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Microchip-Based Structure Determination of Disease-Relevant p53

Maria J. Solares,

Molecular, Cellular, and Integrative Biosciences Graduate Program, Huck Institutes of the Life Sciences, Department of Biomedical Engineering, and Center for Structural Oncology, Pennsylvania State University, University Park, Pennsylvania 16802, United States

G. M. Jonaid,

Bioinformatics and Genomics Graduate Program, Huck Institutes of the Life Sciences, Department of Biomedical Engineering, and Center for Structural Oncology, Pennsylvania State University, University Park, Pennsylvania 16802, United States

William Y. Luqiu,

Center for Structural Oncology, Pennsylvania State University, University Park, Pennsylvania 16802, United States; Department of Electrical and Computer Engineering, Duke University, Durham, North Carolina 27708, United States

Yanping Liang,

Frail Biomedical Research Institute at VTC, Roanoke, Virginia 24016, United States

Madison C. Evans,

Department of Biomedical Engineering, Pennsylvania State University, University Park, Pennsylvania 16802, United States

William J. Dearnaley,

Department of Biomedical Engineering and Center for Structural Oncology, Pennsylvania State University, University Park, Pennsylvania 16802, United States

Zhi Sheng,

Frail Biomedical Research Institute at VTC, Roanoke, Virginia 24016, United States

Deborah F. Kelly

Department of Biomedical Engineering and Center for Structural Oncology, Pennsylvania State University, University Park, Pennsylvania 16802, United States

Corresponding Author: Deborah F. Kelly – Debkelly@psu.edu. Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.analchem.0c03599>.

Supporting Information

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Materials and methods and description for the supporting movies (PDF)

EM structure of the p53 monomer with rotation (MOV)

Cross sections through the p53 monomer structure (MOV)

EM structure of the p53 tetramer with rotation (MOV)

Cross sections through the p53 tetramer structure (MOV)

Model for a putative p53 tetramer formation upon double-stranded DNA (MOV)

The authors declare no competing financial interest.

Abstract

The tumor suppressor protein TP53 (p53) plays a multifaceted role in all cells of the human body. Mutations in the *TP53* gene are often involved in cancer induction and disease progression. Despite its important role in health and development, structural information for p53 remains incomplete. Here, we present a microchip-based technology to facilitate structural studies of p53 assemblies derived from human cancer cells. These devices do not introduce foreign sequences to the *p53* gene and maintain naturally occurring post-translational modifications. Using cryo-electron microscopy, structures for the p53 monomer (~50 kDa) and tetramer (~200 kDa) were resolved to ~4.8 and ~7 Å, respectively. These structures revealed new insights for flexible regions of p53 along with biologically relevant ubiquitination sites. Collectively, the convergence of nanotechnology tools and structural imaging builds a strong framework to understand the oncogenic impact of p53 in human tissues.

Graphical Abstract

INTRODUCTION

Tumor suppressor protein TP53 (p53) plays an integral role in cell cycle processes and is implicated in ~50% of all human cancers. Under normal operating conditions, the famous “guardian of the genome” detects stress signals for DNA damage to induce intricate networks of cellular communication, arrest, and apoptosis.^{1–3} P53 is often deregulated in cancer and heavily involved in tumor progression and reoccurrence. In the case of glioblastoma multiforme (GBM), the deadliest and most common form of malignant glioma, *TP53* mutations account for 22% of all cases.⁴ Some mutations are thought to convey gain-of-function attributes associated with tumor invasion and immortality.^{3,5} Importantly, *TP53* mutations frequently correlate with poor prognosis and chemotherapeutic resistance.^{3,6}

At the molecular scale, detailed information for full-length p53 remains incomplete. The protein comprises three essential segments broadly described as the N-terminal domain, the central core, and the C-terminal domain (CTD). Most crystallographic studies of p53 have focused on the central core (residues 94-292) due to its stability and function.^{7–9} This region of the protein interacts with the genetic material through its DNA binding domain (DBD)

and plays a critical role in DNA repair.^{8–10} Each codon in the DBD is associated with cancer-related mutations, with 25% of mutations residing at six “hotspots” in GBM.^{2,10,11} While studies focused on these mutation-rich regions are essential for understanding disease progression, structural observations of the whole protein play an important role in drug discovery and therapeutic development.

Cryo-electron microscopy (EM) has emerged as an optimal technique to study protein structures of flexible macromolecules.¹² Specimen-related barriers have prevented a full range of structures determined from natively sourced complexes. One reason for this barrier is the difficulty in obtaining highly purified native proteins from cancer cells for structural studies. To overcome this limitation, we recently developed a highly reproducible microchip-based approach to enrich for native p53 complexes from human cancer cells. The new technology showed initial promise in its previous implementation to yield native p53 molecules in their dimeric functional state.^{13,14} Here, we present major technical advancements to achieve higher resolution structures of the p53 monomer (~50 kDa) and tetramer (~200 kDa) bound to damaged DNA. These structures revealed, for the first time, the p53 N-terminal domain primed for DNA repair.

MATERIALS AND METHODS

Protein Enrichment and SDS-PAGE Analysis.

Protocols to isolate p53 complexes from GBM cells (U87MG line) have been previously described.¹³ Briefly, using the NE-PER kit (Thermo Fisher Scientific), cytoplasmic and nuclear fractions were separated from GBM cell pellets (~100 μ L/pellet). The nuclear fraction was incubated with Ni-NTA (nickel-nitrilotriacetic acid) agarose beads (Qiagen) for 1 h at 4 °C. Beads were then washed with five bed volumes of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-salt buffer (20 mM HEPES, (pH 7.2), 140 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, and 5 mM imidazole). Complexes were eluted with the same HEPES buffer, supplemented with 60 mM imidazole. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gels through SimplyBlue SafeStain (Invitrogen) or western blot.^{13,14} Western blot analysis used antibodies against p53 (DO-1; Santa Cruz Biotechnology, sc-126) to detect complexes.

Cryo-EM Data Collection.

For cryo-EM specimen preparation, we followed previously described vitrification procedures using silicon nitride (SiN) microchips¹⁴ (Protochips, Inc.). Frozen specimens were clipped into a 626-specimen holder (Gatan, Inc.) and maintained at –180 °C while transferring to a transmission electron microscope. Chips were imaged using a Talos F200C EM (Thermo Fisher Scientific) operating at 200 kV under low-dose conditions (~5 electrons/Å²/s). Each image was recorded for 1 s using a CMOS camera (Thermo Fisher Scientific) at a magnification of 92,000 $\times$ for a final sampling of 1.5 Å/pixel at the specimen level (Supporting Information, Materials and Methods).

Image Processing Procedures.

We implemented standard processing procedures in the RELION software package.¹⁵ Individual particles were selected from images, and refinement procedures were conducted using an initial model of the p53 DBD (pdb code, 2AC0)⁷ filtered to 20 Å. This model comprises only ~60% of the p53 monomer structure, and the experimental data guided refinement. For the monomer reconstruction, the A chain of the crystal structure was used in the first round of refinement while chains A–D were used in the initial alignment for tetramer reconstruction. The final tetramer map contained particles selected from drift-free images while applying C2 symmetry, bringing the total particle views to 9558. The final resolution for the tetramer map was ~7 Å, according to the 0.5-FSC cutoff as well as gold-standard FSC (0.143) criteria. For the monomer structure, 7335 particles were selected from drift-free images using autopicking procedures in RELION, and the best particles were used to reconstruct the monomer structure. The final resolution of the map following postprocessing procedures was ~4.8 Å, according to the 0.5-FSC cutoff and the gold-standard FSC (0.143) criteria. Resolution values were independently confirmed using the RMEASURE program.¹⁶ The Chimera software package¹⁷ was used to visualize the EM maps at appropriate threshold values for each structure.

P53 Model Building and Movie Production.

We used the I-TASSER protein server to model the p53 N-terminal domain (residues 1–93).¹⁸ The best predicted model utilized the template model ZHOU chain A and had a C-score of –1.81, indicating medium confidence but still allowing us to interpret the EM map. Movies were produced using the Chimera software package.¹⁷

RESULTS AND DISCUSSION

Biochemical Isolation of p53 from Brain Tumor Cells.

To develop a rapid strategy to isolate p53 from human cancer cells, we drew upon knowledge of its domain organization and post-translational modifications (PTMs). The flexible N-terminal domain of p53 contains transactivation domains (TADs) that interact with key regulatory proteins such as MDM2^{2,8} (Figure 1A). TAD1 and TAD2 play a complex role in gene transcription following DNA damage. The central region of the protein contains DBDs, while the distal oligomerization domain (OD) and C-terminus are intrinsically disordered but play vital roles in tetramer formation, nuclear localization, and nuclear export.^{19–21} PTMs can further alter the physical properties of proteins along with their binding specificities.^{2,11} For example, proteins rich in phosphorylation sites are known to interact with metal cations.^{22,23} Our rapid biochemical procedure exploits the phosphorylation-rich nature of p53 to extract it from the nuclear material of human cancer cells and enrich the native protein through IMAC (immobilized metal affinity chromatography) strategies.

Briefly, we utilized the NE-PER kit (Thermo Fisher Scientific) to separate the cytoplasmic and nuclear fractions of U87MG glioblastoma cells (ATCC) that were cultured under standard growth conditions. The soluble nuclear fractions (~1 mg/mL) were incubated with Ni-NTA agarose beads (Qiagen) for 1 h at 4 °C in 20 mM HEPES buffer (20 mM HEPES,

(pH 7.2), 140 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, and 5 mM imidazole) (Figure 1B). Complexes were eluted using the same HEPES buffer supplemented with 60 mM imidazole. SDS-PAGE analysis showed that p53 monomers migrated at ~50 kDa while tetramers migrated at ~200 kDa. Western blots probed with primary antibodies against p53 (DO-1, Santa Cruz Biotechnology) revealed corresponding bands that confirmed the presence of p53 (Figure 1B).

Microchip-Based Enrichment of p53 Assemblies.

To enrich the p53 fractions for cryo-EM, we used silicon nitride (SiN)-based microchips containing integrated microwells (Figure 1C). The microchips were coated with Ni-NTA films that help enrich and further purify p53 complexes. These SiN microchips, also known as Cryo-Chips (Protochips, Inc.), can effectively concentrate a variety of biological samples ranging from viruses to RNA-bound complexes.^{14,24,25} The square frames of the microchips are 2 mm × 2 mm, and the *z*-dimension is 0.3 mm. Arrays of transparent imaging windows (10 μm × 10 μm) are etched into a larger window frame of 500 μm × 200 μm. Protein samples can occupy an imaging window that is 5–10-fold larger than holey carbon substrates commonly used for cryo-EM. The Cryo-Chips fit easily into commercial side-entry specimen holders, and they are currently being adapted for use in auto-loader devices.

Aliquots (3 μL) of the p53 eluates prepared in 20 mM HEPES buffer were incubated with the coated microchips and vitrified in liquid ethane using a Mark III Vitrobot. Cryo-EM images of frozen hydrated specimens were collected using a CMOS camera (Thermo Fisher) integrated in an Talos F200C TEM operating at 200 kV (Figure 1C). Images were acquired under low-dose conditions (~5 electrons/Å²) with no objective aperture at a nominal magnification of 92,000×. Final sampling at the specimen level was 1.5 Å/pixel (Figure 1C).

Defining PTMs on p53.

As PTMs played an important role in isolating native p53, we closely examined the distribution of ubiquitination and phosphorylation sites along the primary sequence of the protein (Figure 2). There are 18 known ubiquitination sites on p53 and more than 35 possible phosphorylation sites. Many of these modifications occur in a stress-dependent manner.^{26,27} One example of this effect happens during cellular oxidation. Oxidative breaks in DNA are quickly attended by a host of repair proteins including p53, MDM2, BRCA1, and many others. Under these circumstances, p53 is ubiquitinated while bound to the injured DNA. In the case of cancer cells that thrive under hypoxic conditions, ubiquitinated p53 is prevalent in the nucleus.

Equally important to ubiquitination modifications is phosphorylation events. These PTMs stabilize and activate p53 in response to genotoxic events. Phosphorylation “profiles” drive cell-specific responses and can serve as a framework to influence other signaling molecules.²⁶ In fact, many cancer treatments rely on p53 phosphorylation by Chk1/2 to trigger cell cycle arrest and apoptotic signaling through ionizing radiation.²⁸ In the current study, we exploited the heavily phosphorylated state of nuclear p53 to facilitate its biochemical separation via IMAC substrates. This naturally occurring effect permitted us to enrich for active assemblies in our cryo-EM structural studies.

Cryo-EM Structure of the p53 Monomer.

We used the RELION software package¹⁵ to perform single particle image processing analysis on p53 assemblies. Monomer and tetramer structures were easily separated using 3D classification procedures carried out through 25 iterations of refinement. Upon inspecting the reconstruction for the p53 monomer, there were two distinct globular domains with a connecting segment. Smaller density is consistent with the size of the N-terminal domain of p53 containing residues 1–93 (cyan, Figure 3A), while the larger domain can accommodate more structured and distinct DBD, comprising residues 96–278 (dark blue, Figure 3A). The I-TASSER protein server was used to model the p53 N-terminal domain¹⁸ which was placed into the smaller density of the map. The predicted model had a *C*-score of –1.81, indicating medium confidence but still allowing us to interpret the EM map. The most flexible region in the N-terminus contained residues 1–14. The density extruding from the globular region accommodated this domain, and the total length of the N-terminal model within the map was ~30 Å.

The lower half of the density map contained the DBD that fully occupied this region in only one position. Residue S96 of the DBD was in close proximity to residue L93, which is the final amino acid in the model of the N-terminus. There was sufficient unoccupied density between the two closely positioned domains to house missing residues 94–95, but they could not be modeled with high confidence. The total length of the DBD within the map was ~40 Å. In combination with the N-terminal region, the approximate length of the map was ~70 Å. Cross sections through the structure show the quality of the model fit within the density (Figure 3B; Movies S1 and S2). Individual monomers having consistent dimensions were visible in the EM images and class averages. Notably, the 4.8 Å map was not limited in particle orientations (Figure 3C,D), and it is the highest resolved structure of a ~50 kDa protein determined using affinity capture techniques. The C-terminal portion of the monomer (~12 kDa) could not be modeled in the EM map as there was limited density beyond the rigid DBD. Structurally, we cannot define regions of the map that were phosphorylated, and ubiquitin adducts are thought to occur on higher-ordered forms of p53 at sites of DNA damage.²⁹

p53 Tetramer Structure is Primed for DNA Repair.

In addition to the p53 monomers, we also identified tetramer assemblies in our samples that migrated at ~200 kDa according to SDS-PAGE analysis (Figure 1B). We performed the same image processing procedures for the tetramers as described for the monomers. The EM reconstruction of the tetramer assembly was interpreted using multiple models for each domain of the structure. We used the crystal structure of the DBD to interpret the lower half of the density map (Figure 4; dark blue, pdb code 2AC0, all chains). The N-terminal domain model was placed in the density with respect to the DBD as defined by the p53 monomer (Figure 4A; Movies S3 and S4).

The upper region of the map also contained two small regions of additional density. According to lower-resolution structures of the p53 dimer,¹⁴ this region was attributed to ubiquitin moieties. We fit single ubiquitin chains (8 kDa) at the two different densities, and each of the models were adjacent to amino acid K24 in the N-terminus of p53 (Figure 4;

yellow, pdb code 1UBQ³⁰). This lysine residue has been identified as a ubiquitination site for p53 during DNA repair processes.³¹ Tetramers having consistent dimensions were easily visualized in class averages, and the ~7-Å reconstruction was not limited in particle views, although some preferred orientations were noted (Figure 4B,C). Cross sections through the structure demonstrate a high-quality fit for each model in the density map (Figure 4D; Movie S4). Minor areas of unoccupied density in the central region of the tetramer are likely attributed to flexible PTMs that we cannot model at the given resolution.

The DNA fragment within the tetramer assembly showed features consistent with a double-stranded break in the midsection of the helical strand. Structural results suggested that nonubiquitinated p53 monomers can engage the genetic material. Ubiquitin molecules that signal for DNA repair occur at p53 N-termini in larger assemblies formed along the length of the DNA strand. Using this knowledge, we developed a new model to interpret the step-wise formation of p53–DNA binding events. The potential sequence for ubiquitination is also provided in Figure 5 and Movie S5. Having multiple ubiquitin molecules lined up in a linear fashion may increase the likelihood to attract repair proteins to damaged sites during DNA surveillance. We expect ongoing experiments aimed at determining high-resolution structures of p53 dimers to shed light on these mechanistic details.

CONCLUSIONS

Expanding our view of p53 structures allows us to develop new disease-based signatures at the protein level. Ubiquitination patterns of p53 in wild-type and disease models may reveal undiscovered opportunities for therapeutics aimed at targeting specific states that best correlate with cancer induction.³² Also, as p53 is subject to phosphorylation at many different residues, other modifications are likely to occur in cancer cells through disease progression.³³ Putting a face to p53 under a variety of conditions provides new 3D insights to complement biochemical evaluations of this highly mutated molecule.

In the current work, we showed that custom-designed microchips are a powerful tool to harvest p53 from cancer cells while preserving native oligomeric states. As PTMs on p53 are involved in gene activation and nuclear repair processes,²⁶ the captured assemblies suggest new snapshots of the tumor suppressor performing one of its most essential duties. Understanding the conformational changes to p53 that occur during these activities is key to dissecting DNA binding events, which may serve to vanquish the growth and proliferation of cancer cells.⁷

Here, we interpreted the findings of our previous p53 dimer structure,¹⁴ along with the current data, to develop a physiologically relevant model for p53–DNA assembly. P53 monomers bind to sites of DNA damage to form dimeric assemblies that are readily ubiquitinated. Tetramers are formed as dimers continue to assemble along the DNA strand. Additional ubiquitin moieties are added to p53 subunits by E3-ubiquitin ligases. The present work sheds light on the p53 monomer structure and its role in the assembly process. We also demonstrate multiple ubiquitin linkages in higher-ordered p53 assemblies for the first time. Overall, the advancements developed here lay the foundation to analyze functional differences in p53 as well as other tumor suppressors as molecular targets of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Biochemical isolation and enrichment of native p53 from GBM cells. (A) Primary sequence of p53 highlighting its domain organization. The N-terminal domain contains TAD1/2 motifs, followed by a central DBD. A predicted model for the N-terminus is shown (cyan) along with one chain of the DBD structure (blue; pdb code, 2AC0, A chain).⁷ The OD and CTD (orange) comprise a highly flexible region of the protein. (B) P53 was enriched from the nuclear material of the U87MG cells by noncovalent interactions with Ni-NTA agarose resin, using IMAC principles. SDS-PAGE analysis and western blots show enriched p53 monomers (~50 kDa) and tetramers (~200 kDa) eluted from the resin in buffer solution containing 60 mM imidazole. (C) P53 fractions were incubated on SiN microchips coated with the Ni-NTA substrate. Microchip frames were 2 mm $\times$ 2 mm, and imaging windows (500 μm $\times$ 200 μm) were etched down to contain microwells (10 μm $\times$ 10 μm). The microwells were ~20–50 nm thick and can accommodate a liquid thickness of 150 nm. The microchips fit easily into side-entry specimen holders and can be vitrified and imaged for cryo-EM data collection. The scale bar is 25 nm.

Figure 2.

Notable PTMs to the p53 protein. Two frequently noted PTMs to the p53 protein are ubiquitination and phosphorylation.³¹ Proposed ubiquitination sites along the p53 primary sequence are indicated with yellow markers and are listed in the upper table. Prominent phosphorylation sites are noted in the lower green-labeled table.³¹ An atomic model of a single ubiquitin molecule is highlighted in yellow (pdb code 1UBQ)³⁰ and shown in a magnified view.

Figure 3.

Cryo-EM structure of the p53 monomer. (A) Different rotational views of the EM structure (white density) of p53 isolated from GBM cells. The map accommodates models of the N-terminal domain (cyan) and the DBD (blue; pdb code, 2AC0, A chain).⁷ The scale bar is 10 Å. (B) Cross sections (1–4) through the structure demonstrate the model fit within the map. (C) Cryo-EM image and class averages of the p53 monomer show particles with features consistent with the 3D structure. (D) According to the 0.5-FSC cutoff and the gold-standard (0.143) criteria, the density map for the monomer was resolved to ~4.8 Å. Particles that

comprised reconstruction did not show limited views according to the angular distribution plot of particle orientations.

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Figure 4.

Cryo-EM structure of the ubiquitinated-p53 tetramer bound to DNA. (A) Magnified view of the p53 tetramer structure shown in different views. The EM map (white) was interpreted using the N-terminal domain model (cyan) along with the full tetramer structure of the DNA-binding-domain (blue; pdb code 2A0C, all chains). The density map accommodates two ubiquitin monomers (yellow; pdb code, 1UBQ)³⁰ and suggests a biologically relevant configuration for attachment. The DBD also surrounds a DNA helix (dark blue) containing a double stranded break. The scale bar is 20 Å. (B) Class averages of the tetramers show a variety of particle orientations. (C) Resolution of the EM map was ~7 Å according to the gold-standard Fourier shell correlation (0.143) criteria. The reconstruction was not limited in its angular distribution of particle views although some preferred orientations were noted.

(D) Cross sections through the structure indicate a near full occupancy of the map. Minor areas of unoccupied density in the central region of the tetramer are likely attributed to PTMs that cannot be modeled at the given resolution.

Figure 5.

Step-wise model for p53 assembly and ubiquitination upon DNA. A nicked strand of DNA (white backbone; pdb code 2AC0; chains F and H)⁷ is shown in step 1. In step 2, the DBD (dark blue; pdb code 2AC0; A chain) of a p53 monomer interacts with the injured DNA. The N-terminal domain of the monomer (light blue) is positioned according to the cryo-EM structure. A second monomer binds to the opposite DNA strand at the injured site to form an active dimer that can be ubiquitinated (step 3). Another set of monomers assemble upon the same strand of DNA to form a tetramer (step 4). An alternative view along the DNA helix shows the linear arrangement of ubiquitin models (yellow; pdb code UBQ1)³⁰ that were placed in biologically relevant positions among the p53 assemblies (Movie S5).