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 31st and August 7th.