Atomic resolution cryo-EM structure of a native-like CENP-A nucleosome aided by an antibody fragment

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Genomic DNA in eukaryotes is organized into chromatin through association with core histones to form nucleosomes, each distinguished by their DNA sequences and histone variants. Here, we used a single-chain antibody fragment (scFv) derived from the anti-nucleosome antibody mAb PL2-6 to stabilize human CENP-A nucleosome containing a native α-satellite DNA and solved its structure by the cryo-electron microscopy (cryo-EM) to 2.6 Å resolution. In comparison, the corresponding cryo-EM structure of the free CENP-A nucleosome could only reach 3.4 Å resolution. We find that scFv binds to a conserved acidic patch on the histone H2A-H2B dimer without perturbing the nucleosome structure. Our results provide an atomic resolution cryo-EM structure of a nucleosome and insight into the structure and function of the CENP-A nucleosome. The scFv approach is applicable to the structural determination of other native-like nucleosomes with distinct DNA sequences.
Results

An antibody fragment and nucleosome stabilization. Antibodies and their fragments have often been used to stabilize macromolecules for structural determination. Anti-nucleosome antibodies are present in patients with systemic lupus erythematosus autoimmune disease. Notably, the PL2-6 antibody, isolated from autoimmune mice with lupus-like nephritis, has been suggested to bind the conserved acidic patch of the nucleosome surface. We engineered a single-chain fragment (scFv) from the PL2-6 antibody, which includes the variable heavy (Hv) and light (Lv) chains connected by a flexible linker (Fig. 1a and Supplementary Fig. 1a). Gel shift assay and isothermal titration calorimetric experiments showed that scFv bound to the NCP containing Drosophila core histones and 147 bp W601 DNA, termed NCPH3, W601, with 2:1 stoichiometry and a dissociation constant (Kd) of ~190 nM for each binding site (Supplementary Fig. 5 and Supplementary Table 1). Gel shift assay and isothermal titration calorimetric experiments showed that scFv bound to the NCP containing Drosophila core histones and 147 bp W601 DNA, termed NCPH3, W601, with 2:1 stoichiometry and a dissociation constant (Kd) of ~190 nM for each binding site (Supplementary Fig. 5 and Supplementary Table 1). Gel shift assay and isothermal titration calorimetric experiments showed that scFv bound to the NCP containing Drosophila core histones and 147 bp W601 DNA, termed NCPH3, W601, with 2:1 stoichiometry and a dissociation constant (Kd) of ~190 nM for each binding site (Supplementary Fig. 5 and Supplementary Table 1) as well as its ability to obtain high-quality diffraction crystals for X-ray crystallography analysis is strongly dependent on the DNA fragment used for NCP assembly. Single particle cryo-EM provides an alternative way to determine atomic resolution structures without requiring crystals; however, nucleosomes tend to dissociate during cryogenic sample preparation, which apparently limits the resolution of structures of NCPs determined. To date, single particle cryo-EM studies of free nucleosomes or those in complex with other proteins have mainly relied on the Widom “601” (W601) DNA, which was selected in vitro for high affinity binding to the core histones.

Here, we overcome this hurdle by using a single-chain antibody fragment (scFv) to stabilize the nucleosome. We determine the cryo-EM structure of the human centromeric nucleosome containing CENP-A and a native α-satellite DNA sequence at 2.6 Å resolution. Our study reveals structural features and provides insights into the structure and function of the centromeric nucleosome. The results and the scFv method present here pave an avenue for the structural determination of nucleosomes with natural DNA sequences at atomic resolution by cryo-EM.

Interactions between the nucleosomes and the scFv. In both structures of the scFv-NCP_CENP-A, NAS and scFv-NCP_CENP-A, W601 complexes, the residues in the variable loops of the scFv interact with the H2A–H2B region including the acidic patch (Figs. 1b and 2a, c). scFv residue Arg124 serves as an “anchor” by insertion into the pocket of the acidic patch, forming salt bridges as well as hydrogen bonds with acidic patch residues Glu61, Asp90, and Glu92 of H2A (Fig. 2c). The arginine at this location is conserved and binds to the acidic patch similarly in previously studied nucleosome–protein complexes (Supplementary Fig. 7). Additional electrostatic interactions are formed between scFv Arg126 and Glu113 of H2B and between scFv Arg188 and Glu64 of H2A. Unique to this complex, many scFv residues form hydrogen bonds with residues in H2A and H2B (Fig. 2a, c), including scFv Asn52 and Tyr76 with H2A Glu91 and Asn94, respectively; scFv Tyr74 with H2A Glu91, scFv Ser123 with H2A Asp90, scFv Tyr54 and Ser127 with H2B Glu105 and H109, respectively; and scFv Tyr190 with H2A Glu64. These tight and specific interactions might help stabilize the nucleosome structure during cryo-sample preparation by preventing the first step of nucleosome dissociation, involving H2A–H2B interactions with the DNA end regions of the nucleosome.

Binding of the scFv does not perturb nucleosome structures. Comparison of our cryo-EM NCP_CENP-A, NAS structure with the CENP-A NCP crystal structure consisting of a palindromic one-half of the human α-satellite DNA (PAS) revealed that the histone octamer conformations (in the defined regions of both structures) to be very similar, with a root mean square deviation of less than 0.6 Å (Supplementary Fig. 8); thus, scFv binding had little perturbation to the NCP structure. We further determined the cryo-EM structure of the free NCP_CENP-A, NAS in the absence of the scFv (Supplementary Fig. 9 and Supplementary Table 1) as a control. The free CENP-A nucleosome showed substantial dissociation comparing to the scFv bound nucleosomes.

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Structural features of the native-like CENP-A nucleosome. In our NCpCENP-A, NAS structure, the DNA regions at the entry and exit sites are well defined; residue Lys49 in the αN helix of the CENP-A and Arg42 of the proceeding region interact with the backbone phosphates of the DNA near the entry and exit sites, while CENP-A residue Arg43 inserts into a DNA minor groove and Arg44 interacts with the backbone phosphate near the dyad (Fig. 3a). By contrast, the 13 bp DNA at each corresponding end and Arg44 interacts with the backbone phosphate near the dyad while CENP-A residue Arg43 inserts into a DNA minor groove backbone phosphates of the DNA near the entry and exit sites, CENP-A and Arg42 of the proceeding region interact with the DNA in our cryo-EM structure. Moreover, 145 bp DNA pattern for the entry/exit DNA is similar to that of the DNA radical foot-printing results, which showed that the cleavage DNA in our cryo-EM structure is supported by previous hydroxyl radical digestion experiment. Notably, the ordered DNA ends are also observed in the cryo-EM structures of the NCpCENP-A, NAS and crystal CENP-C motif (Supplementary Fig. 13b). The two Lys residues are missing in the CENP-A crystal structure (Fig. 3c). It is likely that crystal packing and lower resolution of the crystal structure may contribute to these observed differences.

Fig. 2 Overall structure of the scFv-NCpCENP-A, NAS complex and interactions between scFv and NCpCENP-A, NAS. a The overall structure of the scFv-NCpCENP-A, NAS complex. Enlarged region shows the detailed interactions between scFv and the H2A–H2B dimer. Dashed lines show hydrogen bonds with distances less than 3.0 Å. Side chains are shown in sticks. Oxygen and nitrogen atoms are colored in red and blue, respectively. Residues in scFv, H2A, and H2B are labeled in magenta, black, and red, respectively. b Illustration of density maps for assignment of typical DNA base pairs, and a region in αN-helix of CENP-A. Maps were prepared in Chimera. c Cryo-EM density maps of the scFv and H2A–H2B residues that form direct interactions as shown in (a), contoured at 3σ. Both (a) and (c) were prepared using PyMOL.
CENP-A to move outward to form interactions with the end DNA (Figs. 3a and 4a). The more closed DNA conformation in the NCPH3.1, PAS structure is unlikely caused by crystal packing as the cryo-EM structure of H3 nucleosome displays the same conformation (Supplementary Fig. 14).

**Discussion**

Our cryo-EM NCP<sup>CENP-A, NAS</sup> structure has implications for the structure and function of centromeric chromatin. The outward shifts of the DNA at the entry and exit sites in the CENP-A NCP would lead to a more open conformation of the linker (or flanking) DNA in the nucleosome in comparison with those in the H3 nucleosome bound to a linker histone (Fig. 5a)<sup>26,27</sup>. Binding of the linker histone to the more open CENP-A nucleosome would require additional bending of the linker DNA, which is energetically unfavorable. Thus, our structure provides a possible explanation for the earlier observation that linker histones bind weakly to the CENP-A nucleosome and are largely absent at the centromeric chromatin<sup>19</sup>. Since linker histones help condense chromatin, the lack of linker histones would lead to a less condensed chromatin structure at the centromere, which in turn could make the CENP-A nucleosome more accessible for binding of kinetochore proteins such as CENP-C<sup>12</sup> and CENP-N<sup>21,22</sup> for kinetochore assembly. We note that the more open DNA end conformation of the CENP-A nucleosome is determined by the residues of the CENP-A histone (Fig. 4b)<sup>18–20</sup> instead of DNA sequence. Thus, the active centromeric nucleosome containing CENP-B box DNA sequence, which is recognized by CENP-B<sup>28</sup>, is
likely to have an open structure as well. Consistent with this hypothesis, the lack of linker histones at centromere was previously shown to be required for mitotic fidelity as kinetochore assembly at the centromere is critical for chromosome segregation. Notably, the lack of linker histones at centromere was previously attributed to the flexibility of DNA ends in the CENP-A nucleosome based on the crystal structure of the NCP\textsuperscript{CENP-A, PAS} and the ~20 Å resolution cryo-EM 3D models of the CENP-A nucleosome\textsuperscript{19}. In this work, we demonstrate that the scFv can be used to stabilize nucleosomes for determination of atomic resolution native-like nucleosome structures by cryo-EM. We have used this method to provide insights into the structure and function of the human CENP-A nucleosome with native DNA sequence. Determination of nucleosome structures by cryo-EM avoids the crystal packing at the DNA ends, which may alter the number of nucleotides associated with core histones\textsuperscript{1-3}. Three factors may have contributed to the high resolution density map of the scFv–nucleosome complex: (i) scFv binding reduces the background noise of the cryo-EM micrograph by preventing nucleosome dissociation; (ii) the unique well-defined hydrogen bond network formed between the scFv and NCPs could make the structure of the complexes more rigid, allowing accurate alignment of the particles; (iii) the larger size of the scFv–nucleosome complex (relative to the free nucleosome) increases the signal/noise ratio of the particle data.

The binding properties of the scFv to the nucleosome parallels the described interactions of mAb PL2-6 with the "epichromatin" region of interphase nuclei from diverse eukaryotic species\textsuperscript{29}. As acidic patch residues are broadly conserved, we anticipate the scFv can be applied to the structural determination of nucleosomes from different species. For example, the scFv approach may be readily used to determine the structures of a number of nucleosomes that play important functional roles: the centromeric nucleosome of budding yeast containing the AT-rich DNA\textsuperscript{30}, the nucleosome containing the specific binding site of pioneer transcription factors\textsuperscript{31,32}, the nucleosome with DNA sequences favored by intasomes for integration of virus DNA into human genomes\textsuperscript{33,34}. It is expected that the scFv should also be helpful for structural determination of nucleosomes bound to other protein factors whose binding sites on the nucleosome do not overlap with that of the scFv, for example, the chromatosomes\textsuperscript{36,37}. Finally, high-resolution structures of NCPs with differing native DNA sequences are ultimately required to understand how DNA sequences affect nucleosome positioning and dynamics in the genome.

**Methods**

**scFv design, cloning, expression, purification, and refolding.** Design of scFv was based on a previous single chain antibody structure (PDB ID: 2GKI)\textsuperscript{9}. The DNA sequence encoding the mouse mAb PL2-6 antibody heavy chain variable region (GenBank id: X60334) and light chain variable region (GenBank id: X60341) and a linker with three repeats of GGGS\textsuperscript{5} were optimized for Escherichia coli usage, synthesized commercially (Bio Basics, NY) and was subcloned into pET His\textsubscript{6}-TEV vector from Addgene plasmid #48284. Expression of recombinant scFv was made using BL-21 RIL Prolon Plus cells. Protein expression was induced by adding 0.2 mM isopropyl- β-D-1-thiogalactopyranoside (IPTG) for 18 h at 25 °C. Cells were then lysed by suspension in B-per Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, IL). Inclusion bodies that contain scFv were harvested by centrifugation at 15,000×g for 30 min. Refolding of antibody fragments from inclusion bodies followed the procedures described previously\textsuperscript{35}. Briefly, the inclusion bodies were denatured and reduced in GTE buffer (6 M Guanidine. 100 mM Tris–HCL, pH 8.0, 2 mM EDTA) with 10 mg/ml 1,4-dithioerythritol (Millipore Sigma, MO). Refolding was carried out by quickly mixing reduced/denatured antibody fragment solution with 100-fold buffer A (100 mM Tris–HCL, pH 9.5, 1 mM EDTA, 0.5 M arginine, 551 mg/L oxidized glutathione) at 10 °C for 48 h. The refolding solution was then dialyzed against 50-fold buffer B (20 mM Tris–HCL, pH 7.4, 1 mM EDTA and 150 mM NaCl) at 4 °C.

**Preparation of nucleosomal DNAs.** The 145 bp human α-satellite DNA was amplified by PCR from a previous plasmid (a gift from professor Ben E. Black). To increase the yield of the α-satellite DNA, we generated a modified pUC18 plasmid harboring 2 copies of 145 bp human α-satellite DNA sequence isolated from EcoRV (GATATC) cleavage sites. The plasmid used to produce 147 bp Widom 601 DNA was a gift from professor Tan Song. Plasmids bearing human α-satellite DNA or Widom 601 DNA were produced in Alpha-Select Chemically Competent cell (Bioline). Harvested cells were treated with lysis buffer (1% SDS + 0.2 M NaOH) for 30 min. The lysis buffer was replaced by 50 mM potassium acetate, 24 mM potassium acetate, after dialysis, the sample was precipitated and concentrated by chloroform extraction. 145 bp human α-satellite DNA or 147 bp Widom 601 DNA were cleaved from the plasmid by EcoRV (NEB) digestion\textsuperscript{36}.

The sequence of the 145 bp human α-satellite DNA is as follows: ATCAATATCCACCTGCAGATTCTACCAAAAGTGTATTTGGAAACTGC. The sequence of the 145 bp human α-satellite DNA was amplified by PCR from a previous plasmid (a gift from professor Ben E. Black).

**Histones purification and nucleosomes reconstitution.** Drosophila or human core histones H2A, H2B, H3, H4 were produced as recombinant protein in *E. coli* BL21–CodonPlus(DE3)–RIL (Novagen). Expressed histones were purified from the inclusion body using Hitrap SP column with AKTA FPLC (GE Healthcare) under non-denaturing condition\textsuperscript{18}. Human CENP-A was expressed with an N-terminal 6×His tag under denaturing condition\textsuperscript{18}. His6 tag were removed by thrombin protease (GE Healthcare) cleavage under non-denaturing condition (5 mM Tris–HCL, pH 7.4 and 5 mM 2-mercaptoethanol). All histones were further purified by using metal-affinity chromatography with a 5-mL His-trap column (GE Healthcare) using AKTA FPLC system. The folded scFv was concentrated and purified using a Superdex 75 10/300 GL gel filtration column in buffer (20 mM Tris–HCL, pH 7.4, 1 mM EDTA and 150 mM NaCl) at 4 °C.
Preparation of the scFv and nucleosome complex. The ratio of scFv to nucleosome was empirically determined by titration of scFv with nucleosomes and visualized by native PAGE gel. scFv (~5 μM) was added to nucleosome (~0.5 μM) in 10 mM Tris–HCl pH 7.4 and 1 mM EDTA with 30 mM NaCl. The complex was concentrated to ~10 μM for cryo-EM analysis.

Electrophoretic mobility shift assay. Binding reactions of scFv and NCP were carried out on ice for 10 min in buffer containing 10 mM Tris, 1 mM EDTA, 30 mM NaCl, 1% Ficoll 6000. Reactions contained 100 nM CENP-A, and either 100, 200, or 300 mM scFv. 10 μl of the solution was analyzed on a 5% acrylamide gel in 0.2× TBE, and run at 100 V for 120 min at 4°C. After electrophoresis, gels were stained with Midori Green Advance (Bulldog) and the gel images were visualized using ImageJ (http://imagej.nih.gov).

Stability assay of CENP-A NCP with native DNA in complex with scFv. 100 nM CENP-A NCP in 3% sucrose and 50 mM NaCl was incubated in reaction solution (10 mM Tris pH 7.4, 1 mM EDTA, 1% Ficoll 6000) with 0, 50, 100, or 150 mM NaCl overnight at 4°C. 10 μl of each reaction was added on a 5% acrylamide gel in 0.2× TBE and run at 100 V for 120 min at 4°C. The gel was imaged as described above.

3D cryo–electron microscopy. ITC experiments were performed with a PEAQ-ITC micro-calorimeter (Malvern) at 25°C. scFv and nucleosome samples were extensively dialyzed against the ITC buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA) and degassed before loading into the syringe and the cell. In a typical titration experiment, 0.5 μM of the nucleosome was titrated with 11.3 μM scFv in the ITC buffer. The ITC data was analyzed using MicroCal PEAQ-ITC data analysis software (Malvern). Binding curves were generated by plotting the heat change of the binding reaction against the ratio of the total concentration of scFv to the total concentration of the nucleosome. The association constant (Ka) and the stoichiometry of binding (n) were determined by fitting the observed binding curves to the model that two molecules scFv bind to the two sides of one nucleosome independently.

Cryo-electron microscope preparation and imaging. Cryo-EM grid preparations were performed by applying 3 μl of freshly prepared NCP or scFv-NCP complexes at a concentration of 7.5 μM to a glow-discharged Quantifoil R1.2/1.3 500 mesh carbon coated 300 mesh copper grids. The grids were vitrified by plunging into liquid ethane with a Vitrobot Mark IV (FEI) operated at 22°C and 70% humidity. The frozen grids were stored in liquid nitrogen until data collection. Cryo-EM data collection were acquired on a FEI Titan Krios operated at 300 kV and equipped with a K2 Summit direct detector (Gatan). Movies were recorded at super-resolution mode at a dose rate of 2.63 e/Å²/s with a total exposure time of 15.2 s, for an accumulated dose of 40 e/Å². Intermediate frames were recorded at every 0.4 s for a total number of 38 movie frames per micrograph. Defocus value ranged from ~0.8 to ~2.0 μm with a step size of 0.2 μm. The physical pixel sizes of the H3 and CENP-A NCPs were 1.72  Å and 1.358 Å, respectively.

Model building and structural analysis. The atomic models of the W601 DNA (PDB ID: 3LZ0)15, octamer histones (PDB ID: 1X5S)16, octamer CENP-A histones (PDB ID: 3AN2)44, scFv (PDB ID: 2GKI)15 as rigid body were fitted into the corresponding 3D density maps using UCSF Chimera47. The coordinates including DNA, histone octamer, and scFv were further adjusted manually using COOT15. Most of the side-chain densities were clearly visualized throughout the map, which allowed unambiguous building and refining of the model. Mutations were also made according to the actual residues or DNA sequence used in this study. Structure models were further refined using Real Space Refined module in the Phenix suite15. EM maps and fitted structural models were deposited in EMDB and PDB. Figures were made using UCSF Chimera15, ChimeraXX44, or PyMOL 2.1.1 (The PyMOL Molecular Graphics System, Version 2.0 Schrodinger, LLC). Struc−

data deviations were calculated using rms command in PyMOL. Electrostatic potential of nucleosome surface was calculated using APBS (http://www.poissonboltzmann.org) plugin in PyMOL. Local R.M.S.D. was calculated using colorbyrmsd.py (http://pymolwiki.org/index.php/ColorByRMSD) in pyMOL.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Three-dimensional cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-8938 (scFv-NCP-H3, W601), EMD-8945 (scFv-NCP-CENP-A, NAS), and EMD-8586 (NCPCENP-A, NAS). The coordinates of atomic models have been deposited in the Protein Data Bank under accession numbers 6DZT (scFv-NCP-H3, W601), 6E0C (scFv-NCP-CENP-A, NAS), and 6O1D (NCPCENP-A, NAS). All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request. The source data underlying Supplementary Figs. 1a, 2a, 5a, 6c, 9c, and 11c are provided as a Source Data file. A reporting summary for this Article is available as a Supplementary Information file.

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**Author contributions**
Y.B., D.E.O., and A.L.O. conceived the antibody-nucleosome structure project. B.-R.Z. engineered scFv, prepared all NCP or scFv-NCP complexes, and performed biochemical/biophysical studies. Y.B. and P.Z. initiated the cryo-EM study. K.N.S.Y. conducted the cryo-EM experiments with P.Z., observed the antibody-nucleosome stabilization effect under cryo-EM, and worked together with B.-R.Z. to optimize the stabilization condition. K.N.S.Y. and B.-R.Z. built the initial structural models using cryo-EM maps reconstructed in RELION 2.0 under the guidance of P.Z. B.C. collected cryo-EM data set for nucleosomes to initiate reprogramming. *Cell* **161**, 555–568 (2015).
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