

Characterization of the NF1 role in microtubule dynamics through Cryo-EM and CL-MS: rediscovering a tumor suppressor

1. BACKGROUND AND RATIONALE

Neurofibromin 1 (NF1) is a tumour suppressor, originally identified as the gene responsible for neurofibromatosis type 1 disease, but increasingly recognized as a somatic driver of numerous cancers¹. NF1 protein is well known as a regulator of RAS GTPase activity and this role is extensively covered in literature^{1,2}. NF1 biochemistry has remained understudied for several years due to the difficulties in its manipulation; recently solved structure has revealed important details on its interaction with RAS, but many other interactions suggested over the years remain underexplored^{3,4}.

We have accumulated extensive evidence pointing to a role for NF1 in regulating microtubule (MT) dynamics through a RAS-independent mechanism. In particular, using CRISPR-engineered models of breast cancer and biochemical assays on purified recombinant NF1, we demonstrated that NF1 physically interacts with microtubules and regulates their dynamics both *in vitro* and *in vivo*. In particular, NF1 appears to protect cells from microtubule damage induced by a specific class of microtubule-depolymerizing drugs, maytansinoids, which are currently routinely used as payloads in approved-antibody-drug conjugates⁵.

Our final goal is to unveil the structure of the NF1-MT complex and to identify NF1 domains essential for the control of microtubule dynamics.

2. SIGNIFICANCE, INNOVATION AND OBJECTIVES

Although the structure of the NF1 protein, either alone or bound to RAS, is well characterized, the conformation that NF1 adopts once it binds to microtubules remains unknown. This lack of knowledge limits our molecular understanding about how NF1 regulates microtubule dynamics and repair. Part of my lab's work is focused on obtaining the topology and the structure of the NF1-MT complex that will reveal key mechanistic insight in Neurofibromatosis physiology and pathology.

OBJECTIVE 1. Characterization of the structural basis for NF1-MT interaction with Cryo-EM approaches. We successfully developed reagents and methods for the high-yield purification of tag-free recombinant NF1 from insect cells (fig 1 A-C). Purified NF1 showed multiple biochemical properties of canonical MAPs: co-sedimentation with polymerized MTs, induction of MT bundles, enhancement of tubulin polymerization and enhancement of multiple parameters of MT dynamics measured by TIRF microscopy (fig 1 D-J). Notably, we collected preliminary Cryo-EM images of the NF1-MT complex (fig 2A). Preliminary data show that NF1 indeed decorates the surface of Taxol stabilized-MT, nevertheless the pattern was not sufficiently homogeneous to attempt a reconstruction. To further advance our structural studies, we seek access to Cryo-EM grid screening and high-resolution data collection for the NF1-MT complex. Additionally, in order to reduce the complexity of the system, we aim to perform Cryo-EM screening for NF1 in combination with different types of MT (subtilisin treated-MT, GMPCPP MT, dolastatin treated-MT) and with $\alpha\beta$ tubulin dimers.

OBJECTIVE 2. Identification of NF1 domains essential for the control of microtubule dynamics. We already performed CL-MS on NF1-MT complex crosslinked with Sulfo-SDA. Preliminary data identified few proximal residues (fig 2B) that are outside the commonly described Tubulin binding domain (TBD), but the crosslinking needs further optimization to obtain higher crosslinking identification

coverage. Additionally, in order to reduce the complexity of the system, we aim to perform CL-MS on NF1 with $\alpha\beta$ tubulin dimers. For this, the access to the Orbitrap Eclipse CL-MS/MS to perform CL-MS analysis will be crucial.

3. RESEARCH DESIGN AND METHODOLOGY

We set-up the recombinant production of the NF1 protein in insect cells. Our purification procedure (affinity purification, tag removal and size exclusion chromatography (SEC)) led to the production of pure and homogeneous recombinant NF1 protein in milligram quantity (fig 1A). Recombinant NF1 was also demonstrated to be around 640 KDa and to form dimers (fig 1B-C), as expected from the literature. We then focused on the NF1-MT sample optimization for Cryo-EM and we managed to achieve a high decoration of microtubule by the NF1 protein. A small data collection in linear mode gave microtubules that displayed an extra density (fig 2C) but the pattern was not sufficiently homogeneous to attempt a reconstruction. Further processing using the MIRP pipeline are ongoing. We are also testing different strategies to simplify the visualization of NF1 on microtubules using various types of microtubules, including a) subtilisin-treated Taxol-stabilized microtubules b) shorter microtubules stabilized with a GTP analog (GMPCPP), and c) circular microtubules treated with dolastatin. These different types of microtubules have already been used for other biochemical assays (co-sedimentation, negative staining and TIRF microscopy) in the lab but have not yet been tested in CryoEM because of the limited access to the screening microscope. Moreover, to further simplify the system, we are also optimizing the complex NF1- $\alpha\beta$ tubulin although the interaction appears to be much weaker compared to the one with microtubules.

4. ENVIRONMENT

The proposed experimental plan is based on two pillars: i) the infrastructure and support of my hosting institution which offers state-of-the-art internal technological units. The lab will work in close contact with the Biochemistry and Structural Biology Unit (BSU, <https://tinyurl.com/BSUnit>) that will assist my junior scientists with high-level technical support, training and equipment in protein biochemistry, biophysics and Cryo-EM structure determination. The BSU is equipped with state-of-the-art technology for recombinant protein production and purification. For EM studies, a glow discharge and the Vitrobot Mark IV are available for in house Cryo-EM grid preparation, while grid screening and preliminary data collection are limiting to date because of spot access to a 200 kV Talos Arctica with DED (University of Milan). ii) the established collaboration with the senior researcher dr. Sami Chaaban from Carter Lab at the MRC Laboratory of Molecular Biology (<https://www2.mrc-lmb.cam.ac.uk/groups/cartera/>) who will support us in processing the collected data in house using a multi-GPU workstation with the most updated software for CryoEM structure determination.

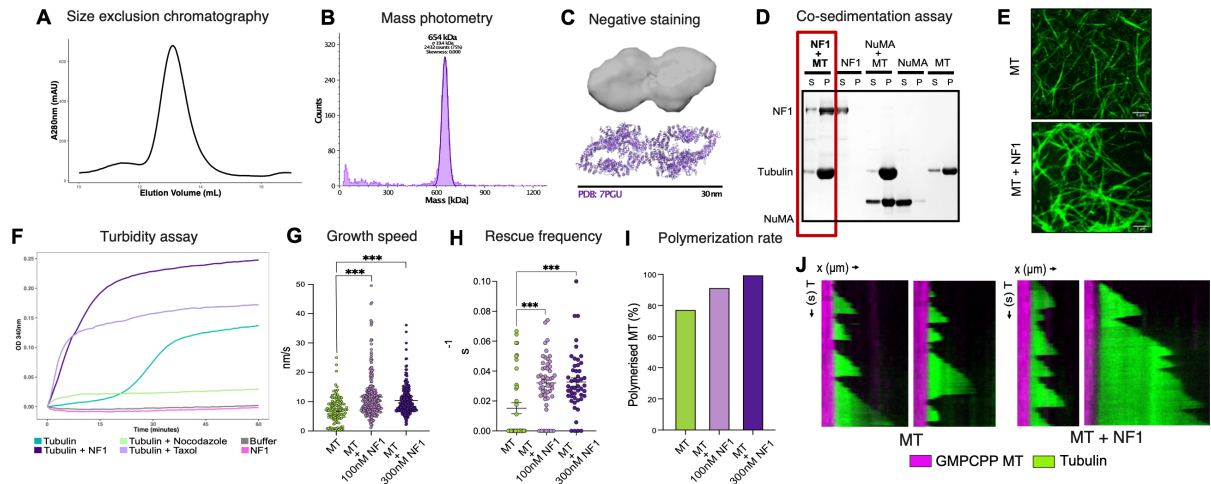


Figure 1: (A) NF1 protein purifies as a single peak by size-exclusion chromatography. (B) Mass photometry histogram of purified NF1. (C) Negative staining low resolution density map obtained from our NF1 preparation compared with previously determined cryoEM structure. (D) Microtubule co-sedimentation assay; the microtubule interactor NuMA is used as positive control. (E) Representative images of microtubules polymerized in vitro with 488-tubulin +/- recombinant NF1. (F) In vitro tubulin polymerization assay +/- recombinant NF1 or Nocodazole and Taxol as controls. (G-I) in vitro measurement of microtubule dynamics by TIRF microscopy. (J) Representative kymographs of microtubules +/- recombinant NF1.

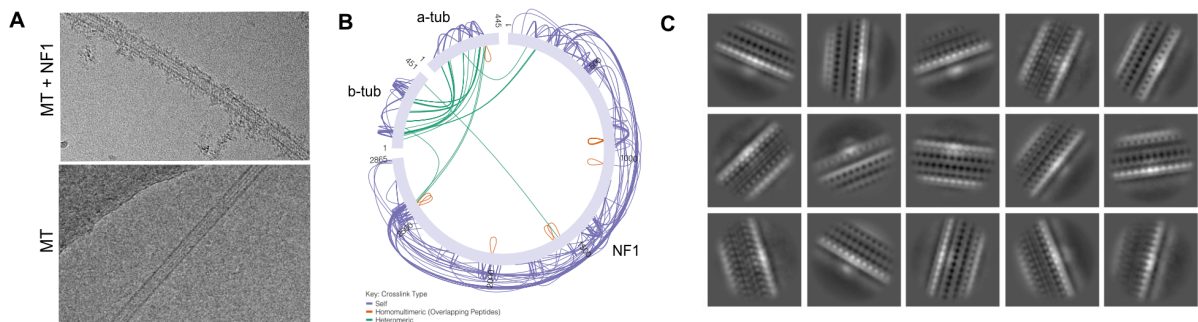


Figure 2: (A) Representative micrograph of the NF1-MT complex and of MT alone collected on a Glacios 200 kV. (B) Connectivity map of the cross-linked amino acids in the NF1-MT complex. (C) 2D classification of selected particles (32,861) of the NF-MT complex.

1. Bergoug, M. *et al.* Neurofibromin Structure, Functions and Regulation. *Cells* **9**, (2020).
2. Gutmann, D. H., Wood, D. L. & Collins, F. S. Identification of the neurofibromatosis type 1 gene product. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9658–9662 (1991).
3. Lupton, C. J. *et al.* The cryo-EM structure of the human neurofibromin dimer reveals the molecular basis for neurofibromatosis type 1. *Nat. Struct. Mol. Biol.* **28**, 982–988 (2021).
4. Chaker-Margot, M. *et al.* Structural basis of activation of the tumor suppressor protein neurofibromin. *Mol. Cell* **82**, 1288–1296.e5 (2022).
5. Duso, B. A. *et al.* A novel, RAS-independent role for NF1 in microtubular dynamics and damage repair dictates sensitivity to T-DM1 in HER2-positive breast cancer. *bioRxiv* 2023.12.06.569572 (2023).