EUROPEAN SYNCHROTRON RADIATION FACILITY
INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

	Experiment title: Structure solution of full-length NEDD4 E3 ligase	**Experiment number**: **MX-2506**
Beamline: CM-01	**Date of experiment**: from: 25 January 2023 to: 28 July 2023	**Date of report**: 15 November 2023
Shifts:	**Local contact(s)**: Eaazhisai KANDIAH	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Simona Polo[1,2]

1) IFOM ETS, The AIRC Institute of Molecular Oncology, Milan, Italy; 2) Dipartimento di Oncologia ed Emato-oncologia, Università degli Studi di Milano, Milan, Italy. Electronic address: simona.polo@ifom.eu.

Report:

Glacios Dataset

To prepare the cryo-EM grids, 3 ul of the NEDD4 sample [diluted from 5.7 mg/ml to 0.6 mg/ml in Dilution buffer (20mM TrisHCl pH 8.0, 100mM NaCl, 1mM EDTA, 1mM DTT)] were applied to a Quantifoil Au-C 300 mesh 1.2/1.3 carbon grids (Quantifoil), and then blotted (force 4) for 5 s and plunge-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific) present at the CM01 facility at ESRF (Grenoble) operated at 6°C and 100% humidity. CryoEM data were acquired through Glacios; specifics are listed in **Table 1**.

Table 1

Microscope: Glacios @200kV
Data collection software: serialEM
camera type: Gatan K2
acquisition mode: counting
compression: TIFF LZW
gain normalized: no
pixel size 0.902 Ang/pixel
exposure time 4.4 s
total dose per movie 41 electron/Ang^2
number of frames per movie 40
defocus spread -1.0 to -2.3 um in 0.2 um step.

Image processing was performed in the Biochemistry and Structural Biology Unit (BSU) at the European Institute of Oncology (IEO - Milan) with CryoSPARC v4.2.1. All movie frames were gain corrected during data processing with a gain reference file acquired in the same microscope session. Micrographs showing non vitreous ice, thick ice, high anisotropy or heavy contamination were discarded. In total 2276 images were processed. Initial picking was performed using a blob picker with a

minimum and maximum particle diameter respectively of 40 Å and 120 Å. An NCC score of 0.3 and power score between 5 and 566 were initially applied, resulting in almost 5 million particles that were extracted with a box size of 200x200 pixels and subjected to several rounds of 2D classification for template generation. In all the 2D classification jobs, the number of online-EM iteration was set to 40, the batchsize per class was increased to 400 and a circular mask diameter was set to 120 Å. The templates were used for template-based picking using a particle diameter of 100 Å yielding a total of 4 million particles that were subjected to iterative 2D classification (using the same parameters described above) that resulted in 246'842 particles (**Figure 1A**).

The selected particles clearly showing protein features, were used for ab-initio 3D reconstruction using 5 classes (**Figure 1A**). The first and the last out of five 3D classes gave shapes of three lobes radiating from a central region, compatible with the shape of the HECT domain (**Figure 1A**). However, the first class presented an additional density if compared to the HECT suggesting the presence of additional domains. This class was selected for further heterogeneous and non-uniform refinement (**Figure 1B**). The refinement process produced a final 9.4Å resolution density map (**Figure 1C**) where the structure of T-inverted HECT domain (PDB: 4BBN) was initially rigid body fitted. After fitting the HECT domain, the extra density adjacent to it matched the size of either the C2 or WW domains. For simplicity, and based on previously published NMR data (PMID: 25438670), the C2 domain (PDB: 3B7Y) was fitted into the density to generate a preliminary model of NEDD4 in its auto-inhibited form (**Figure 1D**). Prompted by this preliminary observation, we carried out high-resolution data collection under a Titan Krios microscope with the aim of getting the high-resolution structure of the NEDD4 in its auto-inhibited state.

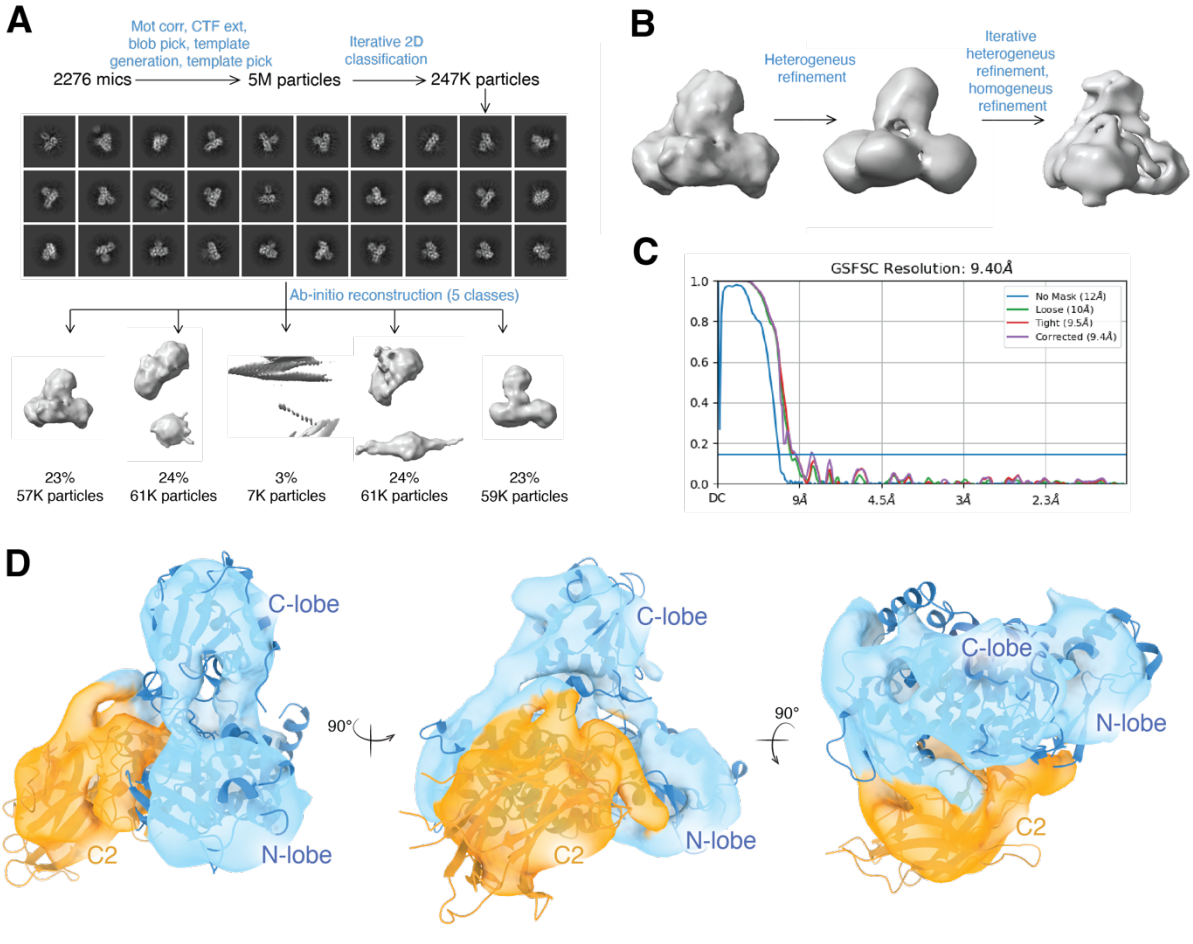

Figure 1. Glacios dataset: CryoEM analysis workflow and NEDD4 3D volume. *A) CryoEM data analysis workflow performed with Cryosparcv4.2.1. Number of selected micrograph and picked particles are showed together with 2D class averages and ab-initio reconstruction classes. B) 3D volume showing additional density to the HECT was selected for homogeneous and heterogeneous refinement. C) Gold-standard Fourier shell correlation (GSFSC) plot for refined cryo-EM map of NEDD4. cutoff value for resolution (0.143) is indicated with a blue line. D) Structures of the HECT domain (PDB: 4BBN) and C2 domain (PDB: 3B7Y) fit into the density map.*

Krios Dataset

Multi-grid data collection was performed first on the sample diluted in Dilution buffer to 0.4mg/ml sample (grid type Quantifoil Au-C 300 mesh 1.2/1.3, blot force 0 and blot time 2sec) and a second one on the 0.6mg/ml sample (grid type Quantifoil Au-C 300 mesh 1.2/1.3, blot force 4 and blot time 5sec). Parameters of acquired images are listed in **Table 2**.

A total of 25'174 micrographs were collected, of which 24'627 were accepted and used for analysis. The particles extracted from these two data sets were combined. All analysis was performed using CryoSPARC v4.2.1 including motion correction and CTF estimation.

Template picking using a template generated from the Glacios dataset allowed the selection of 19 million particles which were extracted and processed as previously described. Iterative 2D classification resulted in 3.5 million particles which were used to generate 3 classes of ab-inition reconstruction (**Figure 2A**). Two of them were compatible with the HECT shape, with one of the two (34.7% of the particles) showing a more compact shape with additional protruding density (**Figure 2A**). The particles from this class were further subjected to iterative heterogeneous refinement, global and local CTF correction, and non-uniform refinement resulting in a map with an estimated resolution of approximately 3.8 Å based on the Fourier shell correlation plot (**Figure 2B**).

Table 2

Microscope: Krios
camera type: Gatan K3
acquisition mode: counting
compression: TIFF LZW
gain normalized: yes
magnification 105k
pixel size 0.84 Ang/pixel
electron dose ~18 electrons/pixel/s
exposure time 1.6 sec
total dose per movie ~ 43electron/Ang^2
number of frames per movie 40
dose/frame 1.03 e/A^2
defocus spread -1 to -2.4 um in 0.2 um step.
energy filter: Gatan Quantum LS
slit width: 20 eV
phase plate: no
Obj Aperture: 100um

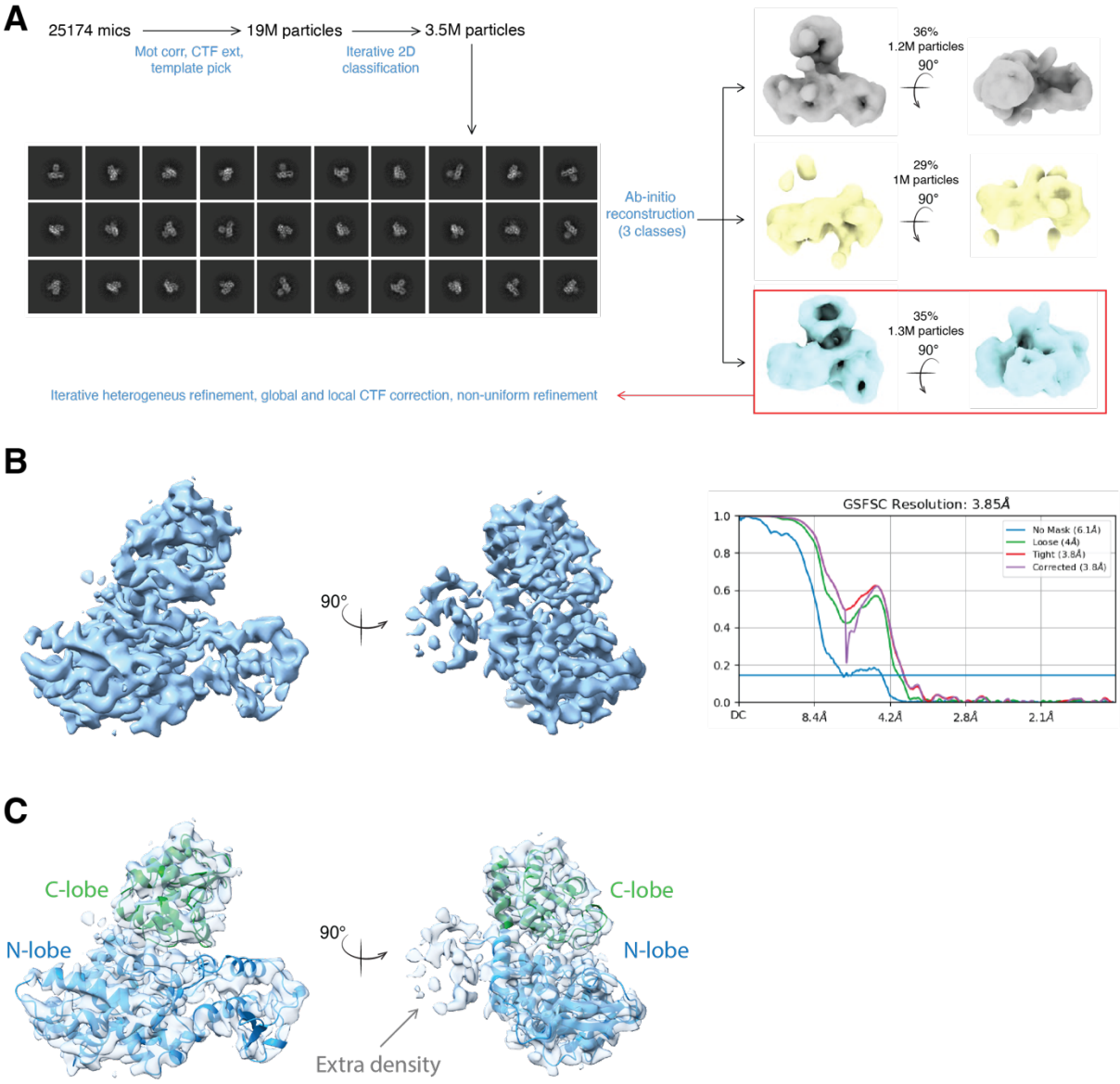

Figure 2. Krios dataset: CryoEM analysis workflow and NEDD4 3D volume. *A) CryoEM data analysis workflow performed with Cryosparcv4.2.1. Number of selected micrograph and picked particles are showed together with 2D class averages and ab-initio reconstruction classes. Red squared density was selected for further refinement B) 3D volume showing additional density to the HECT was selected for homogeneous and heterogeneous refinement (left) and Gold-standard Fourier shell correlation (GSFSC) plot for refined cryo-EM map of NEDD4 (right); cutoff value for resolution (0.143) is indicated with a blue line. C) Structure of N-lobe (blue) and C-lobe (green) of the HECT domain (PDB: 2XBF) fit into the density map.*

The high-resolution map was used for model building by rigid body fitting the existing structure of the HECT domain. Using the isolated C-lobe and N-lobe of the HECT crystal structure (PDB: 2XBF), we could unambiguously fit the HECT domain in a compact and closed conformation (**Figure 2C**). An additional weaker density was present at N-terminus of the HECT domain but definitively assigning a unique secondary structure proved challenging. The relatively low affinity and flexibility between interacting domains or the adoption of different conformations may have hindered their unambiguous assignment onto the HECT domain density. Interestingly, while intrinsic flexibility exists between the HECT N- and C- lobes, cryo-EM analysis revealed both lobes, indicating constrained flexibility and the presence of a major conformational state distinct from the crystal structure of the isolated HECT domain (PDB: 2XBF). Currently, local refinement and 3D classification

using focused mask on the extra density region are ongoing. This effort aims to accurately trace the residues within this particular area.