**Supplementary Figure S1.** Structural characterization of USP1-UAF1. (**a**) SEC-SAXS trace for the crystallized ubiquitin-bound USP1-UAF1 construct. (**b**) Guinier plot of buffer subtracted, averaged SAXS measurements. (**c**) Fit of different USP-UAF1 crystal structures to SAXS measurements. The better resolved chains A and B of the ubiquitin-free (USP1-UAF1) and chains A, B, and C (USP1^{Ub}-UAF1) of the ubiquitin-bound structure were used for fitting. (**d**) The two USP1-UAF1 complexes in the asymmetric unit of the ubiquitin-free (brown) and ubiquitin-bound (blue) crystal structures aligned by UAF1. The

better resolved chains A and B of the ubiquitin-free (USP1-UAF1) and chains A, B, and C USP1^{Ub}-UAF1) of the ubiquitin-bound structure were used for analysis unless otherwise stated.
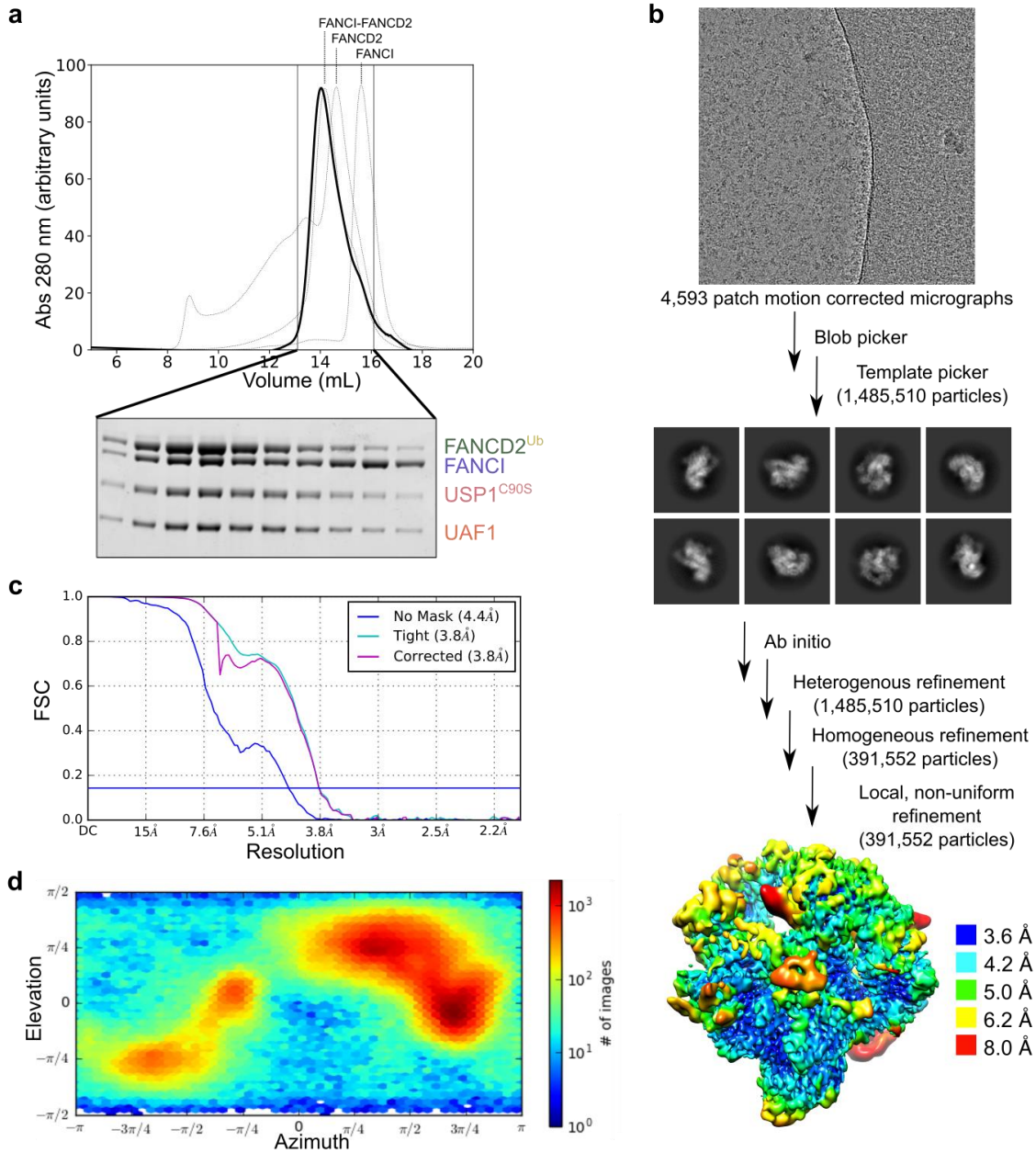

Supplementary Figure S2. Cryo-EM data processing. (**a**) Gel filtration profile of the assembled USP1^{C90S}-UAF1-FANCI-FANCD2^{Ub} complex and associated SDS-PAGE and Coomassie staining. (**b**) Flow diagram for analysis of the cryo-EM data by single particle analysis (see also Methods). The locally filtered map is

shown. (**c**) Unmasked, masked, and corrected Fourier Shell Correlation curves. (**d**) Viewing direction distribution.
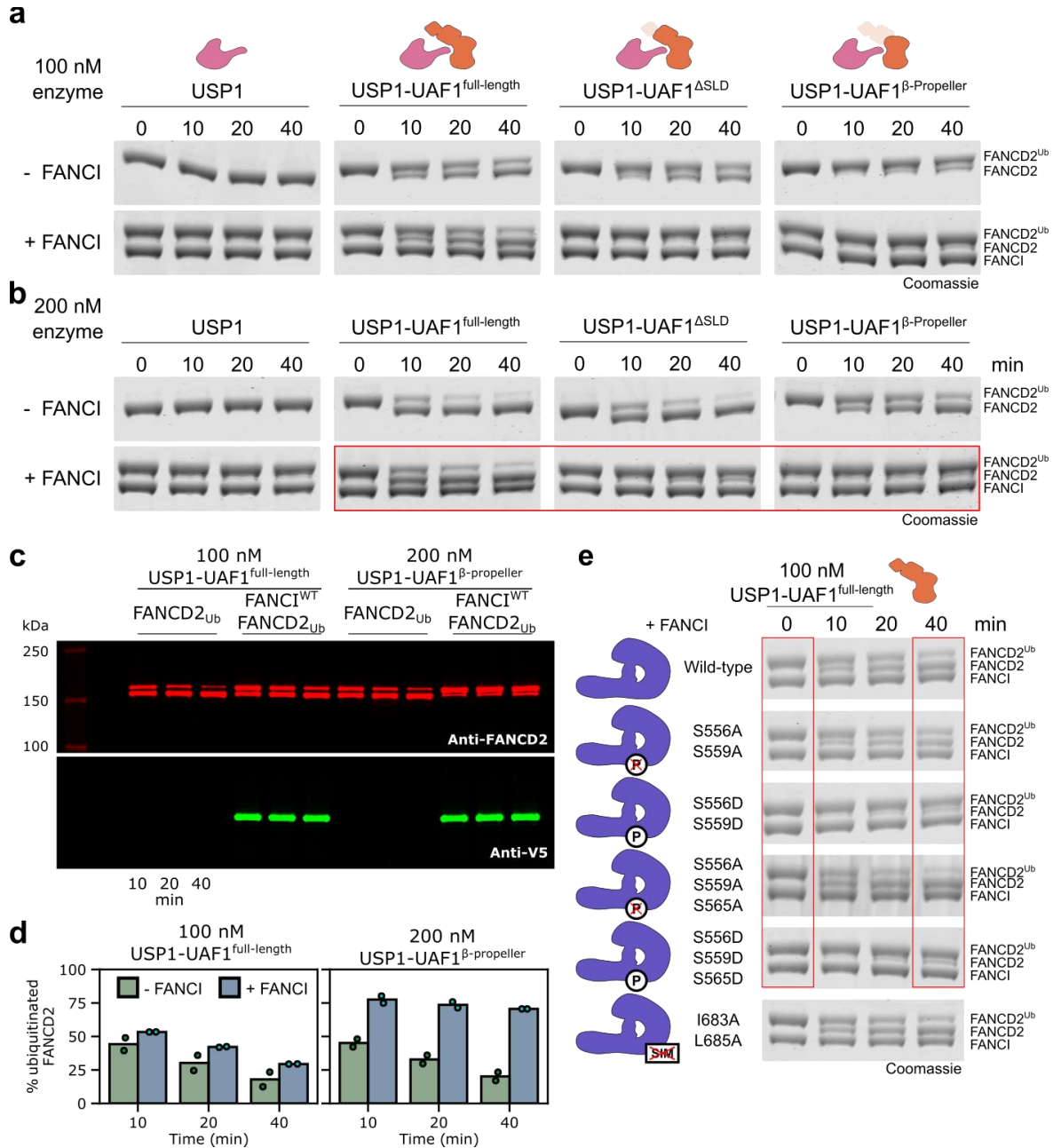
Supplementary Figure S3. Deubiquitination assays of FANCD2 by USP1-UAF1. (**a**) Deubiquitination time-courses for full-length USP1 alone and with the addition of various UAF1 truncations, at 100 nM USP1, 100 nM UAF1 as assessed by SDS-PAGE and Coomassie staining. (**b**) Deubiquitination time-courses for USP1 alone and with the addition of various UAF1 truncations, at 200 nM USP1, 200 nM UAF1 as

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assessed by SDS-PAGE and Coomassie staining. (**c**) Deubiquitination time-courses as assessed by Western blot. (**d**) Quantification of Western blots. Mean values are represents as bars with the individual replicates shown as points. (**e**) Deubiquitination time-courses for full-length USP1 and full-length UAF1 with ubiquitinated FANCD2 and various FANCI mutants as assessed by SDS-PAGE and Coomassie staining. For all assays 1 µM ubiquitinated FANCD2 was used, and 1 µM FANCI where included. All assays were in the presence of 4 µM 61 base pair dsDNA, and performed at least twice (two technical replicates). Red boxes indicate results displayed in Figure 3.
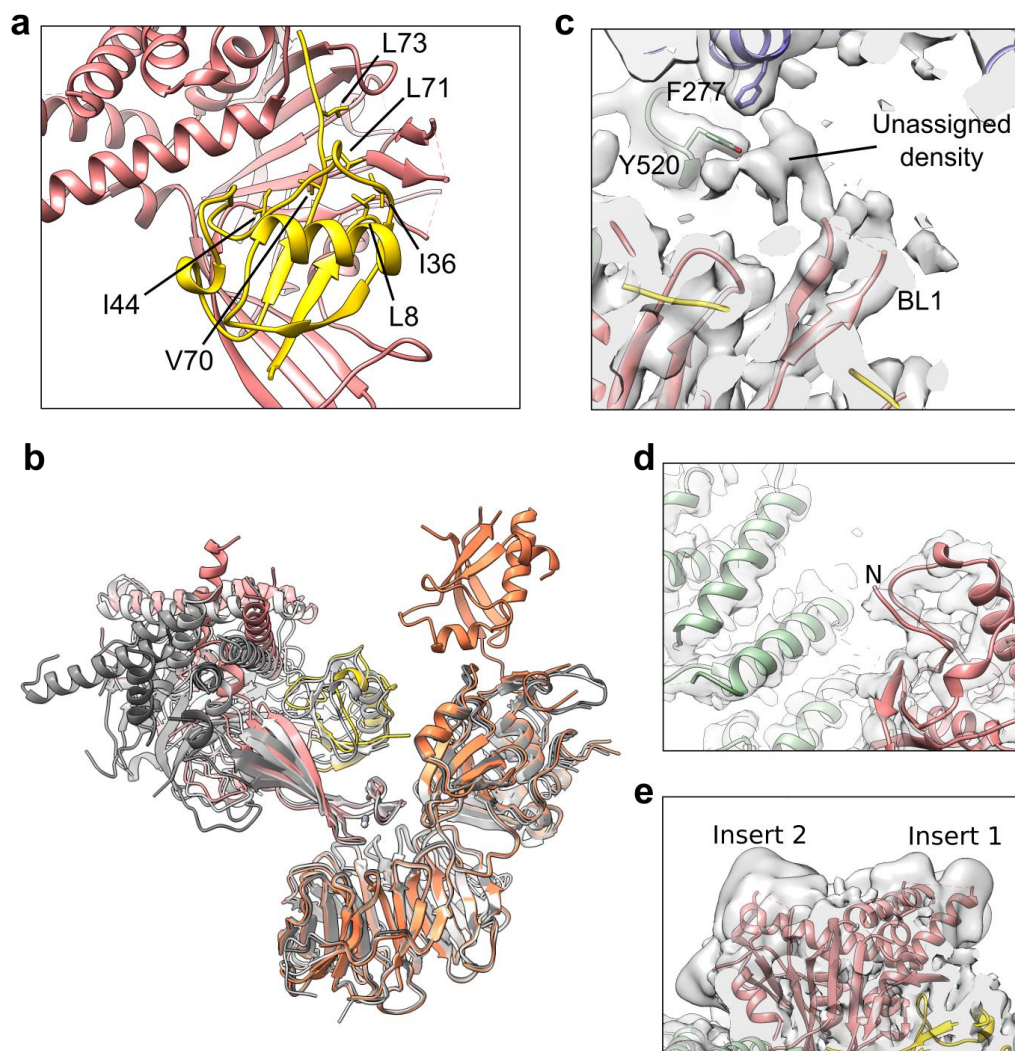
Supplementary Figure S4. The structure of USP1-UAF1 when bound to FANCI-FANCD2^{Ub}. (**a**) The USP1-ubiquitin interface. Residues of the hydrophobic patch of ubiquitin are highlighted. (**b**) Alignment of

USP1-UAF1 structures by the UAF1 subunit. The ubiquitin-free (dark gray) and ubiquitin-bound (light gray) crystal structures, and the FANCI-FANCD2[Ub]-bound cryo-EM structure (colored) are shown. (**c**) An unassigned blob of density is present adjacent to FANCI, FANCD2 and the BL1 of USP1. The locally filtered map is shown at a threshold of 0.07. (**d**) The N-terminus of USP1 is not well resolved. The DeepEMhancer map is shown at a threshold of 0.1. (**e**) Inserts 1 and 2 of USP1 are not well resolved. The locally filtered map is shown at a threshold of 0.02.

Supplementary Table S1. Crystallography data collection and model refinement statistics[a]

	Ubiquitin-free USP1-UAF1	Ubiquitin-bound USP1-UAF1
Data collection and processing		
Beamline	DLS I04	DLS I04
Wavelength (Å)	0.9795	0.9795
Unit cell	a=b=119.57 Å, c=195.46 Å	a=b=134.24 Å, c=274.67 Å
	$\alpha=\beta=\gamma=90°$	$\alpha=\beta=90°, \gamma=120°$
Space group	$P4_1$	$P6_5$
Resolution range (Å)	84.549 – 3.602 (3.754 – 3.602)	91.56 – 3.20 (3.31 – 3.20)
Ellipsoidal resolution (Å) (direction)	4.312 (0.902 a* + 0.433 b*)	N.A.
	4.381 (0.879 a* + 0.476 b*)	
	3.601 (0.246 a* + 0.469 b* + 0.848 c*)	
Unique reflections	25470 (1264)	46123 (4501)
Multiplicity	5.7 (5.4)	10.5 (10.1)
Completeness (%)		
Spherical	80.3 (34.2)	100 (100)
Ellipsoidal	95.0 (100)	N.A.
Mean I/sig(I)	6.4 (1.7)	11.6 (1.7)
Wilson B-factor (Å^2)	102.7	62.8
R_{meas}	0.258 (1.698)	0.193 (1.657)
R_{pim}	0.108 (0.727)	0.059 (0.519)

$CC_{1/2}$	0.992 (0.565)	0.997 (0.581)

Refinement

R_{work} / R_{free}	0.235 / 0.266	0.204 / 0.234
Number molecules in asymmetric unit		
USP1	2	2
UAF1	2	2
Ubiquitin	0	2
Disorder model	One TLS group per protein molecule + residual isotropic B-factors	One TLS group per protein molecule + residual isotropic B-factors
Bond length rmsd (Å)	0.003	0.004
Bond angle rmsd (°)	0.808	0.909
All-atom clashscore	7.3	3.9
Ramachandran plot		
Outliers (%)	0.51	0.31
Favored (%)	93.79	95.91
Rotamer outliers (%)	1.39	0.07
PDB ID	7AY0	7AY2

[a]Values in parentheses correspond to the highest resolution shell, apart from Ellipsoidal resolution

Supplementary Table S2. Cryo-EM data collection and model refinement statistics

Data collection and processing

Microscope	Cryoarm
Detector	DE64
Magnification	120,000x
Voltage (kV)	300
Electron Dose ($e^-/Å^2$)	65
CTF Estimated Defocus range (μm)	0.22 – 3.7

Pixel Size (Å)	1.015
Symmetry imposed	C1
Consensus map resolution (Å)	3.8
FSC threshold	0.143
Map resolution range (Å)[a]	3.6-10
FSC threshold	0.5
EMDB ID	EMD-11934

Refinement

Initial models used	6VAF, 5K1A, ubiquitin-bound USP1
Map sharpening B-factor (Å^2)	94.6
Correlation coefficient (mask)[b]	0.83
Bond length rmsd (Å)	0.006
Bond angle rmsd (°)	0.973
All-atom clashscore	9.1
Ramachandran plot	
Outliers (%)	0.04
Favored (%)	93.61
Rotamer outliers (%)	0.07
PDB ID	7AY1

[a]1% and 99% quantiles

[b]Calculated in phenix

Supplementary Movie S1. Morph between FANCI-FANCD2^{Ub} on its own (6VAF) and bound to USP1-UAF1. FANCD2 – green; FANCI – violet; ubiquitin – yellow; USP1-UAF1 – transparent.

Supplementary Movie S2. 3D variability of the cryo-EM dataset of USP1-UAF1-FANCI-FANCD2^{Ub}. First eigenvector of variability in the dataset. Viewed from above with respect to Figure 2, with FANCD2 on the left hand side.

Supplementary Movie S3. 3D variability of the cryo-EM dataset of USP1-UAF1-FANCI-FANCD2^{Ub}. Second eigenvector of variability in the dataset. Viewed from above with respect to Figure 2, with FANCD2 on the left hand side.