nature portfolio

Peer Review File

The sequence-structure-function relationship of intrinsic ERalpha disorder

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Referee #1

A. Estrogen receptor alpha (ER) is a transcription factor that plays critical roles in many physiological and disease processes. This is especially true for breast cancer, where 70% of patients are characterized by their expression of this nuclear hormone receptor and their disease relies on estrogen receptor transactivation to maintain a high proliferative capacity. Estrogen receptor typically depends on estrogenic hormone binding to spur on these oncogenic transcriptional programs. Because ER activities are critical for progressive breast cancer, it remains a mainstay therapeutic target. However, a wealth of evidence over the past several decades has shown that ER can engage in ligand-independent activities to maintain breast cancer pathology and reduce the efficacy of targeted therapies, especially aromatase inhibitors and tamoxifen. The phosphorylation of serine at position 118 (S118) in ER's N-terminal domain (NTD) by cyclin-dependent kinase 7 (CDK7) and MAPK are well known to engage such activities. While the structure-function activities of ER's ligand binding and DNA binding domains are well characterized, our understanding of these relationships for the NTD are less well understood, especially in the context of S118 phosphorylation.

In this study, the Yang Laboratory uses a comprehensive suite of structural and biological tools to understand how S118 phosphorylation enables hormone-independent ER activities in breast cancer cells. They show compelling evidence that clusters of hydrophobic residues confer transient and compact pseudo-structures within the NTD, which are disrupted upon S118 phosphorylation. This is accomplished through comprehensive mutational analysis to not only mimic the charge of the phospho group at 118 but to also disrupt the hydrophobicity of these two regions. Each of these mutations is also studied in the context of the breast cancer cell to gain a better understanding of how S118 phosphorylation and NTD dynamics impacts the function of full-length ER.

B. This is a highly important study that will be of significant interest to a large and diverse audience of scientists, not just those who study ER but more generally to anyone interested in nuclear hormone receptors. I believe that it presents a new paradigm for our understanding of the role played by the NTD in ER activity that is not only fundamental to our understanding of receptor biology, but also potentially therapeutically relevant as S118 phosphorylation is well known to enable resistance to certain first-line hormone therapies in breast cancer.

C. The approaches used to characterize this interaction are empirical and robust. ER is a difficult protein to work with for biophysical experiments, especially the NTD. The Yang group's use of NMR to study its dynamics then incorporating that into functional studies within the breast cancer cell is the optimal way to study this. The data appear to be of high quality. The figures and paper are well presented in this body of work. One minor suggestion would be to increase the font size where sequences are shown to make it easier to read on a page.

D. Statistics are shown and appear to be the appropriate tests. Description of the star values and statistical test used should be included in the figure legends, though.

E. The conclusions are well supported by their studies.

F. Suggested improvements: The only issue I have with this work is that they used indirect methods to prove the hydrophobic clustering hypothesis. First by increasing the osmotic strength of their buffer to disprove that it was through electrostatic repulsion. Second by comprehensive mutational analysis across several different residues. However, they did not, from what I can tell, manipulate the hydrophobicity of the buffer. If it is possible, it would be ideal to include an experiment with one or two detergents present below their CMC to better directly probe the role of hydrophobicity and its disruption by S118 phosphorylation.

G. References are appropriate.

H. This is a very well written manuscript. It is mentioned in the introduction that no drugs target the ER NTD. However, selective estrogen receptor degraders/downregulators are known to target the NTD by triggering receptor degradation. That is why SERDs will show benefit in the first-line hormone therapy-resistant ER+ setting.

Referee #2

The manuscript “The sequence-structure-function relationship of intrinsic ERalpha disorder” by Zhanwen Du et al. unveils the molecular mechanism for the activation of the estrogen receptor (ER) through the phosphorylation of S118, placed in the N-terminal transactivation domain (NTD). The relevance of this mechanism is exemplified by the current development of kinase inhibitors aiming at reducing the amount of pS118 to target breast cancer. Although the relevance of this phosphorylation was known, the molecular mechanism leading to activation, which could eventually drive to new treatments, was unknown.

By combining NMR (CS, Relaxation and PREs) and SAXS, the authors unambiguously show that in the native state the ER NTD is disordered but contains a complex network of intramolecular interactions, which render the conformational ensemble relatively compact. They show that the nature of these contacts is mainly hydrophobic. They also demonstrate that this network of contacts can be partially perturbed upon phosphorylating S118, mutating it (S118D) or reducing the hydrophobicity through point mutants (F120A and L121A). These changes modify the interaction with the coactivator TIF2 and affect gene transcription and cell proliferation. These findings establish hydrophobic disruption as a critical regulatory mechanism for ER and they hypothesize that the same mechanism could be general in nuclear receptors, which contain dispersed hydrophobic residues in their transactivation domain.

The biological question addressed in the manuscript is relevant, the techniques applied are well suited, and the conclusions are robust and based on the experimental observations. The manuscript is easy to read. There is still, in my opinion, several improvements that would reinforce the study.

- Although the mechanism of hydrophobic perturbations is well documented by the authors, it is unclear how much the network of intramolecular contacts is affected by the phosphorylation of S118 or its mutation (S118D). For instance, PRE profiles are perturbed (Figs. 2f, S3e, and S9), but the intensity ratio does not come back to 1.0. Moreover, the relaxation profile still displays the two clusters upon phosphorylating S118. These observations suggest that part of these contacts is still present even in the ‘active’ state, so a simple on/off mechanism does not apply here. The authors should discuss about this point, probably in relation with the next point.

- (Related) The compactness of the ER NTD and the expansion upon mutating (S118D) is not well characterized. The authors should do a proper modelling of the ensembles using SAXS data. I would suggest the use of EOM for the interpretation of all SAXS datasets (wt, pS118, S118D…) in order to have a clear picture of the compactness of the ensemble corresponding to the different states with respect to the random coil model (possibly derived from Flexible-Meccano). Note that the synthetic P(r) displayed in Fig. 1b does not provide the same information.

- The selection of the reduced hydrophobicity mutants (S118A/F120A and S118A/L121A) seems a bit arbitrary. Would other mutants reducing the hydrophobicity have the same biophysical and biological properties as the two selected by the authors? This would reinforce the concept of hydrophobic cluster.

- When looking at Fig. 2e, it seems that the maximum of the R2/R1 profile corresponds to the alanine-rich segment, which the authors identify as partially helical. This observation suggests that this fragment could have a relevant role in the network of hydrophobic interactions. Would the compactness of the protein be affected if the structure and/or sequence of this long and hydrophobic a-helix is perturbed?

- Several experimental and computational studies have connected chain compactness induced by the presence of intramolecular interactions to the capacity of the protein to form condensates in vitro and in vivo. Moreover, the presence of hydrophobic residues and alanine-rich sequences often induce phase separation, especially in transcription factors. It could be even tempting to hypothesize that the regulation mechanism described by the authors could be explained through a perturbation of the capacity of the protein to phase separate. The authors should comment on this possibility.

- The authors hypothesize about the generality among the nuclear receptor family (and other IDPs) of the mechanism unveiled for ER-NTD due to the patterning of hydrophobic residues in their sequence. This is a very attractive hypothesis that the authors could substantiate by analyzing few of the sequences displayed in Fig. 6b and Fig. S17 by looking at their compactness by SAXS or by performing coarse-grained simulations. This information would, in my opinion, increase the impact and relevance of the study.

Minor point:

- Error bars should be displayed for the relaxation and PRE data along the manuscript.

Referee #3

General comments:

Du et al. present a beautiful illustration of the power of NMR and SAXS to answer challenging questions about structural features of intrinsically disordered proteins (IDPs), whose structural ensembles currently elude AI-based approaches such as AlphaFold and ESMFold. Phosphorylation of IDPs is a general phenomenon given their extended nature, and has been well established to dramatically alter their structural features including folding upon binding (Bah et al., Nature, 2015) or coacervation (Aumiller et al., Nat. Chem., 2015). Here, Du et al. argue that phosphorylation of the IDP ERalpha creates long-range conformational changes, disrupting the close proximity of two aromatic-rich clusters. They report that, surprisingly, this is not charge-mediated, but rather mediated by the disruption of a hydrophobic environment. This is a strong, novel claim, and their main argument hinges upon a salt titration experiment (Fig. 3). The manuscript can be greatly strengthened via the inclusion of additional point mutations to bolster their case. If further validated this is a significant finding in the context of the IDP field.

Major comments:

One of the authors' main claims is that phosphorylation of S118 causes long-range effects not due to electrostatics, but due to a less hydrophobic environment. The authors demonstrate that altering the salt concentration does not alter long-range chemical shift perturbations (Fig. 3c). This would be made more convincing if the authors produced the mutation E56Q. If the authors' claim is correct, E56Q (unphosphorylated) should behave like the WT construct, whereas E56Q-pS118 (phosphorylated) should show similar behaviour to WT-pS118 for both NMR (chemical shifts) and SEC-SAXS.

The authors observe a 3 angstrom difference between the WT and S118D. While significant, the authors' word choice of 'closed' vs 'open' conformations is very strong and misrepresents the dynamic ensemble of conformations (evidenced by relaxation measurements). 'Closed' implies folding, which is not the case as R1 and R2 measurements between 'open' and 'closed' conformations are highly similar. The authors should consider using other language such as 'slightly compacted' and 'more extended' for 'closed' and 'open' states, respectively.

The authors' claim that pS118 induces long-range conformational changes would be greatly strengthened by the inclusion of SEC-SAXS data of pS118. If that is not possible, could the authors perform DOSY measurements for WT and pS118?

Fig. 1g very clearly shows that there are long-range interactions in the central region of ERalpha. How did the authors define clusters I and II? What determined the cut-off?

It is unclear what error bars represent in Fig. 1b.

The authors should propagate error bars in Fig. 1e.

The authors should show the full spectra of the experiments presented in Figs 5a and S15 as supplementary figures.

Figs 2c, 3b, and S7 all appear to have delta chemical shifts that are cut off, particularly around residue 120. Full data should be shown.

The title of the work is uninformative. I much prefer the authors' bioRxiv title: 'Phosphorylation modulates estrogen receptor disorder by altering long range hydrophobic interactions'.

In Fig. 3e, do the authors have an explanation for the difference in PRE between L121A and WT in the first 30 and last 20 residues?

The equation for the correlation time, tau_c, is incorrect and not applicable to IDPs (Figs S3b and S8b). It only holds true for globular proteins in the absence of internal motions. Plotting the R1 and R2 ratios is sufficient.

Minor comments:

Often the authors employ self-congratulatory language which I suggest they remove: e.g. we made the pivotal discovery', 'remarkably', 'revelation'.

How delta chemical shifts are calculated should be included in captions for Figs 2 and 3.

Fig 3e: The label for F120A and L121A should go in the legend with the other labels. In its current form, it is very confusing.

In the NMR literature, the use of an asterisk (*) to denote 'complex points' is unconventional. The standard notation typically

involves just indicating the number of complex points directly without an asterisk.

Often the authors use the phrase ‘residual structure' (multiple times on pp 5 and 8).... the phrase ‘secondary structural propensity’ is more representative of the heterogeneous conformational ensembles adopted by IDPs.

Why are the cross peaks in the newly obtained NMR spectra better resolved than previously obtained spectra (p 5)? The authors should explain.

Word missing on p 8: ‘These results suggest that…arise from the formation of amino acid clusters’ Perhaps aromatic amino acid clusters?

In Fig. 2b do the authors have any idea why G57 moves differently between S118D and pS118?

In Fig. 2b, near residue 130 there is a dramatic change between R2/R1 ratios of WT and ps118. What is happening? Is it Y131? Can the authors connect this to Fig. 5a?

‘Fig. 9a’ on p 8 should be ‘Fig. S9a’.

‘Resonance reassignments’ should be ‘resonance assignments’.

Fig. 2d: It is hard to see the IDP structure.

Page 10: ‘reassignments’ should be ‘assignments’.

Without a comparison to proteins outside the family, Fig. 6b does not demonstrate the significance of hydrophobic residues. The authors should consider swapping Fig. 6b with Fig. S18 and include the 16 proteins mentioned in the text.

Page 14, “These strategically…focussed assessment of the impact of reduced hydrophobicity on ER function”: This does not seem to be the reason for choosing these mutations, as the authors have already mutated the phosphorylation site. Perhaps: “Residues F120 and L121 were mutated to alanine to reduce the hydrophobicity of the environment around S118, whilst in the presence of a S118A mutation.”

Page 15, “These findings support the notion that the F120A… hydrophobic mechanism”: This is a circular argument, best rephrased.

Clarify in figure legend of Fig. 5a that the plots are of the NTD variants (and not TIF2).

Page 20, “it is less expected to involve two separate regions within a disordered protein”: Rather than less expected, perhaps “there are yet to be many examples found of…” is more accurate.

Could the authors address why targeting AR and MSI-1436 were not a challenge?

“This revelation offers a new strategy that shifts the paradigm for designing small molecules…”: This is a great point; could the authors spell out what should be targeted and how it will be beneficial for therapeutic development?

Version 1:

Reviewer comments:

Referee #2

The authors have properly addressed all my comments an have performed the vast majority of the demanded validations and changes. Therefore, in my opinion the study can be published as it is. I think that this is a very important contribution in the field of IDPs and IDP-regulated biological mechanisms.

Referee #3

Du et al. have performed extensive experiments to bolster their story, including SEC-SAXS on pS118, the inclusion of four point mutants, and two double mutants, and the inclusion of experiments in the presence of DDM. The text, figures, and captions now have increased clarity.

Only a few minor comments remain:

1) Pg 10, line 223: Fig. 1d should be Fig. S2d.

2) Fig. 1e error bars require explanation in the caption.

3) The authors should include the discussion of future CPMG relaxation dispersion experiments with respect to Y131 in the text.

4) All NMR data (spectra and assignments) should be made open access.

Author Rebuttal To Initial Comments:

We greatly appreciate the reviewers' constructive feedback and have addressed each point comprehensively below.

- Reviewer comments in black
- Our response in blue
- Major changes in the main text are highlighted.

Following the Author's guidelines, we have reorganized the Extended Data and Supplementary information.

Referee #1:

A. Estrogen receptor alpha (ER) is a transcription factor that plays critical roles in many physiological and disease processes. This is especially true for breast cancer, where 70% of patients are characterized by their expression of this nuclear hormone receptor and their disease relies on estrogen receptor transactivation to maintain a high proliferative capacity. Estrogen receptor typically depends on estrogenic hormone binding to spur on these oncogenic transcriptional programs. Because ER activities are critical for progressive breast cancer, it remains a mainstay therapeutic target. However, a wealth of evidence over the past several decades has shown that ER can engage in ligand-independent activities to maintain breast cancer pathology and reduce the efficacy of targeted therapies, especially aromatase inhibitors and tamoxifen. The phosphorylation of serine at position 118 (S118) in ER's N-terminal domain (NTD) by cyclin-dependent kinase 7 (CDK7) and MAPK are well known to engage such activities. While the structure-function activities of ER's ligand binding and DNA binding domains are well characterized, our understanding of these relationships for the NTD are less well understood, especially in the context of S118 phosphorylation.

In this study, the Yang Laboratory uses a comprehensive suite of structural and biological tools to understand how S118 phosphorylation enables hormone-independent ER activities in breast cancer cells. They show compelling evidence that clusters of hydrophobic residues confer transient and compact pseudo-structures within the NTD, which are disrupted upon S118 phosphorylation. This is accomplished through comprehensive mutational analysis to not only mimic the charge of the phospho group at 118 but to also disrupt the hydrophobicity of these two regions. Each of these mutations is also studied in the context of the breast cancer cell to gain a better understanding of how S118 phosphorylation and NTD dynamics impacts the function of full-length ER.

We greatly appreciate this positive and constructive feedback and have used it to improve our Introduction and Discussion sections. We have updated these sections to better explain the critical role of ER in breast cancer and the importance of our study.

Our study contributes to this field by using a comprehensive suite of structural and biological tools to investigate how S118 phosphorylation enables hormone-independent ER activities in breast cancer cells. We demonstrate that clusters of hydrophobic residues create transient structures in the NTD, which are disrupted upon S118 phosphorylation. Through extensive mutational analysis, we examine how mimicking the phosphorylation charge and disrupting the hydrophobicity of these regions impact the function of full-length ER in breast cancer cells.

This framing highlights the significance of our study in advancing the understanding of ER function in breast cancer, particularly in the context of treatment resistance and ligand-independent activation. By elucidating the structural changes induced by S118 phosphorylation and their functional consequences, our work provides new insights into the mechanisms of ER-driven breast cancer progression and potential avenues for therapeutic intervention.

B. This is a highly important study that will be of significant interest to a large and diverse audience of scientists, not just those who study ER but more generally to anyone interested in nuclear hormone receptors. I believe that it presents a new paradigm for our understanding of the role played by the NTD in ER activity that is not only fundamental to our understanding of receptor biology, but also potentially therapeutically relevant as S118 phosphorylation is well known to enable resistance to certain first-line hormone therapies in breast cancer.

We are deeply grateful for the reviewer's evaluation of our study. This insightful analysis highlights the broader implications of our findings, extending beyond ER-specific research to the broader field of nuclear hormone receptors. We are particularly encouraged by the reviewer's assessment that our work presents a new paradigm for understanding NTD function in ER activity. Their recognition of the potential therapeutic relevance, especially in the context of hormone therapy resistance in breast cancer, underscores the clinical significance of our research. This feedback validates our study's importance and inspires us to explore the translational aspects of our findings in the future.

C. The approaches used to characterize this interaction are empirical and robust. ER is a difficult protein to work with for biophysical experiments, especially the NTD. The Yang group's use of NMR to study its dynamics then incorporating that into functional studies within the breast cancer cell is the optimal way to study this. The data appear to be of high quality. The figures and paper are well presented in this body of work. One minor suggestion would be to increase the font size where sequences are shown to make it easier to read on a page.

We greatly value the reviewer's comprehensive assessment and constructive feedback. The recognition of the inherent challenges associated with ER biophysical experiments, particularly regarding the NTD, validates our methodological choices. We are particularly encouraged by the endorsement of our integrated approach, combining NMR dynamics studies with functional analyses in breast cancer cells, as an optimal strategy for investigating this complex protein.

The reviewer's positive evaluation of our data quality and overall presentation is deeply appreciated. It reinforces our confidence in the robustness and clarity of our findings and affirms the rigor of our experimental design and execution.

Regarding the suggestion for improved readability, we have increased the font size of the amino acid sequences in Fig. 1a. We appreciate this practical recommendation, which enhances the visual accessibility of our presentation. This detailed feedback is invaluable in ensuring our work is as clear and impactful as possible.

D. Statistics are shown and appear to be the appropriate tests. Description of the star values and statistical test used should be included in the figure legends, though.

We appreciate the reviewer's careful examination of our statistical reporting. In response to this feedback, we have thoroughly updated the figure legends for Fig. 4, Fig. 5, Extended Data Fig. 7, and Extended Data Fig. 8. Each legend now includes the specific statistical test used for each analysis and p-values corresponding to the significance levels indicated by asterisks.

E. The conclusions are well supported by their studies.

We greatly appreciate the reviewer's affirmation that our conclusions are well supported by our studies. This validation from an expert in the field is invaluable and reinforces our confidence in the robustness of our experimental approach and data interpretation. We have strived to ensure that our conclusions are firmly grounded in the evidence presented, and it is gratifying to know that this effort is recognized. This feedback encourages us to maintain this high standard of scientific rigor in our ongoing and future research endeavors.

F. Suggested improvements: The only issue I have with this work is that they used indirect methods to prove the hydrophobic clustering hypothesis. First by increasing the osmotic strength of their buffer to disprove that it was through electrostatic repulsion. Second by comprehensive mutational analysis across several different residues. However, they did not, from what I can tell, manipulate the hydrophobicity of the buffer. If it is possible, it would be ideal to include an experiment with one or two detergents present below their CMC to better directly probe the role of hydrophobicity and its disruption by S118 phosphorylation.

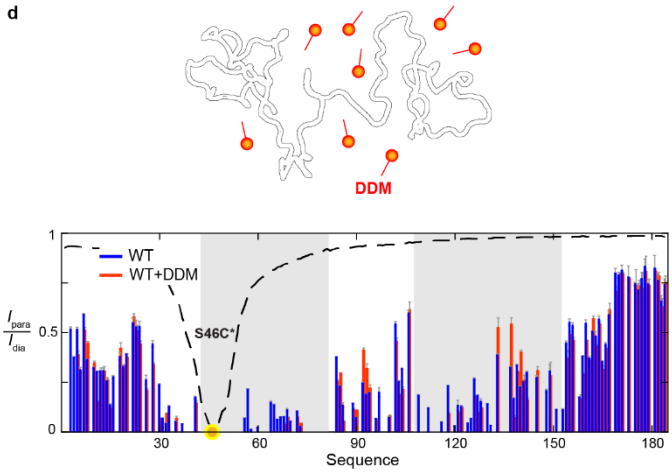
We appreciate the reviewer's insightful suggestion to probe the role of hydrophobicity directly. In response, we conducted additional experiments using n-dodecyl β-D-maltoside (DDM), a non-ionic detergent that modulates hydrophobic interactions by competing with them.

PRE measurements with S46C spin labeling (30 μM) were performed with and without DDM (120 μM, below its critical micelle concentration or CMC). The results showed modest but substantial changes in PRE profiles upon introducing DDM, with PRE ratio shifts from 0.14 to 0.22 in specific regions within Cluster-II (Extended Data Fig. 2d; see also below). These changes provide direct evidence that a non-ionic detergent influences long-range interactions within the protein, supporting our hypothesis that hydrophobic interactions play a crucial role in maintaining ER-NTD conformation.

Only non-ionic detergents can disrupt hydrophobic interactions, but options are limited. We explored Triton X-100, a weaker alternative, but faced challenges due to the 30 μM minimum protein concentration required for PRE measurements. The minimum protein concentration of 30 μM for PRE measurements constrained our experiments. Maintaining a protein-detergent molar ratio higher than the 1:4 used for DDM (120 μM) proved difficult within the narrow range between 120 μM and the detergent's CMC.

We have updated the main text (page 10) to incorporate these findings:

" To directly probe hydrophobic interactions, we conducted PRE measurements using n-dodecyl β-D-maltoside (DDM), a non-ionic detergent that competes with hydrophobic sidechain interactions. ER-NTD with S46C spin labeling (30 μM) exhibited modest yet significant changes in PRE profiles upon DDM addition (120 μM), particularly in Cluster-II regions (Extended Data Fig. 1d). This evidence suggests hydrophobic interactions play a role in maintaining ER-NTD conformation."



Extended Data Figure 2. (**d**) Effect of non-ionic detergent DDM (120 μM) on long-range interactions probed by spin labeling at S46C. Protein concentration: 30 μM; yellow circle: spin label position.

G. References are appropriate.

H. This is a very well written manuscript. It is mentioned in the introduction that no drugs target the ER NTD. However, selective estrogen receptor degraders/downregulators are known to target the NTD by triggering receptor degradation. That is why SERDs will show benefit in the first-line hormone therapy-resistant ER+ setting.

Thanks for this insightful comment. We acknowledge the significant role of SERDs in indirectly targeting the ER-NTD. To address this, we have included the following point in our Discussion section:

“Current ER modulators, including small molecule degraders[59,60], affect NTD indirectly. Our study establishes a framework for direct ER-NTD targeting, proposing strategies to modulate NTD conformation and inhibit its activation.”

Referee #2:

The manuscript “The sequence-structure-function relationship of intrinsic ERalpha disorder” by Zhanwen Du et al. unveils the molecular mechanism for the activation of the estrogen receptor (ER) through the phosphorylation of S118, placed in the N-terminal transactivation domain (NTD). The relevance of this mechanism is exemplified by the current development of kinase inhibitors aiming at reducing the amount of pS118 to target breast cancer. Although the relevance of this phosphorylation was known, the molecular mechanism leading to activation, which could eventually drive to new treatments, was unknown.

By combining NMR (CS, Relaxation and PREs) and SAXS, the authors unambiguously show that in the native state the ER NTD is disordered but contains a complex network of intramolecular interactions, which render the conformational ensemble relatively compact. They show that the nature of these contacts is mainly hydrophobic. They also demonstrate that this network of contacts can be partially perturbed upon phosphorylating S118, mutating it (S118D) or reducing the hydrophobicity through point mutants (F120A and L121A). These changes modify the interaction with the coactivator TIF2 and affect gene transcription and cell proliferation. These findings establish hydrophobic disruption as a critical regulatory mechanism for ER and they hypothesize that the same mechanism could be general in nuclear receptors, which contain dispersed hydrophobic residues in their transactivation domain.

The biological question addressed in the manuscript is relevant, the techniques applied are well suited, and the conclusions are robust and based on the experimental observations. The manuscript is easy to read. There is still, in my opinion, several improvements that would reinforce the study.

We greatly appreciate the reviewer's thorough and positive assessment of our manuscript. The reviewer has provided an accurate summary of our key findings regarding the molecular mechanism of estrogen receptor activation through S118 phosphorylation in the NTD. We are pleased that the reviewer found our study relevant, methodologically sound, and presented.

The reviewer's recognition of our robust conclusions and their potential implications for nuclear receptors is particularly encouraging. We are glad they highlighted our use of NMR and SAXS to reveal the complex interactions within the ER-NTD, and how we linked structural changes to functional outcomes.

- Although the mechanism of hydrophobic perturbations is well documented by the authors, it is unclear how much the network of intramolecular contacts is affected by the phosphorylation of S118 or its mutation (S118D). For instance, PRE profiles are perturbed (Figs. 2f, S3e, and S9), but the intensity ratio does not come back to 1.0. Moreover, the relaxation profile still displays the two clusters upon phosphorylating S118. These observations suggest that part of these contacts is still present even in the ‘active’ state, so a simple on/off mechanism does not apply here. The authors should discuss about this point, probably in relation with the next point.

Thank you for this insightful feedback. We have addressed these concerns about the intramolecular contacts in the following ways:

1. We have revised our terminology to reflect the nuanced conformational changes more accurately. Instead of "open" and "closed" states, we now use terms like "more compact" and "more extended" conformational ensembles to better represent the dynamic nature of the system (page 6, Fig. 2g, page 8, page 20).

2. We have added a discussion on residual structures, clarifying that charged residues at both termini likely contribute to maintaining partial compactness even in the more activated state. (page 18).
3. We have updated our discussion section to emphasize that the changes represent a shift in the conformational ensemble rather than a binary switch.

- (Related) The compactness of the ER NTD and the expansion upon mutating (S118D) is not well characterized. The authors should do a proper modelling of the ensembles using SAXS data. I would suggest the use of EOM for the interpretation of all SAXS datasets (wt, pS118, S118D…) in order to have a clear picture of the compactness of the ensemble corresponding to the different states with respect to the random coil model (possibly derived from Flexible-Meccano). Note that the synthetic P(r) displayed in Fig. 1b does not provide the same information.

We appreciate the reviewer's suggestion and have implemented the Ensemble Optimization Method (EOM) modeling for our SAXS data. The EOM ensembles' averaged p(r) functions have been added to Fig. 1b (blue line; see below). The EOM-derived p(r) function is similar to the GNOM calculations (circles in Fig. 1b), as both fit the same SAXS intensity well.

Significantly, the EOM-derived p(r) functions differ markedly from the p(r) determined from Flexible-Meccano simulations (dashed line in Fig. 1b), which assume a random coil model. This difference underscores the presence of residual structures in ER-NTD and quantifies the ensemble compactness in different states relative to a random coil model.

While SAXS data demonstrates ER-NTD compactness and expansion upon S118D mutation, it lacks residue-level resolution. Our PRE data complements the SAXS analysis by providing this detailed information for all states. The PRE-revealed variations in distances between hydrophobic clusters offer a mechanistic explanation for the conformational changes observed in our SAXS measurements.

These results have been incorporated into our revised manuscript, providing a more comprehensive characterization of ER-NTD compactness.

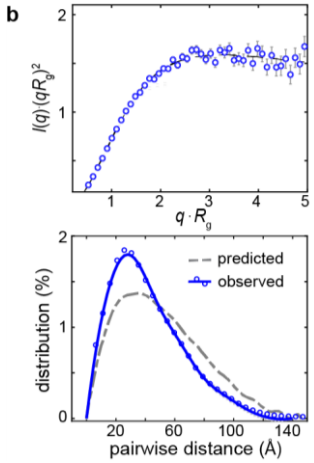


Fig. 1. (**b**) Kratky plot of SEC-SAXS data collected at 4°C. $I(q)$: scattering intensity (normalized by zero-angle scattering); q: amplitude of scattering vector; R_g: radius of gyration. Error bars represent the propagated uncertainties from I(q) measurements. Lower panel: pairwise distance distribution. Blue line indicates SAXS-derived conformational average via Ensemble Optimization Method (EOM)[40]; circles represent GNOM calculations[41]; dashed line shows calculations from flexible-meccano simulations[39].

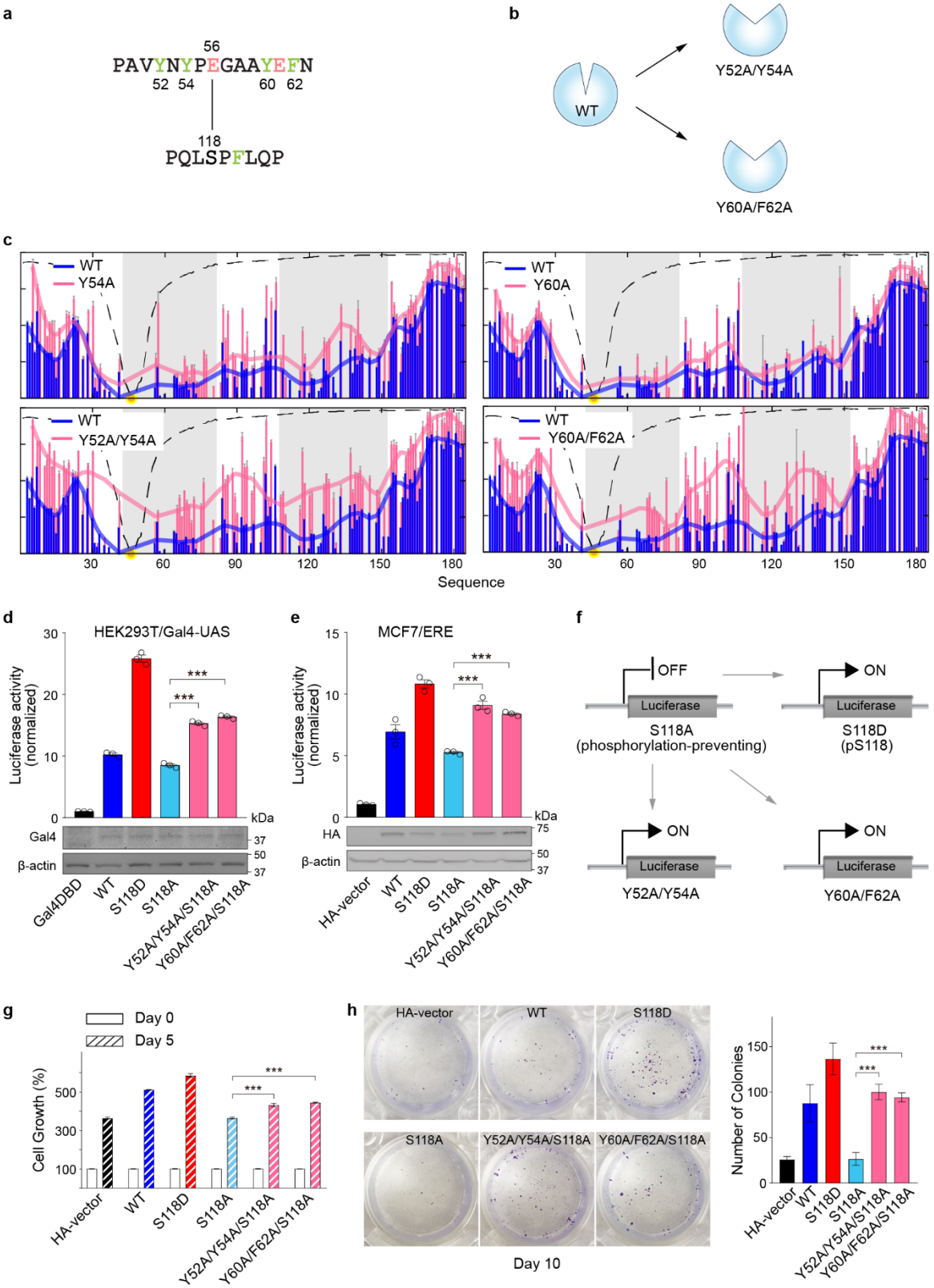
- The selection of the reduced hydrophobicity mutants (S118A/F120A and S118A/L121A) seems a bit arbitrary. Would other mutants reducing the hydrophobicity have the same biophysical and biological properties as the two selected by the authors? This would reinforce the concept of hydrophobic cluster.

We appreciate the reviewer's insightful suggestion. To strengthen our proposed mechanism, we expanded our mutational study beyond the initial F120A and L121A mutations, testing the hypothesis that collective interactions among multiple hydrophobic amino acids are crucial for maintaining nonlocal hydrophobic clustering.

We introduced additional mutations near E56: single mutations (Y54A and Y60A) and double mutations (Y52A/Y54A and Y60A/F62A). Biophysical analysis (Extended Data Fig. 8a-c; below) revealed a clear trend: single mutations increased the inter-cluster distance compared to wild-type, while double mutations showed a more pronounced effect. This progressive impact supports the critical role of collective hydrophobic interactions in maintaining nonlocal clustering.

Functional studies on these variants complemented our biophysical findings. Mutations (Y52A/Y54A and Y60A/F62A in S118A background) significantly affected ER transcriptional activity in both ER-NTD and full-length ER (Extended Data Fig. 8d-f), as well as cellular phenotypes including growth (Extended Data Fig. 8g) and colony formation (Extended Data Fig. 8h).

Our expanded analysis reinforces the concept of hydrophobic cluster-mediated regulation in ER-NTD, demonstrating that reducing hydrophobicity in either cluster leads to similar biophysical and biological outcomes. These findings, summarized in Extended Data Fig. 8 and incorporated into the Results section (pages 10, 14, 17), provide a comprehensive view of hydrophobic interactions in ER-NTD function and regulation, strongly supporting our original hypothesis.



Extended Data Figure 8. Alanine substitutions of hydrophobic aromatic residues near E56 induce conformational changes in ER-NTD, altering transcriptional activity and cellular phenotypes.

(**a**) Four hydrophobic aromatic residues (Y52, Y54, Y60, F62; green) near E56 (red) are proximal to serine 118.

(**b**,**c**) Y52A/Y54A or Y60A/F62A double mutants in non-phosphorylated NTD induce greater conformational changes than single-site mutations, assessed by PRE measurements (S46C spin labeling). Yellow circle: spin labeling position; solid lines: visual aid. HSQC spectra: 850 MHz, 4°C.

(**d**,**e**) Y52A/Y54A or Y60A/F62A restore reporter gene activity impaired by S118A in ER-NTD (HEK293T cells) and full-length ER (MCF7 cells). Bottom: western blots of transfected proteins.

(**f**) Schematic: Y52A/Y54A or Y60A/F62A rescue transcription activity of phosphorylation-deficient S118A mutant.

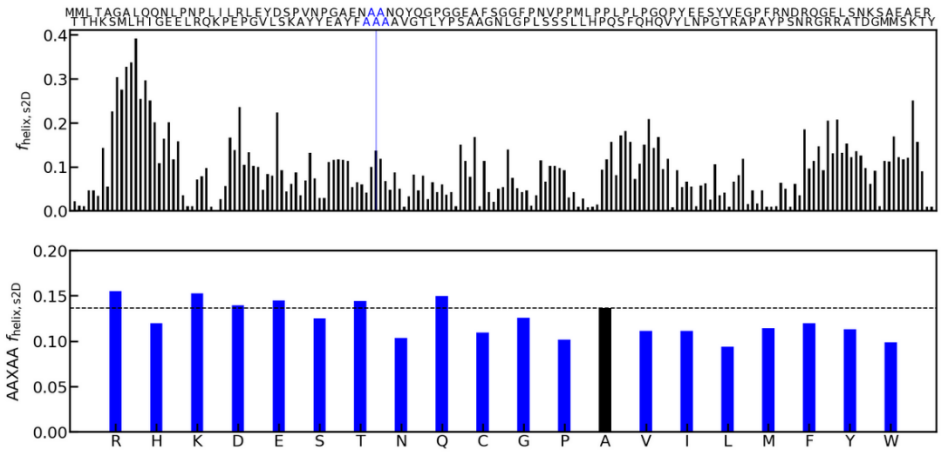
(**g**,**h**) Y52A/Y54A or Y60A/F62A restore impaired cell growth and colony formation of S118A mutant in MCF7 cells. Growth in DMEM; colonies quantified by crystal violet (absorbance at 450 nm). One-way ANOVA followed by Dunnett's test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

- When looking at Fig. 2e, it seems that the maximum of the R2/R1 profile corresponds to the alanine-rich segment, which the authors identify as partially helical. This observation suggests that this fragment could have a relevant role in the network of hydrophobic interactions. Would the compactness of the protein be affected if the structure and/or sequence of this long and hydrophobic a-helix is perturbed?

We appreciate the reviewer's insightful observation regarding the peak of cluster-I in the R2/R1 profile and its proximity to the alanine-rich segment (residues 64-68, AAAAA). While this suggests a potential role for this segment in the hydrophobic interaction network, directly testing the impact of secondary structure propensity on hydrophobic clusters presents several challenges:

1. Low secondary structure propensity: The AAAAA region exhibits a relatively low helical fraction (<30%) based on our chemical shift measurements (Fig. 1d), making it difficult to alter the secondary structure through mutagenesis significantly.
2. Computational analysis: Using the s2D tool [Sormanni et al., 2015, J. Mol. Biol. 427:982, PMID: 25534081], we estimated secondary structure changes upon mutating the central alanine in the AAAAA segment. Across all 19 possible single mutations, the helical fraction changed by less than 5% (see Supporting Figure below).
3. Experimental limitations: Given these computational predictions, experimental testing of mutants would likely not yield substantial changes in secondary structure propensity (SSP). It would be challenging to distinguish between effects arising from changes in SSP versus alterations in amino acid hydropathy.

As such, while the alanine-rich segment may contribute to the protein's hydrophobic interaction network, the subtle nature of its structural propensities makes it challenging to perturb and analyze in isolation. Our study, therefore, focused on regions where we could more definitively manipulate and observe the effects of hydrophobic interactions.



Supporting Figure. Helical propensity analysis of ER-NTD and AAAAA segment.

Top: Predicted helical fraction along the NTD sequence using s2D. The AAAAA segment (residues 64-68) is highlighted.

Bottom: Helical propensity predicted after each single-site substitution at position 66 (central residue of AAAAA segment). Wild-type alanine (A) is indicated in black. All mutations result in <5% change in helical fraction.

- Several experimental and computational studies have connected chain compactness induced by the presence of intramolecular interactions to the capacity of the protein to form condensates in vitro and in vivo. Moreover, the presence of hydrophobic residues and alanine-rich sequences often induce phase separation, especially in transcription factors. It could be even tempting to hypothesize that the regulation mechanism described by the authors could be explained through a perturbation of the capacity of the protein to phase separate. The authors should comment on this possibility.

We thank the reviewer for highlighting the potential connection between chain compactness, hydrophobic interactions, and liquid-liquid phase separation (LLPS) in transcription factors. This is indeed an important area of research, and we appreciate the opportunity to address it in the context of our findings and current literature.

Recent studies have shown that ER exhibits weak LLPS behavior but enhances interaction with Mediator MED1 and is incorporated into MED1 condensates (Boija et al., Cell 2019). This suggests a cooperative effect rather than strong independent phase separation by ER. It is worth noting that LLPS mechanisms vary across the nuclear receptor family. For instance, in the androgen receptor (AR), the DNA-binding domain is crucial for RNA-dependent phase separation (Ahmed et al., Protein Sci. 2021).

Our study identified hydrophobic clusters in ER-NTD, which may contribute to ER's weak LLPS behavior and its interaction with phase-separating proteins like MED1. While we focused on structural features and hydrophobic clusters, we acknowledge that these elements could play a role in LLPS-related functions.

To address this critical point, we have added a brief comment to the Discussion along the line above (page 20) as follows:

“The identified hydrophobic patterns may contribute to ER's weak phase separation behavior and its interactions with phase-separating proteins like MED1[65], although mechanisms vary across nuclear receptors[66].”

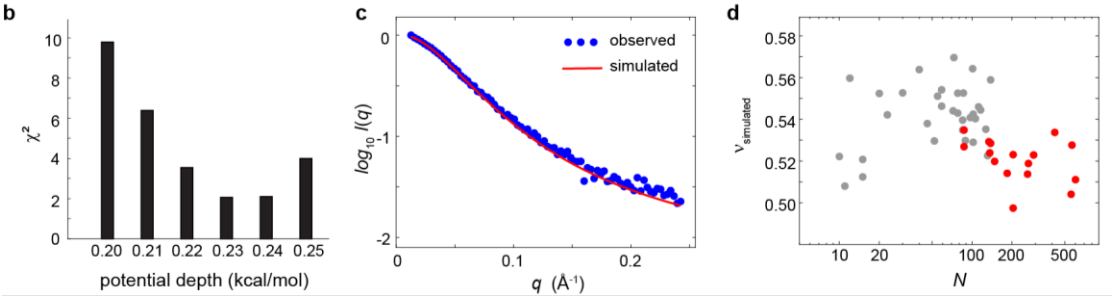
- The authors hypothesize about the generality among the nuclear receptor family (and other IDPs) of the mechanism unveiled for ER-NTD due to the patterning of hydrophobic residues in their sequence. This is a very attractive hypothesis that the authors could substantiate by analyzing few of the sequences displayed in Fig. 6b and Fig. S17 by looking at their compactness by SAXS or by performing coarse-grained simulations. This information would, in my opinion, increase the impact and relevance of the study.

We appreciate the reviewer's suggestion to investigate the generality of our findings across the nuclear receptor family. To address this, we conducted a comprehensive analysis using coarse-grained simulations.

Our methodology involved adjusting the CALVADOS2 model [Tesei and Lindorff-Larsen, 2022, Open Res. Eur. 2:94] to match experimental SAXS data of ER-NTD (Extended Data Fig. 10b,c; see below). We then simulated NTDs of all 48 nuclear receptor proteins, computing radius of gyration (R_g) and Flory exponent (v) for each (Extended Data Fig. 10d and Supplementary Table 1).

Key findings from this analysis were twofold. First, we identified 15 NTDs with similar hydrophobic patterning (Fig. 6c) and charged residue fractions to ER-NTD (Extended Data Fig. 10d). Second, these 15 NTDs showed smaller Flory exponents (indicating more collapsed structures) and longer chain lengths on average, suggesting hydrophobic cluster formation.

These results support the generalizability of the hydrophobic-driven compaction mechanism across the nuclear receptor family. We have included this analysis in the Discussion (page 20) and provided detailed methods in the Supplementary Methods section.



Extended Data Figure 10. (**b**) CALVADOS2 model optimization for ER-NTD using SEC-SAXS data, showing deviation (χ2) vs. pairwise interaction potential depth (ε) in CALVADOS2[60]. χ^2: root-mean-square deviation between simulated and experimental SAXS intensities, normalized by the experimental errors. Optimal ε = 0.23 kcal/mol. Simulation conditions: 310K, 150 mM ionic strength, pH 7.4, 1 μs total (800 ns analyzed after 200 ns equilibration).

(**c**) Comparison of simulated and experimental SAXS intensities for ER-NTD using CALVADOS2 simulations (ε = 0.23 kcal/mol).

(**d**) Analysis of 48 NTDs: *N* (residue count) vs $\nu_{simulated}$ (Flory exponent) obtained from an adjusted CALVADOS2[60] simulation model (ε = 0.23 kcal/mol) and power-law relation between R_g and N[99]. Red: NTDs with fraction of charged residues (FCR) < 0.22 and sequence hydrophobic patterning (SHP) > 0.6, similar to ER-NTD; gray: remaining NTDs. Two sequences lacking DNA-binding domains were excluded. FCR and SHP values for all 48 NTDs are provided in Supplementary Table 1.

Minor point:

- Error bars should be displayed for the relaxation and PRE data along the manuscript.

We have updated all relevant figures throughout the manuscript in response to the reviewer's request for error bars on relaxation and PRE data. Specifically:

1. Relaxation data: Error bars with gray caps have been added to Fig. 1e, Fig. 2b, and Extended Data Fig. 3.
2. PRE data: Error bars with gray caps have been incorporated into Fig. 1g, Fig. 2e, Fig. 3e, Fig. 3g, Extended Data Fig. 1b,c,d, Extended Data Fig. 6c,d, and Extended Data Fig. 8c.

Referee #3:

General comments:

Du et al. present a beautiful illustration of the power of NMR and SAXS to answer challenging questions about structural features of intrinsically disordered proteins (IDPs), whose structural ensembles currently elude AI-based approaches such as AlphaFold and ESMFold. Phosphorylation of IDPs is a general phenomenon given their extended nature, and has been well established to dramatically alter their structural features including folding upon binding (Bah et al., Nature, 2015) or coacervation (Aumiller et al., Nat. Chem., 2015). Here, Du et al. argue that phosphorylation of the IDP ERalpha creates long-range conformational changes, disrupting the close proximity of two aromatic-rich clusters. They report that, surprisingly, this is not charge-mediated, but rather mediated by the disruption of a hydrophobic environment. This is a strong, novel claim, and their main argument hinges upon a salt titration experiment (Fig. 3). The manuscript can be greatly strengthened via the inclusion of additional point mutations to bolster their case. If further validated this is a significant finding in the context of the IDP field.

We sincerely thank the reviewer for their insightful and constructive suggestions. We appreciate the recognition of our work's significance in demonstrating the power of NMR and SAXS for studying the disordered ER-NTD, particularly where AI-based approaches like AlphaFold are currently limited. We are gratified by acknowledging our findings' novelty and potential impact, especially regarding the unexpected mechanism involving hydrophobic environment disruption rather than charge-mediated effects.

We agree that additional experimental evidence strengthens our claims about the hydrophobic-driven mechanism. The new experiments and analyses we have conducted provide multiple lines of evidence supporting our proposed mechanism, addressing the reviewer's concerns and further validating our findings' significance in the context of IDP research.

We are grateful for the reviewer's valuable input, which has significantly enhanced the robustness and impact of our study. We are confident that these improvements address the concerns raised and strengthen our manuscript substantially.

Major comments:

One of the authors' main claims is that phosphorylation of S118 causes long-range effects not due to electrostatics, but due to a less hydrophobic environment. The authors demonstrate that altering the salt concentration does not alter long-range chemical shift perturbations (Fig. 3c). This would be made more convincing if the authors produced the mutation E56Q. If the authors' claim is correct, E56Q (unphosphorylated) should behave like the WT construct, whereas E56Q-pS118 (phosphorylated) should show similar behavior to WT-pS118 for both NMR (chemical shifts) and SEC-SAXS.

We appreciate the reviewer's insightful suggestion regarding the E56Q mutation. To address this, we conducted new experiments with the E56Q-pS118 variant, comparing it to pS118 using SEC-SAXS and PRE measurements, which are more suitable for detecting long-range conformational changes.

Our SEC-SAXS results (Fig. 3f below) reveal that the radius of gyration (R_g) of E56Q-pS118 is virtually identical to that of pS118, both exhibiting larger R_g values compared to WT. This indicates that E56Q alone does not alter the phosphorylation-driven expansion of NTD, suggesting that the conformational change is not due to repulsive interactions between charged E56 and phosphorylated S118.

To further investigate, we used PRE measurements with spin labeling at S46C to probe the distance between the two hydrophobic clusters. This labeling position, outside the identified hydrophobic clusters, minimizes the potential impact on their interactions. Our results (Fig. 3g) show that the inter-cluster distance increases similarly for pS118 and E56Q-pS118 upon phosphorylation, in contrast to unphosphorylated WT. This confirms that the charge on E56 is unnecessary for the phosphorylation-driven conformational change in ER-NTD.

These new experiments with the E56Q mutant strongly support our original claim. E56Q-pS118 (phosphorylated) exhibits behavior similar to pS118 in both SEC-SAXS and PRE measurements. These results reinforce our hypothesis that the long-range effects of S118 phosphorylation are primarily due to changes in the hydrophobic environment rather than electrostatic interactions.

It is worth noting that chemical shift perturbation is not ideal in assessing the role of charged E56 in this context. Mutating E56 inevitably alters the local chemical environment, complicating the distinction between local effects and non-local interactions caused by S118 phosphorylation. Moreover, E56Q (unphosphorylated) does not directly address our hypothesis of the hydrophobic-driven mechanism upon phosphorylation, unlike the E56Q-pS118 variant discussed above.

We have updated the Results section (pages 10 and 12) as follows:

“We extended this approach by introducing alanine substitutions for four aromatic amino acids near E56 in the other cluster (Extended Data Fig. 8a). PRE measurements revealed a gradual increase in inter-cluster distance correlating with the number of mutations: single mutants (Y54A and Y60A) showed modest increases, while double mutants (Y52A/Y54A and Y60A/F62A) exhibited more pronounced effects (Extended Data Fig. 8b,c). This trend highlights the critical role of collective hydrophobic interactions from multiple amino acids from both clusters in maintaining nonlocal hydrophobic clustering.

To rule out electrostatic interactions as the primary driver of these conformational changes, we employed a charge-neutralizing E56Q mutation. SEC-SAXS analysis demonstrated that E56Q-pS118 and pS118 exhibit comparable radius of gyration (R_g) values, both significantly larger than wild-type (Fig. 3f). PRE measurements with S46C spin labeling corroborated these findings, showing similar increases in inter-cluster distance for pS118 and E56Q-pS118 relative to unphosphorylated WT (Fig. 3g). These results demonstrate that the charge on E56 is not essential for the phosphorylation-driven conformational change in ER-NTD.”

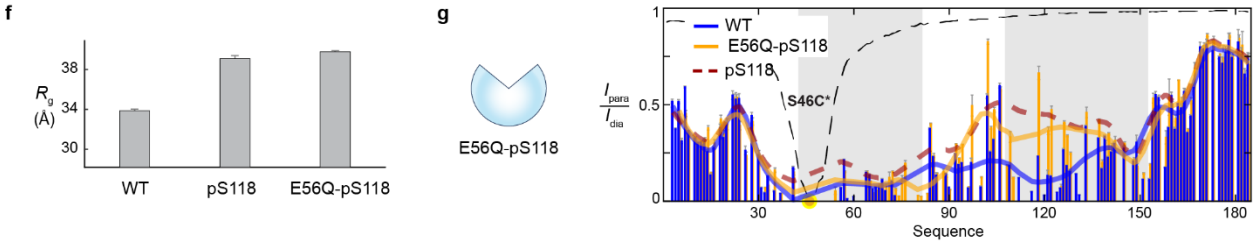


Fig. 3. (**f**,**g**) The charge-neutralizing E56Q mutation has minimal impact on R_g (measured via SEC-SAXS; see Extended Data Fig. 1) and cluster distance separation (assessed via PRE with S46C spin labeling) compared to pS118.

The authors observe a 3 angstrom difference between the WT and S118D. While significant, the authors’ word choice of ‘closed’ vs ‘open’ conformations is very strong and misrepresents the dynamic ensemble of conformations (evidenced by relaxation measurements). ‘Closed’ implies folding, which is not the case as R1 and R2 measurements between ‘open’ and ‘closed’ conformations are highly similar. The authors should consider using other language such as ‘slightly compacted’ and ‘more extended’ for ‘closed’ and ‘open’ states, respectively.

We appreciate this feedback on our terminology and structural data interpretation. We concur with the assessment and have revised our manuscript accordingly. We have replaced the terms "closed" and "open" with "more compacted" and "more extended," respectively, throughout the manuscript. This change more accurately reflects the observed 3-angstrom difference between the WT and S118D variants and avoids implying a folding event. Additionally, we have emphasized the dynamic ensemble nature of the protein's conformations, acknowledging that the observed differences represent shifts in the conformational ensemble rather than discrete structural states. We have also revised our discussion to reflect the similarities between the conformational states better and avoid over-interpreting the data. These revisions provide a more nuanced and accurate description of our findings, aligning with the dynamic nature of intrinsically disordered proteins.

The authors' claim that pS118 induces long-range conformational changes would be greatly strengthened by the inclusion of SEC-SAXS data of pS118. If that is not possible, could the authors perform DOSY measurements for WT and pS118?

We appreciate the reviewer's suggestion and have accordingly acquired new SEC-SAXS data for pS118, which significantly strengthens our claim about pS118-induced long-range conformational changes. The new results are presented in Fig. 2f (below) and Extended Data Fig. 1e (also below). To incorporate this additional data, we have made the following updates to our manuscript:

1. Updated Fig. 2f with relevant findings from the SEC-SAXS analysis.
2. Expanded the Methods section to include detailed SEC-SAXS experimental procedures.
3. Deposited the new SEC-SAXS data in the SASBDB database (accession code SASDVC5).
4. New acknowledgments were added to recognize contributions related to this data.

These additions support our original claim and address the reviewer's concern by including SEC-SAXS data for pS118. This new data significantly enhances the strength and validity of our findings regarding the conformational changes induced by S118 phosphorylation.

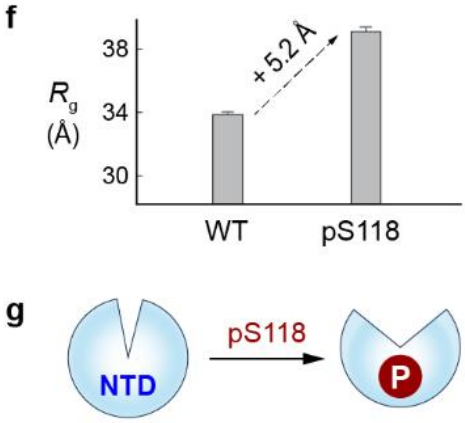
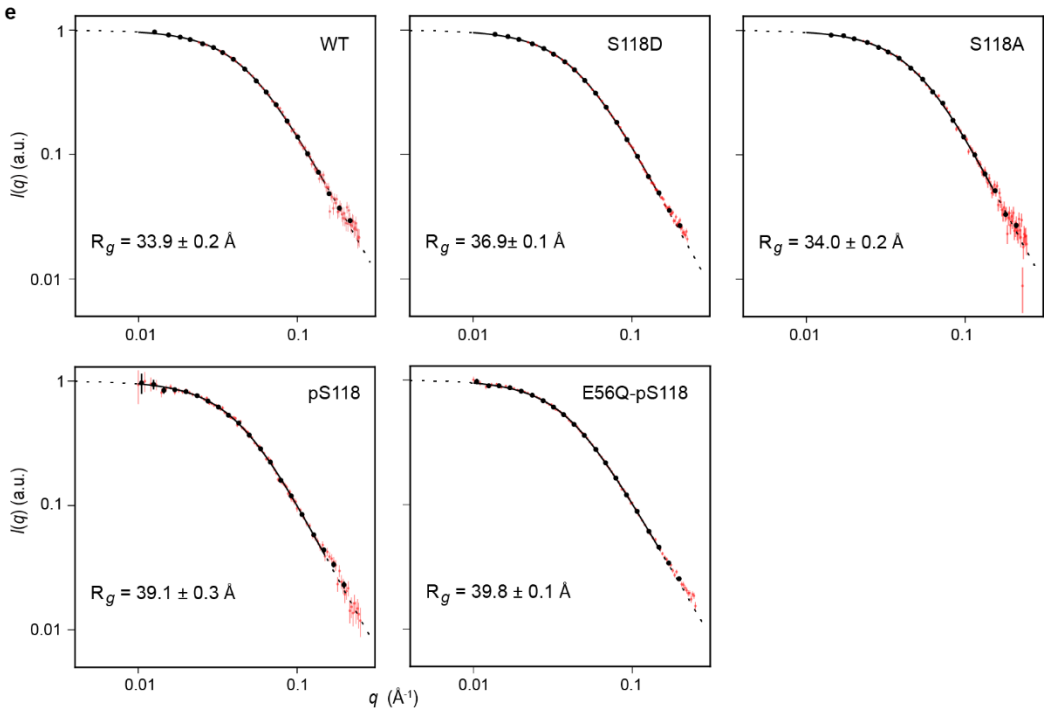


Fig. 2. (**f**) Ser118 phosphorylation increases R_g by 5.2±0.4 Å (Extended Data Fig. 1e.).

(**g**) Schematic: Ser118 phosphorylation expands ER-NTD conformation.



Extended Data Figure 1. (e) Radius of gyration (R_g) for ER-NTD variants (WT, S118D, S118A, pS118, E56Q-pS118) from SEC-SAXS at 4°C. Dashed lines: empirical molecular form factor (MFF) method fits[33].

Fig. 1g very clearly shows that there are long-range interactions in the central region of ERalpha. How did the authors define clusters I and II? What determined the cut-off?

We defined hydrophobic clusters I and II using the quantitative approach adapted from Klein-Seetharaman et al. (2002, Science 295:1719). Our analysis showed that two Gaussian distributions effectively fit the data corresponding to the two clusters. The boundaries of each were determined using the Full Width at Half Maximum (FWHM) of its respective Gaussian function as a natural cutoff. We have included the fitting equation and relevant parameters in the Supporting Methods and updated the legend of Fig. 1e accordingly.

It is unclear what error bars represent in Fig. 1b.

We have updated Fig. 1b's legend to clarify: "Error bars represent the propagated uncertainties from $I(q)$ measurements."

The authors should propagate error bars in Fig. 1e.

Error bars in Fig. 1e have been included with propagation from relaxation data (plotted in Extended Data Fig. 3).

The authors should show the full spectra of the experiments presented in Figs 5a and S15 as supplementary figures.

Full HSQC spectra for the experiments presented are now provided in Supplementary Figure 1.

Figs 2c, 3b, and S7 all appear to have delta chemical shifts that are cut off, particularly around residue 120. Full data should be shown.

We have expanded the y-axis range in Fig. 2c, Fig. 3b, and Extended Data Fig. 6a,b (previously referred to as Fig. S7) to display the full range of delta chemical shifts, including those around residue 120. This adjustment ensures that all data points are visible, representing the chemical shift changes across all residues.

The title of the work is uninformative. I much prefer the authors' bioRxiv title: 'Phosphorylation modulates estrogen receptor disorder by altering long range hydrophobic interactions'.

We appreciate this feedback on the title. We agree that the bioRxiv title offers more technical detail. The current title was chosen to appeal to a broader audience. We are open to using either title and welcome the editor's recommendation on which would be more appropriate for this journal's readership.

In Fig. 3e, do the authors have an explanation for the difference in PRE between L121A and WT in the first 30 and last 20 residues?

Our analysis centered on L121A's effect of increasing separation between the two main clusters compared to wild-type. Although we observed PRE differences in terminal regions for L121A, these changes were absent in the physiologically relevant pS118 variant. Since L121A primarily demonstrates the hydrophobic mechanism, exploring its additional effects falls outside our focus on phosphorylation-induced changes.

The equation for the correlation time, tau_c, is incorrect and not applicable to IDPs (Figs S3b and S8b). It only holds true for globular proteins in the absence of internal motions. Plotting the R1 and R2 ratios is sufficient.

We have removed the tau_c equation (and associated figures/Results), as this analysis is not applicable here. The R1/R2 ratio plots are retained. We appreciate this insight, which has enhanced the accuracy and rigor of our study.

Minor comments:

Often the authors employ self-congratulatory language which I suggest they remove: e.g. we made the pivotal discovery', 'remarkably', 'revelation'.

The manuscript has been revised to use more neutral language throughout. We've replaced self-congratulatory phrases with more modest alternatives in all sections, including the Introduction, Results, Discussion, Abstract, and Conclusion. Fundamental changes include substituting descriptive terms like "found" or "important" for more emphatic words such as "pivotal" or "novel" and removing unnecessary modifiers that overstate the impact of findings.

How delta chemical shifts are calculated should be included in captions for Figs 2 and 3.

We have added the chemical shift calculation details to the figure legends of Fig. 2c, Fig. 3b, and Fig. 5a.

Fig 3e: The label for F120A and L121A should go in the legend with the other labels. In its current form, it is very confusing.

We have addressed this issue in several ways. We removed the pS118 lines and updated the cartoon to improve clarity. Additionally, we edited the figure legend to include the F120A and L121A labels, as suggested.

In the NMR literature, the use of an asterisk (*) to denote 'complex points' is unconventional. The standard notation typically involves just indicating the number of complex points directly without an asterisk.

We have removed the asterisks and presented the complex point values directly.

Often the authors use the phrase ‘residual structure' (multiple times on pp 5 and 8).... the phrase ‘secondary structural propensity’ is more representative of the heterogeneous conformational ensembles adopted by IDPs.

We appreciate this valuable point about terminology. We agree that 'residual structure' alone does not fully capture the dynamic nature of ER-NTD's conformational ensemble. To address this, we have made the following changes throughout the manuscript:

1. "potential secondary structural preferences" (page 4)
2. "more compact conformation" (page 6)
3. "residual structures with long-range interactions" (page 7)
4. "a more expanded NTD conformation" (page 8)

These revisions better reflect the dynamic, heterogeneous nature of ER-NTD conformational ensembles.

Why are the cross peaks in the newly obtained NMR spectra better resolved than previously obtained spectra (p 5)? The authors should explain.

The enhanced cross-peak resolution in our new NMR spectra stems from several factors: higher protein concentration (400 µM) and non-uniform sampling (NUS) for improved signal-to-noise ratio and resolution in indirect dimensions, respectively; use of a high-field spectrometer (850 MHz) for increased chemical shift dispersion; extended acquisition times (100 ms in both 1H and 15N dimensions); optimized pulse sequences and data processing methods; and meticulous sample preparation at 4°C. We have updated the text accordingly (page 4).

Word missing on p 8: ‘These results suggest that…arise from the formation of amino acid clusters’ Perhaps aromatic amino acid clusters?

We have fixed this oversight. The addition of 'aromatic' provides greater specificity and accuracy in describing the nature of the amino acid clusters involved.

In Fig. 2b do the authors have any idea why G57 moves differently between S118D and pS118?

The divergent behavior of G57 in S118D versus pS118 likely arises from their differing chemical properties. Phosphoserine (pS118) is larger and carries an additional negative charge compared to aspartate (S118D), potentially influencing local protein structure or interactions. While this explanation is plausible, further research is necessary to confirm these hypotheses, though such investigations are beyond the current scope.

In Fig. 2b, near residue 130 there is a dramatic change between R2/R1 ratios of WT and ps118. What is happening? Is it Y131? Can the authors connect this to Fig. 5a?

The elevated R2/R1 ratio for Y131 in the pS118 variant suggests additional microsecond-timescale conformational exchange, possibly linked to the chemical shift changes seen in Fig. 5a. This hints at a structural or dynamic connection between the phosphorylation site and this region. However, characterizing this phenomenon for a single residue is challenging and would require further experiments like CPMG relaxation dispersion. Additional studies are needed to understand this effect and its structural implications fully.

‘Fig. 9a’ on p 8 should be ‘Fig. S9a’.

We have fixed the incorrect figure reference.

‘Resonance reassignments’ should be ‘resonance assignments’

Fixed.

Fig. 2d: It is hard to see the IDP structure.

We have enhanced the clarity of the ER-NTD illustration.

Page 10: ‘reassignments’ should be ‘assignments’

Fixed.

Without a comparison to proteins outside the family, Fig. 6b does not demonstrate the significance of hydrophobic residues. The authors should consider swapping Fig. 6b with Fig. S18 and include the 16 proteins mentioned in the text.

We appreciate the reviewer's insightful suggestion. To address this, we have made the following changes:

1. Integrated former Supplementary Fig. S18 into the main text as the new Fig. 6b, presenting a comparative analysis of hydrophobic residue distribution.
2. Repositioned the original Fig. 6b to Fig. 6c to accommodate this change while maintaining compliance with space limitations for Extended Data Figures.

These modifications provide a more comprehensive comparison, demonstrating the significance of hydrophobic residues in nuclear receptors relative to other protein families.

Page 14, “These strategically…focused assessment of the impact of reduced hydrophobicity on ER function”: This does not seem to be the reason for choosing these mutations, as the authors have already mutated the phosphorylation site. Perhaps: “Residues F120 and L121 were mutated to alanine to reduce the hydrophobicity of the environment around S118, whilst in the presence of a S118A mutation.”

We appreciate the reviewer's insight. We agree that our initial statement was imprecise and have revised it to accurately reflect the rationale for these specific mutations.

Page 15, “These findings support the notion that the F120A… hydrophobic mechanism”: This is a circular argument, best rephrased.

We have rephrased it: "Both Ser118 phosphorylation and the F120A mutation reduce ER-TIF2 interactions, suggesting that alterations in local hydrophobicity can modulate coregulator binding.”

Clarify in figure legend of Fig. 5a that the plots are of the NTD variants (and not TIF2).

We appreciate the attention to detail. We have revised the Fig. 5a legend and the corresponding main text (page 16) to specify that the plots represent the chemical shifts of ER-NTD variants.

Page 20, “it is less expected to involve two separate regions within a disordered protein”: Rather than less expected, perhaps “there are yet to be many examples found of…” is more accurate.

Thanks for highlighting this critical point. We have revised the statement to reflect the current state of research more accurately. The updated phrasing now reads: “examples of phosphorylation affecting multiple regions within an IDP remain scarce.”

Could the authors address why targeting AR and MSI-1436 were not a challenge?

We appreciate the reviewer highlighting this critical point. We have revised our manuscript (page 19) to clarify the challenges of targeting IDPs by focusing on the exemplary case of AR-NTD. Developing effective small molecule modulators for AR-NTD required over a decade of intensive research following its initial identification. This extended timeline illustrates the complexities inherent in targeting IDPs.

“This revelation offers a new strategy that shifts the paradigm for designing small molecules…”: This is a great point; could the authors spell out what should be targeted and how it will be beneficial for therapeutic development?

Thanks for highlighting this point. We have updated the text (page 20) to clarify two potential strategies for targeting ER-NTD: (1) stabilizing the inactive conformation and (2) destabilizing the active/pS118 state. The revised passage now reads: "This hydrophobic-driven insight highlights a new strategy for designing small molecules specifically targeting the disordered ER-NTD, potentially modulating its conformation and preventing ER activation."

Author Rebuttal to First Revision:

We thank the reviewers for their constructive feedback and thoughtful evaluation of our manuscript. Below we address all comments, with reviewer remarks followed by our responses.

Referee #2:
The authors have properly addressed all my comments and have performed the vast majority of the demanded validations and changes. Therefore, in my opinion the study can be published as it is. I think that this is a very important contribution in the field of IDPs and IDP-regulated biological mechanisms.
We are grateful for the reviewer's thorough evaluation and endorsement of our study.

Referee #3:
Du et al. have performed extensive experiments to bolster their story, including SEC-SAXS on pS118, the inclusion of four point mutants, and two double mutants, and the inclusion of experiments in the presence of DDM. The text, figures, and captions now have increased clarity.
We appreciate the reviewer's assessment of our expanded experiments and manuscript improvements.

Only a few minor comments remain:
1) Pg 10, line 223: Fig. 1d should be Fig. S2d.
We have corrected this figure reference.

2) Fig. 1e error bars require explanation in the caption.
We have added the following to the Fig. 1e caption:
“Error bar: standard deviation propagated from R_1 and R_2 uncertainties.”

3) The authors should include the discussion of future CPMG relaxation dispersion experiments with respect to Y131 in the text.
We have added: “... to be explored by future CPMG measurements.” This addition acknowledges future dynamic characterization while maintaining focus on our current findings.

4) All NMR data (spectra and assignments) should be made open access.
We have deposited NMR assignments in BMRB (all will be made public upon publication): accession codes 51972 (wild-type), 51976 (S118D), and 51977 (pS118). Representative NMR spectra are provided in Fig. 1c, Fig. 2a, Extended Data Fig. 2a, Extended Data Fig. 5a, and Supplementary Figure 6.