Mechanistic insights into enhancement or inhibition of phase separation by different polyubiquitin chains
Thuy Dao, Yiran Yang, Maria Presti, Michael Cosgrove, Jesse Hopkins, Weikang Ma, Stewart Loh, and Carlos Castañeda
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Corresponding author(s): Carlos Castañeda (cacastan@syr.edu)

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Dao et al. use microscopy, NMR, and other biophysical methods to study how ubiquitin chains of varying length and linkage impact UBQLN2 liquid-liquid phase separation (LLPS). The mechanistic contribution of UBQLN2 and other UBL-UBA proteins as a ubiquitin shuttle factor has remained somewhat elusive but recent studies, including by the Castaneda lab, have indicated their importance in driving LLPS. A controversy in the field has been whether ubiquitin chains promote or inhibit shuttle factor mediated LLPS. This manuscript is focused around addressing the nuances of ubiquitin chain modulation of LLPS. The authors demonstrate that at high ubiquitin chain concentrations, LLPS is inhibited, with this inhibition occurring at lower concentrations for chains of compact structure (K48 and K11 linked) compared to those with extended structures (K63, M1, and HOTag6-G10-Ub). One concern is that the authors argue this difference to be substantial, but the data seem to show more subtle differences. The authors find that at low concentration, ubiquitin chains can promote LLPS at certain temperatures, and show data suggestive that this effect is stronger for chains of extended structures although again the differences seem more subtle than perhaps concluded by the authors. The authors further demonstrate that UBQLN2 condensates are formed during in vitro enzymatic assembly of K63-linked chains and that Ubc13, which appears to be ubiquitinated, is recruited to the droplets. The study is well-done overall, invoking multiple methodologies, including the innovative invocation of HOTag6-G10-Ub, clearly presented, and impactful, with relevance to the broad field of ubiquitin signaling as well as the newer field of LLPS. Concerns over how the authors interpret their data however exist for this reviewer. It's possible that these concerns can be addressed with changes to the text and/or greater explanation and without further experiments.

**Major comments:**

- Are the key conclusions convincing?

The authors present the data and methods clearly and use multiple approaches. One aspect that is not convincing to this reviewer concerns the conclusion that compact chains destabilize UBQLN2 phase separation whereas extended chains promote it, as written in the second highlight and also throughout the text. In particular, all chains seem to inhibit UBQLN2 condensates at high enough concentration (which the authors do mention at some points in the text) and K48 chains seem to promote it at certain temperatures and chain lengths. For example in Fig. 1B, at 36\(^\circ\)C and 40\(^\circ\)C, K48-Ub3 appears to promote condensates at low Ub:UBQLN2 molar ratios. In addition in Fig. 1A the amount of total condensate in the field of view for the K63-Ub3 appears lower than that of K48-Ub3 at 0.1:1 Ub:UBQLN2 ratio. This data seems to contradict the statement on p. 7: "Conversely, like Ub and K48-Ub2, K48-Ub3 also inhibited LLPS at all concentrations." To address these concerns, the data in Fig. 1A should be quantified as amount of total condensate to field of view. This same concern applies to Fig. 5A. An aspect that could be discussed further is why the condensates change in morphology with Ub chain addition.
The data in Fig 1A don't seem to correspond well to that of Fig. 1B for K48-Ub4. Condensates are observed at 30°C in Fig. 1A up until 1.2:1 Ub:UBQLN2 ratio. If the difference is caused by Fig 1A being at 50 micromolar UBQLN2 compared to 30 micromolar in Fig 1B then microscopy data should be provided to validate and complement Fig 1B. This data is also needed for the highest temperatures to rule out effects from aggregation/protein unfolding. What is the melting temperature for the UBQLN2 UBA domain? Presumably when unfolded it can no longer bind to Ub. Ubiquitin has a high melting temperature and an alternative possibility is that reduced turbidity at the high ubiquitin concentrations and higher temperature measurements is caused by the presence of the soluble protein (Ub chains) in the solution.

The plots in Fig. 1C are somewhat deceptive as there is no data between 0.8 and 2 Ub:UBQLN2 ratio (unless it's not shown?), but the plot gives the impression that there is. If the data is available but not shown, it should be included in Fig. 1B.

The value calculated of 16 micromolar for UBQLN2 binding to K63 Ub4, a value indicating 5-fold weaker affinity than calculated for monoUb, seems inconsistent with the model of binding to four independent and available binding sites in the extended chain (p. 10). What would the mechanism of inhibited binding relative to monoUb be given that monoUb modules exist within this chain type? This concern also applies to the Kd values provided on p. 13 for the other Ub chain types. Providing that raw data for these plots would be helpful. Are chemical shift perturbations a true measure of binding in these cases? Were the amino acids used in the binding site or peripheral? Were broadening effects observed that complicate the quantification?

Another concern is the possibility that the loss of condensates with the Ub chains at higher molar ratios is caused by some type of aggregation process.

For the comparative analyses such as in Fig 5A measuring the Ub chain concentrations accurately would be essential. How are these concentrations being measured? Were equivalent amounts loaded for Fig. S1? Why are there upper molecular weight bands for the K48 chains?

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

Some of the interpretations seem inconsistent with the data, as mentioned above, and warrant greater explanation.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Greater experimental support for the indicated binding constants is needed. In addition, some type of experimental evidence is needed to evaluate whether the loss of condensates with high levels of ubiquitin chains is due to aggregation/unfolding mechanism.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

Yes, if the authors still have the samples then these experiments should be straightforward and not too time consuming.

- Are the data and the methods presented in such a way that they can be reproduced?

The data and methods are presented clearly and could be reproduced.
2. Significance:

Significance (Required)

The advance resolves controversies in the ubiquitin field regarding the role of ubiquitin chains in promoting LLPS. If the concerns noted above can be addressed then this work is of high impact.

My background is in the field of ubiquitin signaling and biophysics.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

**Summary:**

This is a very interesting paper showing that different polyubiquitin chains can resolve or induce the phase separation state of UBQLN2. The same group previously showed that high concentrations of UBQLN2 spontaneously phase separate by its own multivalent interactions, and that the UBQLN2 droplets are resolved by the addition of ubiquitin monomer or K48-linked polyubiquitin (polyUb) chains. In this study, the authors unexpectedly found that M1-, K11-, K63-linked polyUb chains and multiply monoubiquitinated substrates can induce UBQLN2 phase separation. By subsequent NMR titration and SAX analyses, the authors determined a new region of UBQLN2 which may discriminate different polyUb chains and showed that
The affinity between UBQLN2 and different polyUb chains do not correlate with their phase separation efficiency. The authors concluded that the higher-order structure of polyUb chains, or more precisely, the flexibility of polyUb chains and the availability of ubiquitin interface, is important for the co-phase separation. The experiments were performed only in vitro, but the phase separation assays, NMR, SAX analyses were well controlled and the presented data are solid. Collectively, as ubiquitin-dependent phase separation is currently attracting attention and the functional units of polyUb chains are not well defined, this paper is very timely and would provide new insights into the ubiquitin code.

**Major comments:**

1. In the enzymatic assembly of K63-linked polyUb chains, UBQLN2 and ubiquitinated MMS2 undergo phase separation (Figure 6). Control experiments are needed to determine if phase separation does not occur with K48-polyUb chain system, using E2-25K or appropriate enzymes. Since the intracellular concentration of UBQLN2 is 1-2 uM and there are interacting molecules such as molecular chaperones and membrane protein substrates, the homotypic phase separation of UBQLN2 as shown in Figure 1 may not occur in the cell. In other words, this homotypic phase separation ability may be mainly for the co-phase separation with polyUb chains, and locally growing K48-polyUb chains may sufficiently induce the co-phase separation. This control experiment is necessary because it is an important paper that will influence future studies.

2. FRAP experiments are required for Figure 5. The authors nicely showed that different ubiquitin chains and multiply monoubiquitinated protein form UBQLN2 droplets of different sizes. However, the authors only evaluated the final products and did not analyze droplet dynamics, such as fusion and fluidity. Although the final conclusion may not change, it will be interesting to analyze whether the addition of ubiquitin chains increases the fluidity of UBQLN2 and whether the fluidity is maintained in larger droplet sizes.

**Minor comments:**

1. The word "sticker" is not familiar to most readers and needs to be briefly explained.
2. A brief explanation is needed why the authors used urea for the UBQLN2 purification. UBQLN2 is a soluble multidomain protein, which is known to adopt a closed conformation through UBL-UBA interaction. Did the authors confirm that the structure was renatured after desalting?
3. Another technical concern is that fluorescent labeling was done by dye-NHS-ester, which would modify important Lys residues of the ubiquitin (especially K6) and the UBQLN2 UBA domain. At least, the authors should confirm the UBQLN2-polyUb chains interactions before and after the fluorescent labeling.

2. **Significance:**

**Significance (Required)**

Ubiquitin-dependent phase separation is thought to be involved in the formation of ubiquitin-positive inclusion bodies observed in most neurodegenerative diseases, and is likely to attract significant attention in the near future. In addition, the functional units of ubiquitin modification, such as linkage-type, chain length, and their combinations, are still unclear, and the decoding mechanism by most ubiquitin-binding proteins also remains unknown. Therefore, the findings that different types of polyubiquitin regulate the phase separation state of UBQLN2 is a great conceptual advance.

Audience: Since UBQLN2 mutations are causative of ALS and phase separation studies are very active, this
paper will be of interest to a wide audience.

My expertise: Biochemistry.

3. **How much time do you estimate the authors will need to complete the suggested revisions:**

   **Estimated time to Complete Revisions (Required)**

   **(Decision Recommendation)**

   Between 1 and 3 months
1. General Statements

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

We thank the reviewers for their excitement towards our findings and their comments/suggestions that significantly improved our manuscript. Please see below for individual responses to the reviewers’ comments. Here, we quickly summarized the major changes to the manuscript:

1. We refit our NMR titration data for $K_d$ values using TITAN 2D NMR lineshape analysis to account for not only changes in chemical shift perturbations (CSPs) but also peak broadening. Peak broadening, resulting in intermediate exchange, was due to the substantial size increase in the bound UBQLN2:polyUb complexes especially involving K11-Ub4 and K48-Ub4.

2. We incorporated new data from fluorescence anisotropy measurements as a complementary technique to obtain $K_d$ values for UBQLN2 and polyUb chains. The $K_d$ values from NMR and fluorescence anisotropy data agree reasonably well to show that there are no significant differences in $K_d$ values among the different chains that would account for the significant differences in how the chains affect UBQLN2 phase separation. Fluorescence anisotropy also confirmed the differences in rotational tumbling of the different polyUb chain types.

3. We performed new microscopy and NMR experiments at higher temperature to show UBQLN2 and Ub still bind and do not aggregate at higher temperatures.

4. We performed fluorescence recovery after photobleaching (FRAP) experiments to determine how the polyUb chains affect UBQLN2 mobility inside the droplets.

5. We carried out in vitro K48-linked polyUb chain reaction in the presence of UBQLN2 to show that UBQLN2 did not condense, in contrast with results from our in vitro K63-linked polyUb chain reaction.

6. We revised our language to be more precise and to explain more technical terms for a generalized audience.
2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

Response to Reviewer #1 (Evidence, reproducibility and clarity (Required)):
Dao et al. use microscopy, NMR, and other biophysical methods to study how ubiquitin chains of varying length and linkage impact UBQLN2 liquid-liquid phase separation (LLPS). The mechanistic contribution of UBQLN2 and other UBL-UBA proteins as a ubiquitin shuttle factor has remained somewhat elusive but recent studies, including by the Castaneda lab, have indicated their importance in driving LLPS. A controversy in the field has been whether ubiquitin chains promote or inhibit shuttle factor mediated LLPS. This manuscript is focused around addressing the nuances of ubiquitin chain modulation of LLPS. The authors demonstrate that at high ubiquitin chain concentrations, LLPS is inhibited, with this inhibition occurring at lower concentrations for chains of compact structure (K48 and K11 linked) compared to those with extended structures (K63, M1, and HOTag6-G10-Ub). One concern is that the authors argue this difference to be substantial, but the data seem to show more subtle differences. The authors find that at low concentration, ubiquitin chains can promote LLPS at certain temperatures, and show data suggestive that this effect is stronger for chains of extended structures although again the differences seem more subtle than perhaps concluded by the authors. The authors further demonstrate that UBQLN2 condensates are formed during in vitro enzymatic assembly of K63-linked chains and that Ubc13, which appears to be ubiquitinated, is recruited to the droplets. The study is well-done overall, invoking multiple methodologies, including the innovative invocation of HOTag6-G10-Ub, clearly presented, and impactful, with relevance to the broad field of ubiquitin signaling as well as the newer field of LLPS. Concerns over how the authors interpret their data however exist for this reviewer. It's possible that these concerns can be addressed with changes to the text and/or greater explanation and without further experiments.

>>> We thank the reviewer for their overall positive feedback on this manuscript. We understand the reviewer's concerns as we were ambiguous in parts. Please see the responses below.

The authors present the data and methods clearly and use multiple approaches. One aspect that is not convincing to this reviewer concerns the conclusion that compact chains destabilize UBQLN2 phase separation whereas extended chains promote it, as written in the second highlight and also throughout the text. In particular, all chains seem to inhibit UBQLN2 condensates at high enough concentration (which the authors do mention at some points in the text)

>>> The reviewer correctly pointed out that the compact Ub4 chains do not completely destabilize LLPS at all concentrations. We have revised the text and the second highlight to reflect that compact Ub4 chains do not significantly enhance LLPS at lower concentrations.
However, we believe that our data showed significant differences in how the different chain types affect UBQLN2 LLPS. Specifically, a mixture of UBQLN2 and extended polyUb chains (K63-polyUb, M1-polyUb, and a multi-monoubiquitinated mimic, HOTag6-Ub) permit UBQLN2 to phase separate over wide concentration ranges far more than that of compact polyUb chains (K48-polyUb and K11-polyUb). This tracks with the overall shape and conformations of the polyUb chains. As shown in the first panel of Figure 1C, 12.5 µM UBQLN2 does not phase separate on its own until at least a temperature of 50 °C is reached. Strikingly, K63-Ub4 (but not K48-Ub4) lowered the onset temperature of UBQLN2 LLPS to physiological conditions (~37 °C) and phase separation occurred over a wide range of K63-Ub4 concentrations. This is an illustration of how K63-Ub4 promotes UBQLN2 LLPS, as the concentration and temperature threshold for UBQLN2 phase separation is significantly reduced when K63-Ub4 is added, but not when K48-Ub4 is added. At other UBQLN2 concentrations, the extent of the temperature/concentration phase diagram that contains phase-separated K63-Ub4 and UBQLN2 is far larger than that of K48-Ub4 and UBQLN2.

We modified our explanation of the behavior that all polyUb chains inhibit phase separation at high enough concentration in the first paragraph on pg. 7. For phase-separating systems that are driven by heterotypic interactions (e.g. two protein components and protein/RNA condensates), excess of one protein component over the other will eliminate phase separation of the system and has been demonstrated experimentally (Choi et al., 2019; Dignon et al., 2020; Xu et al., 2020). For the Ub4-UBQLN2 system described here, we expect that an excess of Ub4 would eventually inhibit UBQLN2 phase separation if heterotypic interactions are at play. Ub4 can act as a scaffold to bring the UBQLN2 molecules together (which already have a propensity to phase separate due to homotypic interactions) with each Ub binding to a UBQLN2 molecule (if it is sterically possible for 4 UBQLN2 to bind to a Ub4 at once). The ‘inhibition’ of Ub4-UBQLN2 condensates at high Ub4 concentration occurs when there are too many Ub4 molecules such that not all Ub units bind to UBQLN2 molecules at once. At high enough Ub4 concentration, Ub4 might act to pull the UBQLN2 apart instead of bringing them together.

We have refined our language in the manuscript to emphasize the difference in concentration ranges for UBQLN2 phase separation in the presence of the different polyUb chains. Consistent with this, we now have included an additional ubiquitination experiment where we monitored whether UBQLN2 formed condensates in the presence of K48-polyUb chain formation (to complement the in vitro experiment where we monitored K63-polyUb chain formation). We did not observe UBQLN2 condensate formation under similar conditions as for K63-polyUb chain formation (Figure S9).

K48 chains seem to promote it at certain temperatures and chain lengths. For example in Fig. 1B, at 36{degree sign}C and 40{degree sign}C, K48-Ub3 appears to promote condensates at low Ub:UBQLN2 molar ratios.

>>> We apologize for oversimplifying the trends. We did discuss the points mentioned by the reviewer in the discussion. We have changed the text in the results to make these points clearer. We also now include raw turbidity assay data as new Fig. S1B (and copied into Reviewer Fig. 1 below) to show that even though K48-Ub3 does slightly enhance UBQLN2
LLPS at 0.04:1 and 0.08:1 ratios, the level of enhancement is much less than what is observed for K63-Ub3.

Reviewer Fig. 1. Raw spectrophotometric turbidity traces for different ratios of K48-Ub3 or K63-Ub3 and UBQLN2. UBQLN2 concentration was 30 µM.

In addition in Fig. 1A the amount of total condensate in the field of view for the K63-Ub3 appears lower than that of K48-Ub3 at 0.1:1 Ub:UBQLN2 ratio. This data seems to contradict the statement on p. 7: "Conversely, like Ub and K48-Ub2, K48-Ub3 also inhibited LLPS at all concentrations."

>>> We intended the microscopy images to show the overall trend that UBQLN2 is still forming droplets and not aggregating in the presence of Ub chains. To obtain the images shown in Figures 1 and 5, we examined several locations on the coverslip, took a few images for each sample, and randomly selected one to include in the figure. We wanted to be fairly objective and not nitpick images to show as much differences as possible. For example, we acquired two and three images for K48-Ub3 and K63-Ub3, respectively, at 0.1:1 ratio with UBQLN2 (full images shown below). Overall, they looked quite similar, with K63-Ub3 appearing to have more droplet coverage, consistent with the turbidity assays (Reviewer Fig. 1). To address the reviewer concern, we changed the image for K63-Ub3 in the new Figure 1A. We have also changed the language in the text to more accurately reflect the data.
An aspect that could be discussed further is why the condensates change in morphology with Ub chain addition.

Morphologically, the main difference is that the droplets appear larger with K63-Ub4 than with K48-Ub4 chains since there are more droplets that fuse to make bigger and settle to the coverslip surface. We have discussed this point more on pg. 5. Additionally, we collected FRAP experiments, as requested by Reviewer 2 and addressed below.

To address these concerns, the data in Fig. 1A should be quantified as amount of total condensate to field of view. This same concern applies to Fig. 5A.

We acquired the microscopy images mainly to show general trends and to confirm that we indeed were observing heterotypic phase separation of UBQLN2 and polyUb. There are a couple of caveats with quantifying our microscopy data. First, we imaged droplets on the coverslip and not in solution due to focusing issues in solution (droplets moving frequently in and out of frame at a single z position). For example, the droplets do not settle onto the coverslip completely evenly. Some areas of the coverslip might have more coverage than others. We tried our best to move around the coverslip to make sure what we imaged was a good representation of what was in the sample to be as objective as possible. Moreover, since we imaged at the coverslip, we could see droplets that settled onto an empty spot, or settled and then fused, or fused right on top of another droplet. However, the total area of two individual droplets on the coverslip is not the same as a fused droplet (Reviewer Fig. 3). For these reasons, we complemented our microscopy data with more quantitative phase diagrams of UBQLN2 phase separation with polyUb chains obtained from turbidity assay data using multiple replicates.
The data in Fig 1A don't seem to correspond well to that of Fig. 1B for K48-Ub4. Condensates are observed at 30°C in Fig. 1A up until 1.2:1 Ub:UBQLN2 ratio. If the difference is caused by Fig 1A being at 50 micromolar UBQLN2 compared to 30 micromolar in Fig 1B then microscopy data should be provided to validate and complement Fig 1B.

>>> We performed most microscopy experiments at 30 °C since this temperature is closer to physiological temperature, but we needed 50 µM UBQLN2 to see droplets at 30 °C. The increase in protein concentration necessary for microscopy is expected given the phase diagrams in Figure 1C, where the temperature onset of UBQLN2 LLPS decreases as protein concentration increases. For the turbidity assays, we could not use 50 µM UBQLN2 because many of the samples would phase separate at the lowest temperature, preventing accurate determination of the cloud point temperatures for our phase diagrams. However, our data in Figure 1 indicate that the range of Ub:UBQLN2 ratios at which phase separation is observed increases with UBQLN2 concentration. Please compare the different panels in Figure 1C. To address reviewer concerns, we imaged different ratios of K48-Ub4 and UBQLN2 at 30 µM at 60 °C (Reviewer Fig. 4). As shown, there is very little phase separation as we reached 1.2:1 ratio, consistent with the 30 µM panel in Figure 1C.

This data is also needed for the highest temperatures to rule out effects from aggregation/protein unfolding. What is the melting temperature for the UBQLN2 UBA domain? Presumably when unfolded it can no longer bind to Ub. Ubiquitin has a high melting temperature.
and an alternative possibility is that reduced turbidity at the high ubiquitin concentrations and higher temperature measurements is caused by the presence of the soluble protein (Ub chains) in the solution.

We imaged a few Ub:UBQLN2 ratios of K63-Ub4 with UBQLN2 at 30 µM and 60 ºC and observed no aggregation (Reviewer Fig. 5). Moreover, we always checked our cuvettes at the end of each turbidity assays for signs of aggregation; the solutions for these assays were always clear. When there is visible aggregation, we observe spikes in our turbidity traces, as in Reviewer Fig. 6 below, instead of the smooth traces as seen in Reviewer Fig. 1. None of our polyUb assays contained traces like those in Reviewer Fig. 6.

Reviewer Fig. 5. Mixtures of 30 µM UBQLN2 (full-length) with varying Ub:UBQLN2 concentrations (top) at 60 ºC.

Reviewer Fig. 6. Representative turbidity curve for a UBQLN2 mutant that, upon induction of phase separation, forms aggregates visible by eye and under a microscope.

In our previous work (Dao, Martyniak et al., 2019 Structure), we confirmed that UBQLN2 450-624 remains folded over the temperature range 25-55 ºC in the absence or presence of 200 mM NaCl. (We could not go to higher temperatures due to limitations on the cryoprobe on our spectrometer). We showed that the amide resonances of the UBA domain remain well-dispersed at 55 ºC and that the overall HSQC fingerprint is very similar across the 25-55 ºC temperature range. We concluded that the UBA domain is still folded at 55 ºC. In response to this reviewer, we repeated those experiments here using the UBQLN2 450-624 construct at 50
µM with and without 150 µM ubiquitin (i.e. 3:1 Ub:UBQLN2 ratio) at three temperatures (25, 40 and 55 ºC). We chose these concentrations as these are the conditions at which we observed saturation in the amide resonance titration curves. Our NMR data show that UBQLN2 and ubiquitin still interact and form a complex at 55 ºC. We have included these data as Fig. S1C.

The plots in Fig. 1C are somewhat deceptive as there is no data between 0.8 and 2 Ub:UBQLN2 ratio (unless it's not shown?), but the plot gives the impression that there is. If the data is available but not shown, it should be included in Fig. 1B.

>>> We apologize for this confusion. We do have the raw data for those experiments. Since we ran these turbidity assays to build our phase diagrams, we occasionally used different ratios between K48 and K63 to build as complete a phase diagram as possible. For example, 1:1 ratio of K48-Ub4:UBQLN2 was the highest that we could go to obtain baselines for our turbidity data so that we could reliably fit for the cloud point temperatures. However, we didn't need 1:1 ratio for K63-Ub4 and therefore did not run it. So when we made Figure 1B, we chose the common ratios for all the different chains.

The value calculated of 16 micromolar for UBQLN2 binding to K63 Ub4, a value indicating 5-fold weaker affinity than calculated for monoUb, seems inconsistent with the model of binding to four independent and available binding sites in the extended chain (p. 10). What would the mechanism of inhibited binding relative to monoUb be given that monoUb modules exist within this chain type? This concern also applies to the Kd values provided on p. 13 for the other Ub chain types. Providing that raw data for these plots would be helpful. Are chemical shift perturbations a true measure of binding in these cases? Were the amino acids used in the binding site or peripheral? Were broadening effects observed that complicate the quantification?

>>> We thank the reviewer for astutely pointing out the potential problems with our fitting of the binding constants. In the previous version of our manuscript, our NMR-derived binding constants were determined using the traditional weighted CSP equation that assumes fast exchange between the unbound and bound states:

\[
\delta_{\text{obs}} = \delta_{\text{free}}(1 - f_{\text{bound}}) + \delta_{\text{bound}}f_{\text{bound}}
\]

This assumption breaks down when significant peak broadening is observed during the course of the titration, as that is an indication of intermediate exchange. We did observe significant peak broadening whenever we added Ub4 to our UBQLN2 samples, as is evident by the significant reduction in peak intensity of all UBA resonances upon binding Ub4 (Figure 2B and 4D). When peak broadening is observed across the whole UBA domain, this is indication of a significant slow-down in rotational tumbling rate due to increased size of the bound complex (Kovrigin 2012, Williamson et al. 2013). To correct for these observations, we took the following steps:

1) We re-analyzed all of our NMR titration data using the 2D NMR lineshape analysis program TITAN (Waudby et al. 2016) that accounts for both chemical shift perturbation and peak broadening to obtain binding affinities between UBQLN2 and the chains. For each titration, we selected resonance trajectories for >10 residues near the Ub-UBA interface and performed a global fit analysis (new Supplementary Figure S6). Using a single site binding model where \( K_d \) and the kinetic off-rate, \( k_{\text{off}} \), were global fitting parameters (Figure 4H, Table
S1), we determined that the $K_d$ values were much closer across the four different polyUb chains used in this study (K11-Ub4, K63-Ub4, M1-Ub4, and HOTag6-Ub) than when only CSPs were used, and were similar to the $K_d$ for monoUb binding to UBQLN2 450-624 (~3 µM). The $K_d$ determined for K48-Ub4 was weaker (~25 µM) than for the other Ub4s. However, together with fluorescence anisotropy data (shown below), we reasoned that the high $K_d$ resulted from K48-Ub4 having to open before binding could happen and leading to an artificially high apparent $K_d$. Indeed, we provide evidence that K48-Ub4 needs to “open” in order to make the Ub interface accessible for binding to UBQLN2 (Figure 3). All of these results are now shown in Figure 4H and summarized in Table S1. We observed differences in the fitted $k_{off}$ rates, particularly between the compact K11 and K48-Ub4 chains vs. the extended K63, M1, or HOTag6-Ub chains. We surmise that the differences in $k_{off}$ rates are reflecting differences in the overall size of the UBQLN2-bound species (see complementary fluorescence anisotropy data in point 2 below). Specifically, the complex of UBQLN2 bound to individual Ub units in compact K11-Ub4 or K48-Ub4 tumbles more slowly compared to UBQLN2 bound to the more freely tumbling Ub units of K63-Ub4, M1-Ub4, or HOTag6-Ub, where the Ub units adopt beads-on-a-string conformations. Indeed, a comparison of $^1$H 1D spectra of compact K48-Ub4 vs. extended K63-Ub4 collected at identical concentrations and NMR experimental parameters (Reviewer Fig. 7) show that proton peaks are broadened for many resonances in K48-Ub4 compared to those resonances in K63-Ub4.

2) To complement the NMR titration experiments, we collected new data using fluorescence anisotropy to determine the binding constants between UBQLN2 and Ub4 chains. First, we labeled UBQLN2 with Alexa Fluor 488 by engineering a Cys mutation at position 624 (on the C-terminal end of the UBA domain that is not directly involved in Ub-binding), and titrated in monoUb and all five polyUb chains. The $K_d$ values determined with monoUb, K63-Ub4, M1-Ub4, and HOTag6-Ub were comparable to those determined using NMR (Fig. 4H and Table S1). However, FA-derived $K_d$ values for K11-Ub4 and K48-Ub4 were significantly stronger (~0.4 µM) than those obtained by NMR analysis. Additionally, we observed significant increase in anisotropy when either K11-Ub4 or K48-Ub4 chains were titrated into UBQLN2. We hypothesized that the large increase in anisotropy was due to the large difference in rotational tumbling properties of the compact K11 or K48-Ub4 chains vs. the beads-on-a-string conformation of the extended chains. In other words, when UBQLN2 binds to the Ub units in the compact chains, the rotational tumbling of UBQLN2 is significantly slowed, whereas when UBQLN2 binds to the Ub units of the extended polyUb chains (e.g. K63-Ub4, M1-Ub4), the rotational tumbling of UBQLN2 is not as slowed given the increased flexibility of the Ub units in the extended chains (SAXS data from Figure 4B). To address the possibility that the large increase in size of the binding complex is affecting the FA-derived $K_d$ value, we labeled a single Ub unit in K48-Ub4 and K63-Ub4 and titrated in UBQLN2. For a single Ub unit in K48-Ub4, we observed a significant decrease in anisotropy upon binding UBQLN2 likely due to breaking the Ub-Ub interface in K48-Ub4 to accommodate binding to UBQLN2 (Figure 2H). The $K_d$ obtained from this experiment was ~3 µM, which matches the $K_d$ between monoUb and UBQLN2. Interestingly, when the similar experiment was performed using labeled K63-Ub4, the change in anisotropy was minimal. We surmise that UBQLN2 binding to the Ub unit in K63-Ub4 did not significantly change the overall tumbling properties of K63-Ub4, as it already adopted an extended beads-on-a-string like conformation.

In summary, these additional experiments have added support that the binding between UBQLN2 and the Ub units in these chain types is likely similar, and that the differences in
binding are due to the differences in overall shape and conformation of the polyUb chains themselves.

Another concern is the possibility that the loss of condensates with the Ub chains at higher molar ratios is caused by some type of aggregation process.

>>> As indicated in our responses above, we do not observe visible aggregation in our microscopy experiments at the higher Ub:UBQLN2 ratios. Furthermore, we showed that the UBQLN2:Ub complex remains folded and bound over the temperature range of 25-55 °C. There is no indication that either component is unfolding. For the turbidity experiments, we always look at the cuvettes post-assay to ensure that there is no visible aggregation.

For the comparative analyses such as in Fig 5A measuring the Ub chain concentrations accurately would be essential. How are these concentrations being measured? Were equivalent amounts loaded for Fig. S1? Why are there upper molecular weight bands for the K48 chains?

>>> For all Ub4 chains (K11-Ub4, K48-Ub4, K63-Ub4, and M1-Ub4), concentrations are measured identically. As noted in the Methods section, we use standard spectrophotometric determination (A_{280}) measurements of protein concentration using a molar extinction coefficient of 5960 for Ub4 (1490 * 4 Tyr). As part of our routine quality check of samples, we check the overall protein concentration using NMR spectroscopy as well. Shown below is a ¹H 1D overlay of K48-Ub4 and K63-Ub4 at identical concentrations (50 µM). Differences in peak positions are expected given more inter-Ub interactions within K48-Ub4 than exists within K63-Ub4. Spectra were collected with identical acquisition settings (number of scans, receiver gain, acquisition time, etc.). Additionally, SAXS experiments were also collected at identical concentrations for K48-Ub4, K63-Ub4, and M1-Ub4, as indicated in Table S2 and Figure S5. Size exclusion chromatogram traces (in Figure S5) show similar peak area and peak width for these three proteins.

Our original Figure S1 was intended to show that the constructs used in this study are clean and therefore did not calculate the exact amounts loaded on the gel. We have made a new Figure S1 to show that now. K48 chains of different lengths are a little harder to separate using SEC and cation exchange column. There is always a small amount of contamination as observed by the very faint band above the main Ub4 species.
Reviewer Fig. 7. ¹H 1D overlay of K48-Ub4 (blue) and K63-Ub4 (orange) at identical concentrations (50 µM).

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

Some of the interpretations seem inconsistent with the data, as mentioned above, and warrant greater explanation.

>>> We hope with the new data supplied that we have addressed many of the reviewer’s concerns on this front. Additionally, we have revised our text as noted above to be more precise in our language describing how the chains affect UBQLN2 phase separation properties.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Greater experimental support for the indicated binding constants is needed. In addition, some type of experimental evidence is needed to evaluate whether the loss of condensates with high levels of ubiquitin chains is due to aggregation/unfolding mechanism.

>>> As mentioned in our responses above, we have used fluorescence anisotropy as a complementary technique to verify binding affinity between UBQLN2 and polyUb chains. In addition, we have re-analyzed our NMR titration data to account for peak broadening using TITAN. We now show the affinity between UBQLN2 and polyUb chains are relatively
unchanged, as we originally concluded. In this revision, we also have provided evidence for no aggregation or unfolding of the protein components.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.
  
  Yes, if the authors still have the samples then these experiments should be straightforward and not too time consuming.

- Are the data and the methods presented in such a way that they can be reproduced?
  
  The data and methods are presented clearly and could be reproduced.

- Are the experiments adequately replicated and statistical analysis adequate?
  
  Yes.

Reviewer #1 (Significance (Required)):

The advance resolves controversies in the ubiquitin field regarding the role of ubiquitin chains in promoting LLPS. If the concerns noted above can be addressed then this work is of high impact.

My background is in the field of ubiquitin signaling and biophysics.

Response to Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary: This is a very interesting paper showing that different polyubiquitin chains can resolve or induce the phase separation state of UBQLN2. The same group previously showed that high concentrations of UBQLN2 spontaneously phase separate by its own multivalent interactions, and that the UBQLN2 droplets are resolved by the addition of ubiquitin monomer or K48-linked polyubiquitin (polyUb) chains. In this study, the authors unexpectedly found that M1-, K11-, K63-linked polyUb chains and multiply monoubiquitinated substrates can induce UBQLN2 phase separation. By subsequent NMR titration and SAX analyses, the authors determined a new region of UBQLN2 which may discriminate different polyUb chains and showed that affinity between UBQLN2 and different polyUb chains do not correlate with their phase separation efficiency. The authors concluded that the higher-order structure of polyUb chains, or more precisely, the flexibility of polyUb chains and the availability of ubiquitin interface, is important for the co-phase separation. The experiments were performed only in vitro, but the phase separation assays, NMR, SAX analyses were well controlled and the presented data are solid. Collectively, as ubiquitin-dependent phase separation is currently attracting attention and the functional units of polyUb chains are not well defined, this paper is very timely and would provide new insights into the ubiquitin code.

Major comments:
1. In the enzymatic assembly of K63-linked polyUb chains, UBQLN2 and ubiquitinated MMS2
undergo phase separation (Figure 6). Control experiments are needed to determine if phase separation does not occur with K48-polyUb chain system, using E2-25K or appropriate enzymes. Since the intracellular concentration of UBQLN2 is 1-2 uM and there are interacting molecules such as molecular chaperones and membrane protein substrates, the homotypic phase separation of UBQLN2 as shown in Figure 1 may not occur in the cell. In other words, this homotypic phase separation ability may be mainly for the co-phase separation with polyUb chains, and locally growing K48-polyUb chains may sufficiently induce the co-phase separation. This control experiment is necessary because it is an important paper that will influence future studies.

>>> We completely agree with the reviewer that the cellular environment is more complex and that the behavior of UBQLN2 is likely regulated by multiple factors, rather than just polyUb chains. In fact, one of our current projects is examining UBQLN2 behavior in the presence of other protein quality control components including the proteasome and chaperones. The control experiment suggested by the reviewer is tricky because E2-25K is not as efficient at making K48 chains as Mms2 and Ubc13 are at making K63 chains. To achieve a comparable chain-making rate (by looking mainly at the rate of Ub disappearance), we used more E2-25K and monitored the reaction for longer time. Over a 2-hour period, we observed clear formation of K48-linked chains up to Ub4-5, as well as ubiquitinated GST-E2-25K, as demonstrated by SDS-PAGE gels. However, we did not observe phase separation in enzymatically-active solutions that contained UBQLN2 and that were actively producing K48-linked polyUb chains. We now include these data in new Fig. S9 and briefly discuss these results on pg. 21.

2. FRAP experiments are required for Figure 5. The authors nicely showed that different ubiquitin chains and multiply monoubiquitinated protein form UBQLN2 droplets of different sizes. However, the authors only evaluated the final products and did not analyze droplet dynamics, such as fusion and fluidity. Although the final conclusion may not change, it will be interesting to analyze whether the addition of ubiquitin chains increases the fluidity of UBQLN2 and whether the fluidity is maintained in larger droplet sizes.

>>> The FRAP results, shown in new Supplementary Figure S8, are consistent with our dilute phase concentration data. We performed FRAP experiments for solutions containing UBQLN2 and K48-Ub4, K63-Ub4, M1-Ub4 or HOTag6-Ub4 at 0.5:1, 1:1 and 2:1 Ub:UBQLN2 ratios. We compared these experiments with FRAP experiments of phase-separating solutions that contained only UBQLN2. We found that K48-Ub4 did not affect the fluorescence recovery rate of UBQLN2 whereas the other three chains decreased the recovery rate of UBQLN2. These data are consistent with our findings that K48-Ub4 is barely in UBQLN2 droplets and therefore should not affect the behavior of UBQLN2 in the droplets. On the other hand, K63-Ub4, M1-Ub4 and HOTag6-Ub are enriched in UBQLN2 droplets and likely form cross-linking interaction networks with UBQLN2 that slow down UBQLN2 movement. One experimental caveat is that we used different concentrations of UBQLN2 for these measurements, depending on the chains, so that we would have similar degree of phase separation, i.e. similar droplet sizes. For example, for UBQLN2 by itself, we used 60 µM UBQLN2. For UBQLN2 with K48-Ub4 at 1:1 ratio, we used 75 µM of each protein. For UBQLN2 with HOTag6-Ub at 1:1 ratio, we used 30 µM of each protein. If we had used the same concentration of UBQLN2 throughout these experiments, we would have very few and small droplets for UBQLN2 with K48-Ub4 at 1:1 ratio.
but the whole coverslip surface would be coated with UBQLN2 with HOTag6-Ub at 1:1 ratio, preventing the execution of the experiments. We purposefully chose similar sized droplets (~ 10 µm) and same bleach area to be consistent across samples. The reviewer had wondered about the effect of droplet sizes on the fluidity of the UBQLN2. We tried FRAP experiments on different droplet sizes with solutions containing only UBQLN2 and found no noticeable differences in fluorescence recovery rates using droplet sizes between 5 and 10 µm, as long as the bleach areas were kept relatively similar.

One thing to note is that we are looking at droplets on the coverslip and not in solution. As time goes on, the droplets in solution settle and fuse to make bigger droplets. In solution, when phase separation first occurs, the droplet sizes are comparable for the different UBQLN2 mixes with different chains. So, the different sizes shown in Figure 5A are not the intrinsic properties of the different polyUb:UBQLN2 mixes. Rather the larger droplets are a result of more droplets in solution that subsequently settle and fuse into larger droplets. For FRAP experiments, we only examine droplets at the coverslip, as the droplets no longer move when settled (unless there’s a fusion event). This is a prerequisite for the photobleaching experiment so that we can subsequently track fluorescence recovery over time.

Minor comments:
1. The word "sticker" is not familiar to most readers and needs to be briefly explained.
>>> We thank the reviewer for pointing this out. We added an explanation on pg. 7.

2. A brief explanation is needed why the authors used urea for the UBQLN2 purification. UBQLN2 is a soluble multidomain protein, which is known to adopt a closed conformation through UBL-UBA interaction. Did the authors confirm that the structure was renatured after desalting?
>>> We apologize for this oversight. We did not use urea to dissolve the pellets for this work. That error has been corrected in the text. (We did use urea in the past for proteins for another manuscript when we had a few mutations that led to highly viscoelastic pellets and urea made it a lot easier to solubilize the pellets. As controls, we ran NMR experiments and performed turbidity assays on WT UBQLN2 samples from both urea-dissolved and buffer-dissolved pellets and obtained the same results.)

3. Another technical concern is that fluorescent labeling was done by dye-NHS-ester, which would modify important Lys residues of the ubiquitin (especially K6) and the UBQLN2 UBA domain. At least, the authors should confirm the UBQLN2-polyUb chains interactions before and after the fluorescent labeling.

>>> We would like to point out that it is unlikely that labelling Ub with Alexa Fluor 488 (AF488) NHS ester affects the binding between Ub and UBQLN2 UBA since the structure of the Ub/UBQLN1 UBA complex (PDB ID 2JY6) shows that Ub lysines are not found at the binding interface between the two proteins.
Nevertheless, we carried out an NMR experiment where we compared the chemical shifts of the $^{15}$N-labelled UBA domain in the presence and absence of AF488-labelled Ub at roughly 1:1 Ub:UBA stoichiometric ratio. The observed CSPs are similar to what we observe when we titrate Ub into UBA under similar conditions.

![Graph of weighted amide CSPs](image)

**Reviewer Figure 8.** Weighted amide CSPs of the UBQLN2 UBA domain with Ub (black) or AF488-labelled Ub at 1:1 Ub:UBA stoichiometry. The concentration of the UBA domain was kept fixed at 50 µM, and experiments were collected at pH 6.8 in 20 mM NaPhosphate buffer.

**Reviewer #2 (Significance (Required)):**

Significance: Ubiquitin-dependent phase separation is thought to be involved in the formation of ubiquitin-positive inclusion bodies observed in most neurodegenerative diseases, and is likely to attract significant attention in the near future. In addition, the functional units of ubiquitin modification, such as linkage-type, chain length, and their combinations, are still unclear, and the decoding mechanism by most ubiquitin-binding proteins also remains unknown. Therefore, the findings that different types of polyubiquitin regulate the phase separation state of UBQLN2 is a great conceptual advance.

Audience: Since UBQLN2 mutations are causative of ALS and phase separation studies are very active, this paper will be of interest to a wide audience.

My expertise: Biochemistry.
Dear Dr. Castañeda

Thank you for the submission of your research manuscript to our journal. It has been sent back to the same referees that have evaluated it for Review Commons and we have now received their reports (copied below).

As you will see, both referees acknowledge that the revised manuscript has been strengthened and they support publication in EMBO Reports after some textual changes.

We now need you to format the manuscript according to our journal guidelines. Once we have received the revised version, we will perform a number of editorial checks, including a check of figure legends, figures and statistics. (We need .docx and individual figure files for this). To ensure that all criteria are met and the process runs smoothly, please carefully consider all points listed below.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2” etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called “Appendix”, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2” etc. See detailed instructions regarding expanded view here:

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See also <https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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The following points MUST be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
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See also the guidelines for figure legend preparation:
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My concerns by the reviewers have been adequately addressed. The changes made have strengthened the manuscript. I feel that it is now acceptable for publication in EMBO reports.

Referee #2:

The Authors have addressed all of my concerns and have strengthened the manuscript. My only remaining comment is that the Summary should be written for a general audience and in the spirit of this recommendation that the terms 'homotypically' and 'heterotypically' in the context of phase separation should be defined. Otherwise, I am highly supportive of the publication of this manuscript.
Post revision reviewer comments

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>>> We have revised the Abstract to make it less technical.
Dear Carlos,

Thank you for submitting your revised manuscript to EMBO Reports. I have now checked all revisions and the source data you provided, and everything looks good, except for a few minor formatting issues. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once these few remaining minor issues/corrections have been addressed, as follows.

1) We generally recommend arranging the figures in a way that the panels can be called out in an ordered fashion. In this context we note the following and recommend a reordering, if possible (certainly for the Appendix):
   - Fig 4B is called out after 3A. Fig 7A is called out after 5D.
   - Appendix Fig S2A+B are called out after S1A.

2) Please also add callouts to the following figures and panels where appropriate:
   - Fig 7B callout is missing.
   - Appendix Figs S4, S5, S6, S7, S8 + S9 panel callouts are missing.
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3) Appendix: Please remove the movie legend and provide it as a text file that is zipped together with the movie.

4) The figure legends should follow after the Reference section.

5) Abstract: Please describe your findings in present tense

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New_manu_number: EMBOR-2022-55056V3
Corr_author: Castañeda
Title: Mechanistic insights into enhancement or inhibition of phase separation by different polyubiquitin chains
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- If n=5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- A statement of how many times the experiment shown was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - Common tests, such as t-test (please specify whether paired vs. unpaired), simple \( \chi^2 \), Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Exact statistical test results, e.g., \( P \) values > x but not \( P \) values < x;
  - Definition of 'center values' as median or average;
  - Definition of error bars as s.d. or s.e.m.

### Materials

| Newly Created Materials | Information included in the manuscript? | In which section is the information available? |
|-------------------------|----------------------------------------|---------------------------------------------|
| New materials and reagents need to be available; do any restrictions apply? | Not Applicable | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

| Antibodies | Information included in the manuscript? | In which section is the information available? |
|------------|----------------------------------------|---------------------------------------------|
| For antibodies provide the following information: Commercial antibodies: RRID (if possible) or supplier name, catalogue number and clone number. Non-commercial RRID or citation | Not Applicable | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

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| Short novel DNA or RNA including primers, probes: provide the sequence. | Not Applicable | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

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| Primaries used in the experimental system | Information included in the manuscript? | In which section is the information available? |
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| Include a statement about blinding even if no blinding was done. | Yes | Figures |
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| Studies involving human participants: Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | No | (Reagents and Tools, Table, Materials and Methods, Figures) |
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| Could your study fall under dual use research restrictions? Please check biosafety documents and list of select agents and toxins (CDC): https://www.selectagents.gov/index.jsp. | No | (Reagents and Tools, Table, Materials and Methods, Figures) |
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| Have human clinical and genomic datasets deposited in a public-access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | No | Data Availability Section |
| Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | No | Data Availability Section |
| If publicly available data were reused, provide the respective data citations in the reference list. | No | Data Availability Section |