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Custom Cryo-Chips as a method of enriching and imaging disease-related oncoproteins

Maria Solares, G.M. Jonaid and Deborah Kelly

Pennsylvania State University, University Park, Pennsylvania, United States

Even 40 years after its discovery, much about tumor protein TP53 (p53) remains a mystery. The "guardian of the genome" is integral to cell cycle arrest, DNA repair, and apoptosis processes. However, p53 is often deregulated in cancer. Just in glioblastoma multiforme (GBM), 22% of cases contain a *TP53* mutation that affects cancer progression and reoccurrence [1]. The primary sequence of a p53 monomer can be described in three broad regions: N-terminal domain (NTD), core or DNA-binding domain (DBD), and C-terminal domain (CTD). Most of the structural information available is on the DBD, a highly stable region which is often mutated in cancer. This region plays an integral role in binding the p53 response elements and transcribing critical genes. The remaining NTD and CTD regions are too disorganized and flexible to be fully resolved through NMR and X-ray crystallography methods, leaving researchers unable to access critically important information on p53 regulation and tetramerization.

Cryo-Electron Microscopy (EM) technology is now able to resolve structures of flexible macromolecules in solution. Exploration of natively sourced proteins, however, is limited by the ability to obtain pure and enriched sample from human cancer cell lines. This has forced many researchers to study recombinant proteins produced in model expression system devoid of post-translational modifications (PTMs) that affect protein function. To overcome these limitations, we recently developed a highly reproducible protein purification method, which alongside a microchip-based method, produced novel, higher resolution structures of p53 monomer (~48 kDa) and tetramer (~200kDa) (**Fig. 1**). Our rapid extraction and purification method exploits the inherent nature of p53 and its PTMs to bind metal cations by implementing immobilized metal affinity chromatography (IMAC) methodologies. After separating cytoplasmic and nuclear fractions of GBM cells (U87MG line), soluble nuclear fractions were incubated with Nickel-Nitrilotriacetic acid (Ni-NTA) agarose beads. These fractions were further concentrated for cryo-EM studies using lipid-base Ni-NTA coated Silicon Nitride (SiN)-based microchips containing integrated microwells. These microchips, also known as Cryo-Chips™, hold windows that are 5-10 fold larger than the average holey carbon areas of the grids commonly used to prepare cryo-EM samples[2]. Frozen p53 samples were inserted into a Talos F200 TEM operating at 200 kV. Images were collected using a CMOS camera at low dose conditions (< 5 electrons/A^2/pixel) and at ~92,000x magnification.

Single particle imaging processing using the RELION software package produced monomer and tetramer reconstructions which were easily separated using 3D classification after 25 iterations of refinement. An NTD model was produced through I-TASSER Protein Fold Recognition Server to validate the full-length identity of the resolved p53 complexes [3]. The p53 monomer (~5 Å) and tetramer (~ 7 Å) accommodated the DBD model (blue; pdb code 2AC0 [4]) as well as displayed never seen before extra density that fit the theoretical NTD model, validating the technology's ability to finally capture and immobilize the small and flexible regions of p53 (**Fig 2)** [5]. Further, the tetramer model indicated the presence of a double-stranded DNA break, and extra density which was attributed to ubiquitination (yellow; pdb code 1UBQ [6]) close to K24 in the NTD. We believe we have captured the complex signaling for DNA repair.

Our current work showcases how custom-designed microchips alongside with perfected protein purification methods can harvest and preserve oligomeric p53 states. The models we resolved are snapshots of confirmational and PTM changes that affect protein structure and activity. Detangling of structure-function of p53 is paramount to deciphering associated signaling pathways and how these are deregulated in GBM. These insights would facilitate the development of targeted anti-cancer treatments.

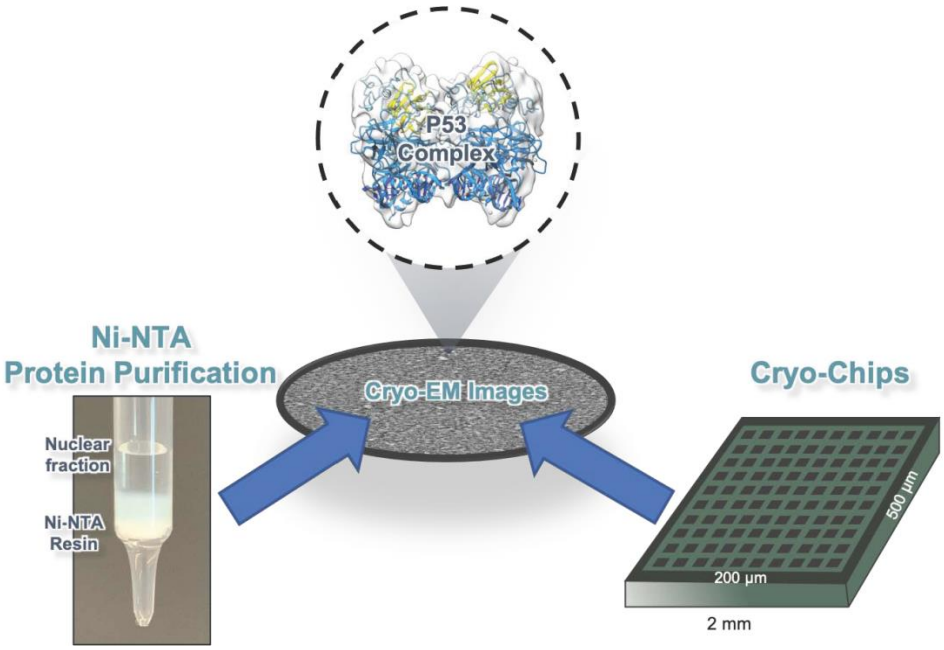

Figure 1. Isolation and enrichment of native p53 complexes from GBM cells. By exploiting p53 and its PTM's affinity towards binding metal, complexes could be isolated and enriched using IMAC principles. Native complexes from U87MG cell's nuclear material interact non-covalently with Ni-NTA agarose resin as an initial enrichment step. These fractions are then incubated on Ni-NTA-coated SiN microchips (Cryo-Chips; Protochips, Inc.) to further concentrate samples. The collaboration between both techniques result in the creation of cryo-EM specimens that produce higher resolution EM maps.

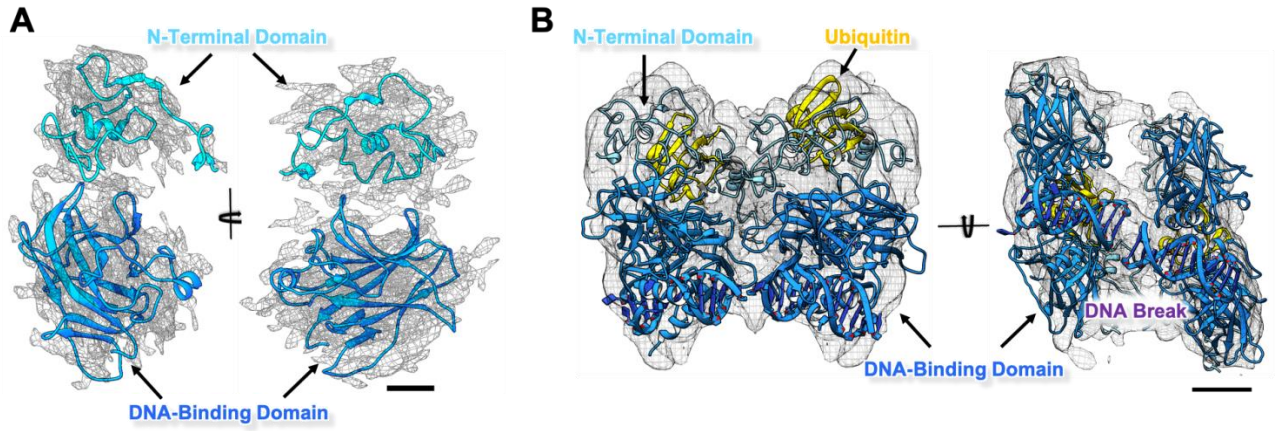

Figure 2. Cryo-EM structure of p53 monomer and tetramer. (A) Different rotational views of EM reconstruction for p53 monomer (white density). The map easily accommodates an NTD (cyan) and DBD (blue; pub code 2AC0, A chain). Scale bar is 10 Å. (B) Different rotational views of EM reconstruction for p53 tetramer (white density).The map accommodated the NTD (cyan) and DBD (blue; pub code 2AC0), alongside biologically-relevant ubiquitins (yellow; pdb code 1UBQ). The model also shows features indicate a double-stranded DNA break in the mid-section of the full-length strand. Scale bar is 20 Å.

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