AAD (lightpink), FCCH (red), SCCH (magenta), UFD (orange), UBC9 (thorium) and FAT10(a) (lime). **f**, Superposition of apo UBE2Z (PDB: 5A4P, grey30) and UBE2Z from the UBA6-UBE2Z-FAT10(a) complex (cyan). Magnified view of the helix α A-UFD interface with the E2s shown as ribbon representation.

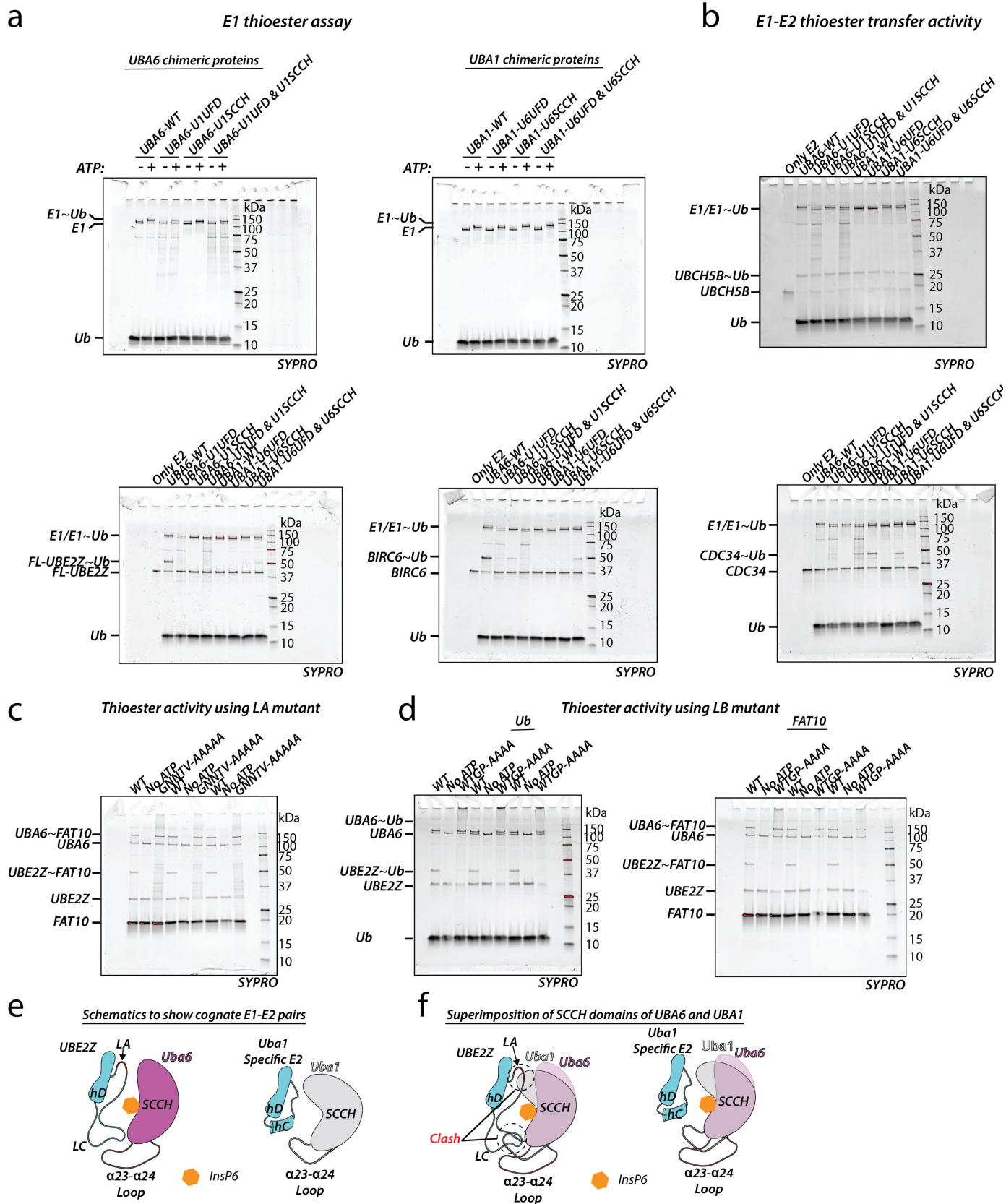


Supplementary Fig. 2 | Cryo-EM data processing tree of the UBA6-UBE2Z/FAT10(a) complex.
Cryo-EM data process flow depicting the major steps of 2D and 3D classification leading to the best map.



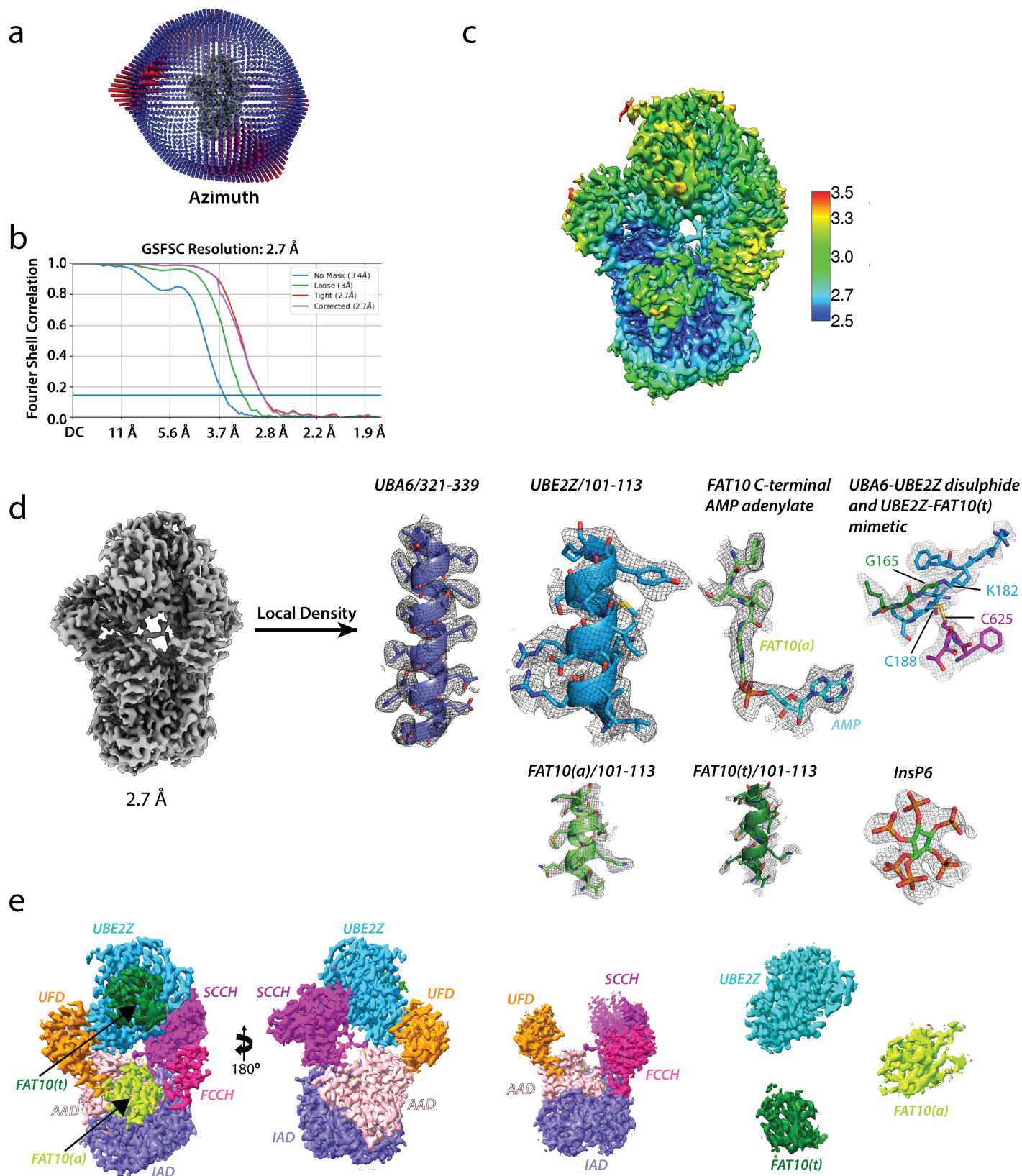
Supplementary Fig. 3 | Structure-function analysis of UBA6-UBE2Z and UBE2Z-FAT10(t) interfaces.

a, Raw data for thioester transfer assay with UFD mutants for UFD:UBE2Z interface presented in Fig. 3c, left. **b**, Raw data for thioester transfer assay for UFD:UBE2Z interface with UBE2Z mutants presented in Fig. 3c, right. **c**, Raw data for thioester transfer assay with UBA6 mutants for SCCH:UBE2Z interface presented in Fig. 3d, left. **d**, Raw data for thioester transfer assay with UBE2Z mutants for SCCH:UBE2Z interface presented in Fig. 3d, right. **e**, Raw data for thioester transfer assay with UBA6 mutants for AAD:UBE2Z interface presented in Fig. 3e, left. **f**, Raw data for thioester transfer assay with UBE2Z mutants for AAD:UBE2Z interface presented in Fig. 3e, right. **g**, Raw data for thioester transfer assay with UBE2Z mutants for UBE2Z:FAT10(t) interface presented in Fig. 7c, bottom. **h**, Raw data for thioester transfer assay with UBE2Z and FAT10(t) mutants for UBE2Z:FAT10(t) interface presented in Fig. 7c, bottom. **i**, Raw data for thioester transfer assay with FAT10(t) mutants for UBE2Z:FAT10(t) interface presented in Fig. 7c, bottom. All the gels presented above are representative results from $n=3$ technical replicates independently repeated with similar results. For 3b, d and f, some of the mutants were not used for analysis. Uncropped gel images can be found in source data file. Source data are provided as source data file.



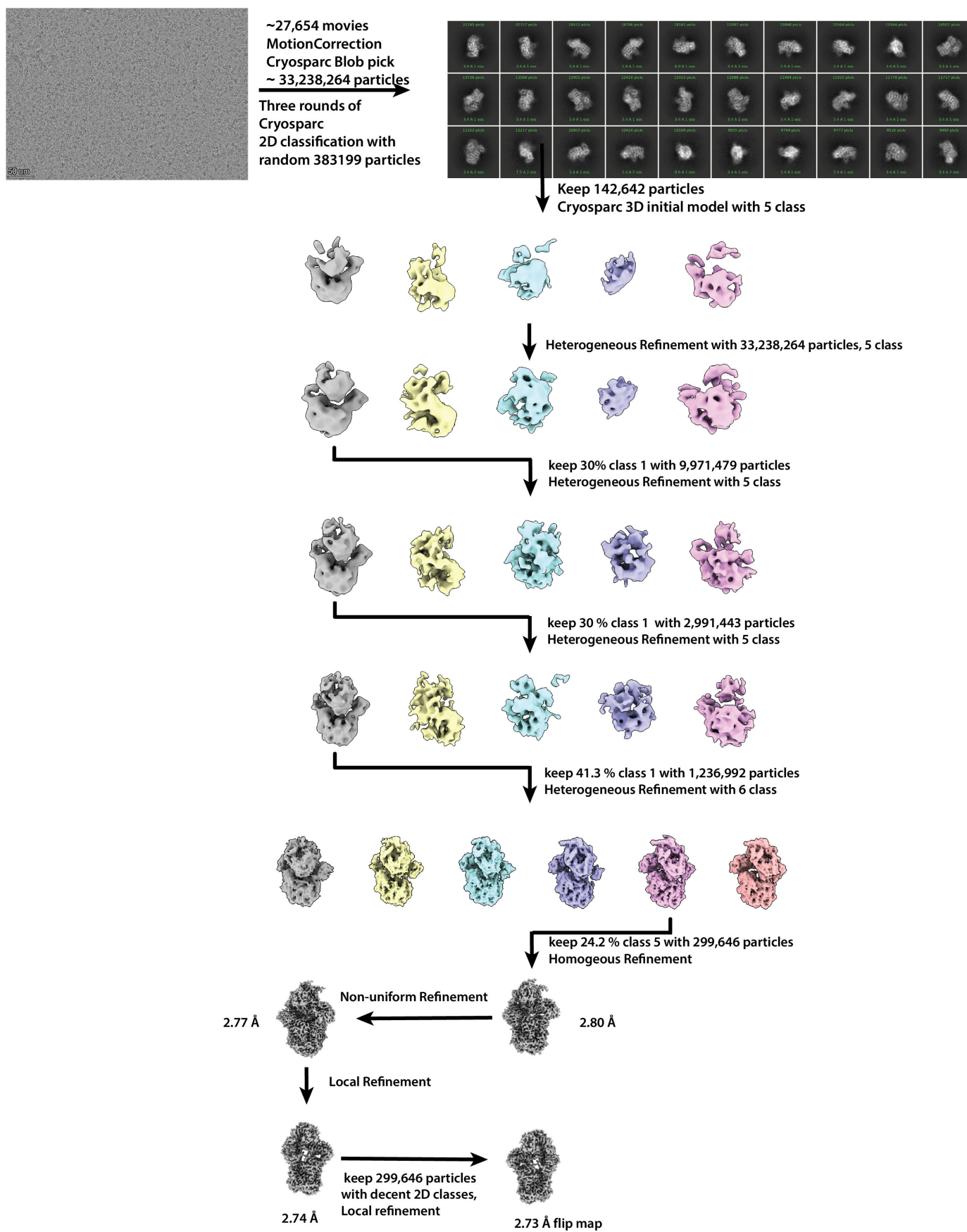
Supplementary Fig. 4 | Functional analysis using chimeric E1 proteins and a panel of E2s.

a, E1 activation assay using UBA6 and UBA1 chimeric proteins. **b**, Raw data for thioester transfer assay with chimeric E1 proteins using UBCH5B, FL-UBE2Z, BIRC6 and CDC34 respectively presented in Fig. 4b. **c**, Raw data for thioester transfer assay using LA mutant of UBE2Z (GNNTV-AAAAA) presented in Fig. 5, bottom. **d**, Raw data for thioester transfer assay using LB mutant of UBE2Z (WTGP-AAAA) with Ub or FAT10 presented in Fig. 7f. Source data are provided as source data file. **e**, Schematics to show cognate E1-E2 pairs with key structural elements labeled. **f**, Superimposition of the SCCH domains as shown in panel e, highlighting the clash between loop LA and LC with the SCCH domain and $\alpha 23-\alpha 24$ loop of UBA1, respectively.



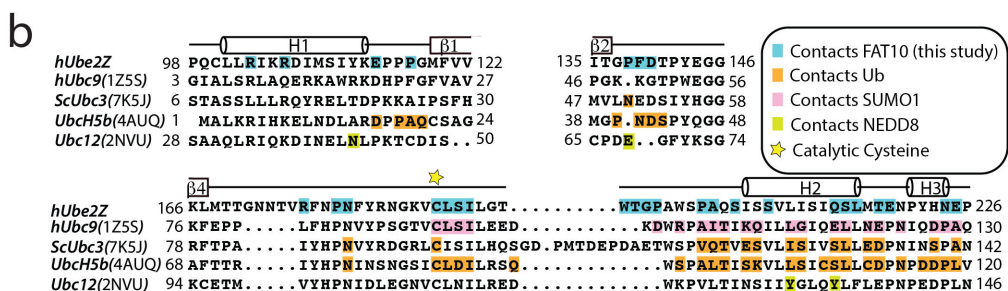
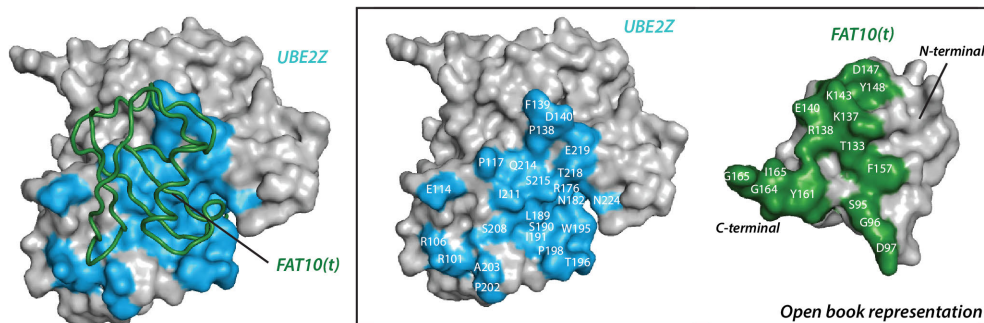
Supplementary Fig. 5 | Quality of the UBA6-UBE2Z-FAT10(t)/FAT10(a) complex cryo-EM reconstruction.

a, The final Euler angle distribution of particles used for 3D refinement. **b**, Fourier Shell Correlation (FSC) plot from two half maps, with an overall resolution of 2.7 Å as determined at 0.143 criterion. **c**, Local resolution map color-coded from lower (red) to higher resolution (blue). **d**, Overall map (left) and detailed views of local cryo-EM density for UBA6, UBE2Z, FAT10(a), FAT10(t), FAT10 C-terminal AMP adenylate and InsP6 regions of the complex. **e**, Cryo-EM map of the UBA6-UBE2Z-FAT10(t)/FAT10(a) complex with two views related by a 180° rotation about the y-axis (left). Maps of the individual subunits are shown in the right with IAD (slate), AAD (lightpink), FCCH (red), SCCH (magenta), UFD (orange), UBE2Z (cyan), FAT10(a) (lime) and FAT10(t) (forest).

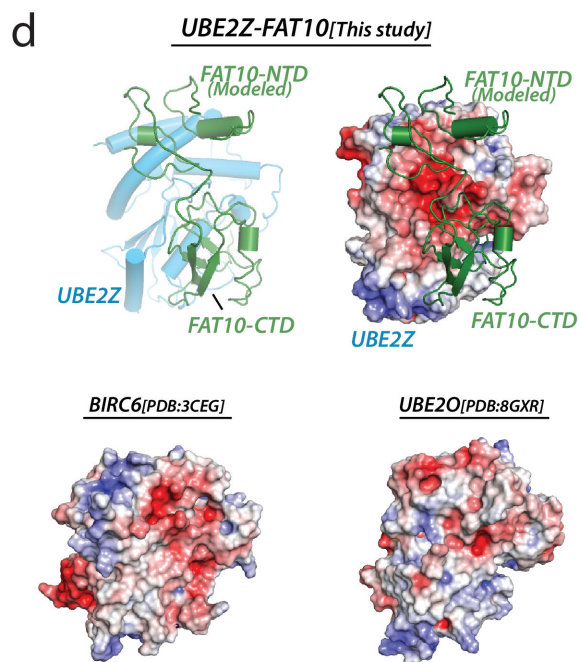
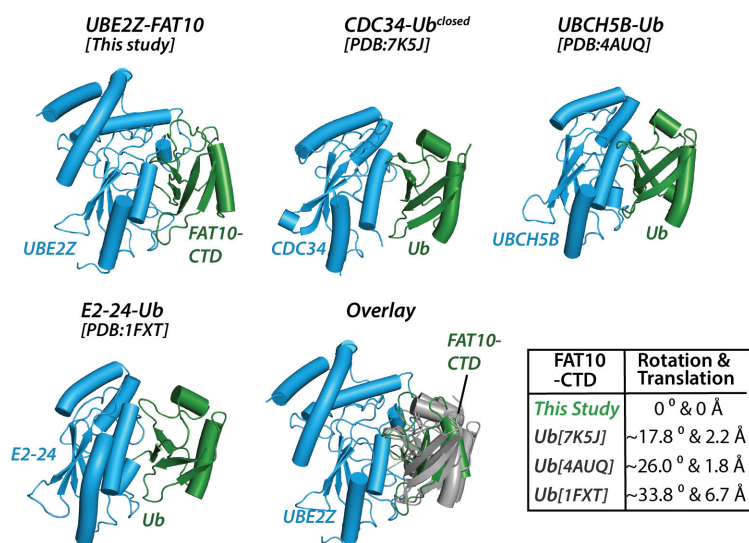


Supplementary Fig. 6 | Cryo-EM data processing tree of the UBA6-UBE2Z-FAT10(t)/FAT10(a) complex.
Cryo-EM data process flow depicting the major steps of 2D and 3D classification leading to the best map.

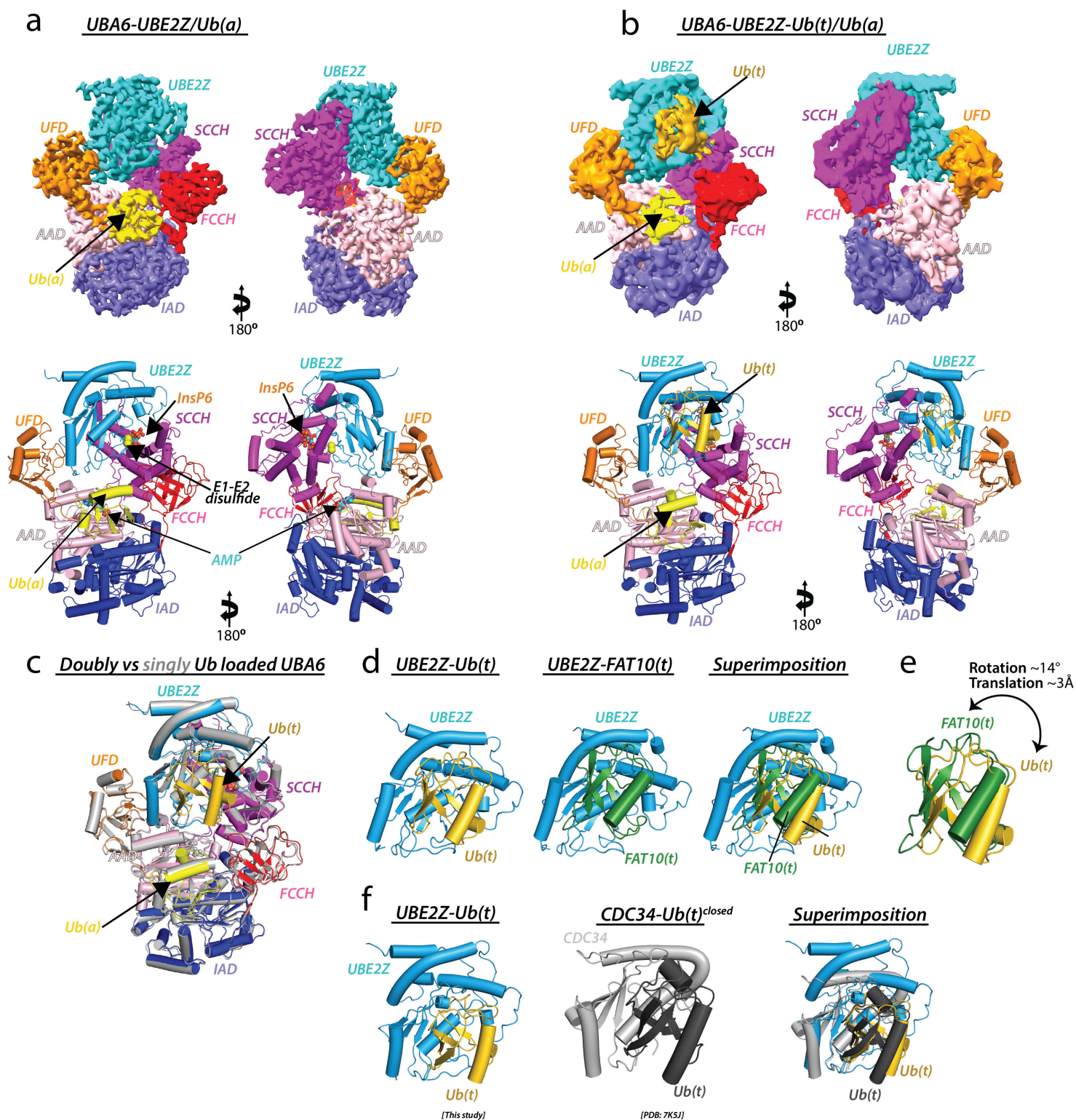
a UBE2Z/FAT10 structure



c Comparing UBE2Z-FAT10 with other E2-Ubl structures

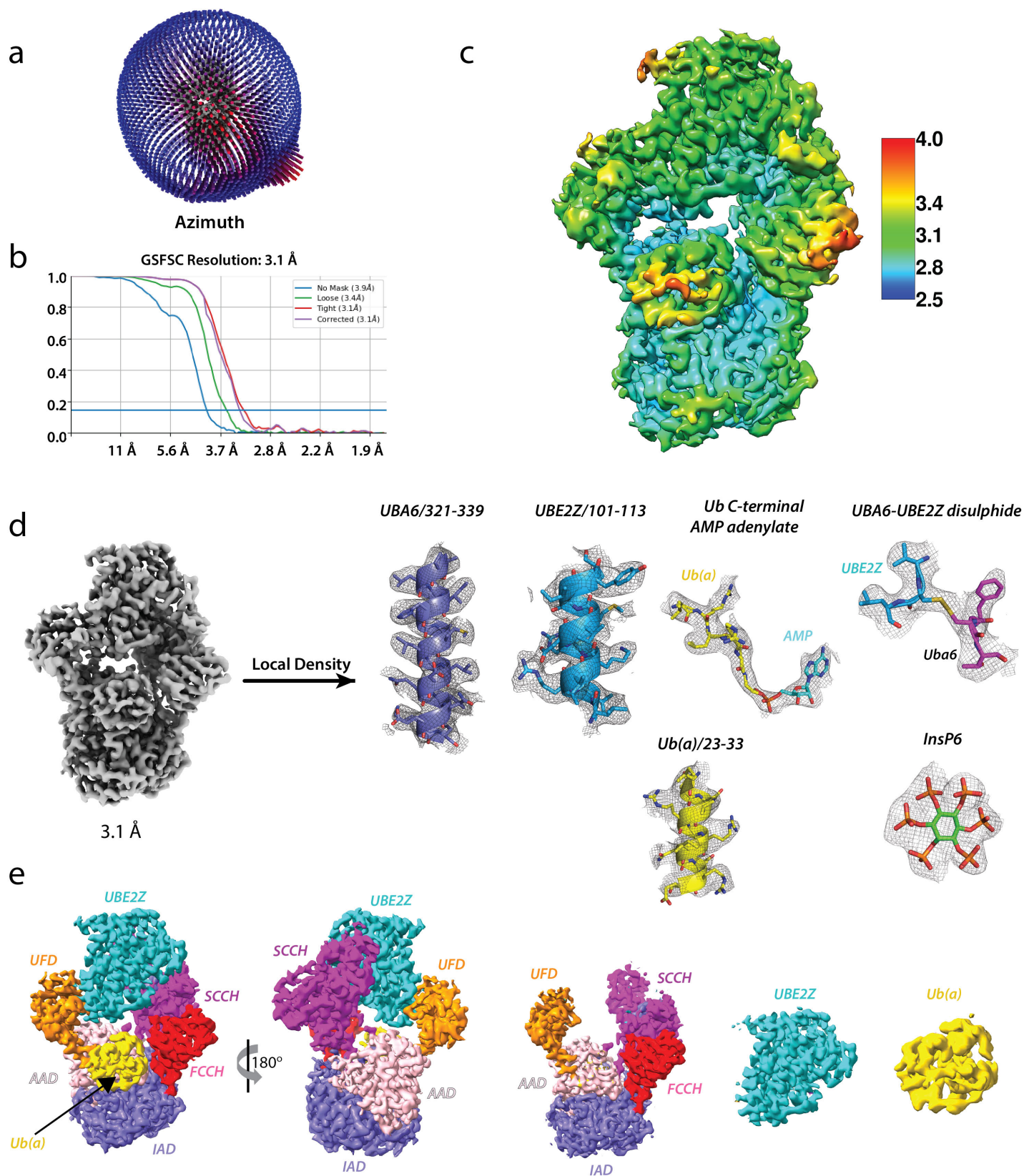


Supplementary Fig. 7 | Interface of UBE2Z-FAT10(t) and comparing it other E2-Ub structures . a, Left, UBE2Z-FAT10(t) structure with UBE2Z shown as grey surface with only contacting residues colored. FAT10(t) is represented as loop and colored green. Right, Surface representations of UBE2Z and FAT10(t) shown in open book representation with residues in the interface colored thorium (E2) and green (FAT10(t)). b, Sequence alignment of E2s across different UbIs with UBE2Z's secondary structure cartoon shown above sequence. Shaded boxes indicate E2s residues that interact with the FAT10(t), SUMO1(t) and NEDD8(t). c, Comparing the UBE2Z-FAT10(t) with other published E2-Ub(t) conjugates. d, UBE2Z-FAT10(t) shown as cartoon on the left and UBE2Z shown as electrostatic surface on the right with FAT10(t) shown as cartoon. Bottom, Electrostatic surface representation of BIRC6 and UBE2O in the same orientation as above.



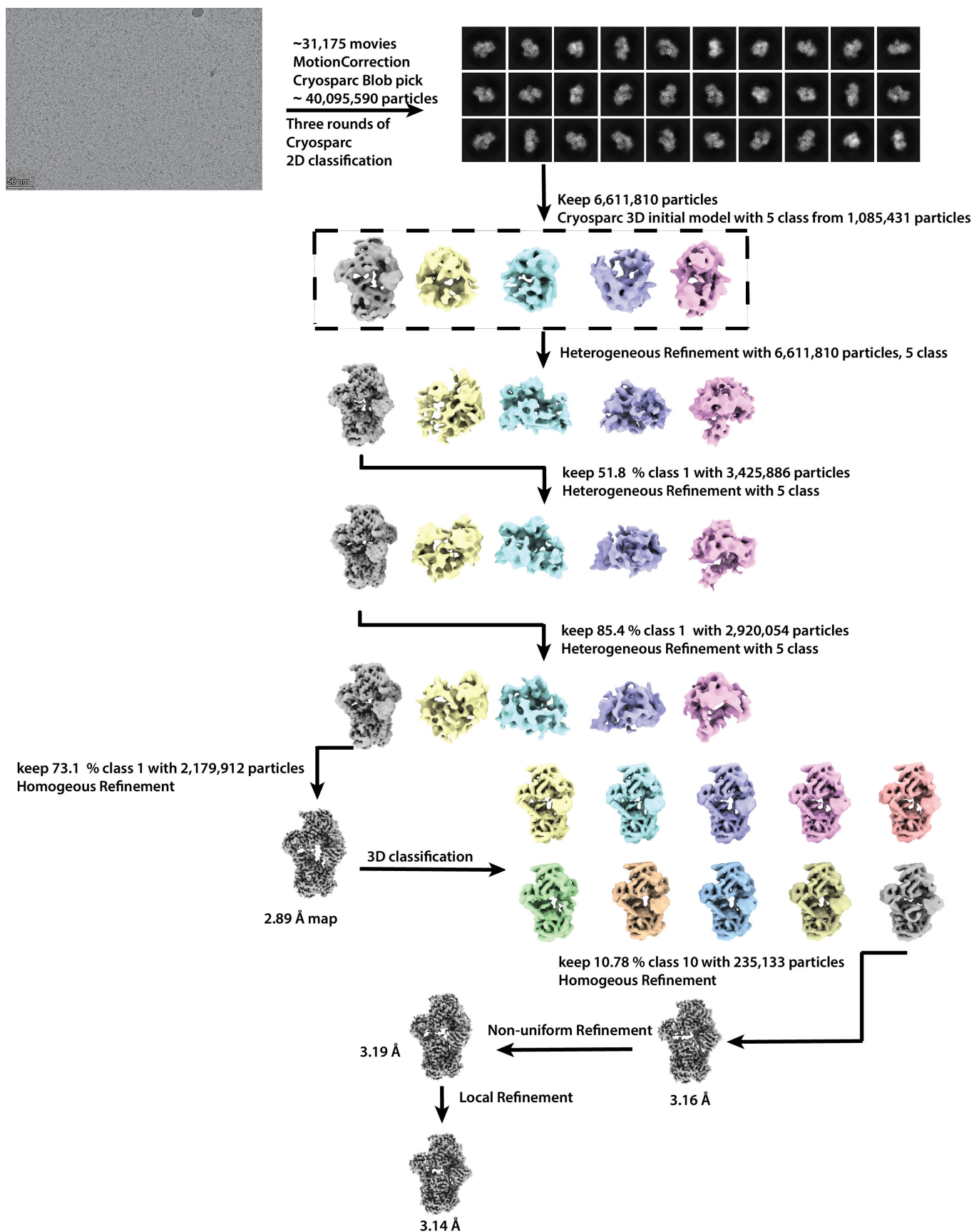
Supplementary Fig.8 | Overall architecture of a UBA6-UBE2Z/Ub(a) and UBA6-UBE2Z-Ub(t)/Ub(a) complex comparison with other E2-Ub structure.

Cryo-EM reconstruction of the UBA6-UBE2Z/Ub(a) complex (**a**) and UBA6-UBE2Z-Ub(t)/Ub(a) complex (**b**) with corresponding cartoon representation in the bottom. The IAD and AAD domains of UBA6 are shown in slate blue and light pink, respectively; the SCCH domain in magenta, FCCH in hot pink, and UFD in orange. UBE2Z is colored cyan, Ub(a) in yellow and Ub(t) in gold. c, Overlay of UBA6-UBE2Z/Ub(a) and UBA6-UBE2Z-Ub(t)/Ub(a) complexes. Single Ub loaded UBA6 is colored grey. d, Comparing the UBE2Z-Ub(t) thioester mimetic with UBE2Z-FAT10(t) in this study. f, Comparing the UBE2Z-Ub(t) thioester mimetic with CDC34-Ub(t) (PDB: 7K5J).



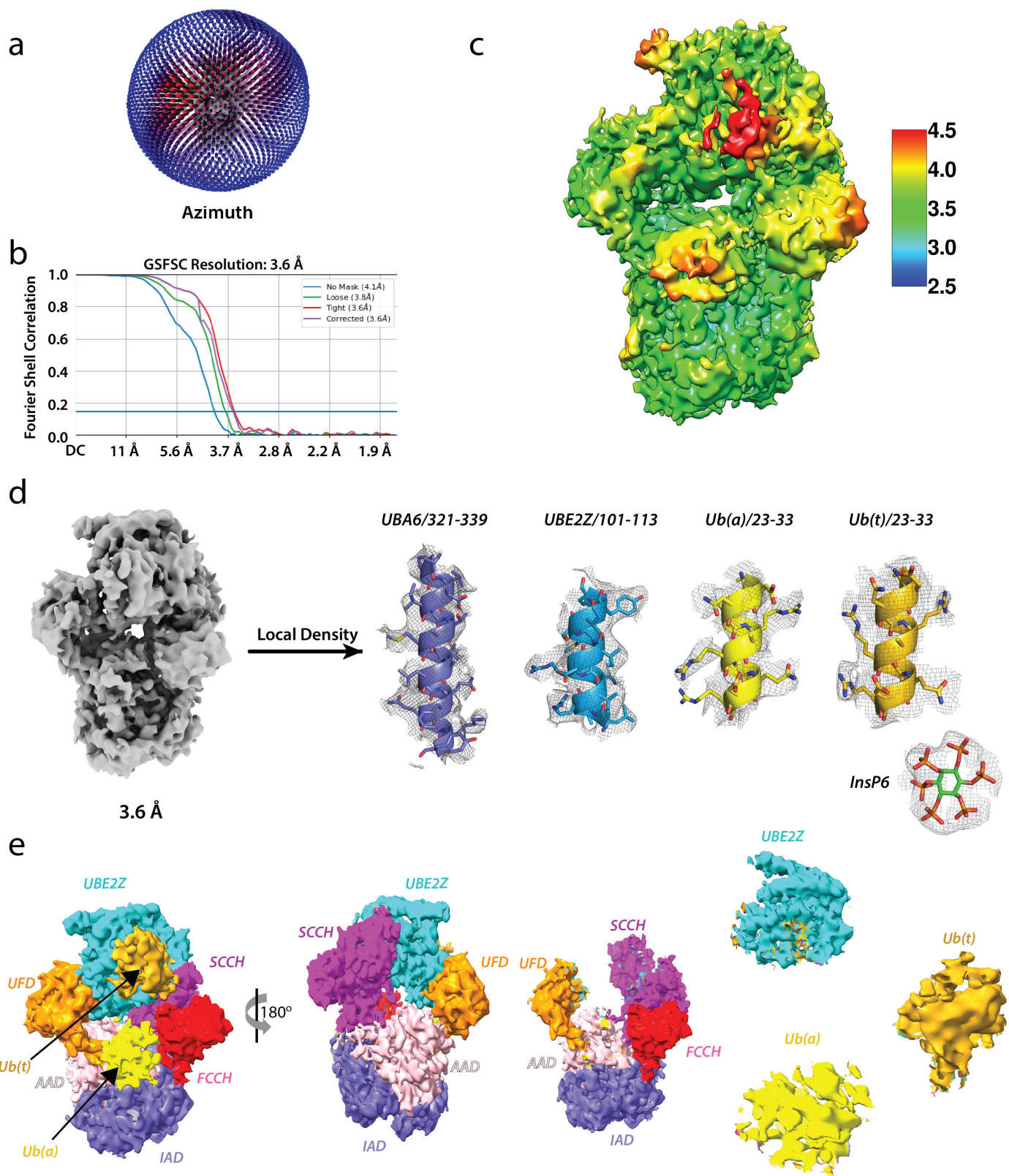
Supplementary Fig. 9 | Quality of the UBA6-UBE2Z/Ub(a) complex cryo-EM reconstruction.

a, The final Euler angle distribution of particles used for 3D refinement. **b**, Fourier Shell Correlation (FSC) plot from two half maps, with an overall resolution of 3.1 Å as determined at 0.143 criterion. **c**, Local resolution map color-coded from lower (red) to higher resolution (blue). **d**, Overall map (left) and detailed views of local cryo-EM density for UBA6, UBE2Z, Ub(a), Ub C-terminal AMP adenylate and InsP6 regions of the complex. **e**, Cryo-EM map of the UBA6-UBE2Z/Ub(a) complex with two views related by a 180° rotation about the y-axis (left). Maps of the individual subunits are shown in the right with IAD (slate), AAD (lightpink), FCCH (red), SCCH (magenta), UFD (orange), UBC9 (thorium) and Ub(a) (yellow).



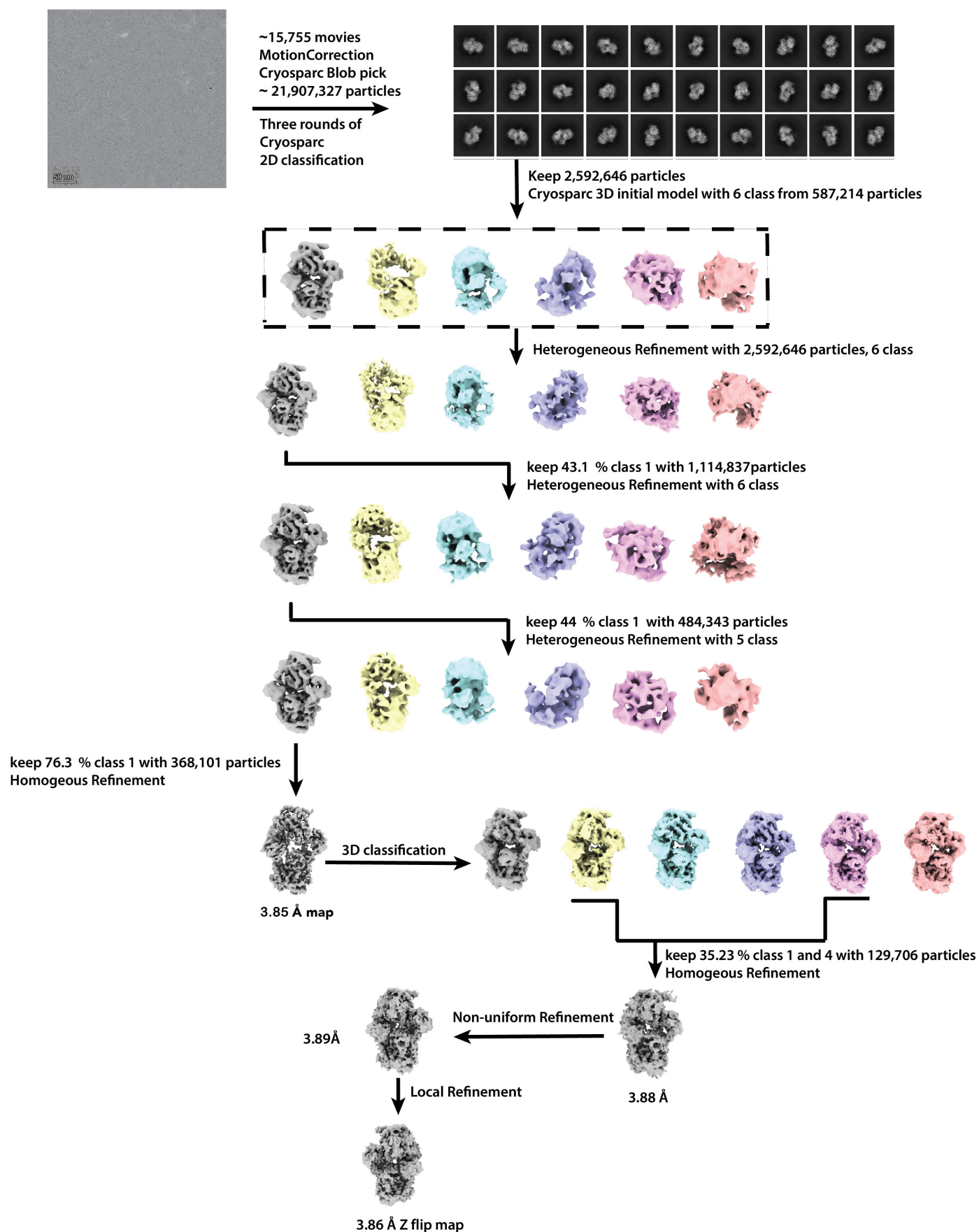
Supplementary Fig. 10 | Cryo-EM data processing tree of the UBA6-UBE2Z/Ub(a) complex.

Cryo-EM data process flow depicting the major steps of 2D and 3D classification leading to the best map.



Supplementary Fig. 11 | Quality of the UBA6-UBE2Z-Ub(t)/Ub(a) complex cryo-EM reconstruction.

a, The final Euler angle distribution of particles used for 3D refinement. **b**, Fourier Shell Correlation (FSC) plot from two half maps, with an overall resolution of 3.6 Å as determined at 0.143 criterion. **c**, Local resolution map color-coded from lower (red) to higher resolution (blue). **d**, Overall map (left) and detailed views of local cryo-EM density for UBA6, UBE2Z, Ub(a), Ub(t) and InsP6 regions of the complex. **e**, Cryo-EM map of the UBA6-UBE2Z/Ub(a) complex with two views related by a 180° rotation about the y-axis (left). Maps of the individual subunits are shown in the right with IAD (slate), AAD (lightpink), FCCH (red), SCCH (magenta), UFD (orange), UBC9 (thorium), Ub(a) (yellow) and Ub(t) (gold).



Supplementary Fig.12 | Cryo-EM data processing tree of the UBA6-UBE2Z-Ub(t)/Ub(a) complex.

Cryo-EM data process flow depicting the major steps of 2D and 3D classification leading to the best map.

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

	Uba6 Double loaded FAT 10 (EMDB- 73059) (9YKV)	Uba6 Single loaded FAT10 (EMDB- 73079) (9YLB)	Uba6 Double loaded Ub (EMDB- 73060) (9YKW)	Uba6 Single loaded Ub (EMDB -73081) (9YLF)
Data collection and processing				
Microscope	Krios	Krios	Glacios	Krios
Voltage (kV)	300	300	200	300
Electron exposure (e- /Å ²)	80	53	50	80
Defocus range (µm)	1.2- 2.5	0.8- 2.5	0.8- 2.0	1.2-2.5
Pixel size (Å)	0.833	1.067	0.87	0.833
Symmetry imposed	C1	C1	C1	C1
Final particle images (no.)	299646	265430	129706	235133
Map resolution (Å) FSC threshold 0.143 (Å)	2.73	3.24	3.86	3.14
Map resolution range (Å)				
Refinement				
Initial model used (PDB code)				
Model resolution (Å) FSC threshold 0.143 (Å)	3.02	3.46	4.44	3.36
Model resolution range (Å)	3.02-5.0	3.46-5.0	4.44-7.0	3.36-5.00
Map sharpening <i>B</i> factor (Å ²)				
Model composition				
Non-hydrogen atoms	10923	10853	10904	10303
Protein residues	1370	1363	1362	1291
Ligands	3	2	8	2
<i>B</i> factors (Å ²)				
Protein	38.38	47.5	67.52	15.88
Ligand	107.79	116.74	61.52	25.92
R.m.s. deviations				
Bond lengths (Å)	0.003	0.006	0.004	0.003
Bond angles (°)	0.559	0.684	0.662	0.540
Validation				
MolProbity score	1.69	1.96	2.79	1.72
Clashscore	8.25	8.71	18.79	8.56
Poor rotamers (%)	0.33	0.66	5.8	0.52
Ramachandran plot				
Favored (%)	96.39	91.73	93.10	96.18
Allowed (%)	3.54	7.90	6.68	3.82
Disallowed (%)	0.07	0.37	0.22	0.00

Supplementary Table 2 | List of primers

UBA6

E233A

F: CATTGTTACTTGCCTTGCGAATCATCCTCACAAACTG

R: CAGTTTGTGAGGATGATTGCGAAGGCAAGTAACAATG

N234A

F: CATTGTTACTTGCCTTGAAGCGCATCCTCACAAACTGGAG

R: CTCCAGTTTGTGAGGATGCGCTTCAAGGCAAGTAACAATG

E572K

F: CAGCATTAGATAATGTGAAAGCCAGGAGATACGTAG

R: CTACGTATCTCCTGGCTTTCACATTATCTAATGCTG

R575E

F: GATAATGTGGAAGCCAGGGAATACGTAGACAGTCGTTG

R: CAACGACTGTCTACGTATTCCCTGGCTTCCACATTATC

E619R

F: GTCATCGGGATCCCCCACGTGAGGAAATACCATTTTG

R: CAAAATGGTATTTCTCACGTGGGGGATCCCGATGAC

F624S

F: CCCAGAAGAGGAAATACCAAGCTGTACTCTAAAATCCTTTCC

R: GGAAAGGATTTTAGAGTACAGCTTGGTATTTCTCTTCTGGG

E680R

F: GTGGACACAGTTTAAGAGGCTGTTTTCAAG

R: CTTGAAAACAGCCTCTTAACTGTGTCCAC

F683A

F: GACACAGTTTAGAAGGCTGTGCGCAAGTTATAAAGTTACTTAGC

R: GCTAAGTAACTTTATAACTTGCGCACAGCCTTCTAACTGTGTC

F722S

F: CAGCTTCTTCACTGTAGCCCTCTTGACATAC

R: GTATGTCAAGAGGGCTACAGTGAAGAAGCTG

F734S

F: GATTAAAAGATGGCAGTTTAAGCTGGCAGTCACCAAAGAGGC

R: GCCTCTTTGGTGACTGCCAGCTTAAACTGCCATCTTTAATC

K991E

F: GTGGTACAGGGAGTCGAAATGCTTTATGTTC

R: GAACATAAAGCATTTCGACTCCCTGTACCAC

H1001A

F: CTGTAATGCCTGGTGCGGCAAAAAGATTGAAG

R: CTTCAATCTTTTTGCCGCACCAGGCATTACAG

D1033R

F: CATTTGCTCCAGACATTCGTGGAGATGAAGATTTGCC

R: GGCAAATCTTCATCTCCACGAATGTCTGGAGCAAATG

E1036K

F: GACATTGATGGAGATAAAGATTTGCCGGGAC

R: GTCCCGGCAAATCTTTATCTCCATCAATGTC

D1037R

F: CATTGATGGAGATGAACGTTTGCCGGGACCTCC

R: GGAGGTCCCGGCAAACGTTTCATCTCCATCAATG

D1033R_E1036K/D1037R

F: GTGTCATTTGCTCCAGACATTCGTGGCGATAAACGTTTGCCGGGACCTCCAGTAAG

R: CTTACTGGAGGTCCCGGCAAACGTTTATCGCCACGAATGTCTGGAGCAAATGACAC

UBE2Z

D157K

F: GTTTCGGTGTCCGCCCAAATATCCCATCCACCCAC

R: GTGGGTGGATGGGATATTTGGGCGGACACCGAAAC

H161A

F: CCCGACTATCCCATCGCGCCACCTCGGGTCAAAC

R: GTTTGACCCGAGGTGGCGCGATGGGATAGTCGGG

K186D

F: CAACTTCTACCGCAATGGGGACGTCTGCTTGAGTATTCTAG

R: CTAGAATACTCAAGCAGACGTCCCCATTGCGGTAGAAGTTG

Y182A

F: GTTTAACCCCAACTTCGCGCGCAATGGGAAAGTCTG

R: CAGACTTTCCCATTTGCGCGCGAAGTTGGGGTTAAAC

N172A

F: CAAACTGATGACAACGGGCGCGAACACAGTGAGGTTTAAC

R: GTTAAACCTCACTGTGTTTCGCGCCCGTTGTCATCAGTTTG

R232A

F: GCTTTGAACAGGAGGCGCATCCAGGAGACAGC

R: GCTGTCTCCTGGATGCGCCTCCTGTTCAAAGC

F228A

F: CACAATGAGCCCGGCGCGGAACAGGAGAGACATC

R: GATGTCTCTCCTGTTCCGCGCCGGGCTCATTGTG

E134A

F: GAACCCCTATCACAATGCGCCCGGCTTTGAACAG

R: CTGTTCAAAGCCGGGCGCATTGTGATAGGGGTTC

E229R

F: CAATGAGCCCGGCTTTTCGTCAGGAGAGACATCCAG

R: CTGGATGTCTCTCCTGACGAAAGCCGGGCTCATTG

K105A

F: GTCTACTCCGGATCGCGCGGGATATCATGTC

R: GACATGATATCCCGCGCGATCCGGAGTAGAC

D127K

F: GTACCTGATACTGTAAAATGACTAAGATTCATG

R: CATGAATCTTAGTCATTTTAACAGTATCAGGTAC

K113E

F: GGATATCATGTCCATTTATGAAGAGCCTCCTCCAGGAATG

R: CATTCTGGAGGAGGCTCTTCATAAATGGACATGATATCC

R106D

F: CAGTGTCTACTCCGGATCAAGGATGATATCATGTCCATTTATAAG

R: CTTATAAATGGACATGATATCATCCTTGATCCGGAGTAGACACTG

R103D

F: CGCCGCAGTGTCTACTCGATATCAAGCGGGATATCATG

R: CATGATATCCCGCTTGATATCGAGTAGACACTGCGGCG

K105E

F: GTCTACTCCGGATCGAACGGGATATCATGTC 3'

R: GACATGATATCCCGTTCGATCCGGAGTAGAC 3'

K105E/R103D/R106D

F: CCGCGCCGCAGTGTCTACTCGATATTGAAGATGATATCATGTCCATTTATAAG

R: CTTATAAATGGACATGATATCATCTTCAATATCGAGTAGACACTGCGGCGCGG

D107R

F: CTA^TCTCCGGATCAAGCGGCGTATCATGTCCATT^TTATAAG
R: CTTATAAATGGACATGATACGCCGCTTGATCCGGAGTAG

E114K

F: CATGTCCATT^TTATAAGAAACCTCCTCCAGGAATG
R: CATTCTGGAGGAGGTTTCTTATAAATGGACATG

F139A

F: CATTGATCACAGGCCCGAGCGGACACTCCTTATGAAG
R: CTTCATAAGGAGTGTCCGCTGGGCCTGTGATCAATG

D140R

F: GATCACAGGCCCATTTCTGACTCCTTATGAAGGGGG
R: CCCCCTTCATAAGGAGTACGAAATGGGCCTGTGATC

E144K

F: CATTTGACACTCCTTATAAAGGGGGTTTCTTCCTG
R: CAGGAAGAAACCCCTTTATAAGGAGTGTCAAATG

Q214S

F: CAGTGCTCATCTCTATCAGCTCCCTGATGACTGAGAAC
R: GTTCTCAGTCATCAGGGAGCTGATAGAGATGAGCACTG

E219R

F: CTATCCAGTCCCTGATGACTCGTAACCCCTATCACAATGAGC
R: GCTCATTGTGATAGGGGTACGAGTCATCAGGGACTGGATAG

R245D

F: CTATAATGAATGTATCGATCACGAGACCATCAGAG
R: CTCTGATGGTCTCGTGATCGATACATTATTATAG

E277R

F: GGAGAAGTCCTTTCTGCGGTATTACGACTTCTATG

R: CATAGAAGTCGTAATACCGCAGAAAGGACTTCTCC

Y278A

F: GAGAAGTCCTTTCTGGAGGCGTACGACTTCTATGAGGTG

R: CACCTCATAGAAGTCGTACGCCTCCAGAAAGGACTTCTC

D280R

F: CCTTTCTGGAGTATTACCGTTTCTATGAGGTGGCCTG

R: CAGGCCACCTCATAGAAACGGTAATACTCCAGAAAGG

F281S

F: CTGGAGTATTACGACAGCTATGAGGTGGCCTG

R: CAGGCCACCTCATAGCTGTCGTAATACTCCAG

K287E

F: CTTCTATGAGGTGGCCTGCGAAGATCGCCTGCACCTTC

R: GAAGGTGCAGGCGATCTTCGCAGGCCACCTCATAGAAG

D288K

F: GAGGTGGCCTGCAAAAAACGCCTGCACCTTCAAG

R: CTTGAAGGTGCAGGCGTTTTTTGCAGGCCACCTC

FAT10**D97K**

F: GTTCTGGTGGAATCTGGCAAAGAAGCAAAACGTCATCTG

R: CAGATGACGTTTTGCTTCTTTGCCAGATTCCACCAGAAAC

R101D

F: GGCGATGAAGCAAAAGATCATCTGCTGCAGG

R: CCTGCAGCAGATGATCTTTTGCTTCATCGCC

K137E

F: GTTACCCTGAATGGTGAACGTCTGGAAGATG

R: CATCTTCCAGACGTTCCACCATTCAGGGTAAC

D147K

F: GATGGTAAAATGATGGCCAAATATGGCATTTCGTAAAGGC

R: GCCTTTACGAATGCCATATTTGGCCATCATTTTACCATC

K143E

F: GTAAACGTCTGGAAGATGGTGAAATGATGGCCGATTATGG

R: CCATAATCGGCCATCATTTCCACCATCTTCCAGACGTTTAC

F157A

F: CGTAAAGGCAATCTGCTGGCGCTGGCCTCTTATTCTATTG

R: CAATAGAATAAGAGGCCAGCGCCAGCAGATTGCCTTTACG

R138D

F: GTTACCCTGAATGGTAAAGATCTGGAAGATGGTAAAATG

R: CATTTTACCATCTTCCAGATCTTTACCATTTCAGGGTAAC

K143E

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