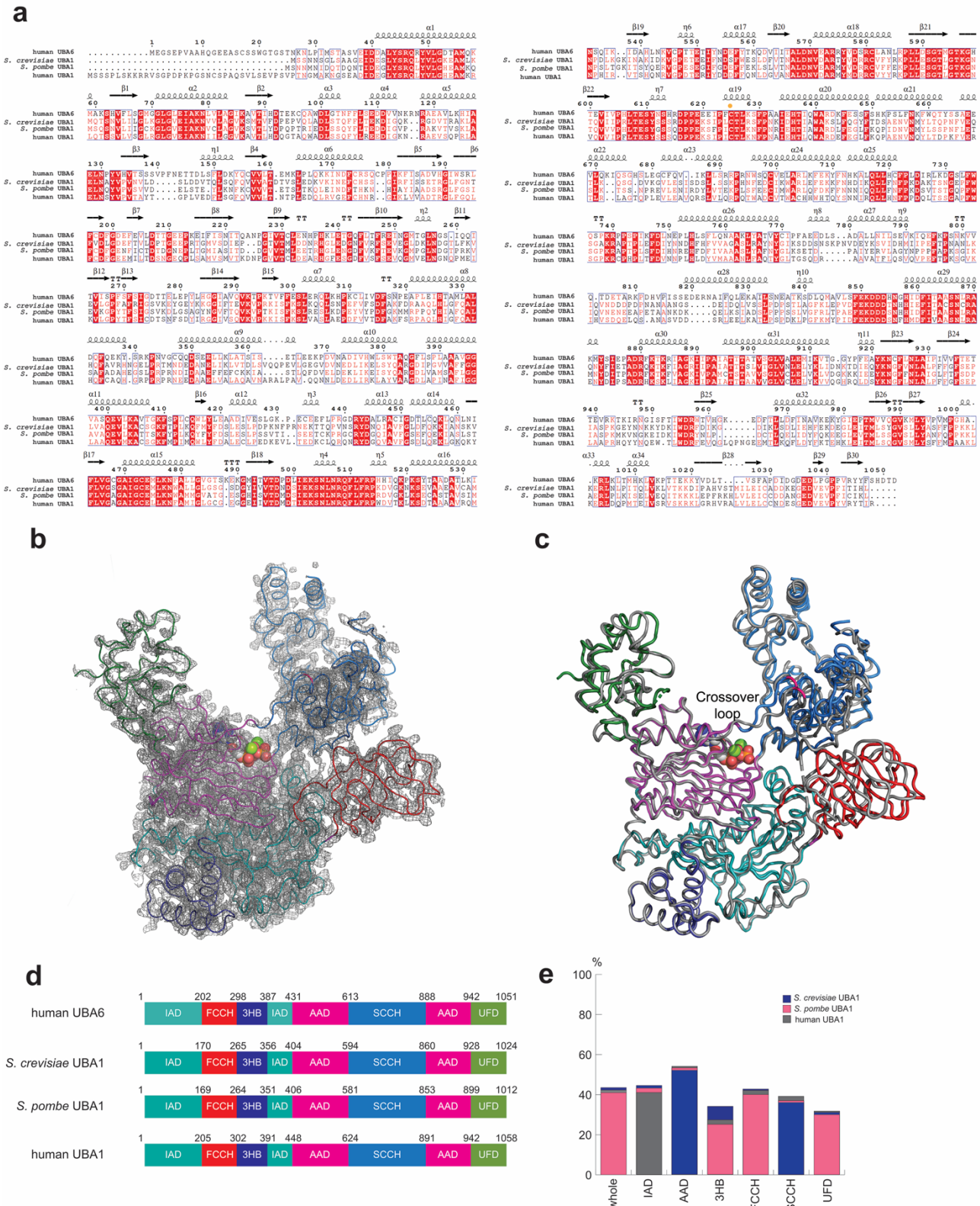
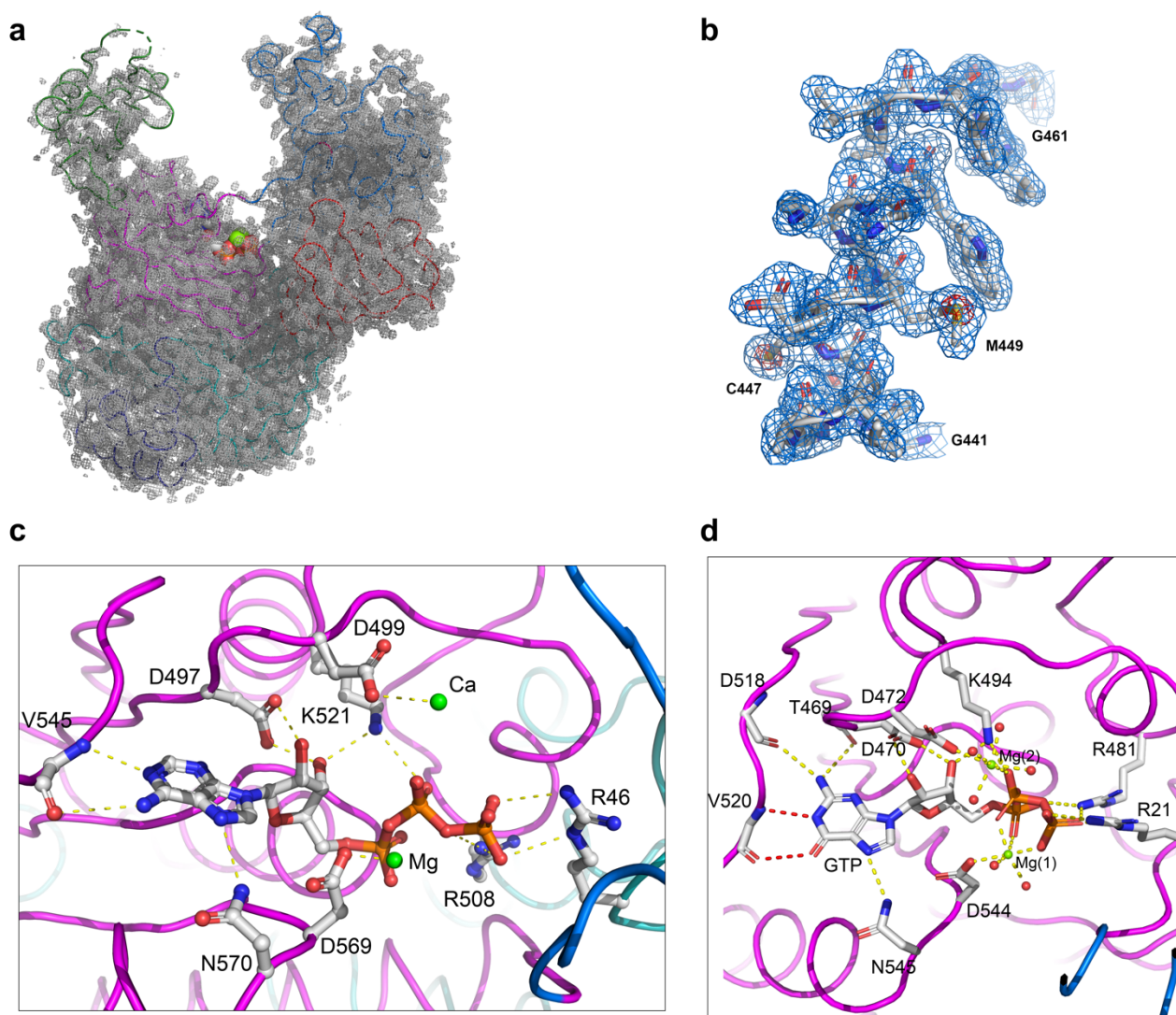


1 **Supplementary Material**



2
3 **Figure S1. Sequence and structural features of UBA6.** **a** Multiple sequence alignment of human
4 UBA6 and the three structurally characterized UBA1 proteins (human UBA1, *S. cerevisiae* and *S.*
5 *pombe* Uba1). Above the alignment, the secondary structure elements present in UBA6 are depicted

6 with helices as spirals (designated with α for α -helices and η for 3_{10} -helices) and β -strands as arrows.
7 Sequences were aligned using Clustal Omega and the figure was generated with ESPript. Red
8 shades indicate conserved residues, while red letters in white boxes indicate similar residues. The
9 catalytically active cysteine (C625 of UBA6) is indicated by a pink dot. **b** SIGMAA weighted $2F_o - F_c$
10 electron density map (gray) of the UBA6-ATP complex at a contour level of 1.0 rmsd. UBA6 is shown
11 in loop representation with domains color coded as in Figure 1a-c and ATP in sphere representation.
12 **c** Superimposition of the two protomers of the UBA6-ATP complex in loop representation with
13 monomer A in domain coloring and monomer B in gray. **d** Domain architecture of UBA6 and UBA1
14 orthologs. **e** Overall and per domain sequence identity of UBA1 orthologs with respect to UBA6.
15



16

17 **Figure S2. Structural features of the Uba1-ATP complex.** **a** SIGMAA weighted $2F_o - F_c$ electron
 18 density map (blue) of the Uba1-ATP complex at a contour level of 1.0 rmsd. Uba1 is shown in loop
 19 representation with domains color coded as in Figure 1a-c for UBA6. **b** SIGMAA weighted $2F_o - F_c$
 20 electron density map of residues G441-G461 in the Uba1-ATP complex at contour levels of 1.3 rmsd
 21 (blue) and 6 rmsd (red), with the latter revealing the positions of the two S-atoms present in this part
 22 of the model. The first and last residue together with the S-containing residues are labeled. **c**
 23 Coordination of the nucleotide cofactor in the UBA6-ATP structure with direct interactions highlighted
 24 as dashed yellow lines. The Mg and Ca ion are indicated but due to the more limited resolution, the
 25 water molecules coordinating the metals have been omitted. **d** Model of the Uba1-GTP complex.
 26 Additional hydrogen bonds involving the exocyclic amino group of guanine and the main chain
 27 oxygen atoms of T469 and D518 are shown in yellow, while repulsive interactions with the main
 28 chain of V520 are shown in red.

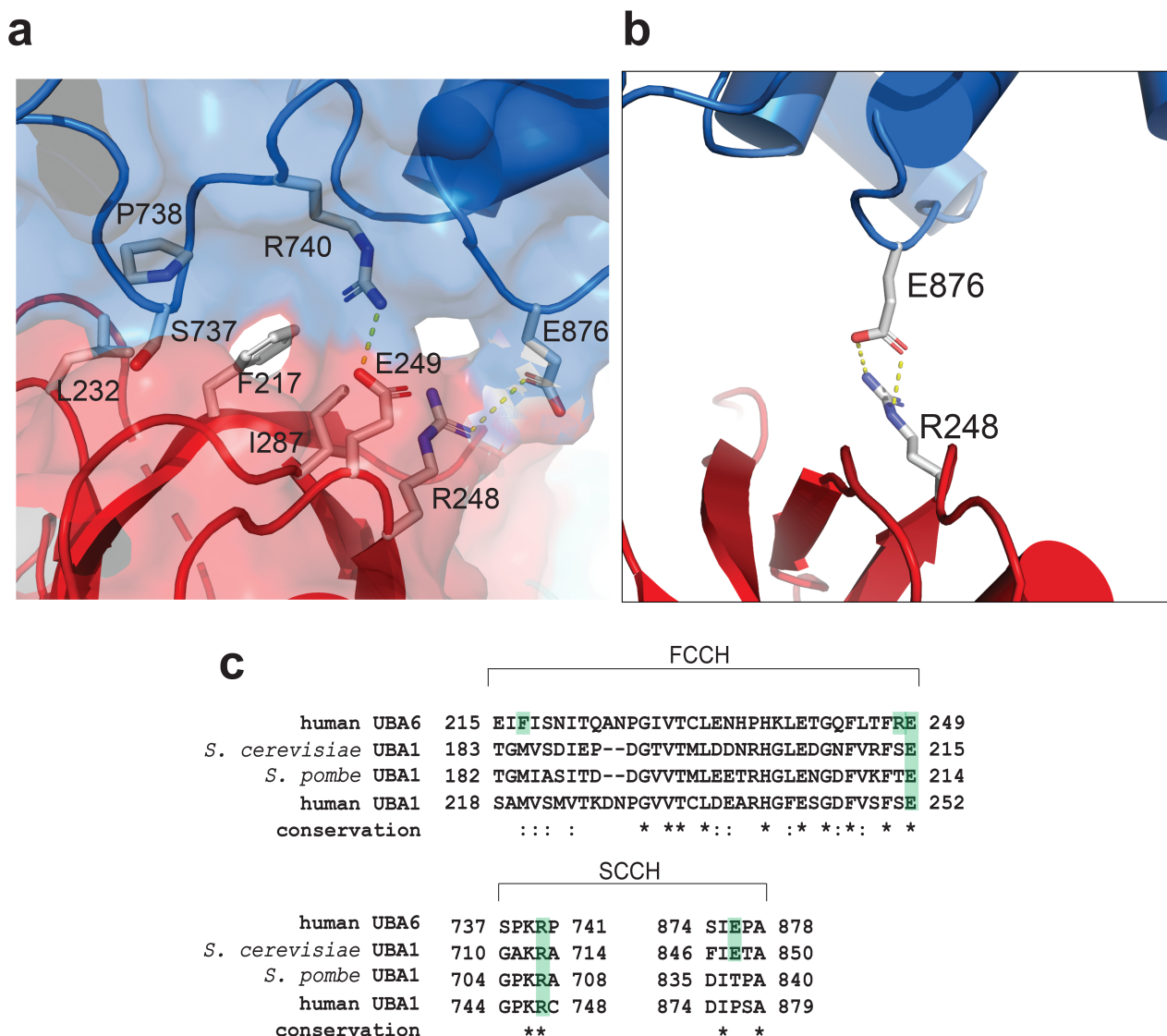
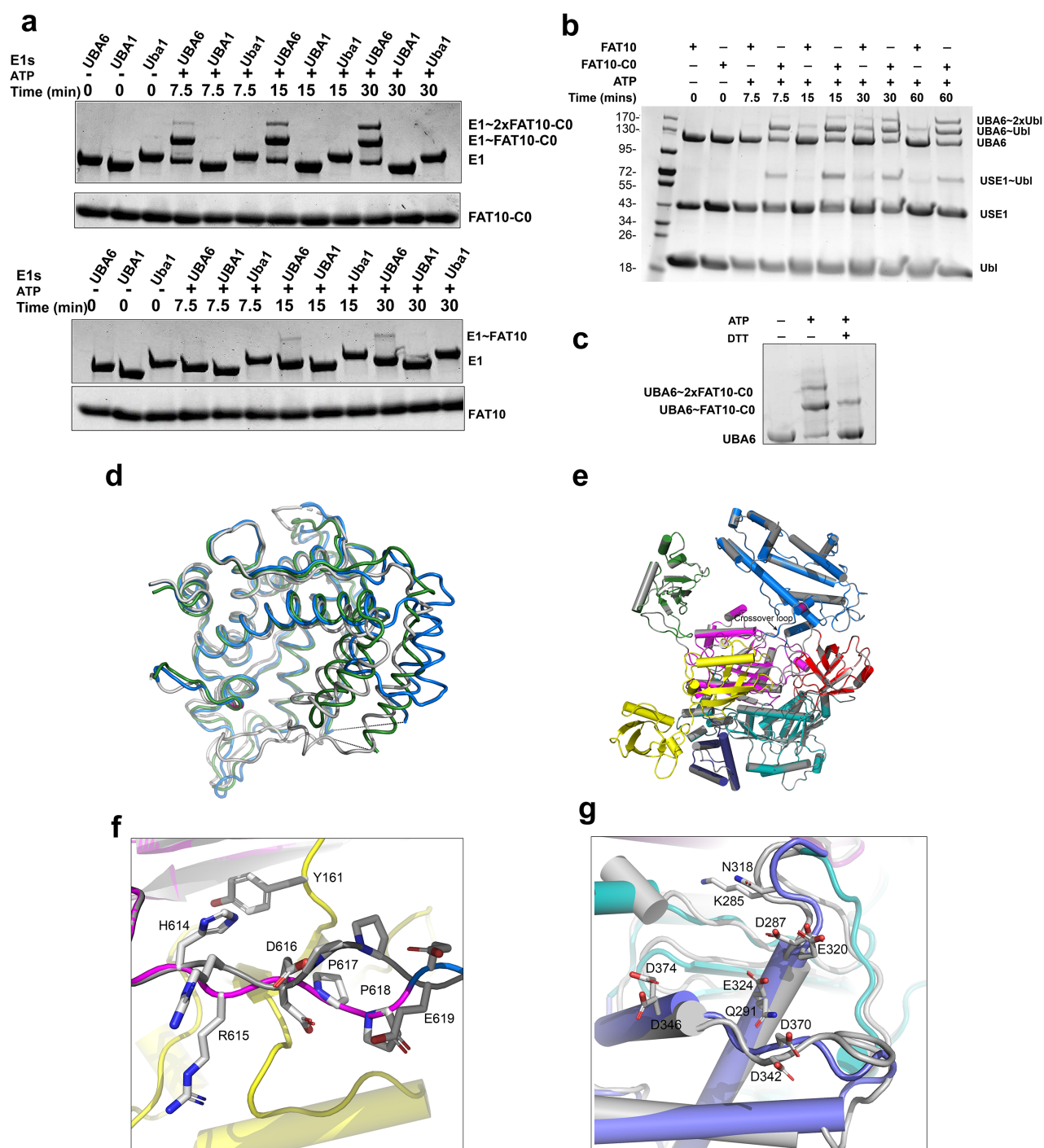


Figure S3. Stabilization of the FCCH in the outward conformation. **a** Interface between the FCCH and SCCH domains of the UBA6-ATP complex (ribbon representation with transparent surface) in monomer B. **b** Ion pair stabilizing the FCCH and SCCH domains in the UBA6-ATP complex in monomer A. **c** Partial sequence alignment of UBA6 and UBA1 orthologs showing the conservation of ionic interactions between the FCCH and SCCH domains.

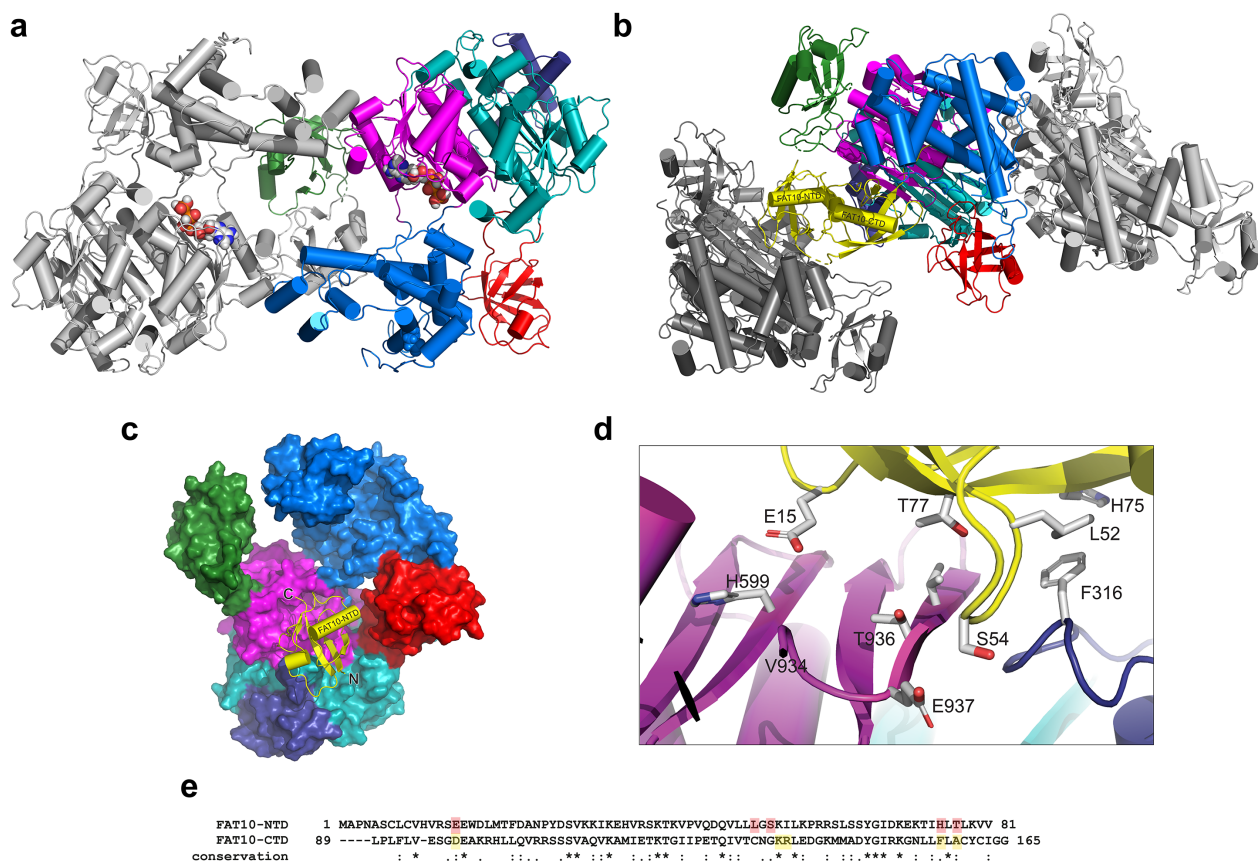


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37 **Figure S4 Activity assays and structural analysis of the UBA6-FAT10 complex.** **a** UBA6, human
38 UBA1 and *S. cerevisiae* Uba1 activity assay comparing the activation of FAT10-C0 (top) and the
39 FAT10 wild-type (bottom). **b** UBA6-USE1 transthiioesterification assay comparing the activation of
40 the FAT10 wild-type and FAT10-C0. **c** UBA6 activity assay run for 40 min in the absence and
41 presence of DTT showing the disappearance of the UBA6+2xC0-FAT10 band upon DTT treatment.
42 The assays in a-c were repeated 3 times with similar results, source data are provided as a Source
43 Data file. **d** Superimposition of the SCCH domains of UBA6 from the UBA6_{chim}-FAT10 complex

44 (marine blue), from the UBA1-ATP complex (green) and the Uba1-ATP complex (gray). **e**
45 Superimposition of the UBA6-FAT10 complex (UBA6 is color coded according to its domains and
46 FAT10 in yellow) with the apo-UBA6 monomer (gray) present in the P1 unit cell. **f** Superimposition
47 of the UBA6-FAT10 complex highlighting the N-terminal region of the crossover loop with side chains
48 undergoing conformational changes upon FAT10 binding, together with Y161 in the C-terminal tail
49 of FAT10. **g** Comparison of the region in the 3HB of UBA6 (color coded according to its domains)
50 interacting with the NTD of FAT10 and Uba1 (gray). Residues of UBA6 interacting with FAT10 and
51 their counterparts in Uba1 are highlighted.

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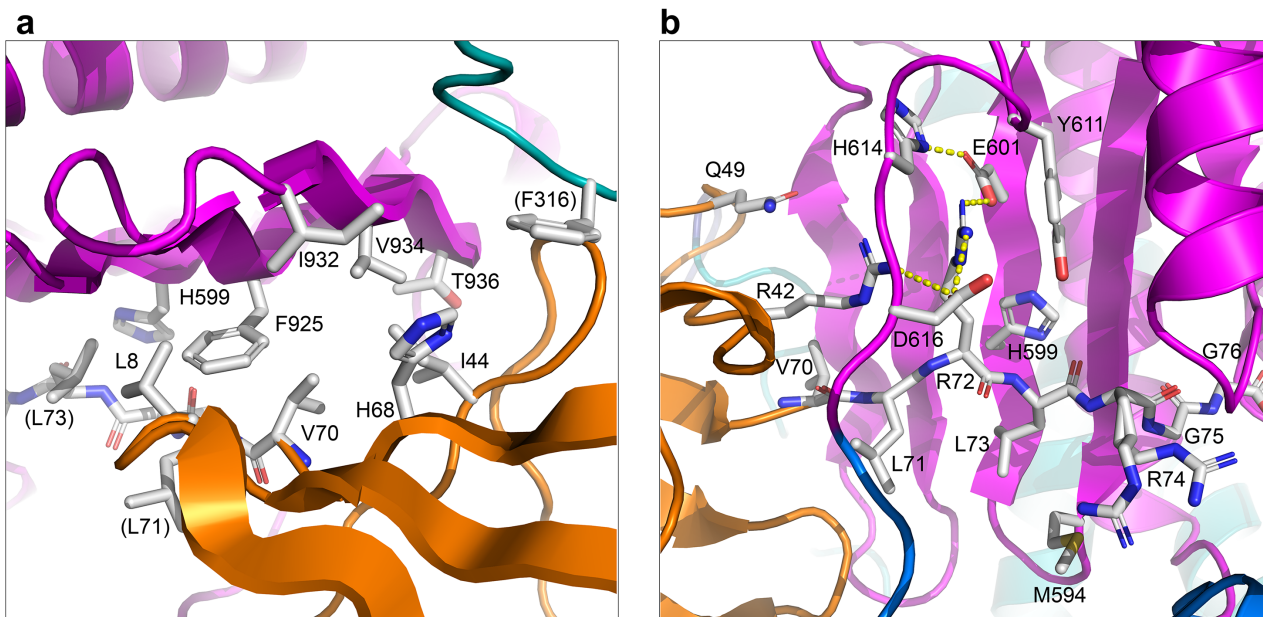


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54 **Figure S5. Analysis of the UBA6-FAT10 complex.** **a** Arrangement of the two UBA6-ATP
 55 complexes in the asymmetric unit of the C2 cell. One complex is color coded according to its domains
 56 and the other is in light gray with ATP in space filling representation. The view is along the non-
 57 crystallographic twofold axis of symmetry. **b** Arrangement of the two UBA6_{chim} molecules (one color
 58 coded according to its domains and the other in light gray) and FAT10 (yellow) in the P1 unit cell.
 59 The two UBA6_{chim} molecules are related by a non-crystallographic twofold axis of symmetry
 60 coinciding with the viewing direction. A symmetry-related UBA6_{chim} molecule is shown in dark gray
 61 to illustrate how FAT10 is simultaneously interacting with the UBA6_{chim} molecule shown in color and
 62 the symmetry-related UBA6 (dark gray). **c** Overall view of the interaction between the symmetry
 63 related UBA6_{chim} molecule from **b** but now shown in surface representation and color coded
 64 according to its domain architecture illustrating its interaction with the NTD of FAT10. **d** Detailed
 65 interactions between the NTD of FAT10 and the symmetry-related UBA6_{chim} molecule. **e** Sequence
 66 alignment of the NTD and CTD of FAT10 highlighting residues interacting with UBA6.

67

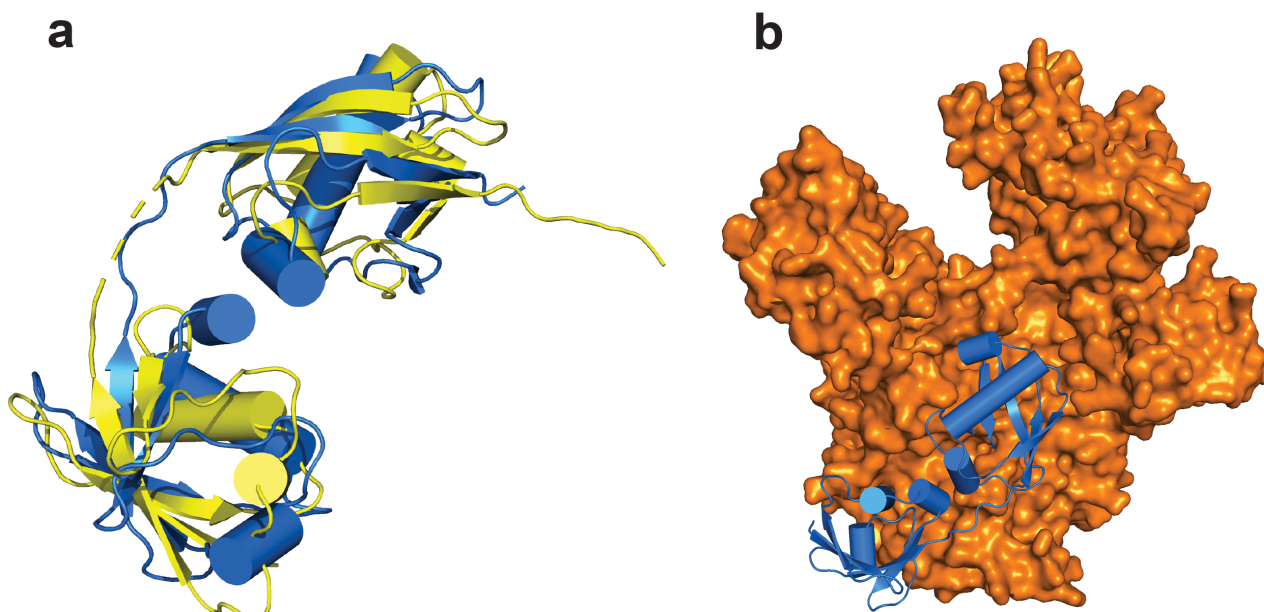
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70 **Figure S6. Features of the UBA6-ubiquitin model.** **a** Hydrophobic interface in the model of the
 71 UBA6-ubiquitin complex. UBA6 is color coded according to its domains while ubiquitin is in orange.
 72 Residues contributing to the hydrophobic interface are shown with their side chains. To minimize
 73 steric clashes the side chain of L8 was modeled with a different rotamer. Residue labels in
 74 parentheses indicate side chains which are not involved in hydrophobic interactions but are included
 75 as reference points. The orientation resembles that of the UBA6-FAT10 complex shown in Figure
 76 4d. **b** Interactions between the C-terminal tail of ubiquitin and UBA6 in the UBA6-ubiquitin model.
 77 H614 and D616 were modeled with different rotamers to allow binding of R72. R74 is represented
 78 by two alternate conformations as observed in the Uba1-ubiquitin complex.

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80

81 **Figure S7 Model of the UBA7-ISG15 complex.** **a** Superimposition of the FAT10 structure (yellow)
 82 as observed in the UBA6_{chim}-FAT10 complex with the crystal structure of ISG15 (PDB entry 1z2m)
 83 (blue) based on both domains. **b** Model of the UBA7-ISG15 complex. UBA7 is shown in surface
 84 representation (orange) and ISG15 in ribbon representation (blue). Based on the UBA6_{chim}-FAT10
 85 complex UBA6 was replaced with the Alphafold model of UBA7 and the CTD of ISG15 was
 86 superimposed with its counterpart in FAT10.

87

88 **Supplementary Table 1**

89

Nr.	Original sequence	Mutation	Sequence
1	pGEX-del34UBA6	F316A fw	tgccttattgtggatgctagcaaccctgaggcacctttag
		F316A rv	cctcaggggtgctagcatccacaataaggcactttgg
2	pGEX-del34UBA6	V934A fw	ccattccaattgtagcatttacagagacaactgaag
		V934A rv	ttgtctctgtaaatgctacaattggaatggctaag
3	pGEX-del34UBA6	E320K fw	gcaaccctaaggcacctttagagattc
		E320K rv	gaatctctaaaggtgccttaggggtgctaaaatcc
4	pGEX-del34UBA6	E324R fw	cctgaggcacctttacggattcacacagctatgc
		E324R rv	gctgtgtgaatccgtaaaggtgcctcagg
5	pGEX-del34UBA6	D370R fw	ggaagagaagcctcgtgtaaatgctgacattgtg
		D370R rv	cacaatgtcagcatttacacgaggcttctctcc
6	pGEX-del34UBA6	D616A fw	caatagtcacgggctccccagaagagg
		D616A rv	cctcttctggggagcccgatgactattg
7	pGEX-del34UBA6	E601Q fw	aagggaacacactcaagttattgtaccgcathtt
		E601Q rv	gtacaataacttgagtgtgtccctagtgtccattg
8	pET28a-ScUba1	D472A fw	gttggaactttcgatcgaagcgttatccgtaacaacgat
		D472A rv	atcgttggtacggataacgcttcgatcgaaaagtccaac
9	pET28a-ScUba1	D472E fw	gacttttcgatcgactcgttatccgtaacaacgatgtaaccg
		D472E rv	cggttacatcgttggtacggataacgagtcgatcgaaaagtc
10	pET28a-ScUba1	D544A fw	tcttgcgtcgacattggctagagcgttggtgac
		D544A rv	gtcaccaacgctctagccaatgtcgacgcaaga
11	pET28a-ScUba1	D544E fw	gttcttgcgtcgacattctctagagcgttggtgac
		D544E rv	gtcaccaacgctctagagaatgtcgacgcaagaac
12	pET28a-ScUba1	K494E fw	cctctgccgctacttcagactcgttcttccgacatccttt
		K494E rv	aaaggatgtcgaaagaacgagtcgtaagtagcggcagagg
13	pET28a-ScUba1	R21A fw	ccaacacataaagttgagcagaataaagactttcatcgatttctcc
		R21A rv	ggagaaatcgatgaaagtctttattctgctcaactttatgtgtggg
14	pET28a-ScUba1	R481A fw	cttgggtctaacaagaactgagcgtttaagttggacttttcgatc
		R481A rv	gatcgaaaagtccaacttaaacgctcagttctgttagaccaaaag
15	pET28a-ScUba1	C600A fw	ttgggaaagaacgtagggtagccaatgggatagacttttctg
		C600A rv	cagaaaagtctatccattggctaccctacgttcttcccaa
16	pGEX-del34UBA6	C625A fw	gaggaaataccatttgcactctaaaatccttcc
		C625A rv	attttagatgacaaatggatttctctctg
17	pGEX-del34UBA6	UBA6chim fw1	aagaggaaataccaatctgtaccctgaagaac
		UBA6chim rv1	tcttcagggtacagattggatttctcttc
	huUba1-pET23b	UBA6chim fw2	aagctgattgcagggaataatactgctatag
		UBA6chim rv2	agcagggtataatttccctgcaatcagcttgc

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