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# Production, Crystallization, and Structure Determination of the IKK-binding domain of NEMO

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**TITLE:**

# Production, Crystallization, and Structure Determination of the IKK-binding Domain of NEMO

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**KEYWORDS:**

NF-κB, NEMO, IκB kinase (IKK), NEMO binding domain (NBD), coiled coil, X-ray crystallography, protein engineering, structure determination, structure-based drug design

**SUMMARY:**

We describe protocols for the structure determination of the IKK-binding domain of NEMO by X-ray crystallography. The methods include protein expression, purification and characterization as well as strategies for successful crystal optimization and structure determination of the protein in its unbound form.

**ABSTRACT:**

NEMO is a scaffolding protein which plays an essential role in the NF-κB pathway by assembling the IKK-complex with the kinases IKKα and IKKβ. Upon activation, the IKK complex phosphorylates the IκB molecules leading to NF-κB nuclear translocation and activation of target genes. Inhibition of the NEMO/IKK interaction is an attractive therapeutic paradigm for the modulation of NF-κB pathway activity, making NEMO a target for inhibitors design and discovery. To facilitate the process of discovery and optimization of NEMO inhibitors, we engineered an improved construct of the IKK-binding domain of NEMO that would allow for structure determination of the protein in the apo form and while bound to small molecular weight inhibitors. Here, we present the strategy utilized for the design, expression and structural characterization of the IKK-binding domain of NEMO. The protein is expressed in *E. coli* cells, solubilized under denaturing conditions and purified through three chromatographic steps. We discuss the protocols for obtaining crystals for structure determination and describe data acquisition and analysis strategies. The protocols will find wide applicability to the structure determination of complexes of NEMO and small molecule inhibitors.

**INTRODUCTION:**

The NF-κB pathway is activated in response to a variety of stimuli, including cytokines, microbial products and stress, to regulate expression of target genes responsible for inflammatory and

immune response, cell death or survival and proliferation[1]. Pathologies including inflammatory and autoimmune diseases and cancer[2–5] have been correlated to hyperactivation of the pathway, which has made modulation of NF-κB activity a prime target for the development of new therapies[6,7].

The canonical NF-κB pathway in particular is distinguished from the non-canonical pathway, responsible for lymphorganogenesis and B-cell activation, by the former's dependence on the scaffolding protein NEMO (NF-κB essential modulator[8]) for the assembly of the IKK-complex with the kinases IKKα and IKKβ. The IKK complex is responsible for the phosphorylation of IκBα (inhibitor of κB) that targets it for degradation, freeing the NF-κB dimers to translocate to the nucleus for gene transcription[1] and is therefore an attractive target for the development of inhibitors to modulate NF-κB activity.

Our research focuses on the characterization of the protein-protein interaction between NEMO and IKKβ, targeting NEMO for the development of small molecules inhibitors of IKK complex formation. The minimal binding domain of NEMO, required to bind IKKβ, encompasses residues 44-111, and its structure has been determined in complex with a peptide corresponding to IKKβ sequence 701-745[9]. NEMO and IKKβ form a four-helix bundle where the NEMO dimer accommodates the two helices of IKKβ(701-745) in an elongated open groove with an extended interaction interface. IKKβ(734-742), also known as the NEMO-binding domain (NBD), defines the most important hot-spot for binding, where the two essential tryptophans (739,741) bury deeply within the NEMO pocket. The details of the complex structure can aid in the structure-based design and optimization of small molecule inhibitors targeting NEMO. At the same time, it is difficult that binding of a small molecule or peptide would recreate in NEMO the full conformational change (i.e., extensive opening of the NEMO coiled-coil dimer) caused by binding of the long IKKβ(701-745), as observed in the crystal, and the structure of unbound NEMO or NEMO bound to a small molecule inhibitor may represent a better target for structure-based drug design and inhibitor optimization.

Full length NEMO and smaller truncation constructs encompassing the IKK-binding domain have proven intractable for structure determination in the unbound form via X-ray crystallography and nuclear magnetic resonance (NMR) methods[10], which prompted us to design an improved version of the IKK-binding domain of NEMO. Indeed, NEMO (44-111) in the unbound form is only partially folded and undergoes conformational exchange and we therefore set to stabilize its dimeric structure, coiled-coil fold and stability, while preserving binding affinity for IKKβ. By appending three heptads of ideal dimeric coiled-coil sequences[11] at the N-and C-termini of the protein, and a series of four point mutations, we generated NEMO-EEAA, a construct fully dimeric and folded in a coiled coil, which rescued IKK-binding affinity to the nanomolar range as observed for full length NEMO[12]. As an additional advantage, we hoped the coiled-coil adaptors (based on the GCN4 sequence) would facilitate crystallization and eventually aid in the X-ray structure determination via molecular replacement. Coiled-coil adaptors have been similarly utilized to both increase stability, improve solution behavior and facilitate crystallization for trimeric coiled coils and antibody fragments[13, 14]. NEMO-EEAA is easily expressed and purified from *Escherichia. coli* cells with a cleavable Histidine tag, is soluble, folded in a stable dimeric coiled coil and is easily

crystallized, with diffraction to 1.9 Å. The presence of the ordered coiled-coil regions of GCN4 could additionally aid in phasing the data from crystals of NEMO-EEAA by molecular replacement using the known structure of GCN4[15].

Given the results obtained with apo-NEMO-EEAA, we believe the protocols described here could also be applied to the crystallization of NEMO-EEAA in the presence of small peptides (like the NBD peptide) or small molecule inhibitors, with the goal of understanding the requirements for NEMO inhibition and structure-based optimization of initial lead inhibitors to high affinity. Given the plasticity and dynamic nature of many coiled-coil domains[16], the use of the coiled-coil adaptors could find more general applicability in aiding structural determination.

**PROTOCOL:**

## 1. Design of construct for crystallography

1.1. Clone the sequence of NEMO-EEAA as in previous publication[12] in a vector for expression in *E. coli* using the T7 promoter, including a N-terminal hexa-histidine tag and a protease cleavage site.

NOTE: In this protocol, we used a vector modified to include a N-terminal hexa-histidine tag and a Tobacco Etch Virus (TEV) cleavage site[10]. This vector facilitates cleavage of the His tag for protein crystallization and leaves only the short extension of GSW residues before the start of the desired protein sequence. The vector from which this was derived, and alternative vectors are listed in the **Table of Materials**. In this protocol, subsequent modifications to the original NEMO(44-111) sequence were introduced stepwise, as described earlier[10], using side directed mutagenesis. We initially attempted to stabilize the NEMO coiled-coil dimer appending the ideal coiled-coil adaptors (in a length of at least three heptads) to the N-terminal or C-terminal end or to both. The double coiled coil was the most promising from earlier crystallization trials and it was subsequently modified introducing mutations to improve crystallization as described previously[12].

## 2. Large scale expression of $His_6$ tagged NEMO-EEAA

2.1. Transform construct into BL21(DE3) competent cells. Store at -80 °C as a cell glycerol stock.

2.2. Day 1 – Prepare a cell starter culture. In a 125 mL Erlenmeyer flask, add 10 mL of Terrific Broth solution and 10 µL of a 100 mg/mL stock of Ampicillin. Add a few microliters of cell glycerol stock (from -80 °C storage of BL21(DE3) competent cells transformed with vector).

2.3. Shake the 10 mL starter culture overnight at 37 °C, 220 rpm (approximately 15 h).

2.4. Day 2 – From the starter, dilute to an $OD_{600}$ = 0.1 in 250 mL of Terrific Broth. Add ampicillin to a final concentration of 100 µg/mL. Grow to an $OD_{600}$ = 0.8-1.0.

2.4.1. Add isopropyl β-D-1-thiogalactopyranoside (IPTG) to 500 µM, and grow for 4 h at 37 °C.

2.4.2. Measure $OD_{600}$ of induced culture after 4 h. Culture should reach an $OD_{600}$ = 6-10.

**3. Purification of $His_6$ tagged NEMO-EEAA**

3.1. Spin cell culture down at 3,800 x *g* for 20 min at 4 °C.

3.2. Save the cell pellet and discard the medium.

NOTE: The cell pellet can be saved and stored at -20 °C at this point, for purification at a later time.

3.3. Resuspend the cells in 40 mL of lysis buffer containing 20 mM Tris, 150 mM NaCl, 10 mM imidazole, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM Dithiothreitol (DTT), and 3 µL of Benzonase Nuclease.

NOTE: The Nickel immobilized metal ion affinity chromatography (IMAC) column utilized is compatible with 2 mM DTT. Alternatively, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP) can be utilized.

3.4. Split resuspended cells into two 20-25 mL aliquots.

3.5. Lyse the cells using French press (approximate pressure 25,000 psi), repeating 2-3 times for each aliquot (in the cold room).

NOTE: Alternatively, cells could be lysed by sonication (not tested in this protocol).

3.6. Add urea to the cell lysate to a final concentration of 8 M, allow to incubate on a rocking platform for a minimum of 2 h or up to overnight. This and all following purification steps, with the exception of the dialysis, can be performed at room temperature.

3.7. Day 3 – Transfer the lysate to ultracentrifuge tubes and balance weight, ensuring lysate fills tubes to at least ¾ full. Spin the lysate at 125,000 x *g* for 30 min at 25 °C. Decant the supernatant into a 100 mL beaker for loading onto column.

NOTE: Centrifuging at 4 °C will cause urea to crash out.

3.8. On a fast liquid chromatography system, remove ethanol from IMAC 5 mL column with 25 mL of ultrapure $H_2O$ at 5 mL/min, followed by 25 mL of elution buffer containing 20 mM Tris, 150 mM NaCl, 500 mM imidazole, 2 mM DTT, pH 8.0, then 25 mL of binding buffer containing 20 mM Tris, 150 mM NaCl, 10 mM imidazole, 2 mM DTT, 8 M urea, pH 8.0.

3.9. Load urea incubated supernatant at 3 mL/min onto IMAC column, collecting the flow through. Wash the column for 10 column volumes with binding buffer at 3 mL/min.

3.10. Refold NEMO-EEAA construct on column by washing column with refolding buffer for 20 column volumes at 3 mL/min, containing 20 mM Tris, 150 mM NaCl, 10 mM imidazole, 2 mM DTT, pH 8.0.

3.11. Perform gradient elution of NEMO-EEAA, from 10 to 500 mM Imidazole over a 12-column volume gradient, collecting all eluate in fraction collection plate (1 mL fractions).

3.12. Continue elution at 500 mM imidazole for two column volumes, continuing to collect.

3.13. Run sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) of fractions to determine NEMO- EEAA presence in elution fractions.

NOTE: We used 10% acrylamide, MES buffer.

3.14. Pool fractions containing pure target protein.

3.15. Measure protein concentration by Bradford assay[17].

NOTE: This is necessary to estimate the amount of protease for tag cleavage.

**4. $His_6$ tag cleavage and purification**

4.1. Add TEV in a 1:10 weight ratio of TEV:NEMO-EEAA protein to cleave the $His_6$ tag. The TEV protease was purified in house.

NOTE: Optimize the amount of TEV, time and temperature required for complete cleavage separately.

4.1.1. Express TEV protease, S219V mutant[18] in BL21(DE3)-RIL cells and purify as described earlier[19]. Briefly grow the cells as described in step 2, lyse by French press and purify using an IMAC column. Store final protein in 25 mM sodium phosphate buffer, pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 20% v/v glycerol.

4.2. Dialyze the sample overnight (approximately 15 h) in 4 L of 20 mM Tris, 150 mM NaCl, 2 mM DTT, pH 8.0, to allow for cleavage and to remove excess imidazole from the sample for the subsequent purification.

4.3. Day 4 – Remove the sample from dialysis. Run an SDS-PAGE gel of the sample from TEV cleavage to ensure cleavage is completed.

4.4. On a fast liquid chromatography system, remove ethanol from an IMAC 5 mL column with 25 mL of ultrapure $H_2O$ at 5 mL/min, followed by 25 mL of elution buffer containing 20 mM Tris, 150 mM NaCl, 500 mM imidazole, 2 mM DTT, pH 8.0, then binding buffer containing 20 mM Tris, 150 mM NaCl, 10 mM imidazole, 2 mM DTT, pH 8.0.

4.5. Load the column at 1 mL/min with TEV-cleaved NEMO-EEAA. Cleaved NEMO-EEAA will elute in the flow-through: collect in a 96 well fraction collection plate (1 mL fractions). Wash the column for five column volumes of 20 mM Tris, 150 mM NaCl, 10 mM imidazole at 1 mL/min, continuing to collect in fraction collection plate.

4.6. Elute TEV and uncleaved $His_6$-NEMO-EEAA with three column volumes of 20 mM Tris, 150 mM NaCl, 500 mM imidazole, 2 mM DTT, pH 8.0, collecting elution in 50 mL conical tubes.

4.7. Run SDS-PAGE gel of flow-through fractions to determine presence of cleaved NEMO-EEAA.

4.8. Pool flow-through fractions containing cleaved NEMO-EEAA construct, and concentrate using a stirred-cell concentrator to 5 mL. Membrane molecular weight cut-off (MWCO) = 3 kDa.

4.9. Using a 3 kDa MWCO membrane, dialyze concentrated sample in 2 L of 20 mM Tris, 100 mM NaCl, 2 mM DTT, pH 8.0 for 2 h. Change the dialysis buffer to 2 L of fresh dialysis buffer for overnight dialysis (approximately 15 h), at 4 °C.

4.10. Day 5 – Load 5 mL of the dialyzed sample on a size exclusion chromatography (SEC) 16 mm x 60 cm column (34 µm average particle size) at 1 mL/min in 2 mM Tris, 100 mM NaCl, 2 mM DTT, pH 8.0. Repeat with additional columns depending on sample volume.

4.11. Pool the fractions corresponding to dimeric NEMO-EEAA.

NOTE: NEMO-EEAA elutes between 60-65 mL, corresponding to a larger molecular weight protein, due to the elongated nature of the dimeric coiled coil.

4.12. Concentrate using a stirred-cell concentrator and a MWCO = 3 kDa membrane to a final concentration of 113 µM (1.65 mg/mL).

4.13. Aliquot the protein and store at 4 °C (stable for over 1 month).

## 5. Sparse matrix screening

NOTE: The protocol performs crystallization trials using commercially available screens and setting up sitting drop experiments using a crystallization robot. Crystal images are collected automatically by an imager.

5.1. Using commercially available sparse matrix screens (see **Table of Materials**), pipette 60 µL of sparse matrix solution into each of the 96 wells of a 2 drop-chamber crystallization plate for sitting drop vapor diffusion (reservoir solution).

5.2. Using a robotic drop setter, dispense 100 nL of protein solution at 1.65 mg/mL in a 1:1 ratio with reservoir solution in drop 1 for a final volume of 200 nL; then 66 nL of protein solution with 134 nL of reservoir solution for a final volume of 200 nL in drop 2 (1:2 ratio).

5.3. Seal the plate using 3-inch-wide sealing tape immediately after dispensing.

NOTE: Drops will dry out if left exposed to atmosphere for longer than 2-3 min.

5.4. Store the trays in the crystallization imager storage, at 20 °C, checking the images collected automatically for crystal presence, starting after two days.

NOTE: Crystallization screening proceeded in parallel with construct optimization. Initial crystals formed in the following conditions (commercial screens listed in the **Table of Materials**): a) 0.1 M Tris, pH 8.0, 30 % v/v polyethylene glycol (PEG) MME 550, 5% poly-γ-glutamic acid, 200-400 kDa low molecular weight polymer (PGA -LM); b) 0.1 M Tris, pH 7.8, 20% w/v PEG MME 2k, 5% PGA-LM; c) 0.1 M Tris, pH 7.8, 20% w/v PEG 3350, 5% PGA-LM. Sparse matrix screen crystals will have poor lattice uniformity; therefore, they will look poor using cross-polarized imaging. Use UV imaging to ensure that the crystals contain protein. The following seed stock generation step is necessary for obtaining the final diffraction quality crystals.

## 6. Seed stock generation

NOTE: We reproducibly obtain crystals for seed generation in 0.1 M Tris pH 8.0, 5% PGA-LM, 3.6% w/v PEG 20k. However, crystals will show high mosaicity and are unsuitable for data collection at this stage.

6.1. Using a kit for seed generation, prepare seed stock by pipetting out entire drop with crystal present, and place into 50 µL of crystallization condition solution in the provided vial.

6.2. Vortex the seed stock for 3 min, pulsing 20 s on and 10 s off.

6.3. Serially dilute seed stock in 1:10 increments down to 1:10,000. Store all dilutions at 4 °C, for further use.

## 7. Fine screens

7.1. Design fine screens varying the conditions for Tris, PGA-LM, and PEG. Vary PEG length for individual trays.

NOTE: The fine screen that produced the crystal utilized for structure determination of NEMO-EEAA employed the following conditions: 0.1 M Tris pH 8; PGA-LM varied from 8 to 0% in columns 1-12. Rows A-H screened different PEGs, with concentrations varying in columns 1-12 as follows. A: PEG 200 (0-40% v/v); B: PEG 400 (0-40% v/v); C: PEG MME 500 (0-40% v/v); D: PEG 1000 (0-30% w/v); E: PEG 3350 (0-30% w/v); F: PEG 6k (0-30% w/v); G: PEG 10k (0-20% w/v); H: PEG 20k (0-20% w/v). The crystal appeared in well H4 (5.45% w/v PEG 20k, 5.8% w/v PGA-LM). In this protocol, a protein crystallography screen builder liquid handler was used to build the screens.

7.2. Seed a protein stock in a 1:25 volume ratio of 1:1,000 seed dilution.

NOTE: Concentration of NEMO-EEAA will drop slightly, but crystals will still form.

7.3. Repeat step 2 using 1:10,000 seed dilution, same 1:25 volume ratio.

7.4. Using the drop setter, dispense 100 nL of protein solution at 1.65 mg/mL, with 1:1,000 dilution of seed stock into a 1:1 ratio with reservoir solution in drop 1 for a final volume of 200 nL. Repeat for drop 2, but with 1:10,000 dilution of seed stock present.

7.5. Seal the plate using 3-inch-wide sealing tape immediately after dispensing.

NOTE: Drops will dry out if left exposed to atmosphere for longer than 2-3 min.

7.6. Store the trays in the imager storage, at 20 °C, checking images collected after two days for crystal presence.

7.7. Check the crystals with the cross-polarizer imager for lattice uniformity, to select for single conditions to set up following trays.

## 8. Generation of crystals for data collection

8.1. Design single condition screens around Tris, PGA-LM, and PEG condition which produced uniform crystals as analyzed by cross-polarized images, and the largest crystals possible.

NOTE: Crystals from these conditions are irregular in shape, mostly rectangular thin sheets. It is key to select a condition where the edges of the crystal are well defined and have the greatest thickness possible.

8.2. Make 20 mL of crystallization condition by hand.

8.3. Using a multi-channel pipettor, dispense 60 µL per well in a 2 drop-chamber, 96 well crystallization plate for sitting drop vapor diffusion.

8.4. Prepare seed stock as described in step 6.2.

8.5. Dispense the protein as described in step 6.3.

8.6. Seal the plate using 3-inch-wide sealing tape immediately after dispensing.

NOTE: Drops will dry out if left exposed to atmosphere for longer than 2-3 min.

8.7. Store the trays in the imager at 20 °C and check the images for crystal presence every day.

8.8. Check cross-polarized images of the crystals for lattice uniformity, to select crystals for data collection.

## 9. Determination of cryo-protectant

9.1. To test the cryo-protectants, create stock solutions corresponding to the crystallization conditions but containing 30%, 20%, 10%, and 5% higher concentration of each component. The addition of the cryo-protectant volume will result in a final concentration of components that is the same as the crystallization conditions.

NOTE: For testing a cryo-protectant at a 30% concentration by volume for a 0.1 M Tris crystallization condition, start with a stock solution of 0.143 M Tris, before adding the cryo-protectant.

9.2. From a cryo-reagents kit, create 10 µL of sample for cryo-protectant test by mixing 30% by volume of cryo condition in 70% of crystallization condition stock solution, for a final concentration of 30% cryo-protectant in original crystallization conditions. Mix thoroughly.

9.2.1. Using a 10 µL pipette, take 5 µL of test cryo-protected solution and plunge the pipet tip into liquid nitrogen. If ice is observed, discard.

9.2.2. Test all cryo-protectants at 30%. For the successful solutions, repeat the process at 20%, then 10% and 5% of cryo-protectant.

9.2.3. Utilizing the successful cryo-protectant solution with the smallest percentage of cryo-protectant, add 0.5 µL of solution into a test drop with crystals present. Observe under microscope, timing how long crystal lasts in the condition, if not indefinite.

NOTE: These crystals are for test only and will be discarded. For NEMO-EEAA, 12% 1,2-propanediol is the optimal cryo-protectant solution. 12% accounts for the dilution the cryo-protectant solution will experience when added to the crystal drop of approximately 100 nL, for an approximate final concentration of 1,2-propanediol of 10%.

## 10. Crystal looping

10.1 Loop crystals 1-2 days before shipment to synchrotron.

NOTE: Crystals will be roughly 60-100 µm in diameter: 0.05 – 0.10 mm loops are ideal for looping.

10.2 Cut the tape from the top of the well.

10.3 Add 0.5 µL of crystallization solution containing 12% 1,2-propanediol cryo-protectant directly to the well.

NOTE: Final concentration of 1,2-propanediol is now at approximately 10%, due to dilution by 100 nL of drop solution (approximate drop volume reduction from initial 200 nL, due to vapor diffusion).

10.4 Loop the crystal from the well.

NOTE: Crystals often grow on the bottom of the well but will dislodge with a gentle nudge from the loop. Once dislodged, loop.

10.5 Store the crystal containing cryo-loops in pucks immersed in liquid $N_2$.

10.6 Store these pucks in liquid $N_2$ in Dewar flask until they are ready for shipment for X-ray diffraction at the synchrotron.

## 11. Data collection

11.1. Collect X-ray diffraction data. In this protocol, use AMX beamline (ID: 17-ID-1), National Synchrotron Light Source II.

NOTE: Data was collected on site but can be collected remotely. Collect data in the region of the crystal which showed lattice uniformity in cross-polarized images. Position the crystals in the loop so that data collection does not involve “poor” regions of the crystal while the crystal rotates in the goniometer. Data which provided the best resolution came from collection on the edge of an extension of a crystal about 5 x 5 µm in size. Use rastering to identify the best areas on the crystal to collect[20].

## 12. X-ray data processing

12.1. Process dataset to highest resolution collected (1.8 Å) with XDS IDXREF program to determine space group, unit cell, and solvent content.

12.2. Integrate data using XDS INTEGRATE program.

12.3. Process scaled intensities from XDS XSCALE program using STARANISO Server, using I/σI cutoff mean of 1.2 for diffraction-limit surface for the data.

NOTE: Data will not be cut in true ellipsoid. Retain all data above the I/σI of 1.2 cutoff. The statistics on the data calculated for spherical completeness will be poor, due to the non-spherical truncation. The elliptical completeness was 88% with highest resolutions of: 1.88 Å, 2.10 Å and 2.55 Å along the a*, b* and c∗ axis, respectively.

## 13. Structure solution

13.1. Utilize the X-ray structure of GCN4 (PDB: 4DMD)[15] as a search model for molecular replacement using MRage[21] in PHENIX[22]. The 4DMD structure was defined in MRage as an "ensemble", and the MRage solution successfully built the structure portion corresponding to the N-terminal coiled-coil adaptor of NEMO-EEAA, homologous to the search model, for both chains in the dimer.

NOTE: Turn off anisotropy correction in PHASER, or data will be further scaled back.

13.2. Utilize successive rounds of Autobuild[23] in PHENIX and manual building (using $2F_o$-$F_c$ and $F_o$-$F_c$ maps, in Coot[24]) to build the remainder of the structure.

13.3. Manually build the residues still missing into the model based on $2F_o$-$F_c$ and $F_o$-$F_c$ maps, using Coot[24].

NOTE: The last stage involved building the 4 N-terminal residues and 4 C-terminal residues for each monomer. Sidechain placement was also manually adjusted as needed.

13.4 Calculate a composite omit map using PHENIX and a 10% omission of the structure.

## 14. Structure refinement

14.1 Refine the structures with PHENIX Refine. Run the initial refinements against bulk-solvent and stereochemistry weights, relaxing RMSbond and RMSangle constraints to 0.01 and 1.0, respectively. Continue refinement on Individual B-factors, TLS parameters, and Occupancies.

## REPRESENTATIVE RESULTS:

### Cloning, expression and purification of the IKK-binding domain of NEMO.

The protocol followed in this study to obtain the final sequence of NEMO-EEAA (**Figure 1A**), which produced diffraction quality crystals, involved the expression and characterization of all the intermediate constructs, including the addition of the coiled-coil adaptors at N- and or C-terminus, the mutations C76A, C95S and the mutations E56A, E57A. **Figure 1B** displays an SDS-PAGE gel of samples collected throughout the purification procedure as described in the Scheme in **Figure 1C**. The protein is overexpressed in *E. coli* and appears as a band approximately at the 14 kDa MW weight marker on the SDS-PAGE gel (lane 3, cells collected at harvest and lysed in Laemmli sample buffer supplemented by 8 M urea). The protein appears pure after the first IMAC column and displays a monomer and a dimer band at the level of the 14 and 28 kDa MW markers on the SDS-PAGE gel (lane 9). TEV cleavage is practically complete following the protocol and the

protein elutes with the flow through during the second IMAC column almost entirely as a dimer, at the expected MW (band below 28 kDa). Size exclusion chromatography displays a single peak eluting between 60-65 mL and corresponding in our experience to the dimer (**Figure 1D**). The dimeric coiled coil always elutes earlier than expected on SEC due to the elongated shape of the coiled coil and the consequently large hydrodynamic radius[10]. NEMO-EEAA in fractions from SEC peak still appears as a monomer and a dimer on SDS-Page gel (lanes 14-15). Utilizing a stirred-cell concentrator is important to prevent sample possible aggregation and precipitation upon concentration.

**Crystallization of NEMO-EEAA**

Initial crystals were obtained from a commercial screen using PGA (see **Table of Materials**), utilizing 1.65 µg/mL of NEMO-EEAA in 2 mM Tris, 100 mM NaCl, 2 mM DTT, pH 8.0. Fine screening produced crystals in 0.1 M Tris pH 8.9, 5% PGA-LM, 3.6% PEG 20k (**Figure 2A**), which were utilized to produce a seed stock. Final crystals were obtained with seeding in 0.1 M Tris pH 8.0, 5.8% PGA-LM, 5.45% PEG 20k (**Figure 2B**).

**Data collection and structure determination**

NEMO-EEAA crystals suffer from mosaicity and anisotropy. Crystals formed in the P 1 $2_1$ 1 space group, with data resolution varying in the a*, b* and c* axis (1.88 Å, 2.10 Å and 2.55 Å). Examples of diffraction profiles are in **Figure 3**. Data was acquired at the AMX (17-ID-1) beamline of NSLS II, with a beam size of 7 x 5 $\mu m^2$. The small beam size was essential to focus on the desired portion of the crystal (**Figure 2B**) and ensure data quality.

The successful protocol for structure determination requires anisotropic truncation of the data using the STARANISO[27] server (http://staraniso.globalphasing.org/cgi-bin/staraniso.cgi), followed by phasing by molecular replacement using MRage[21] within PHENIX[22] and the structure of dimeric GCN4 (PDB: 4DMD)[15] as a search model. In the solution of this structure phasing was initially attempted by labeling the native methionine with SeMet. The anomalous signal was too weak, probably due to the solvent exposed nature of Met95 in the unbound form of NEMO (SeMet95 was successfully used for phasing of the NEMO / IKKβ structure[9]).

Initial data analysis was attempted with spherical truncation of the data to 2.3 Å. This data set could not be successfully phased by molecular replacement but a solution was obtained using MR-ROSETTA[25] and the structure of NEMO(44-111) in complex with IKKβ(701-745) as a search model (PDB: 3BRV)[9]. This initial model could not be refined successfully. We utilized the Diffraction Anisotropy Server at UCLA[26] to elliptically truncate the data and to remove anisotropy by anisotropic scaling. The newly processed dataset could be phased by molecular replacement. To further improve the data, we employed the STARANISO[27] server for anisotropic truncation of the data and amplitudes were corrected with an anisotropic correction factor with Bayesian estimation[28]. This data, resulting in an increase in the number of unique reflections to 19,560, was used for structure refinement. Composite omit maps where calculated with PHENIX, excluding 10% of the atoms at a time, and compared with the final structure model, to confirm that the structure was not biased by the atomic model. The crystallographic model is complete except for the first and last residues in chain A of NEMO-EEAA, for which no electron density was

observed.

NEMO-EEAA is a homo-dimeric, irregular, parallel coiled coil of ~175 Å in length. The regular coiled-coil region encompasses the ideal coiled-coil adaptor sequence at the N-terminus (residues 20-50) and the first two heptads of the NEMO proper sequence (residues 51-65). A regular coiled coil is also present at the C-terminus, starting at NEMO residue 97 and encompassing the C-terminal ideal coil-coil adaptor (**Figure 4A**). The central portion, encompassing NEMO residues 66-98 displays larger interhelical distances (interhelical spacing goes from an average value of 7.6 Å in the regular coiled-coil structure to a maximum of 11.5 Å in the irregular region) and discontinuous hydrophobic interface compared with an ideal coiled coil. This region of NEMO also represents the interface for binding IKKβ and undergoes a conformational change upon ligand binding. The IKKβ-bound structure displays a more open coiled-coil conformation to accommodate the ligand with larger interhelical spacing by 1.0 to 2.2 Å in this region (**Figure 4B**)[12]. In the apo structure residues Leu93, Met94, Lys96, Phe97 and Arg101 are shifted toward the center of the coiled coil to invade the ligand binding pocket (the Cα-Cα distance for Phe97 is tighter by 2.9 Å), with the overall effect of closing the large hydrophobic cleft which hosts the ligand in the IKKβ-bound structure.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Purification of NEMO-EEAA**. **(A)** Sequence alignment of NEMO from *Homo Sapiens*, *Mus Musculus* and *Bos Bovis* and the engineered NEMO-EEAA. The coiled-coil adaptors sequence is underlined, and the mutations are highlighted in orange. **(B)** SDS-PAGE gel of fractions collected during expression and purification (as labeled and referenced in flow chart 1C). 10% Acrylamide, MES buffer. **(C)** A flow chart for the purification of NEMO-EEAA. **(D)** Size-exclusion profile indicating the dimer of NEMO-EEAA (blue). In green the molecular weight markers (kDa) are indicated.

**Figure 2: Morphology and size of NEMO-EEAA crystals. (A)** A crystal of NEMO-EEAA from the initial sparse matrix screen, unseeded. This type of crystal was used for seeding for subsequent crystallization attempts (0.1 M Tris pH 8.0, 5% PGA-LM, 3.6% PEG 20k; with a protein solution in 2 mM Tris, 100 mM NaCl 2 mM DTT, pH 8.0). **(B)** Crystal used for data collection (0.1 M Tris pH 8.0, 5.8% PGA-LM, 5.45% PEG 20k, with a protein solution in 2 mM Tris, 100 mM NaCl 2 mM DTT, pH 8.0). The approximate area used for data collection is circled in red. The scale bar for 100 μm length is defined in the figure.

**Figure 3: Crystallographic data obtained from NEMO crystals**. **(A)** X-ray diffraction profile of early NEMO crystal: elongated streaks indicate mosaicity in the crystal. **(B)** X-ray diffraction profile of an optimized NEMO-EEAA crystal. The resolution ring is drawn at a spatial resolution of 2.5 Å.

**Figure 4: Structure of unbound NEMO. (A)** The NEMO-EEAA dimer is shown as a ribbon, light blue = coiled-coil adaptors, blue = NEMO(51-112). **(B)** Superposition of apo NEMO-EEAA structure (blue, PDB: 6MI3) and NEMO(44-111) from the IKKβ-bound structure (grey, PDB: 3BRV, IKKβ is not displayed), shown as ribbons; the structures are aligned on chain A, region 44-111 only. This

figure has been modified from previous publication[12].

**DISCUSSION:**

Crystallization attempts of NEMO in the unbound form were unsuccessful, including attempts using the full-length protein and several truncation constructs encompassing the IKK-binding domain. Our biophysical characterization of the IKK-binding domain of NEMO (residues 44-111) by circular dichroism, NMR spectroscopy and fluorescence anisotropy indicated that the construct, albeit able to bind IKKβ, existed in a state of conformational exchange, not suitable for crystallization[9, 10]. Our approach involved engineering a construct of the IKK-binding domain of NEMO that adopted a more stable, folded and native-like conformation, eliminating the flexibility that prevented crystallization. Ideal coiled-coil domain adaptors fused to the N- and C-termini of the NEMO(44-111) sequence offer the advantage of stabilizing the dimeric coiled-coil fold of the native NEMO and facilitating crystallization[14]. Behavior of a series of protein constructs was monitored at each step through SDS-PAGE, size exclusion chromatography, circular dichroism, fluorescence anisotropy and NMR spectroscopy, as described earlier[10,12]. Despite the progressive improvement in all desired parameters no construct yielded diffraction quality crystals until the mutations E56A, E57A were introduced. The mutations were the highest impact mutations suggested by the Surface Entropy Reduction Server SERp[29] that did not involve residues implicated in hot-spots of binding for IKKβ, as in the NEMO / IKKβ complex structure. Although other constructs stabilizing the ordered structure of the IKK-binding domain of NEMO through cysteine disulfide linkage and binding IKKβ with high affinity have been described[30], the protocol here described represents the first successful approach to structure determination of the IKK-binding domain of NEMO in the unbound form.

Protein production and purification followed standard procedures in our laboratory. Upon cell lysis a considerable portion of the protein is present in the insoluble fraction, even when growing the cells after induction at 18 °C, therefore the protein was solubilized in 8 M urea after cell lysis and prior to purification, and refolded while bound to the IMAC column. Extensive washing of the column bound protein both under denaturing conditions and after refolding are critical for a pure product after the first purification step. The pure NEMO-EEAA has a higher tendency to precipitate than the previous constructs but is still sufficiently soluble under crystallization conditions.

A critical step in the structure determination was the use of seeding. In an approach similar to microseed matrix screening[31], seeds from crystal obtained during sparse matrix screening were used in an additional screening of conditions, followed by a fine screening round, in order to determine final crystallization conditions. Most of the crystals grown in the final conditions display high mosaicity and are not suitable for data collection. Crystals were screened over several visits to the beamline and best data quality was achieved by collecting datasets at the edge of the crystal, where the newest growth had occurred. As the region which showed lattice uniformity in cross-polarized images was small, it was instrumental to have access to the AMX beamline at NSLS II, due to the small beam size.

We successfully determined the structure of NEMO-EEAA by molecular replacement using as a search model the structure of the dimeric coiled-coil of GCN4[15], which mapped to the ideal coiled-coil adaptors fused at the N- and C-termini of the NEMO sequence. The molecular replacement was successful as the GCN4 adaptors maintain their native structure when fused to NEMO. At the same time, we could verify that NEMO maintains its native like structure by comparing the portions not involved in IKK-binding with the corresponding structure region in the NEMO / IKKβ complex structure. As an alternative, it could be possible to use the structure of NEMO in the NEMO / IKKβ complex (PDB: 3BRV)[9] as a molecular replacement search model, focusing on monomer B, which corresponds to the chain which undergoes the smallest conformational change upon ligand binding. This strategy showed some initial success using the MR-ROSETTA module of PHENIX.

Finally, it was essential for a successful structure determination to utilize all available data in the dataset, by resorting to an anisotropic cut-off of merged intensity data, as provided by the STARANISO[27] server or the Diffraction Anisotropy Server at UCLA[26].

The essential role played by the NEMO/IKK complex in the NF-kB pathway makes it a desirable target for modulation of the pathway for therapeutic intervention. Protein-protein interactions, especially when involving a large binding interface, are challenging to inhibit with small peptides or small molecules, and structure-based inhibitor design can offer a significant advantage. The constructs of the IKK-binding domain of NEMO that we developed overcome the limits of the flexible NEMO(44-111) to facilitate crystallization and structure determination. As the construct easily crystallizes in the unbound form, we envision that the same protocol can be successfully applied in the crystallization of the complex of NEMO with small molecule inhibitors, to determine a structure that would provide details on binding modes and allow further ligand improvements.

An analysis of crystal packing in the crystallization conditions achieved in this work indicates that ligand co-crystallization may be preferred to ligand soaking into apo-protein crystals. While crystal packing provides some space around chain B of the dimer (6 to 10 Å to the nearest chain) and on one face of the ligand binding site (approximately 13 Å in the hot-spot region between Phe82-Phe87), the symmetric binding pocket is completely occluded by nearby chains, preventing ligand binding.

Coiled coils are present in a significant proportion in the proteome and are essential in their function as molecular spacers, scaffolds and in communicating conformational changes[16]. Despite their abundance and relevant roles, there are relatively few structures of full-length coiled-coil proteins. The use of coiled-coil adaptors may aid in the stabilization of structural domains of coiled-coil proteins while preserving their native structure and aid in the structural determination and elucidation of their function.

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**DISCLOSURES:**

The authors declare no competing interests.

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Figure 1

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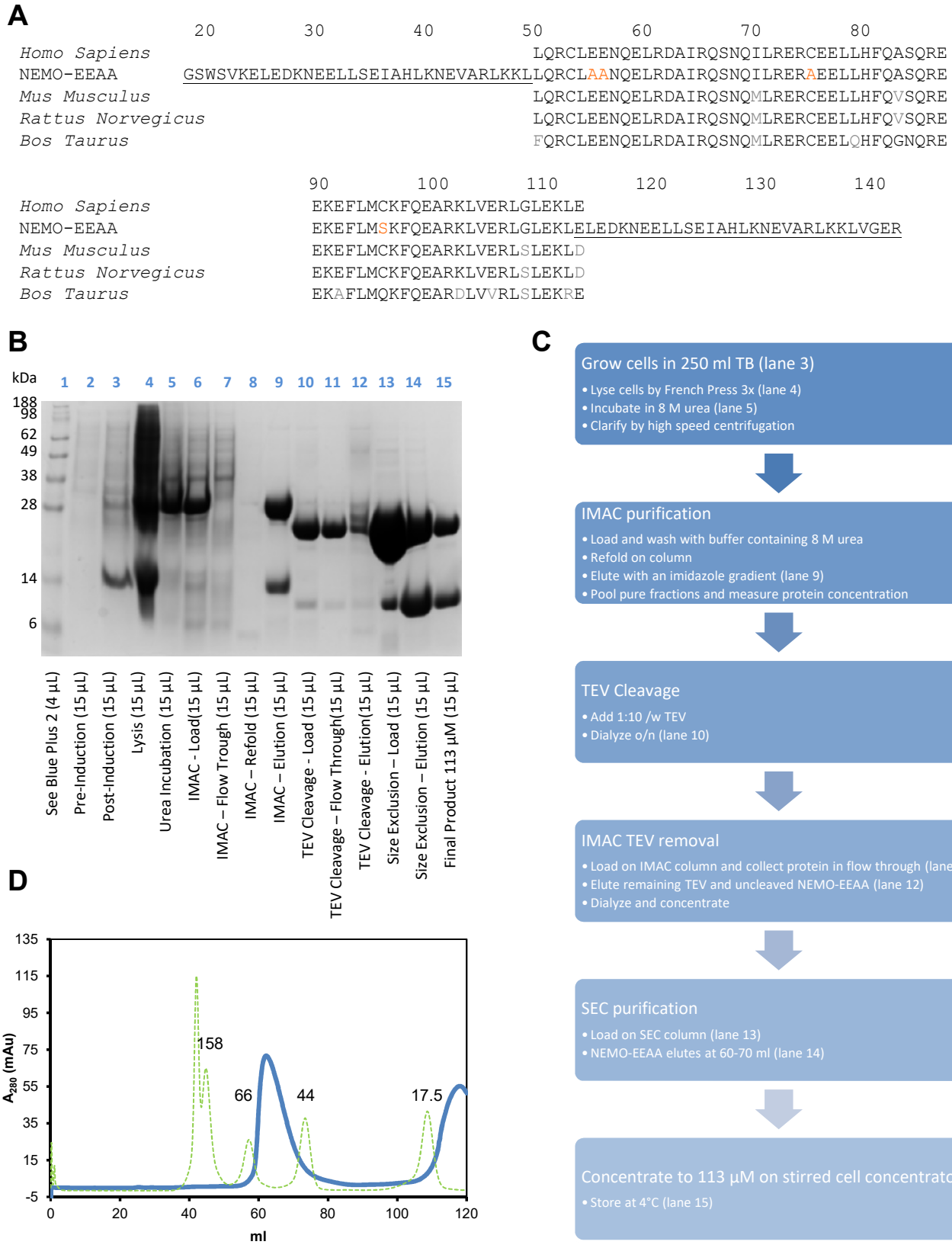

Figure 2

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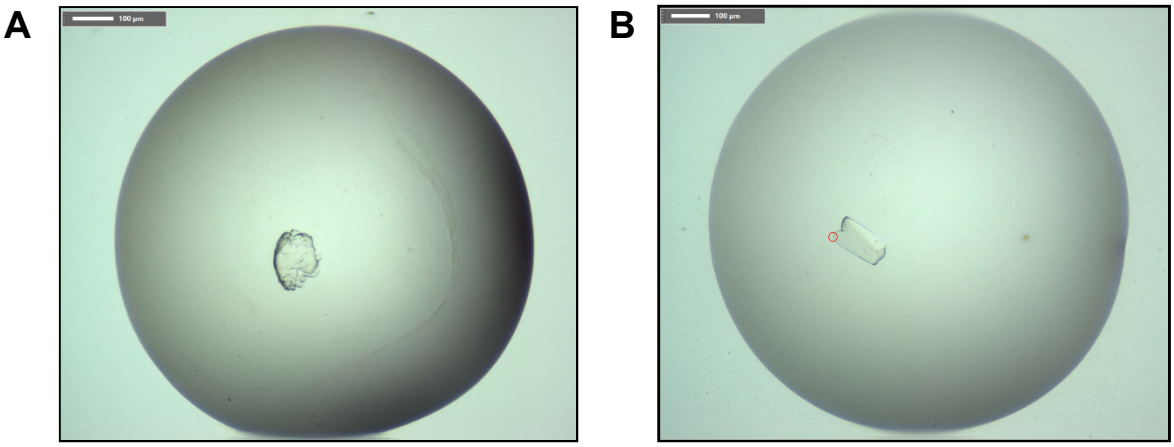

Figure 3

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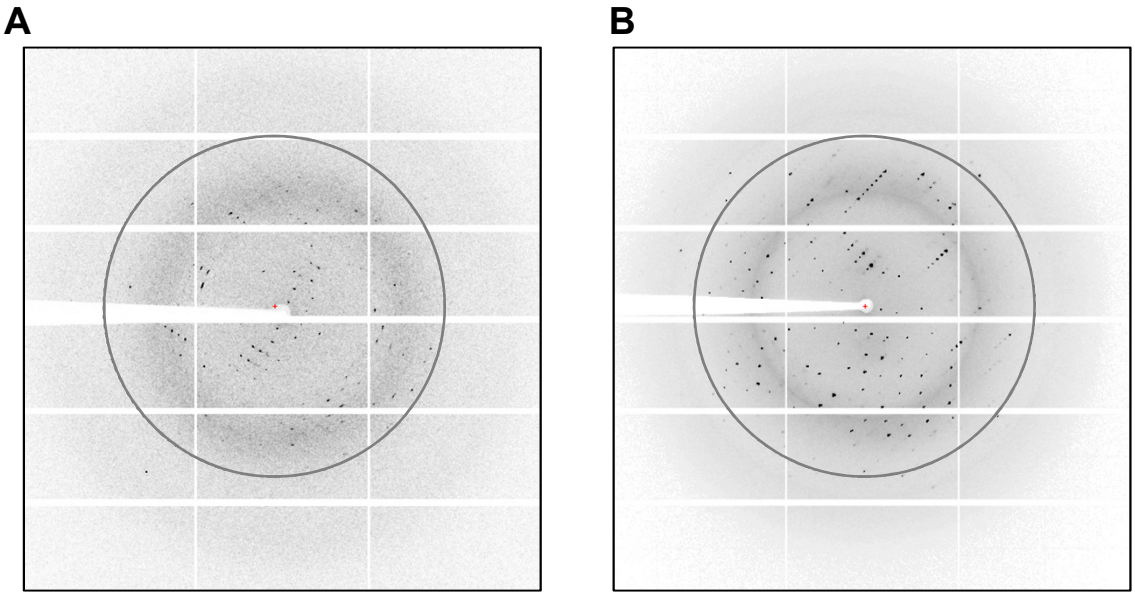

Figure 4

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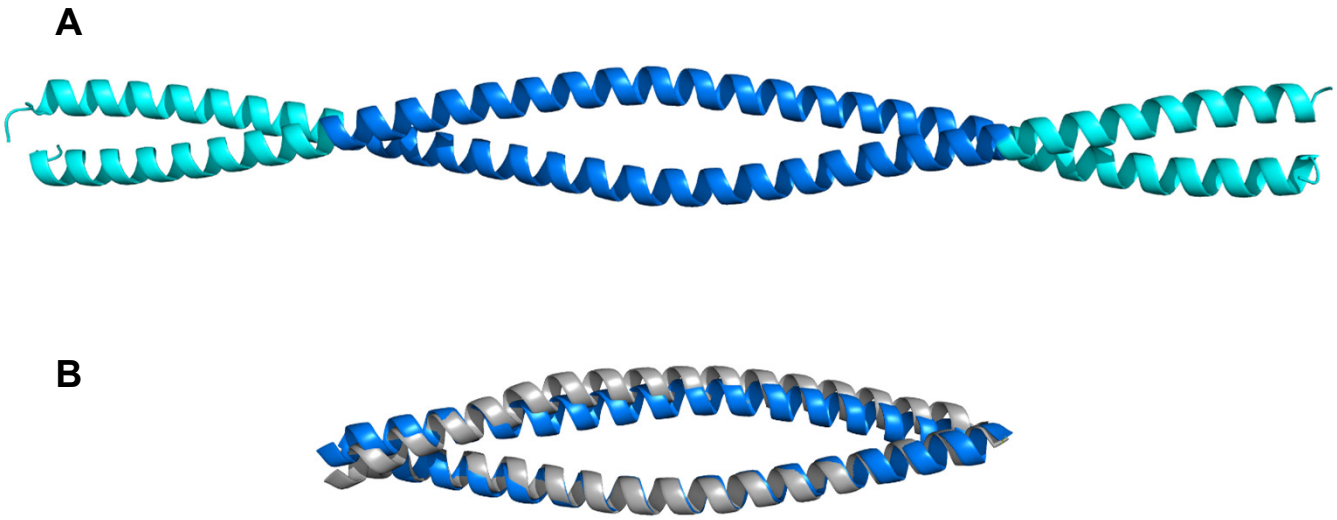

Table of Materials

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| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 20% w/v γ-PGA (Na+ form, LM) | Molecular Dimensions | MD2-100-150 |
| 3.5 kDa MWCO Dialysis Membrane | Spectra/Por | 132724 |
| Amicon Stirred Cell | Millipose Sigma | UFSC 05001 |
| Ammonium Chloride | Millipore Sigma | G8270 |
| Benzonase Nuclease | Millipore Sigma | 9025-65-4 |
| BL21-CodonPlus (DE3)-RIL Competent Cells | Agilent Technologies | Model: 230245 |
| CryoPro | Hampton Research | HR2-073 |
| D-Glucose (Dextrose) | Millipore Sigma | A9434 |
| Difco Terrific Broth | ThermoFisher | DF043817 |
| Dithiothreitol > 99% | Goldbio | DTT25 |
| E. coli: Rosetta 2 (DE3) | Novagen | 71400-3 |
| FORMULATOR | Formulatrix | |
| HCl – 1.0 M Solution | Hampton Research | HR2-581 |
| HiLoad 16/600 Superdex 75 pg | GE Healthcare | 28989333 |
| HisTrap HP 5 mL column | GE Healthcare | 17524802 |
| HT 96 MIDAS | Molecular Dimensions | MD1-59 |
| HT 96 Morpheous | Molecular Dimensions | MD1-46 |
| Imidazole | ThermoFisher | 288-32-4 |
| Isopropyl-beta-D-thiogalactoside | Goldbio | I2481C5 |
| MRC2 crystallization plate | Hampton Research | HR3-083 |
| NT8 - Drop Setter | Formulatrix | |
| pET-16b | Millipore Sigma | 69662 |
| pET-45b | Millipore Sigma | 71327 |
| Phenylmethylsulfonyl fluoride | ThermoFisher | 36978 |
| Polycarbonate Bottle for use in Ultracentrifuge Rotor Type 45 Ti | Beckmann Coulter | 339160 |
| Polyethylene Glycol 20,000 | Hampton Research | HR2-609 |
| pRK793 (TEV) | Addgene | Plasmid 8827 |
| QuikChange XL II | Agilent Technologies | 200522 |
| Required Cap Assembly: | Beckmann Coulter | 355623 |
| ROCK IMAGER | Formulatrix | |
| Seed Bead Kit | Hampton Research | HR2-320 |

| | | |
|---|---|---|
| Sodium Chloride ≥ 99% | Millipore Sigma | S9888 |
| TCEP (Tris (2-Carboxyethyl) phosphine Hydrochloride) | Goldbio | TCEP1 |
| The Berkeley Screen | Rigaku | MD15-Berekely |
| The PGA Screen | Molecular Dimensions | MD1-50 |
| Tris – 1.0 M Solution | Hampton Research | HR2-589 |
| Ultrapure Tris Buffer (powder format) | Thermofisher | 15504020 |
| Urea | ThermoFisher | 29700 |

| Comments/Description |
| --- |
| For fine screen crystallization of NEMO-EEAA |
| For dialysis removal of imidazole |
| For protein concentration |
| |
| For minimal media labeling |
| For digestion of nucleic acid |
| TEV expression |
| Cryo-protectants kit |
| For minimal media labeling |
| For culture growth |
| For reduction of disulfides |
| Expression of unlabeled NEMO-EEAA |
| Liquid handler/ screen builder |
| For fine screen crystallization of NEMO-EEAA |
| For size exclusion purification |
| For purification of His-tagged NEMO-EEAA |
| For sparse matrix screening of NEMO-EEAA |
| For sparse matrix screening of NEMO-EEAA |
| For elution from His-trap column |
| For induction of cultures |
| Crystallization plate |
| Crystallization |
| For cloning of NEMO-EEAA |
| For cloning of NEMO-EEAA |
| For inhibition of proteases |
| Ultracentrifuge bottle |
| For fine screen crystallization of NEMO-EEAA |
| For TEV production |
| Site directed mutagenesis |
| Ultracenttrifuge bottle cap |
| Crystallization Imager |
| Seed generation |

For buffering of purification solutions
Reducing agent
For sparse matrix screening of NEMO-EEAA
For fine screen crystallization of NEMO-EEAA
For fine screen crystallization of NEMO-EEAA
For buffering of purification solutions
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Dear Reviewers,

we are thankful for your comments, observations and suggestions. We believe they have contributed to a stronger and more clear manuscript. We detail in the paragraphs below the changes that we have implemented following the Reviewers' comments, and how they have been incorporated into the revised manuscript. A version of the manuscript with the major changes highlighted in 'Track Changes' was also uploaded.

Best Regards,

Maria Pellegrini

***Editorial comments:***

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

The manuscript was carefully proofread.

*2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."*

The copyright permission information has been uploaded as: Copyright_policy_Scientific_Reports.docx.

*3. Please add a one-line space between each of your protocol steps.*

We added the line spacing.

*4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*

All commercial language was removed from the manuscript, the information added to the Table of Materials.

*5. Step 4.13: Please write this step in the imperative tense.*

Written in imperative tense.

*6. Step 14.1: Please write this step in the imperative tense.*

Written in imperative tense.

*7. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

The steps have been highlighted.

*8. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.*

The scale was added and defined in the legend.

**Response to the Reviewer's comments:**

***Reviewer #1:***

*Minor comments:*
*Regarding the main text:*
*-It may be useful to mention the manufacturers for the chemicals used during the procedure.*
The manufacturer for the chemicals were added to the Table of Materials.
*-It may be useful to mention whether NEMO-EEAA is temperature-sensitive and if the protein purifications should be done at room temperature or on ice, or in a cold room (the temperature requirements are not specified for all purification steps.*
Temperature requirements were added for the purification steps.
*-The authors may also consider specifying the expected shape of the crystals.*
We added a description of the shape of the crystals obtained, which is somewhat variable.
*Regarding the figures:*
*-The authors may consider expanding figure 1 by showing also a multiple-sequences alignment. This alignment would make a great addition to the schematic representation of the primary sequence of the NEMO-EEAA construct.*
We added a sequence alignment of NEMO from *Homo Sapiens* and three different species in Figure 1, to complete the data.

***Reviewer #2:***

*Minor Concerns:*
*1) I recommend expansion of section 1 (Designing construct for crystallography), since this strategy is potentially applicable to other proteins and the most significant aspect of this manuscript. In particular, please give more description on the thought process and selection of the construct in this work. What are the important considerations in adding the N- and C-terminal tags? How many constructs were attempted to arrive at the final construct?*
Indeed, the construct development represented one of the main challenges of this project. We added information on the earlier coiled-coil constructs developed for this effort and we added the reference to our earlier publication that describes their development in detail.
*2) In section 3.2 and elsewhere, volumes have 'nL" as units. Are these correct or the authors meant microliters?*
All volume units were checked and corrected. The drop setter robot dispenses 200 nL volume drops.
*3) The manuscript contains minor grammatical errors here and there. They should be corrected.*
We corrected the errors.

***Reviewer #3:***

*Major Concerns:*
*1. In 101, suggesting commercially available vector(s) in addition to the in-house pET98 vector would be helpful.*
We added suggested alternative vectors to the description.
*2. In 124, the condition for spinning cell culture down has not to be strictly defined. '3,800 x g' or '4,000 x g' would be fine.*

Thank you for noticing the mistake, it was corrected to ‘3,800 x g’

*3. ln 132, French press may be a non-standard method for cell lysis in some laboratories. Mentioning the availability of sonication for this purpose would be helpful.*

We added sonication as an alternative method for cell lysis.

*4. ln 140, GE HisTrap is resistant to 2 mM DTT, while other commercially available Ni-chelating beads are not. This point should be noted just in case.*

We added a note indicating that our column (now specified only in the Table of Materials as per editorial policy) is compatible with 2 mM DTT, and we suggested the alternative use of 0.2 mM TCEP, which we found effective in other protocols.

*5. ln 159, methods of TEV protease preparation need to be described briefly.*

We added a description of the characteristics of the TEV used in this protocol (plasmid listed in the Table of Materials) and added the literature reference for its preparation and a brief description of the protocol used.

*6. ln 226, showing an example of the condition for fine screening would be helpful.*

We added the description of the fine screening utilized to explore different PEG precipitants, that yielded the crystal utilized for structure determination.

*7. ln 408, the side chains of the residues consisting of the hydrophobic cleft should be presented.*

We added a description of the side chains that experience changes in the apo structure versus the IKKβ-bound structure and that result in closing of the hydrophobic ligand binding cleft.

*8. ln 494-497, crystal packing of the crystallization condition described in this paper should be mentioned. Is there any binding space for small compounds in the crystal?*

We added the analysis of crystal packing as it affects the possibility of ligand binding in the crystal. While crystal packing provides some space around chain B of the dimer (6 to 10 Å to the nearest chain) and on one face of the ligand binding site (approximately 13 Å in the hot-spot region between Phe82-Phe87), the symmetric binding pocket is completely occluded by nearby chains, preventing ligand binding.

*9. In Fig 1D, please superpose the elution profile of molecular weight marker(s), or indicate approximate molecular weight(s) in the horizontal axis.*

We added the elution profile of molecular weight markers with corresponding molecular weights.

*10. In Fig 3, circle(s) indicating spatial resolution should be shown (maybe 2 angstrom?).*

We added resolution rings to the diffraction images, for a spatial resolution of 2.5 Å.

*Minor Concerns:*

*1. Please confirm that a single space is inserted between a value and a unit throughout the paper. Also, the usage of 'l' and 'L' should be unified.*

We corrected the manuscript for spaces and correct units.

*2. ln 63, "grove" may be "groove".*

Thank you for noticing, we corrected to “groove”.

*3. ln 117, is "100 µg/mL" a final concentration? Should be clarified.*

We specified that the value indicated the desired final concentration.

***Reviewer #4:***

*Minor comments:*

*1. Design of Construct for Crystallography (page 4, lines 99-109)*

*Here the authors should only describe the construction of the final construct with the GCN4 sequence and point mutations. The actual chronological order the authors used to produce the final optimized construct is not relevant. Interested people can always refer to the Sci. Reports publication from the authors. Indeed, the whole construct can be ordered from a company performing gene synthesis at low cost, saving also time.*

We replaced the step with a streamlined description of the final construct utilized for structure determination. We added a brief description of the history of the construct development as a 'NOTE', as suggested by Reviewer #2.

*2. Page 5, line 153 - add conditions for the SDS-Page gel (% acrylamide, etc).*

The conditions utilized for the SDS-Page gel were added.

*3. Page 5, line 155 - add reference for the Bradford assay.*

The reference for the Bradford assay was added.

*4. Page 6, line 200 - please give examples of sparse matrix screens. In page 20 there are three sparse matric screens referred, but reference here for them.*

We added the three commercial sparse matrix screens utilized to the Table of Materials (editorial policy prevents from referencing commercial products in the text), and the description of the specific conditions that yielded initial crystals to the protocol.

*5. Page 6, lines 215-217 - conditions are mentioned here for crystal formation but not the actual screen and well where this was obtained (eg. JCSG+ B3) This information is very useful.*

We added the exact well conditions of the sparse matrix screens where the initial crystals were observed (the name of the commercial screen is reported in the Table of Materials, as editorial policy prevents from referencing commercial products in the text) and the details of the fine screen that yielded the crystal utilized for structure determination.

*6. Page 10, line 365 - please correct NEMO-EEAA concentration to 113 µM.*

Thank you for noticing, the concentration was corrected.

*7. Data Collection and Structure Determination (page 10, lines 371-409) - the protocol is quite difficult. I wonder if it could be simplified somehow?*

We simplified the description by first describing the simplest protocol to structure determination. Following, is the detailed description of the steps that were actually taken to solve the structure in our laboratory.

*8. It would have been useful to be able to look at the PDB structures (6MI3 and 6MI4) produced using this protocol, but unfortunately, they are not available yet at the PDB. Please make sure that they will be available asap to the general public.*

The PDB structures have been released as of July 31$^{st}$ and August 7$^{th}$.

Response to **Editorial comments:**

*The manuscript has been modified and the updated manuscript, 60339_R1.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.*

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
The manuscript was proof read, please advise of specific additional corrections that need to be applied.

*2. Please do not use more than 1 note for each step.*
We now have just one NOTE for each step.

*3. Please use h, min, s for time units.*
The time units have been corrected throughout.

*4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*
We removed all commercial language. If language that we failed to identify as commercial is still present, please provide us with the details so that we can efficiently correct it.

*5. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*
The highlighted Protocol lines, including heading and spaces are now 120 lines (2.75 pages are 121 lines). As we have no experience in filming a protocol, we would be very happy to incorporate any suggestion to modify/ increase/reduce the portions included in the video. For example, we could remove some of the protein purification steps in favor of more crystallography steps, or similar changes.

*6. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*
We removed the symbols from the table of Materials

*7. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite lines 47-49, 519-527, 540-542.*
The lines have been modified to use original language.

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