Mechanism of histone H2B monoubiquitination by Bre1

Fan Zhao, Chad W. Hicks and Cynthia Wolberger*

Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

*Corresponding author: cwolberg@jhmi.edu

Abstract

Monoubiquitination of histone H2BK120/123 plays multiple roles in regulating transcription, DNA replication and the DNA damage response. The structure of a nucleosome in complex with the dimeric RING E3 ligase, Bre1, reveals that one RING domain binds to the nucleosome acidic patch, where it can position the Rad6 E2, while the other RING domain contacts the DNA. Comparisons with H2A-specific E3 ligases suggests a general mechanism of tuning histone specificity via the non-E2-binding RING domain.

Main

Post-translational modification (PTM) of histones plays a central role in regulating eukaryotic transcription. Monoubiquitination of histone H2B-K123 in yeast, K120 in humans (H2B-Ub), is a hallmark of actively transcribed genes that also plays a role in DNA replication, DNA repair and RNA processing[1-3]. H2B-Ub stimulates methylation of histone H3-K4 and K79, and recruits FACT (Facilitates Chromatin Transcription) to promote efficient transcriptional elongation[4,5]. Bre1 is a dimeric ubiquitin E3 ligase that targets the E2 ubiquitin conjugating enzyme, Rad6, to monoubiquitinate H2B-K123 in yeast[6,7]. In humans, the closely related RNF20/RNF40 heterodimer targets RAD6A/B to ubiquitinate histone H2B-K120[8,9]. Mutations and deletions of RNF20/40 are found in a variety of cancers and are indicators of poor prognoses[10]. The mechanism underlying the specificity of H2B-K123/120 ubiquitination is unknown.

We report here the cryo-EM structure of a Bre1 E3 ligase dimer bound to a nucleosome that reveals the molecular basis for specific ubiquitination of histone H2B. The fragment used in the study, Bre1 591-700, includes the RING domain and a coiled-coil that mediates dimerization[11], and directs specific ubiquitination of H2B-K123[12]. Three distinct states of the Bre1-nucleosome complex were resolved at overall resolutions of 3.80 Å for state 1, 3.85 Å for state 2, and 3.71 Å for state 3 (Extended Data Figs.1,2). In each state, one Bre1 monomer was well ordered, with a local resolution of 3-4 Å, while the other monomer was resolved at resolutions ranging from 4Å to 6Å (Extended Data Fig.2), indicating somewhat higher mobility. The well-resolved Bre1 density map

enabled us to successfully model Bre1 dimer onto nucleosome using its crystal structure[11].

The Bre1 dimer straddles the periphery of the nucleosome, with one RING domain (Bre1-A) contacting the nucleosome acidic patch and the other (Bre1-B) interacting with the DNA at superhelical position (SHL) 6.5 (Figs.1a-b). Residues 632-647 form an α helix that mediates coiled coil interactions with the opposing monomer, followed by the catalytic RING domain, which contains a basic patch comprising residues R679, R681, K682, and K688 (Extended Data Fig.3). The Bre1-A RING domain binds in a similar manner to the nucleosome acidic patch in all three states, with R679 forming salt bridges with H2A residues E61 and D90, and R681 forming salt bridges with H2A residues E61 and E64 (Fig.1b,c). This mode of interaction with the nucleosome acidic patch, termed an “arginine anchor,” has been observed in multiple structures[13]. The position of the Bre1-B RING relative to the DNA backbone varies more between the three states due to a repositioning of the Bre1 coiled coil. In state 1, the Bre1-B RING is positioned such that residues R679 and R681 are in a position to contact the electronegative DNA backbone (Fig.1d, Extended Data Fig.4a). In state 2, the Bre1 dimerization coiled coil is tilted such that the Bre1-B RING rotates by about 10° about the face of the nucleosome and is about 13 Å farther from the DNA. In state 3, the Bre1 dimer is rotated by an additional 5° on the nucleosome relative to state 2, which shifts the Bre1-B RING by about 4 Å relative to state 2 (Fig.1e,f and Extended Data Fig.4). Importantly, there is a bulge in the DNA in state 3 that repositions the sugar-phosphate

backbone 5 Å closer to the Bre1-B RING, placing Bre1-B residue R681 in a position to form electrostatic interactions with the DNA backbone (Fig.1f).

The key roles of Bre1 RING domain residues R679 and R681 are consistent with the deleterious effects of substitutions of these residues on Bre1's binding and H2B ubiquitination activity on nucleosome. Bre1 substitutions R679D and R681D abrogated nucleosome binding in pull-down assays and ubiquitination of H2B K120 in vitro [14]. Bre1 basic patch mutations R675D/R679D and R681D/K682D completely abrogated H2B ubiquitination activity in vivo and resulted in severe growth defects comparable to a *bre1* deletion. Residues R679, R681 and K682 are conserved in the RING domains of human homologues RNF20 and RNF40 (Extended Data Fig.5) [8], suggesting a similar mechanism of H2B ubiquitination in humans.

The positioning of the E2, Rad6, by the Bre1-A RING domain could be modeled based on structural studies of Ubr1, a RING E3 ligase that also binds to Rad6 and polyubiquitinates N-degrons to target substrates for proteasomal degradation[15]. To model the position of Rad6 binding to the Bre1 RING, we aligned the Ubr1 RING domain to the Bre1-A RING domain for all three states of Bre1 bound to the nucleosome. In state 3, this modeling positions the Rad6~Ub thioester directly over histone H2B K120 (corresponding to K123 in the yeast nucleosome), with distances of 2.9 Å between the Sγ of the Rad6 active site cysteine, C88, and Nζ of H2B K120, and 2.6 Å between the carbonyl carbon of Ub G76 and H2B K120 Nζ (Fig.2a, b). The corresponding distances in state 2 are 3.2 Å and 3.9 Å, respectively (Extended Fig.

6a,b). These distances would readily allow ubiquitin transfer from Rad6 to H2B K120. Interestingly, the modeled position of Rad6 in state 1 places the Rad6~Ub thioester farther away from substrate lysine (8.3Å and 11.6Å) (Extended Data Fig. 6c,d). This suggests that the three Bre1-nucleosome complex states each represent a different Rad6 activity status. Bre1 in state1 targets Rad6 and Ub over H2B K120 site but in a "poised" state due to the distance from the substrate lysine, while states 2 and 3 further facilitate an "active" state that promoted ubiquitin transfer to H2B K120.

Both Bre1 and Ubr1 contain N-terminal helical regions that contact Rad6 and enhance E2 discharge, termed RBD (Rad6 binding domain; residues 1-210) in Bre1 and U2BR (Ubc2-binding region) in Ubr1 [12,15]. A recent crystal structure of Bre1-RBD bound to Rad6 revealed that the alpha-helical RBD is a forms an asymmetric homodimer that binds to the Rad6 backside[16]. A superposition of the RBD-Rad6 complex with our modelled Rad6 on bound to the Bre1-A RING shows that the RBD, which extends away from the nucleosome disk, can be readily accommodated without steric clash with the C-terminal Bre1 dimer or the nucleosome (Extended Data Fig.7). An additional ~440 residues that are not present in the RBD or in the Bre1 fragment in the present study bridge the RBD C-terminus and the Bre1 coiled-coil.

Mutations in the human Bre1 homologs, RNF20 and RNF40, have been found in a variety of cancers according to the cancer genomics database of cBioPortal (http://www.cbioportal.org). Of these, 27 map to residues in the RING domain or the α helical regions of RNF20 and RNF40 (Tables 2 and 3; Extended data Fig. 5). These

include RNF20 R955, corresponding to Bre1-R681 that binds the nucleosome acidic patch, which is mutated to histidine in esophagogastric cancer (Extended data Fig.5 and Table 2).

A comparison of the Bre1 complex with that of E3 ligases that ubiquitinate other histone residues points to a pivot-like mechanism for tuning E3 ligase specificity. The positioning on the nucleosome of Bre1, which ubiquitinates H2B-K120/123, is markedly different from that of the heterodimeric E3 ligase, Ring1B/Bmi1[17], which ubiquitinates H2A-K119, and BRCA1/BARD1[18], which ubiquitinates histone H2A K125/127/129. Like Bre1, the RING domain that that recruits the E2, Ring1B or BRCA1, binds to the nucleosome acidic patch with basic residues (Fig.2c-e). It is the orientation of the second RING domain in each complex that governs the positioning of the E2, and hence its specificity. Whereas Bre1 uses the second RING domain to interact with the DNA, Bmi1 and BARD1, respectively, bind to different locations in the globular histone core. Bmi1 caps the C-terminal end of H3 α1 helix via salt bridges formed by K62 and R64 and acidic residues in histones H3 and H4[17], while BARD1 binds to the nucleosome H2B/H4 cleft with its Trp91 inserted[18]. These second RING domains thus play the defining role in determining the E3 ligase specificity for its histone substrate.

METHODS

Protein expression and purification

A polymerase chain reaction (PCR) product of fusion protein Bre1(591-700) -$(GGS)_4$-Bre1(591-700) -$(GGS)_8$-Rad6(1-172) was cloned into vector pET32-a (Novagen) with N-

terminal thioredoxin, a hexahistidine tag (Trx-His), and a Tobacco Etch Virus (TEV) protease site. This plasmid was used to transform BL21(DE3)Rosetta2 *E.coli* strain for protein expression. Briefly, *E. coli* cells were grown in Luria-Bertani (LB) media at 37°C while shaking until the culture reached an OD_{600} of 0.8. The growth temperature was then decreased to 18°C, 0.2 mM IPTG was added to induce protein expression, and the cells grown overnight.

Cells were harvested by centrifugation and resuspended in 20 ml lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 50 μM $ZnCl_2$, 5% glycerol, 5 mM 2-mercaptoethanol, 10 mM imidazole, 100 μM Phenylmethylsulfonyl fluoride (PMSF)) per liter of growth medium. Cells were lysed with a Microfluidizer (Microfluidics) and the cell lysate cleared by high-speed centrifugation. The supernatant was then incubated with 10 mL Ni-NTA beads (QIAGEN) for 90 min at 4°C. The beads were further washed with lysis buffer and the protein eluted with 50 ml lysis buffer supplemented with 250 mM imidazole. A 2 mg sample of TEV protease was added to the Ni-NTA column eluent for overnight cleavage. The resulting protein was further purified using a 5mL HiTrap Heparin column developed with a salt gradient of 100 mM – 600 mM NaCl over 75ml followed by gel filtration on a Superdex 200 10/300 column (GE Healthcare) in buffer 20 mM HEPES, pH 7.5, 150 mM NaCl, 50 μM $ZnCl_2$, 1 mM DTT. The purified protein was concentrated to 10 mg/mL, aliquoted, flash frozen, and stored at −80°C for future use.

Nucleosome reconstitution

Unmodified *Xenopus laevis* histone proteins, H2A, H2B, H3 and H4 were purified as described previously[19]. The pST55−16 × 601 plasmid containing 16 repeats of the 147

base pair Widom 601 positioning sequence was amplified in the *E. coli* strain XL1-Blue. After plasmid extraction, the 601 DNA was excised with *Eco*RV, recovered essentially as described previously[20]. Nucleosomes were reconstituted from purified histones and DNA as previously described[20].

Cryo-EM sample preparation

A sample of 100 nM unmodified nucleosome was incubated with 10 μM Bre1-Bre1-Rad6 fusion protein in cross-linking buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 1 mM DTT) at room temperature for 30 min. An equal volume of 0.15% glutaraldehyde was then added to the sample mixture. After incubation on ice for 1 hour, the cross-linking reaction was quenched by the addition of 100 mM Tris pH 7.5. The sample was dialyzed overnight into quenching buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT) and applied to a Superdex 200 10/300 size exclusion column (GE Healthcare) that was pre-equilibrated with cross-linking buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 1 mM DTT). The peak fractions were concentrated to a final sample concentration of 1 mg/mL.

Quantifoil R2/2 copper 200 mesh grids (Electron Microscopy Sciences) were glow-discharged for 45 seconds at 15 mA, after which, 3 μl of cross-linked sample was applied to the grids and blotted for 3.5 seconds with a blot force of 5, then immediately plunged frozen in liquid ethane using a Vitrobot Mark IV apparatus (Thermo Fisher Scientific) at 4°C, 100% humidity.

Cryo-EM data processing and model building

A cryo-EM dataset was collected at the National Cryo-Electron Microscopy Facility (NCEF) of the National Cancer Institute on a Titan Krios at 300 kV utilizing a Gatan K3 direct electron detector equipped with an energy filter with a 20 eV slit width. A multi-shot imaging strategy (3 shots per hole) was used for data collection. Images were recorded in counting-mode at a nominal magnification of 105,000, a pixel size of 0.855 Å and a dose of 50.02 $e^-/Å^2$ with 40 frames per movie at a defocus range of 1.25-2.5 μM. A total of 4,561 movies were collected.

The dataset was processed using cryoSPARC[21]. After patch-based motion correction and contrast transfer function estimation, 3,778 micrographs were selected for particle picking. Initial particles were picked by Blob picker. After particle inspection and extraction, a total of 721,361 initial particles were applied to 2D classification. After this, the best 2D classes were selected as templates for template picking of particles. Then, a total of 2,134,610 particles were extracted and subjected to two rounds of 2D classification, resulting in a particle set of 481,534 particles. After initial reconstruction and heterogeneous refinement with 3 classes, one 3D class accounting for 305,792 particles was generated and refined. Based on this EM map, a mask encompassing one Bre1 dimer and less than half of nucleosome was generated. After C2 symmetry expansion of particles, a total of 611,584 particles were subjected to 3D classification with the above mask. Among the ten 3D classes, five classes had well-resolved Bre1 dimer, and three states of Bre1-nucleosome binding modes were identified. In these three states, maps were further refined to 3.80 Å for state 1, 3.85 Å for state 2, and 3.71 Å for state 3. Finally, a sharpening B factor of -20 $Å^2$ was applied to maps of state1 and 2, and a sharpening B factor of -5 $Å^2$ was applied to the map of state 3. Although Rad6

was fused to the Bre1 C-terminus in an effort to overcome the low affinity of Rad6 for the Bre1 RING domain[12,22], there was no density corresponding to Rad6 in any of the maps.

PDB structures of Bre1 (PDB: 4R7E) and nucleosome (PDB: 6NOG) were rigid-body fitted in the map using UCSF ChimeraX[23]. The initial Bre1-nucleosome structures were manually rebuilt and subjected to real-space refinement using *Coot*[24] and Phenix[25,26]. Due to the C2 symmetry of the complex, only one Bre1 dimer was modeled in the final structures. These coordinates were further validated using Comprehensive validation (CryoEM) in Phenix before Protein Databank (PDB) database deposition. Figures were created using PyMOL, UCSF Chimera, UCSF ChimeraX and Adobe Illustrator.

Data availability

The reconstructed map is available in the Electron Microscopy Data Bank (EMDB) database under accession No. EMD-29933, EMD-29938, and EMD-29942 for Bre1-nucleosome complex in state 1, 2 and 3.. The atomic model is available in the Protein Databank (PDB) database with accession No. 8GCF, 8GCO and 8GCU for Bre1-nucleosome complex in state 1, 2 and 3 .

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Contributions

C.W. and F.Z. conceived and designed the study. F.Z. and C.W.H. performed experiments, and processed data. F.Z. carried out modeling and map interpretation with assistance from C. W. analysis. F.Z., C.W.H., and C.W. wrote the manuscript. C.W. supervised the study.

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FIGURE 1

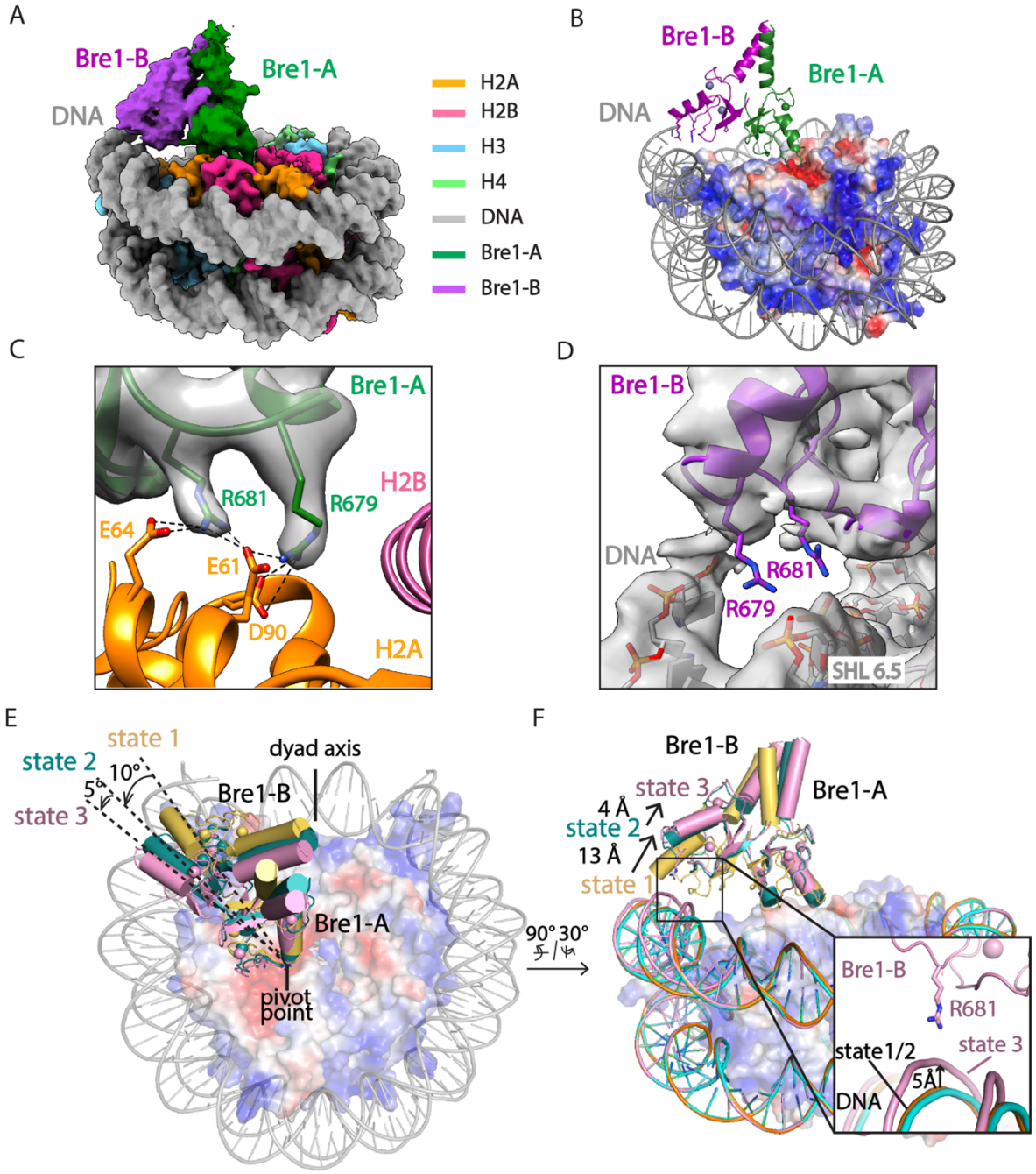


Figure 1. Cryo-EM structures of Bre1 dimer bound to nucleosome reveal multiple conformations. a, Cryo-EM density map of the Bre1 bound to nucleosome (state 1). **b**, View of the interface between Bre1 and nucleosome (state 1). Histone octamer is shown in an electrostatic potential surface representation (red=negative, blue=positive). **c**, Interface between Bre1-A (state 1) and the nucleosome acidic patch superimposed on EM map. **d**, Contact between Bre1-B and the DNA (state 1). The distances of Bre1-B R679 and R681 to DNA backbone are 3 Å and 3.3 Å. **e-f**, Change of Bre1 position in three states. The distance of Bre1-B R681 to DNA backbone in state 3 is 3.3 Å.

FIGURE 2

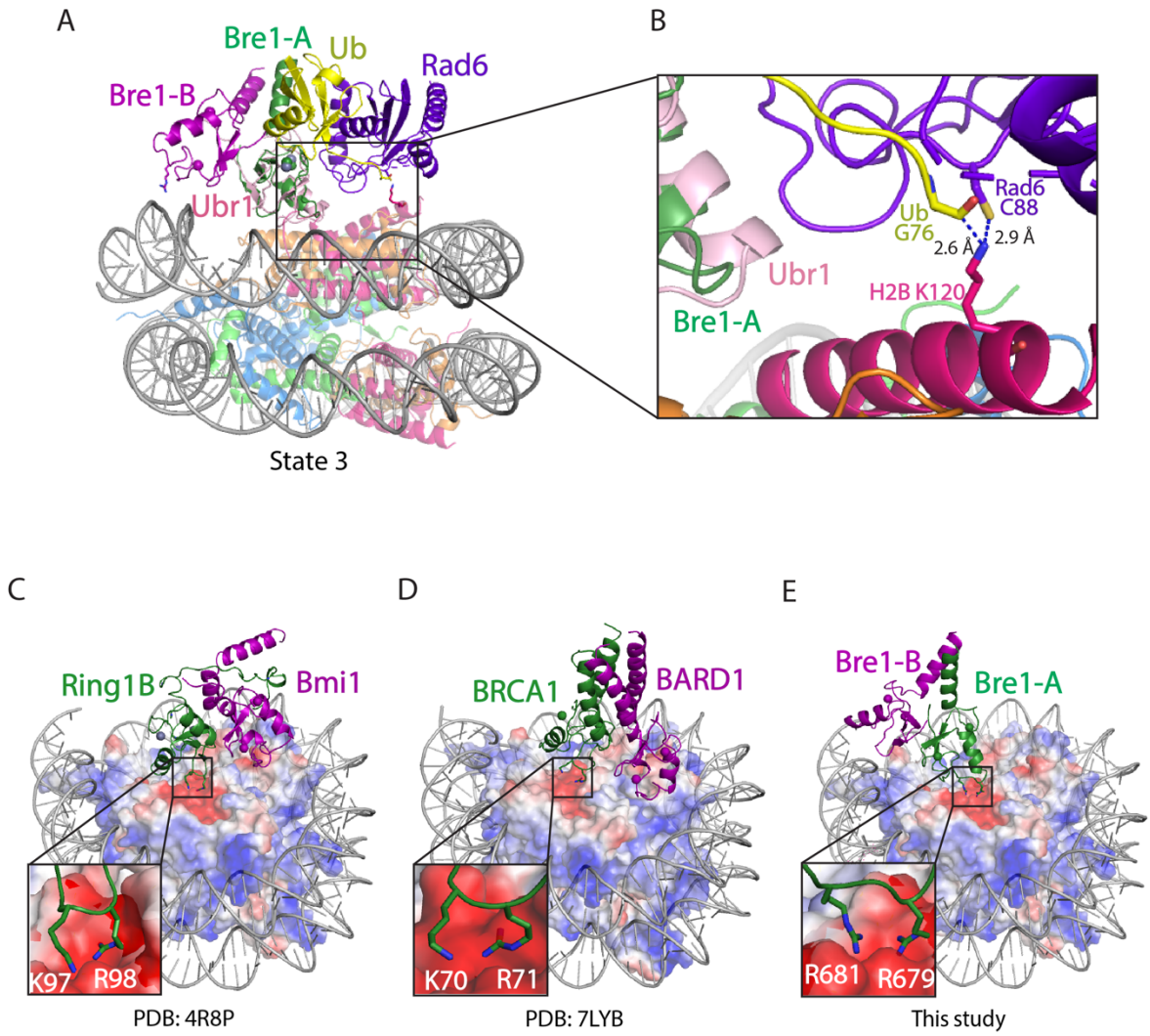


Figure 2. Bre1 orients Rad6 to ubiquitinate histone H2B K120. **a**, Superposition of Bre1-nucleosome state 3 and Ubr1 RING-Rad6-ubiquitin (PDB: 7MEX) via the RING domain **b**, Close-up view of Rad6 active site C88, ubiquitin G76 and H2B K120. **c-e**, Structures of Ring1b-Bmi1 (PDB: 4R8P), BRCA1-BARD1 (PDB: 7LYB), and Bre1 dimer bound to nucleosome. Histone octamer is shown in an electrostatic potential surface representation.

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

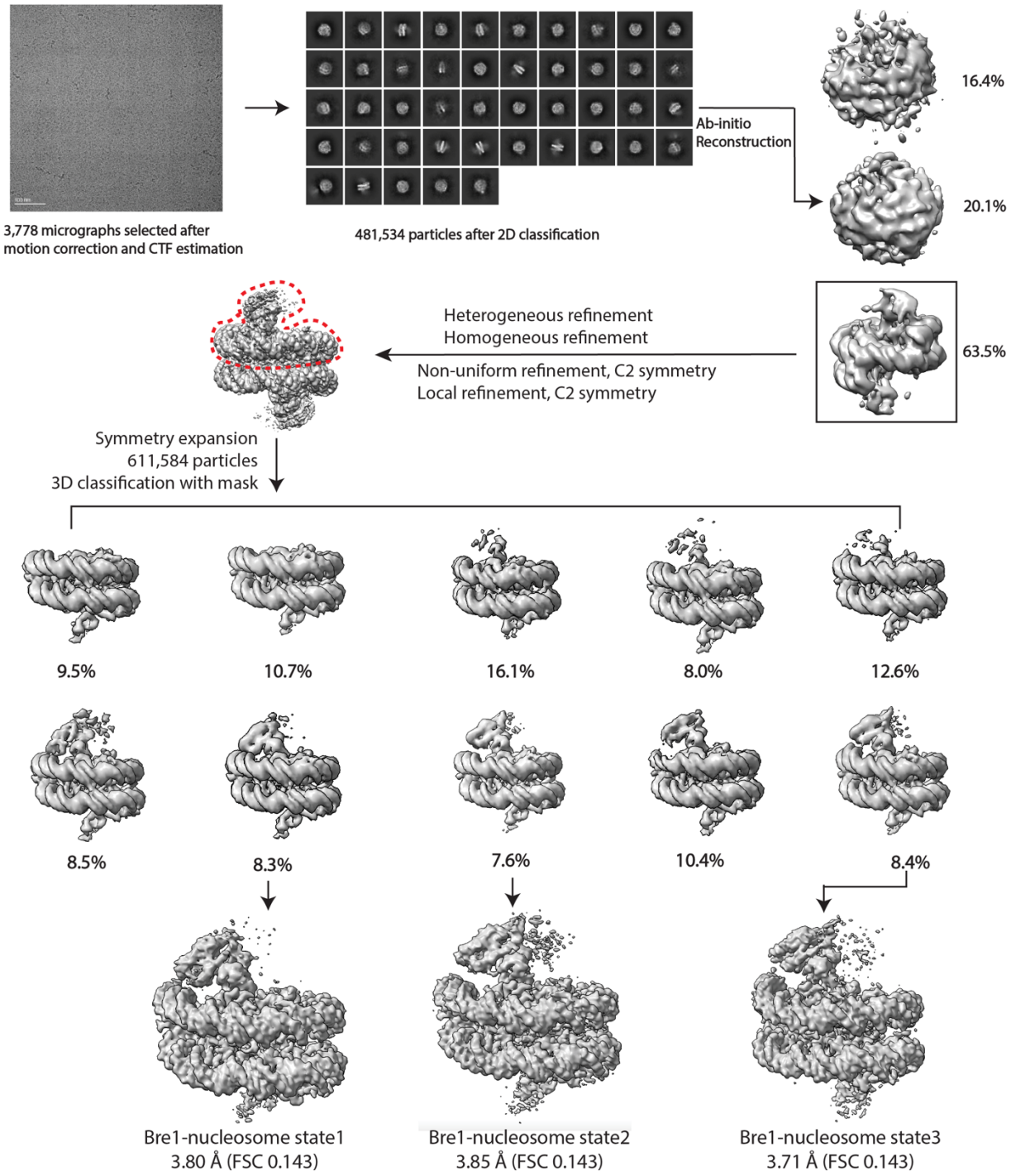
	Bre1-nucleosome complex (state1)	Bre1-nucleosome complex (state2)	Bre1-nucleosome complex (state3)
Data collection and processing			
Magnification	105,000	105,000	105,000
Voltage (kV)	300	300	300
Electron exposure (e^-/Å)	50.02	50.02	50.02
Defocus range (μm)	-1.25 to -2.5	-1.25 to -2.5	-1.25 to -2.5
Pixel size (Å)	0.855	0.855	0.855
Symmetry imposed	C1	C1	C1
Initial particle image (No.)	481,534	481,534	481,534
Final particle image (No.)	50,520	46,504	51,358
Map resolution (Å)	3.80	3.85	3.71
FSC threshold	0.143	0.143	0.143
Refinement			
Map sharpening *B* factor (Å^2)	-20	-20	-5
Model composition			
Nonhydrogen atoms	12981	12971	12971
Protein residues	886	885	885
Nucleotides	292	292	292
Ligands	Zn^{2+}(4)	Zn^{2+}(4)	Zn^{2+}(4)
R.m.s. deviations			
Bond lengths (Å)	0.003	0.003	0.003
Bond angles (°)	0.601	0.619	0.616
Validation			
MolProbity score	1.41	1.53	1.54
Clashscore	6.16	7.74	6.98
Poor rotamers (%)	0.40	0.40	0.40
Ramachandran plot			
Favored (%)	97.69	97.46	97.11
Allowed (%)	2.31	2.54	2.89
Disallowed (%)	0.00	0.00	0.00

Table 2. RNF20 mutations located in RING domain and its preceded α helix.

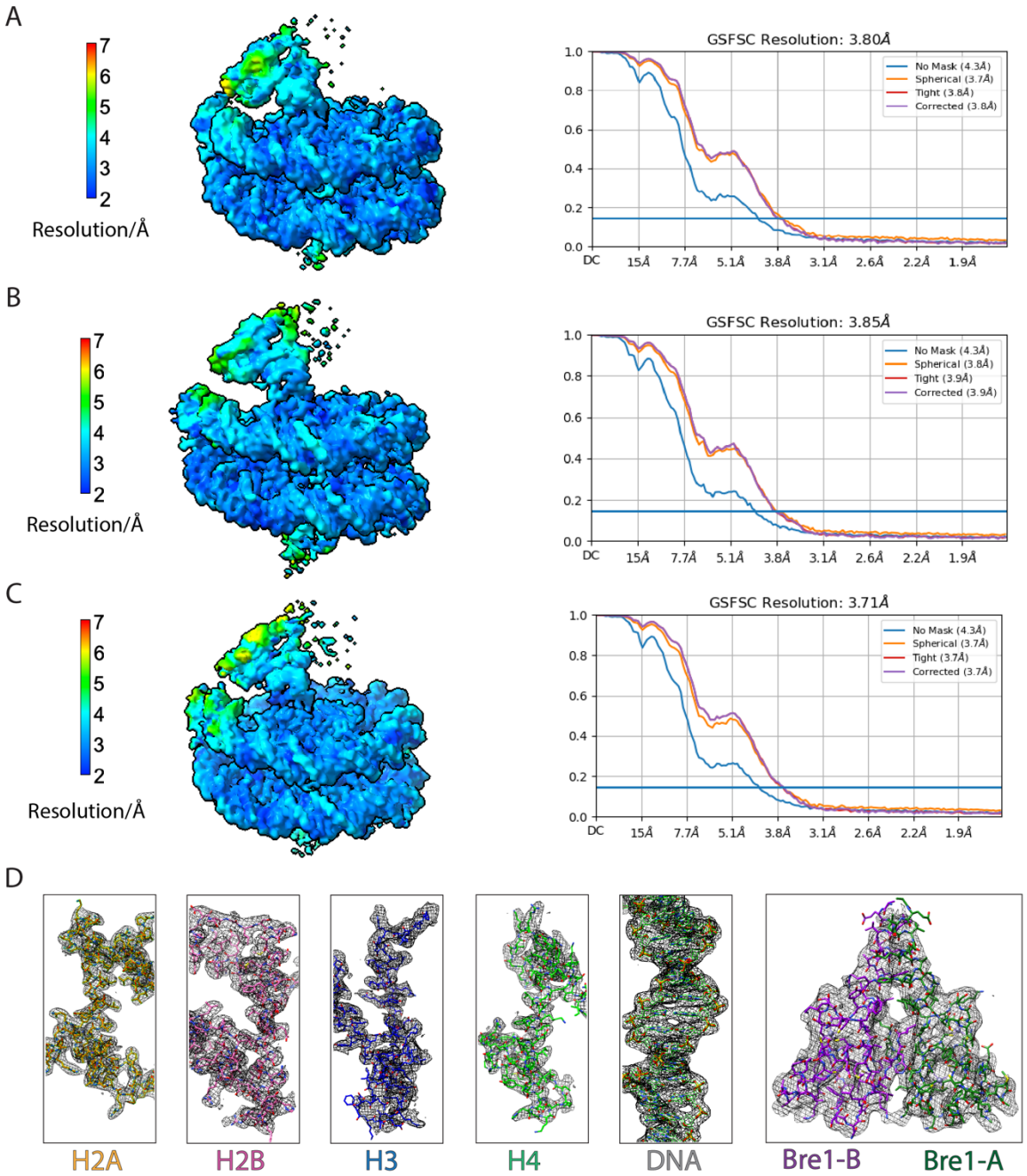
Study of Origin	Cancer Type	Protein Change	Mutation Type
Lung Squamous Cell Carcinoma (TCGA, PanCancer Atlas)	Non-Small Cell Lung Cancer	R949L	Missense_Mutation
Lung Squamous Cell Carcinoma (TCGA, PanCancer Atlas)	Non-Small Cell Lung Cancer	Q954*	Nonsense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	R971H	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	D915Y	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	K929N	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	E907A	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	L909M	Missense_Mutation
Lung Adenocarcinoma (TCGA, PanCancer Atlas)	Non-Small Cell Lung Cancer	C924F	Missense_Mutation
Skin Cutaneous Melanoma (TCGA, PanCancer Atlas)	Melanoma	H970Y	Missense_Mutation
Stomach Adenocarcinoma (TCGA, PanCancer Atlas)	Esophagogastric Cancer	P923L	Missense_Mutation
Stomach Adenocarcinoma (TCGA, PanCancer Atlas)	Esophagogastric Cancer	R955H	Missense_Mutation
Colorectal Adenocarcinoma (TCGA, PanCancer Atlas)	Colorectal Cancer	R949H	Missense_Mutation
Head and Neck Squamous Cell Carcinoma (TCGA, PanCancer Atlas)	Head and Neck Cancer	P923L	Missense_Mutation
Cervical Squamous Cell Carcinoma (TCGA, PanCancer Atlas)	Cervical Cancer	R949C	Missense_Mutation

Table 3. RNF40 mutations located in RING domain and its preceded α helix.

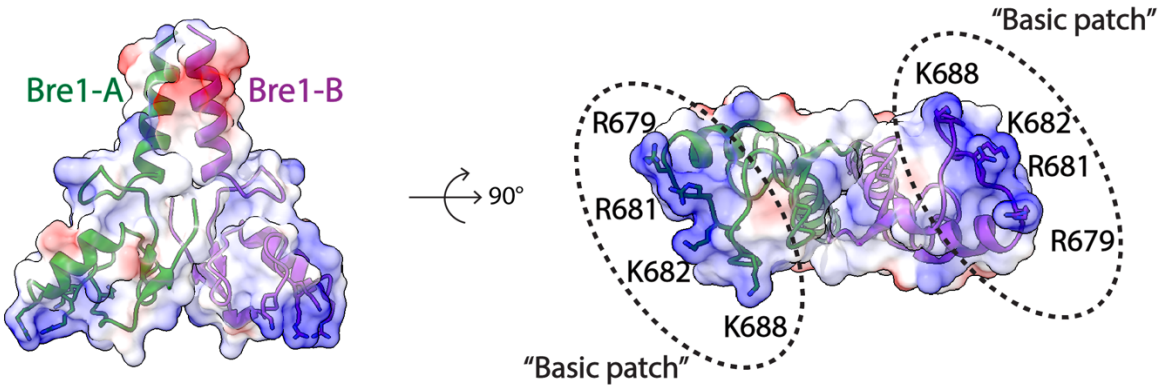
Study of Origin	Cancer Type	Protein Change	Mutation Type
Bladder Urothelial Carcinoma (TCGA, PanCancer Atlas)	Bladder Cancer	X944_splice	Splice_Site
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	R954C	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	R954C	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	R973W	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	E937D	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	A944T	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	X944_splice	Splice_Region
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	A944V	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	F967L	Missense_Mutation
Uterine Corpus Endometrial Carcinoma (TCGA, PanCancer Atlas)	Endometrial Cancer	R997H	Missense_Mutation
Stomach Adenocarcinoma (TCGA, PanCancer Atlas)	Esophagogastric Cancer	R973W	Missense_Mutation
Colorectal Adenocarcinoma (TCGA, PanCancer Atlas)	Colorectal Cancer	V966I	Missense_Mutation
Colorectal Adenocarcinoma (TCGA, PanCancer Atlas)	Colorectal Cancer	R973Q	Missense_Mutation



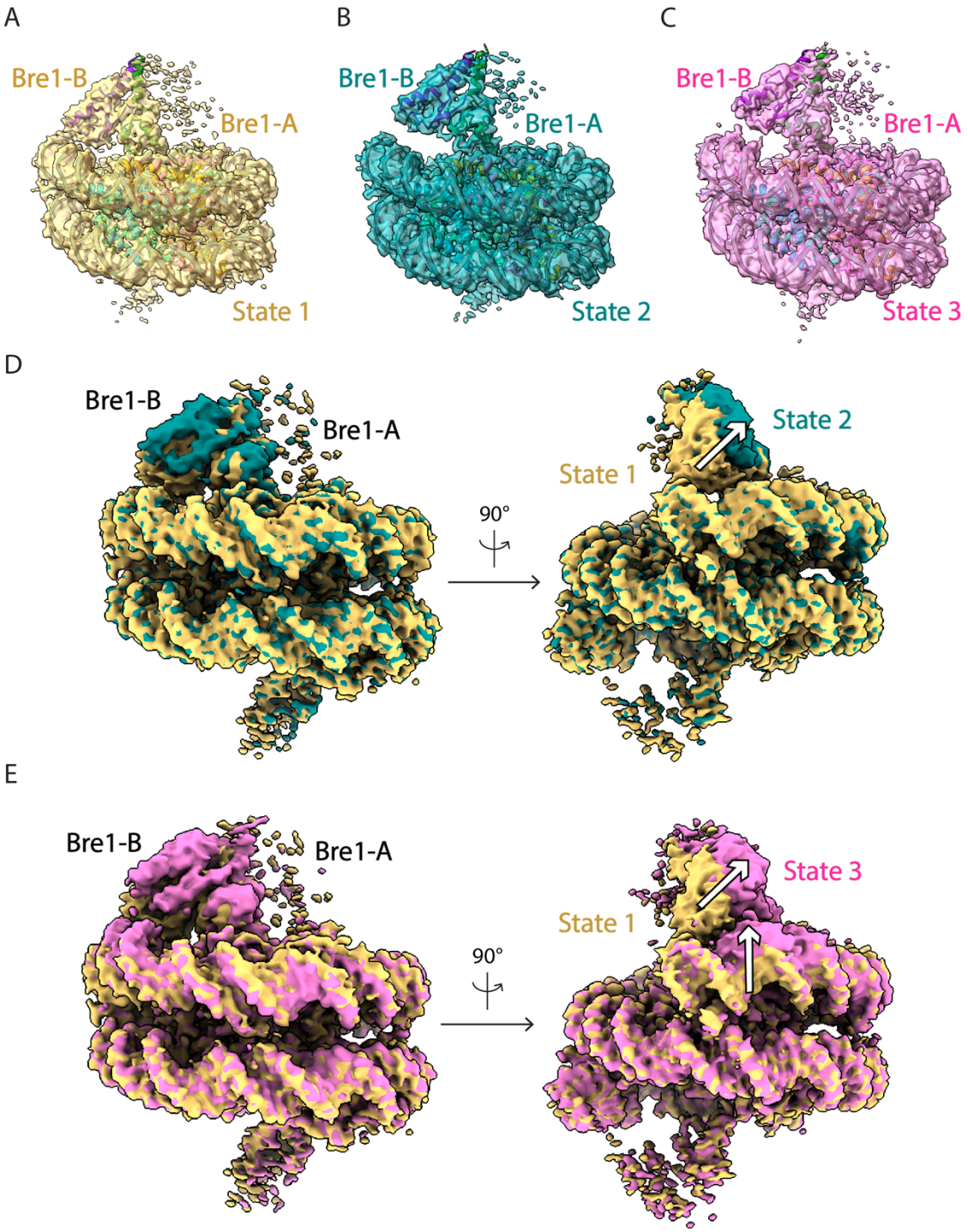
Extended Data Fig.1 Cryo-EM processing workflow. The red dashed line indicates the mask generated for use in 3D classification. Three states of Bre1-nucleosome complex are identified and refined respectively.



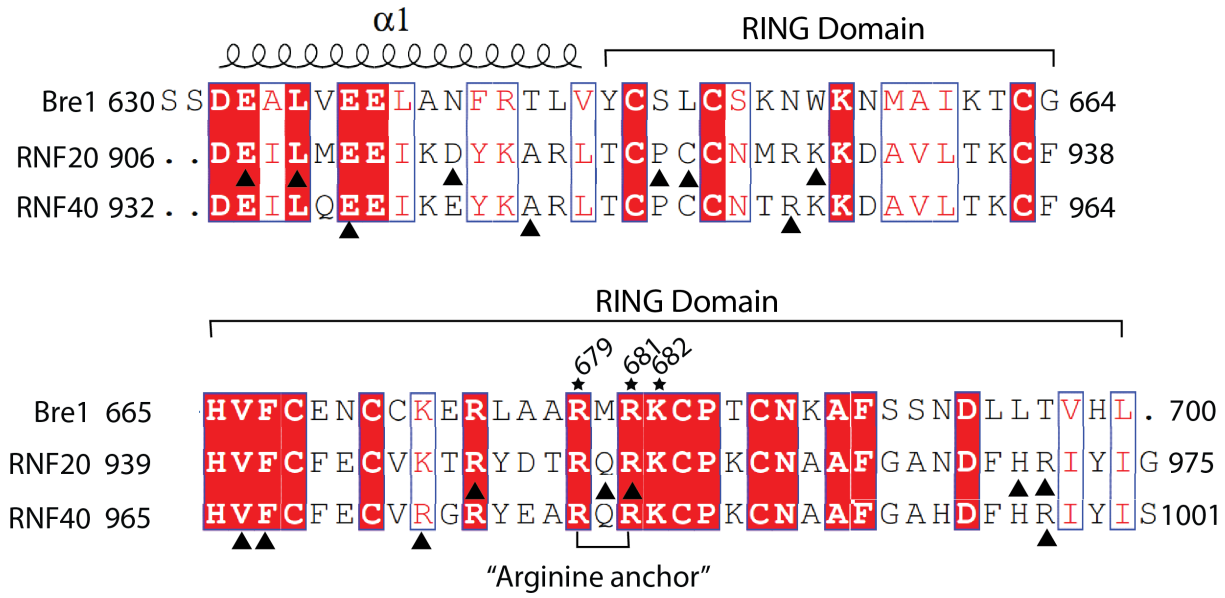
Extended Data Fig.2 Cryo-EM validation and example density. **a-c**, Local resolution estimation and gold-standard Fourier shell correlation (GSFSC) curves at a FSC cutoff of 0.143 for each state of Bre1-nucleosome complex. **d**, Representative cryo-EM density maps for the histones, DNA, and Bre1.



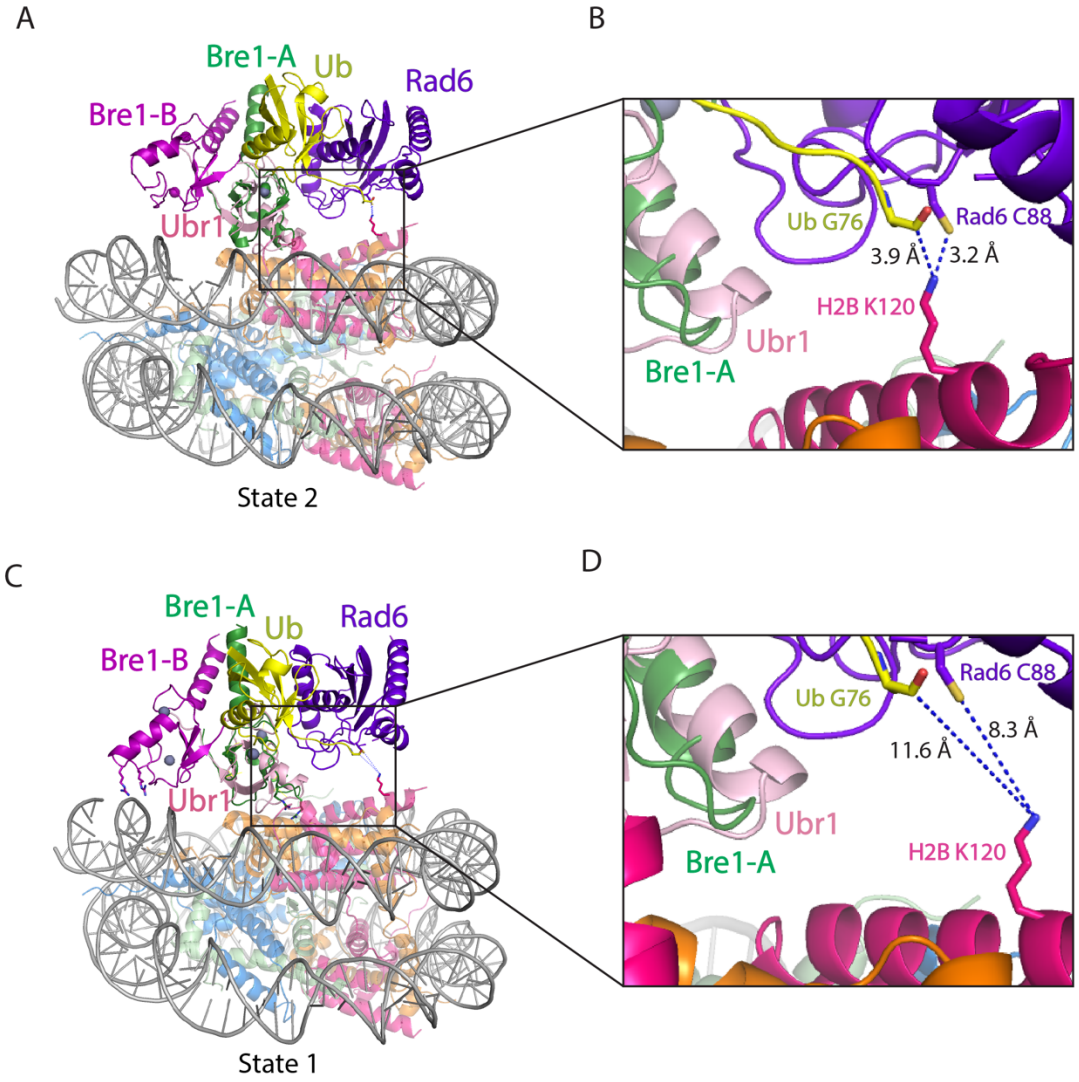
Extended Data Fig.3 Electrostatic surface representation view of Bre1 dimer (PDB:4R7E). Bre1 is shown in a cartoon representation with basic residues highlighted as sticks.



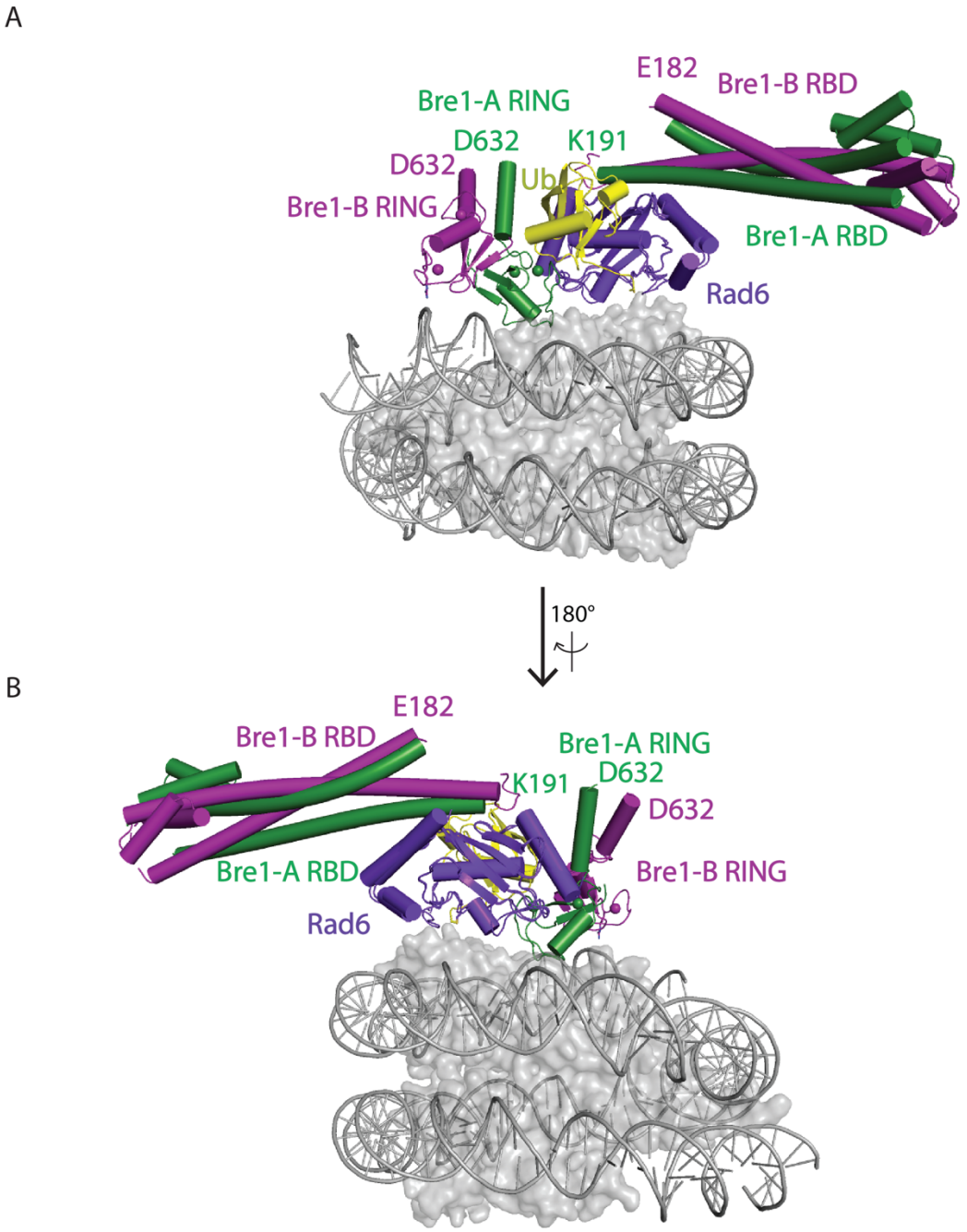
Extended Data Fig.4 Multiple conformations of the Bre1-nucleosome complex. **a-c**, Cryo-EM maps of the Bre1-nucleosome complex with an atomic model fitted to states 1, 2 and 3. **d-e**, Superimpose of Bre1-nucleosome complex maps comparing states 1 and 2 (d), and states 1 and 3 (e). White arrows show the changes between each conformational state.



Extended Data Fig.5 Sequence alignment of coiled coil and RING domains of Bre1, RNF20 and RNF40. Black triangle indicates the mutation sites of RNF20 and RNF40 in cancer genomics.



Extended Data Fig. 6 Superposition of Bre1-nucleosome complex (state 2 and 1) and Ubr1 RING-Rad6-ubiquitin (PDB: 7MEX). **a,c,** Ubr1 RING domain is aligned to Bre1-A RING domain in state 2 (a) and state 1 (c). **b,d**, Close-up view of Rad6 active site C88, ubiquitin G76, and H2B K120 in state 2 (b) and state 1 (d).



Extended Data Fig. 7 Structural alignment of Bre1 RBD-Rad6 complex and Bre1 RING-Rad6-Ub-nucleosome complex (model of state 3)**. a, b,** Rad6 in Bre1 RBD-Rad6 complex is aligned to the Rad6 in Bre1 RING-Rad6-Ub-nucleosome complex (model of state 3).