nature portfolio

Peer Review File

Decoding phospho-regulation and flanking regions in autophagy-associated short linear motifs

Corresponding Author: Professor Elena Papaleo

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This paper presents a comprehensive study of phospho-regulated short linear motifs (SLiMs), using the Optineurin LC3 interacting region as a model system. The authors employed a robust toolkit that includes both computational approaches (molecular dynamics simulations with enhanced sampling techniques, in silico mutational scans, secondary structure analysis, and protein structure network analysis) and biophysical experiments (microfluidic diffusional sizing and surface plasmon resonance). This multi-faceted approach offers novel insights into the structural dynamics and binding properties of phospho-regulated SLiMs and establishes a versatile methodology that could be applied to other protein targets.

Issues:

The abstract refers to "Microfluidic Diffusional Sizing as a novel method to investigate binding affinities…". However, microfluidic diffusional sizing is already a well-established method for quantifying binding affinities. The only new aspect here is its application to SLiMs.

Fig. 1A: The structures of the NEMO-like and UBD domains are depicted as unusually long and perfectly straight helices. This seems more like an idealized AlphaFold prediction than a realistic model.

I am unable to reproduce the authors' calculation of k_off values from k_on and K_d. On line 507, the K_d is reported as 63.5 µM, and on line 509 the corresponding k_on is given as 0.55 x 10^4 M^-1 s^-1. Based on these numbers, I calculate the k_off value as: k_off = Kd x k_on = 63.5 *10^-6 M x 0.55 x 10^4 M^-1 s^-1 = 35 x 10^-2 s^-1. However, the authors report a value of 3.51 x 10^-2 s^-1. This order-of-magnitude discrepancy also appears in the K_d, k_on, and k_off values summarized in the table presented as Fig. 7A.

Fig. 7: There are significant discrepancies betweenthe experimental traces and the fits during the association phases of some SPR experiments (e.g., panel D). Can the authors explain these discrepancies? Since the k_on values are determined from these fits, any errors could also affect the k_off values, which are derived from k_on and K_d. This caveat should be addressed in the paper.

Overall, the writing style is clear and concise. However, the abundance of technical details and the tendency to present the arguments in a chronological rather than logical order occasionally make it difficult to grasp the principal goals and take-home messages.

Reviewer #3

(Remarks to the Author)
In this study, the authors combine a variety of computational methods with two complementary experimental methods to investigate the molecular details underlying the phospho-regulation of the OPTN LIR - LC3B interaction. Starting from experimental structures and AlphaFold models, they perform MD simulations of wild-type (WT), S177-phosphorylated (p1), and pentaphosphorylated (p5) OPTN LIRs bound to LC3B. Using available NMR data, they also verify the conformations generated in these simulations. They describe the molecular changes induced by phosphorylation in great detail and then use the generated conformational ensembles in a computational high-throughput screen. Here, they put great emphasis on

mutations, for which the effect depends on the phosphorylation state of the system. Finally, they test a few selected mutants via Microfluidic Diffusional Sizing (MDS) and Surface Plasmon Resonance (SPR). The paper is clearly structured and well written. The motivation of every section and its contribution to the "big picture" are understandable and easy to follow.

The core subject of the paper, understanding the nuanced regulation of SLiM interactions - both by post-translational modifications and flanking residues in general - is of great interest in multiple fields, with the study of LC3-LIR interactions being one of them. As the authors point out in the introduction, studying these interactions comes with severe challenges, so establishing and testing suited methodologies for the task is a very important contribution to the field. Given the high variety in SLiM residues outside the conserved core residues and the potential combinatorics coming from multiple PTM sites, approaches suitable for high-throughput are especially interesting. The work flow presented in the paper looks very promising in addressing these challenges, but there are some points that should (and some that could) be addressed before publication to further strengthen the manuscript.

Major points:
1) My main concern is that there is little overlap between the residues highlighted in the computational pipeline and the ones tested in experiments. E.g., the authors very nicely highlight some residues that are only important in the context of pentaphosphorylation, e.g., E163 and L166, but do not test those in experiments. That being said, I understand that there were technical problems with the pentaphosphorylated peptide, and I also don't think additional experiments are necessary. Though, as an optional experiment, showing that E163 and L166 residues do not make a difference to the binding affinity in WT and/or p1 constructs - as consistently and very convincingly demonstrated in the computational part - would further strengthen the point of the paper. What I would like to ask for, though, is a detailed analysis of the interactions of M183, one of the core-LIR-flanking residues mutated in the experiments, in the MD simulations, e.g., similar to what was done for E163 and L166 in Fig. 4 B/C. Optionally, such an analysis might also be interesting for some more residues associated with (potentially) disease-related mutations (as listed in Tab. 2).

Points of medium importance:
2) Some of the figure captions are either missing / do only contain the title (Supp. Figs. 1-3) or are possibly still from a previous version of the manuscript (Fig. 4, the caption only features panels A-D, but the figure itself contains A-G). They should be added/updated.

Minor points:
3) Results, lines 150-151 and Fig. 2A: The authors write that the chi squared values plateau after 200-300 ns. However, in the plot it looks like for some atom types plateaus are only reached after appr. 500 ns. Also, it would be good to include the data for the other replicas too, e.g. via a supplementary figure or table.
4) Results, lines 153-159: I wonder about potential reasons for the higher values for the isoleucin methyl group, especially since one ILE residue is part of the core OPTN LIR motif. Would comparing the MD ensembles with the NMR structures give additional insight?
5) Results, lines 173-174 and Fig. 2B/C: In the text it says "within ten and 100-ns time windows", which is also mentioned in the methods section, but in the figure there is only the data for the 100-ns windows. The data for 10 ns should be added or the text in the results/methods changed.
6) Results, line 183: None of the two references (25, 34) seem to discuss LIRs that are disordered in the bound state (but I may have overlooked something going through them).
7) Results, lines 306-310 and Figure S4: In the text, the authors write that neither p1 nor p5 modifications decrease flipping. However, in Fig. S4 it looks like for p1 the flipped state is even more favored (by several kcal/mol). This should be mentioned and discussed.
8) Results, lines 507-509 and Fig. 7A: There are multiple sub-points here:
8a) The k_on value in the text and the one in Fig. 7A are different
8b) An error estimate for the calculated k_off rates based on error propagation should be added.
8c) Is it possible that the k_off rates are off by one order of magnitude? I might be overlooking something, but please check this.
9) Methods, MD simulations: Some detailed information to replicate the simulations is missing, e.g., time step used, thermostat, cutoff-schemes, ...
10) Methods, lines 746-748: The authors say that they also performed reference calculations on the 2LUE PDB structures. I am not sure, though, if the results of these calculations can be found in the manuscript. If they are not, they should be added, and else a reference to where they can be found would be nice.
11) Figure 1A: It's not clear what the source for the shown structures is: Are they AlphaFold models (as hinted at in the text) or experimental? In the first case, pLDDT should be indicated, in the second case RCSB PDB IDs.
12) Figure 1C: Since this is an AlphaFold model, associated confidence metrics (pLDDT and PAE) should be shown.
13) Figure 4A: for E163-R37, the plot is cut off at the left side (I assume this comes from acPSN being 0 for WT and p1?).
14) Figure 5, lines 1256-1258: I am not sure I agree with the authors' conclusion that the importance of E175 and D176 is reduced by p5. The ddG values for them look very similar in all three cases. A more detailed explanation to support the claim by the authors may be needed.
15) Figure 5C: some of the x-axis labels are cut off.

(Potential) Typos:
i) line 301: are showed -> are shown
ii) line 545: showed -> shown
iii) line 991: reference 33 has two years associated with it
iv) Table 2, line E175Q: ddG binding p1 features a range of 1.0-145 kcal/mol. Is this correct or is there a "." missing

somewhere?
v) line 1219: is showed -> is shown
vi) line 1265: (D) S177D -> (D) S177E

Suggestions (nothing that needs to be addressed):
A) Results, lines 278-290: I think the explanation in this paragraph would benefit from a (supp.) figure panel showing the different interactions in the structure together with distance distributions from the simulations.
B) Figure 2D/E: Flipping the legend so that its order matches the one in the plots.

Reviewer #4

(Remarks to the Author)
The authors report on an in-depth investigation of the interaction between the human ATG8 protein LC3B and the selective autophagy receptor Optineurin (OPTN) applying MD simulations, in silico mutational scans and biophysical methods. Specifically, they show how phosphorylated serins in the LC3-interacting region of OPTN influence the flexibility, electrostatic interactions and solvent accessibility of the whole LIR, beyond the core residues, in complex with LC3B. They identify residues important for binding either both, the unphosphorylated and phosphorylated or exclusively one of the variants on LC3B and OPTN. Additionally, disease-relevant mutations of OPTN and their effect on binding the phosphorylated variant to LC3B were investigated. The computational studies are complemented by SPR and Microfluidic diffusional sizing for measuring the affinity between different OPTN LIR variants and LC3B, with the latter being a novel approach in the context of LIR-ATG8 interactions.
While this manuscript provides some new insight on the LC3B-OPTN interaction, which could certainly be useful for studying other phospho-regulated ATG8-LIR interactions, it has several shortcomings.
First, the manuscript appears to be of limited interest in the context of the wider research field. Considering that the interaction between the OPTN LIR and LC3B as well as the influence of phosphorylation has already been thoroughly studied (cited by the authors; Padman et al., 2019; Wild et al., 2011; Rogov et al., 2013), a broader investigation would be preferable. Specifically, it has been shown that OPTN (unphosphorylated) preferentially interacts with the LC3B paralog GABARAP (Rogov et al., 2017, DOI: 10.15252/embr.201643587). While it has been proposed that OPTN phosphorylation induces a switch in affinity toward LC3B, data investigating the affinity of phosphorylated OPTN toward GABARAP (& L1 and L2) is, at least to my knowledge, missing. As residues surrounding the core LIR are known to be important for selective binding, including GABARAP in the investigations (or at least in the discussion) seems logical. Additionally, it has been shown that the LIR of OPTN is also part of the FIP200 interacting region (FIR) of OPTN (Zhou et al., 2021; DOI: 10.1038/s41467-021-21874-1). The authors should at least consider this when discussing disease relevant mutations, as mutations of D176, S177 and F178 have also been shown to disrupt OPTN-FIP200 interaction.
Second, investigations described partially lack consistency in that it is not clear to the reader why certain residues identified in the computational studies were investigated in vitro and others were not (e.g. E163, L166).
Third, several parts of the main text and figure captions are not phrased clearly, limiting comprehensibility for the reader. Details/Examples of this are listed below.

Major comments:

1. Figures and captions are not very clear – e.g. partial captions and/or legends are missing:
• Figure 2: In 2B and C phosphorylated OPTN variants are not labelled clearly – no introduction of abbreviations p1 and p5. 2D and E should be clearly assigned to the respective complex variants and correct special characters should be used for secondary structure legend. Individual plots in 2F are not labelled.
• Figure 3 (& Figure S2 and S3): Caption and corresponding main text (line 200-216) are not clear. E.g. - What do the control values show? Which MD simulation replicates were used? What do the error bars show?
• Figure 4: caption of Figure 4 is missing the description for partial figures E-F. Descriptions for 4 B-D do not seem to match the depicted figures. Not clear which plot represents which variant in B and C.
• Figure 5: B-D – individual plots should be labelled with the corresponding complex variants. The plot in 5C appears to be cropped on the bottom.
• Figure S1: Missing label. Like 2D&E, Figure S1 should clearly indicate which plot represents which model.

2. Figure 1 shows and corresponding results section (line 121-138) show an overview of a published NMR complex structure and Alphafold database models but no new data. The text section appears redundant as more or less the same information is given in the methods section (line 600-619). Figure 1 would be more appropriate as supplemental material.

3. In Figure 2D and E (& Figure S1), the authors investigate secondary structure content of the Optineurin LIR variants in complex with LC3B. They claim increased β-strand propensity in the LIR binding pocket. This heading is somewhat misleading as it suggests structural changes of LC3B, even though they investigated these changes in Optineurin LIR when bound to LC3B. Additionally, while β-strand occurrence indeed slightly increases for F178, it decreases for I181 and R182. Strikingly, the changes in β-strand appear rather insignificant compared to other changes (e.g. α-helix content) between the Optineurin (un-)phosphorylated LIR variants. The authors should reconsider the text corresponding to these figures in the results (line 177-198) and discussion (563-565) sections.

4. Figure 5A and Figure 6A-D as well as the corresponding paragraphs in the main text show the same data. This should be

changed.

5. In line 349-356 the F178W mutation is discussed. An influence of this mutation has been experimentally shown, both in this manuscript and previously (Rogov et al., 2013). The authors should discuss why only structures from one MD replicate show an effect on binding free energy.

6. Some of the in vitro is not well fitted – making the reported Kd values somewhat unreliable (E.g. Fig. 6E, R2: 0.76). While it is good that they mention the limitations of the data in the text, they should also consider resorting to other methods for affinity measurements (e.g. BLI) to generate more reproducible data.

7. In the discussion (line 553-554), the authors claim to be the first to show kinetic measurements of natural ATG8-LIR interactions by SPR. SPR has been employed by others, some showing on- and off-rates and others Kd from steady-state analysis (e.g. Kwon et al., 2016, DOI: 10.1080/15548627.2016.1243199, Schwarten et al., 2009, DOI: 10.4161/auto.5.5.8494). Considering the limitations of the method mentioned by the authors (line 501-503) as well as the reproducibility issues with only one replicate shown (line 531), this claim appears inaccurate. Additionally, the sentence referring to SPR in the abstract (line 57-58) should be rephrased accordingly.

Minor comments:
1. Abbreviations are not consistently introduced when first mentioned (e.g. SASA, RMSF, MDS)
2. Hydrophobic pockets 1 and 2 (HP1 and HP2) are often referred to as HP1 pocket. 'Pocket' is already part of the abbreviation and simply referring to HP1 and HP2 would be correct.
3. Referring to the F178W OPTN LIR as a p62 mimicking LIR (e.g. line 350 & 446) seems outdated, as there are various W-type LIRs. Mutating F178 to W results in a WVEI core LIR, which resembles other LIRs (e.g. the NIX/BNIP3 LIR WVEL) more than the p62 LIR (WTHL).
4. The WT Kon value in Figure 7A (0.52 (M-1s-1)/104) does not match the value in the main text (line 509, 0.55 (M-1s-1)/104).
5. Figure 2A and Table 1 compare MD simulations with published NMR data, thereby validating the applied method rather than showing new results. While this supports the presented data, it might be more appropriate to move this data to the appendix to avoid taking the focus away from new results.
6. In line 206, the authors refer to F178 and I181 of the Optineurin LIR as HP1 and HP2 residues. This is misleading and should be rephrased – e.g. to "residues occupying HP1 and HP2 respectively".
7. Check References. Information referring to reference #63 in line 273-275 could not be found in the publication.
8. Units should be included in supplementary tables where applicable.
9. Line 88: "components of the autophagy machinery" should be changed to ATG8 or LC3/GABARAP as these are the components the authors refer to.
10. Line 90-91 suggest that the core LIR residues do not contribute to paralog specificity. This is not true, see for example Rogov et al., 2017, DOI: 10.15252/embr.201643587.
11. Line 98-100: "…mechanism in which LC3 proteins recruit autophagy factors to drive the autophagosome growth and amplify selective autophagy in an LC3-dependent feedback loop". The second part of the sentence appears redundant.
12. Line 238-239: "It also identifies L166 as another site in the N-terminal flanking region of OPTN that is switched on by phosphorylation". This sounds like L166 is phosphorylated. Should be rephrased accordingly.
13. Line 455-456: "Our findings suggest that the MDS binding assay can identify and validate predicted binding affinity changes exceeding 2 kcal/mol." Is this true? Clear difference between WT OPTN and F178W OPTN can be seen in the MDS (Fig 6 A,E,I). Fig 5B and Table S5 suggest lower values. This should be clarified.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have properly addressed all reviewer comments in their revised manuscript and have clearly responded to all requests. They have provided additional experimental data, clarified previous ambiguities, and corrected critical errors. I recommend the revised manuscript for publication in its present form.

Reviewer #3

(Remarks to the Author)
With the additional analysis and experiments, as well as changes to the text and figures, the authors have done a good job addressing my concerns.

A few remaining, very minor points:
1) line 258: Figure S4A-C -> Figure S4A-D, since the text refers to all 4 residues
2) lines 412-415: Data supporting the claim that R37 only tolerates mutation to K seems to be missing. Or are the statements inferred from the data in Fig. 4? This should me made more clear.

3) Line 539, Fig. 7A: Order of magnitude of k_on is 10^3 in the text but 10^-3 in the figure. For k_off, orders agree between text and figure.
4) Supplementary figures 1-3 still (and new figure S4) seem to be missing captions. Or are they in a different file?

Reviewer #4

(Remarks to the Author)
The revised manuscript is much clearer, and the authors addressed most of my remarks. However, I still have several questions/suggestions regarding main text and figures.

Main Text:
• While the authors have slightly modified the wording regarding their use of experimental methods (SPR and MDS) to determine affinity and kinetics, describing it as novel seems inadequate to me (at least for SPR). It is true that many studies focus on steady state, due to rapid on-off kinetics that the authors of this study also faced, however, applying SPR to study LIR-LDS interaction is not uncommon and there are also examples of selective autophagy receptor LIRs with ATG8 proteins (e.g. Stephani et al., 2020, https://doi.org/10.7554/eLife.58396 & Fumagalli et al., 2016, https://doi.org/10.1038/ncb3423).

“While direct kinetic measurements of LIR interactions remain limited due to their rapid on-off kinetics, some SPR-based studies81–83 have explored both steady-state and kinetic parameters for LC3B interactions - though none specifically focused on autophagy receptors.”
“Moreover, we have generated novel structural 18 insights including binding affinity and size measurements by Microfluidic Diffusional Sizing and kinetic measurements by Surface Plasmon Resonance, both first in the context of natural autophagy receptor LIR-ATG8 interactions.”
• I am missing a reference for the following sentence:
“In contrast, the OPTN LIR on-rate aligns with the one measured for the natural cysteine protease ATG4B LIR’s interaction with LC3B, while the six-fold lower affinity of the autophagy receptor OPTN is largely owing to its increased off-rate.”

• The text referring to Figure S5 is not consistent/clear. The following sentences should be rephrased accordingly.
- “Multiple phosphorylations do not decrease the flipping of F178”
- “..five phosphorylation can decrease the flipping of F178 outside the HP1 of LC3B”
- ”…the multiple phosphorylation cannot rescue the flipping movement of the OPTN phenylalanine residue in the HP1 of LC3B”

Figure 1:
• I find the visualization of the AlphaFold model not ideal as the cartoon representation is simply overlaying the surface, implying the LC3B cartoon to be in front of the optineurin surface. I believe removing the Optineurin surface and making the LC3B surface semi-transparent would make the figure more coherent.
• Caption should clarify that depicted model is trimmed while PAE matrix refers to model of full-length proteins
Figure 3:
• I am missing an explanation on why the control behaves differently than the actual runs (especially evident for D48 accessibility with phospho-variants)
Figure 4/S4:
• Depiction of the atomic contacts with the respective frequencies could be clearer. Due to different number of contacts for each peptide (why are additional ones with 0 occurrence shown?), the residues are not depicted next to each other in the same row. This makes it harder to see differences between the peptides.
Figure 5/6:
• While I understand that keeping the MDS measurements together in Figure 6 is helpful, restructuring the text in a more logical rather than chronological order as mentioned by reviewer 1, would be preferable over showing the same panel twice.

Figure S5:
• X-axis should be in Angstrom, as the main text refers to the distances in Angstrom.

Minor remarks
• “However, the multiple phosphorylation cannot rescue the flipping movement of the OPTN phenylalanine residue in the HP1 of LC3B.” should be ‘multiple phosphorylations’
• “Phosphorylation of OPTN is important to regulate its cellular function, and the structural changes it induces should are integral to understanding the..” The ‘should’ is too much.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have addressed all my comments and improved the manuscript by rephrasing critical parts.

We would like to thank the Editor and Reviewer for the useful and attentive criticisms. Our answers are reported below point by point for sake of clarity and the page numbers refer to the document with track changes.

SPECIFIC COMMENTS FROM EDITOR

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a revised manuscript.

In particular, please note that the following revisions would be necessary for us to contact our referees again:

-If possible, please carry out an experimental test of the E162 and L166 residues, as highlighted by R3 and R4, or alternatively discuss why this was not possible.

We have now included these experiments, unfortunately they provided limited additional value as discussed in the answers below and in the main text and Figure S6

-Please, carry out a detailed analysis of the interactions of M183 as requested by R3.

We have now included a detailed analysis as request and addressed the point by reviewer 3

-Both R1 and R4 seem concerned about the Kd values, which were determined from data that is not well fit. If possible, please use another method for the affinity measurements, or alternatively address the limitations. Also please check the values are correct as pointed out by R1.

We have corrected the figure and text and explained in detail the source of the problem in the response below to the reviewers. It was mostly an error in the figure due to a typos which created confusion on the estimates from SPR.

-Please correct the figure captions appropriately.
-Please tone down the statements highlighted by the reviewers.

We have revised all these points

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This paper presents a comprehensive study of phospho-regulated short linear motifs (SLiMs), using the Optineurin LC3 interacting region as a model system. The authors employed a robust toolkit that includes both computational approaches (molecular dynamics simulations with enhanced sampling techniques, in silico mutational scans, secondary structure analysis, and protein structure network analysis) and biophysical experiments (microfluidic diffusional

sizing and surface plasmon resonance). This multi-faceted approach offers novel insights into the structural dynamics and binding properties of phospho-regulated SLiMs and establishes a versatile methodology that could be applied to other protein targets.

Issues:

The abstract refers to "Microfluidic Diffusional Sizing as a novel method to investigate binding affinities…". However, microfluidic diffusional sizing is already a well-established method for quantifying binding affinities. The only new aspect here is its application to SLiMs.

We have revised the sentence in the abstract at page 2 to avoid this confusion

Fig. 1A: The structures of the NEMO-like and UBD domains are depicted as unusually long and perfectly straight helices. This seems more like an idealized AlphaFold prediction than a realistic model.

We appreciate the reviewer's comment. To clarify the presentation, we have removed the AlphaFold model from panel A. Additionally, in panel C, we have color-coded the AlphaFold multimer model based on its pLDDT score and included its PAE matrix. We have revised the corresponding results section (page 4) and the Figure 1 caption to ensure consistency and clarity.

*I am unable to reproduce the authors' calculation of k_off values from k_on and K_d. On line 507, the K_d is reported as 63.5 µM, and on line 509 the corresponding k_on is given as 0.55 x 10^4 M^-1 s^-1. Based on these numbers, I calculate the k_off value as: k_off = Kd x k_on = 63.5 *10^-6 M x 0.55 x 10^4 M^-1 s^-1 = 35 x 10^-2 s^-1. However, the authors report a value of 3.51 x 10^-2 s^-1. This order-of-magnitude discrepancy also appears in the K_d, k_on, and k_off values summarized in the table presented as Fig. 7A.*

We thank the reviewer for bringing this to our attention. There was an error in **Table 7A**, where the reported *k_on* values were incorrectly listed as 10^4 $M^{-1}s^{-1}$ instead of 10^3 $M^{-1}s^{-1}$. This discrepancy affected the calculation of *k_off* values when using the originally reported *k_on* rates. However, the *k_off* values themselves were correct.

We have now corrected **Table 7A** to reflect the accurate *k_on* values, and we have updated the text accordingly at pages 16-17 to ensure consistency throughout the manuscript.

Fig. 7: There are significant discrepancies between the experimental traces and the fits during the association phases of some SPR experiments (e.g., panel D). Can the authors explain these discrepancies? Since the k_on values are determined from

these fits, any errors could also affect the k_off values, which are derived from k_on and K_d. This caveat should be addressed in the paper.

We thank the reviewer for this observation. We acknowledge that some association phases in the SPR sensorgrams show deviations from the idealized model. This is primarily due to the rapid on-off kinetics of the interaction and the instrument's detection limitations. To address this, we have added an explanation in the text discussing the limitations of the Biacore X100 instrument, particularly regarding the k_off values exceeding its measurement capabilities and k_on values being near the detection limit. Despite these challenges, our global fitting approach remains a robust method for extracting kinetic parameters, as it is standard for interactions with fast kinetics. We relied on measured k_on and steady-state K_d values (corroborated by MDS measurements) to derive k_off rates. Furthermore, the steady-state K_d obtained from SPR aligns well with both MDS-derived values and previous literature, supporting the reliability of our analysis. This discussion has been incorporated into the manuscript (page 16) to clarify the observed discrepancies and ensure transparency regarding the limitations of the experimental setup.

Overall, the writing style is clear and concise. However, the abundance of technical details and the tendency to present the arguments in a chronological rather than logical order occasionally make it difficult to grasp the principal goals and take-home messages.

We have revised different parts of the text to try to avoid technical details in the results section (pages 4 to 17).

Reviewer #3 (Remarks to the Author):

In this study, the authors combine a variety of computational methods with two complementary experimental methods to investigate the molecular details underlying the phospho-regulation of the OPTN LIR - LC3B interaction. Starting from experimental structures and AlphaFold models, they perform MD simulations of wild-type (WT), S177-phosphorylated (p1), and pentaphosphorylated (p5) OPTN LIRs bound to LC3B. Using available NMR data, they also verify the conformations generated in these simulations. They describe the molecular changes induced by phosphorylation in great detail and then use the generated conformational ensembles in a computational high-throughput screen. Here, they put great emphasis on mutations, for which the effect depends on the phosphorylation state of the system. Finally, they test a few selected mutants via Microfluidic Diffusional Sizing (MDS) and Surface Plasmon Resonance (SPR). The paper is clearly structured and well written. The motivation of every section and its contribution to the "big picture" are understandable and easy to follow.

The core subject of the paper, understanding the nuanced regulation of SLiM interactions - both by post-translational modifications and flanking residues in general

- is of great interest in multiple fields, with the study of LC3-LIR interactions being one of them. As the authors point out in the introduction, studying these interactions comes with severe challenges, so establishing and testing suited methodologies for the task is a very important contribution to the field. Given the high variety in SLiM residues outside the conserved core residues and the potential combinatorics coming from multiple PTM sites, approaches suitable for high-throughput are especially interesting. The work flow presented in the paper looks very promising in addressing these challenges, but there are some points that should (and some that could) be addressed before publication to further strengthen the manuscript.

Major points:
1) My main concern is that there is little overlap between the residues highlighted in the computational pipeline and the ones tested in experiments. E.g., the authors very nicely highlight some residues that are only important in the context of pentaphosphorylation, e.g., E163 and L166, but do not test those in experiments. That being said, I understand that there were technical problems with the pentaphosphorylated peptide, and I also don't think additional experiments are necessary. Though, as an optional experiment, showing that E163 and L166 residues do not make a difference to the binding affinity in WT and/or p1 constructs - as consistently and very convincingly demonstrated in the computational part - would further strengthen the point of the paper.

We thank the reviewer for this insightful comment. We acknowledge the limited overlap between the computationally highlighted residues and those tested experimentally. To address this, we tested a longer peptide (OPTN 160-185) that included E163 and L166. However, these constructs led to reduced binding affinity, likely due to increased peptide flexibility affecting interaction stability. We add a new figure of the MDS result as a new supplementary result (Figure S6) and address it in the main article at page 14.

What I would like to ask for, though, is a detailed analysis of the interactions of M183, one of the core-LIR-flanking residues mutated in the experiments, in the MD simulations, e.g., similar to what was done for E163 and L166 in Fig. 4 B/C. Optionally, such an analysis might also be interesting for some more residues associated with (potentially) disease-related mutations (as listed in Tab. 2).

We appreciate the reviewer's suggestion to provide a detailed analysis of the interactions of M183 in the MD simulations. Following this recommendation, we have added a new panel (Figure 4D) that presents the contact analysis for M183, analogous to the analysis performed for E163 and L166 in Figure 4B/C. Furthermore, to address the reviewer's optional request, we have also included a supplementary figure (Figure S4) displaying the results for additional residues associated with disease-related

mutations that are predicted to be pathogenic by EVE or AlphaMissense (E175, D176, F178 and V179). We addressed this analysis at page 8 of the manuscript.

Points of medium importance:
2) Some of the figure captions are either missing / do only contain the title (Supp. Figs. 1-3) or are possibly still from a previous version of the manuscript (Fig. 4, the caption only features panels A-D, but the figure itself contains A-G). They should be added/updated.

We appreciate the reviewer's feedback. We have added the missing captions for supplementary figures S1–S3. Additionally, the caption for Figure 4 was from a previous version of the manuscript, and we have now updated it to reflect the current version of the figure.

Minor points:
3) Results, lines 150-151 and Fig. 2A: The authors write that the chi squared values plateau after 200-300 ns. However, in the plot it looks like for some atom types plateaus are only reached after appr. 500 ns. Also, it would be good to include the data for the other replicas too, e.g. via a supplementary figure or table.

We have now revised the text at page 5 so that it better reflects the atom types with a plateau at 500 ns. We have included in the OSF repository the data from these analyses including the plots for the other three replicates (https://osf.io/6rmeb/files/osfstorage - refer to the folder chemical_shift_analysis). We have clarified in the Materials and Methods at page 22 where to find the plots in OSF.

4) Results, lines 153-159: I wonder about potential reasons for the higher values for the isoleucin methyl group, especially since one ILE residue is part of the core OPTN LIR motif. Would comparing the MD ensembles with the NMR structures give additional insight?

The side chain experimental chemical shifts have been assigned only for the LC3B protein and not the peptide. We have now added a table in the OSF repository (ILE_chemical_shifts_Cdelta_ILE_CD.csv) with the comparison between the experimental and predicted chemical shifts for all the Cδ atoms of each isoleucine of LC3B and discussed the reasons for discrepancies in the text at page 5. In brief, the high values are due to the fact that during the MD simulations also the trans states of the chi2 dihedral angle of ILE34 and 67 are sampled, whereas according to the experimental chemical shifts the two side chains should only sample the minus states. The two residues are not in direct contact with the peptide so this behaviour is not expected to compromise our analyses.

5) Results, lines 173-174 and Fig. 2B/C: In the text it says "within ten and 100-ns time windows", which is also mentioned in the methods section, but in the figure there is only the data for the 100-ns windows. The data for 10 ns should be added or the text in the results/methods changed.

We have now corrected the text at page 6 since it was a typo and we only provided the data with the 100-ns windows, as the reviewer noticed.

6) Results, line 183: None of the two references (25, 34) seem to discuss LIRs that are disordered in the bound state (but I may have overlooked something going through them).

We thank the reviewer for pointing this out. Upon re-evaluation of the cited references, we agree that they do not provide conclusive evidence supporting the presence of disordered LIRs in the bound state. We have thus removed the corresponding statement from the manuscript and rephrased the paragraph at page 6 accordingly to reflect only experimentally supported structural features of LIRs upon binding.

7) Results, lines 306-310 and Figure S4: In the text, the authors write that neither p1 nor p5 modifications decrease flipping. However, in Fig. S4 it looks like for p1 the flipped state is even more favored (by several kcal/mol). This should be mentioned and discussed.

We have revised the text to better explain what is happening with p1 in the enhanced sampling simulation and revised the caption of the old Figure S4 (current Figure S5), accordingly. The text can be found at page 10 of the manuscript.

8) Results, lines 507-509 and Fig. 7A: There are multiple sub-points here:
8a) The k_on value in the text and the one in Fig. 7A are different

We thank the reviewer for pointing this out. The discrepancy was due to an error in Figure 7A, which has now been corrected to match the values reported in the text. The revised figure ensures consistency across the manuscript.

8b) An error estimate for the calculated k_off rates based on error propagation should be added.

We thank the reviewer for this suggestion. We have now included error estimates for the calculated *k_off* values based on error propagation, using the experimental uncertainties for *K_d* and *k_on*. These updated values are now reflected in the corrected Figure 7A, and the text has been revised accordingly on pages 16 to 17.

8c) Is it possible that the k_off rates are off by one order of magnitude? I might be overlooking something, but please check this.

We thank the reviewer for bringing this to our attention. There was an error in Table 7A, where the reported k_on values were incorrectly listed as 10^4 $M^{-1}s^{-1}$ instead of 10^3 $M^{-1}s^{-1}$. This discrepancy affected the calculation of k_off values when using the originally reported k_on rates. However, the k_off values themselves were correct. We have now corrected Table 7A to reflect the accurate k_on values, and we have updated the text accordingly on pages 16 to 17 to ensure consistency throughout the manuscript.

9) Methods, MD simulations: Some detailed information to replicate the simulations is missing, e.g., time step used, thermostat, cutoff-schemes, …

We have added the missing details at pages 20 and 21 in the Method Section for the MD simulations.

10) Methods, lines 746-748: The authors say that they also performed reference calculations on the 2LUE PDB structures. I am not sure, though, if the results of these calculations can be found in the manuscript. If they are not, they should be added, and else a reference to where they can be found would be nice.

We thank the reviewer for bringing this to our attention. We added the data and the analysis of the 2LUE 20 conformers to the OSF repository and mentioned in the text at page 24, they can be found in the folder called nmr_mutatex_analysis in https://osf.io/6rmeb/files/osfstorage#

11) Figure 1A: It's not clear what the source for the shown structures is: Are they AlphaFold models (as hinted at in the text) or experimental? In the first case, pLDDT should be indicated, in the second case RCSB PDB IDs.
12) Figure 1C: Since this is an AlphaFold model, associated confidence metrics (pLDDT and PAE) should be shown.

We appreciate the reviewer's comment. We confirm that model from panel A was indeed an AlphaFold model. To clarify the presentation, we have removed the AlphaFold model from panel A. Additionally, in panel C, we have color-coded the AlphaFold Multimer model based on its pLDDT score and included its PAE matrix. We have revised the corresponding results section at page 4 and the Figure 1 caption to ensure consistency and clarity.

13) Figure 4A: for E163-R37, the plot is cut off at the left side (I assume this comes from acPSN being 0 for WT and p1?).

As the reviewer correctly noted, the plot is cut off on the left side because the acPSN for $LC3B_{1-120}$-$OPTN_{158-191}$ and $LC3B_{1-120}$-$p1OPTN_{158-191}$ were indeed 0. We have revised the plotting script to properly account for 0 values and have updated Figure 4A accordingly.

14) Figure 5, lines 1256-1258: I am not sure I agree with the authors' conclusion that the importance of E175 and D176 is reduced by p5. The ddG values for them look very similar in all three cases. A more detailed explanation to support the claim by the authors may be needed.

We thank the reviewer for the insightful comment. We agree that the data in the heatmaps do not clearly support a reduced importance of E175 and D176 upon p5 phosphorylation. Accordingly, we have removed this claim from the Figure 5 caption to avoid overinterpretation of the results.

15) Figure 5C: some of the x-axis labels are cut off.

We have revised Figure 5C to fix the axis labels

(Potential) Typos:
i) line 301: are showed -> are shown
ii) line 545: showed -> shown
iii) line 991: reference 33 has two years associated with it
iv) Table 2, line E175Q: ddG binding p1 features a range of 1.0-145 kcal/mol. Is this correct or is there a "." missing somewhere?
v) line 1219: is showed -> is shown
vi) line 1265: (D) S177D -> (D) S177E

We have now fixed these typos in the main text

Suggestions (nothing that needs to be addressed):

A) Results, lines 278-290: I think the explanation in this paragraph would benefit from a (supp.) figure panel showing the different interactions in the structure together with distance distributions from the simulations.

This part is now at page 10. We have included all the distance distribution data in the OSF repository and mentioned in the text, they can be found in the folder called R11_distances in https://osf.io/6rmeb/files/osfstorage#

B) Figure 2D/E: Flipping the legend so that its order matches the one in the plots.

We thank the reviewer for their feedback. We have revised Figure 2 by improving the labeling throughout and rearranging panels E and F to better align with the order of presentation in the text, ensuring a more logical flow.

Reviewer #4 (Remarks to the Author):

The authors report on an in-depth investigation of the interaction between the human ATG8 protein LC3B and the selective autophagy receptor Optineurin (OPTN) applying MD simulations, in silico mutational scans and biophysical methods. Specifically, they show how phosphorylated serins in the LC3-interacting region of OPTN influence the flexibility, electrostatic interactions and solvent accessibility of the whole LIR, beyond the core residues, in complex with LC3B. They identify residues important for binding either both, the unphosphorylated and phosphorylated or exclusively one of the variants on LC3B and OPTN. Additionally, disease-relevant mutations of OPTN and their effect on binding the phosphorylated variant to LC3B were investigated. The computational studies are complemented by SPR and Microfluidic diffusional sizing for measuring the affinity between different OPTN LIR variants and LC3B, with the latter being a novel approach in the context of LIR-ATG8 interactions.
While this manuscript provides some new insight on the LC3B-OPTN interaction, which could certainly be useful for studying other phospho-regulated ATG8-LIR interactions, it has several shortcomings.
First, the manuscript appears to be of limited interest in the context of the wider research field. Considering that the interaction between the OPTN LIR and LC3B as well as the influence of phosphorylation has already been thoroughly studied (cited by the authors; Padman et al., 2019; Wild et al., 2011; Rogov et al., 2013), a broader investigation would be preferable. Specifically, it has been shown that OPTN (unphosphorylated) preferentially interacts with the LC3B paralog GABARAP (Rogov et al., 2017, DOI: 10.15252/embr.201643587). While it has been proposed that OPTN phosphorylation induces a switch in affinity toward LC3B, data investigating the affinity of phosphorylated OPTN toward GABARAP (& L1 and L2) is, at least to my knowledge, missing. As residues surrounding the core LIR are known to be important for selective binding, including GABARAP in the investigations (or at least in the discussion) seems logical. Additionally, it has been shown that the LIR of OPTN is also part of the FIP200 interacting region (FIR) of OPTN (Zhou et al., 2021; DOI: 10.1038/s41467-021-21874-1). The authors should at least consider this when discussing disease relevant mutations, as mutations of D176, S177 and F178 have also been shown to disrupt OPTN-FIP200 interaction.

We thank the reviewer for this insightful and constructive comment. We fully agree that the OPTN LIR motif operates in a broader biological context beyond the LC3B interaction alone. In particular, we acknowledge that unphosphorylated OPTN preferentially interacts with GABARAP family members, as previously shown (Rogov et al., 2017), and that phosphorylation may shift its binding preference toward LC3B. Although our study focused on LC3B due to the availability of high-resolution structural

and biophysical data enabling validation of computational predictions, we recognize the importance of exploring the interactions of phosphorylated OPTN with other ATG8 paralogs. We have now added a discussion of this point in the revised manuscript at pages 18-19 to more clearly situate our work within the broader context of selective autophagy. Additionally, we appreciate the reviewer highlighting the functional overlap between the LIR and the FIP200-interacting region (FIR) in OPTN. We have now expanded the discussion of disease-relevant mutations (D176, S177, F178) to consider their dual impact on both LC3B and FIP200 binding. The relevant paragraph in the Discussion at page 19 has been updated accordingly.

Second, investigations described partially lack consistency in that it is not clear to the reader why certain residues identified in the computational studies were investigated in vitro and others were not (e.g. E163, L166).

We thank the reviewer for this insightful comment. We acknowledge the limited overlap between the computationally highlighted residues and those tested experimentally. To address this, we tested a longer peptide (OPTN 160-185) that included E163 and L166. However, these constructs led to reduced binding affinity, likely due to increased peptide flexibility affecting interaction stability. We added a new figure of the MDS result as a new supplementary result (Figure S6) and addressed it in the main article at page 14.

Third, several parts of the main text and figure captions are not phrased clearly, limiting comprehensibility for the reader. Details/Examples of this are listed below.
Major comments:

1. Figures and captions are not very clear – e.g. partial captions and/or legends are missing:
• Figure 2: In 2B and C phosphorylated OPTN variants are not labelled clearly – no introduction of abbreviations p1 and p5. 2D and E should be clearly assigned to the respective complex variants and correct special characters should be used for secondary structure legend. Individual plots in 2F are not labelled.

We have included in the figure caption the explanation of p1 and p5 for clarity. We would like to point out that what p1 and p5 stands for were explained in the text originally for the reader but we understand that it is better to repeat in the caption too. We have left the secondary structure classes according to the DSSP dictionary in the figure since this is what has been used for the analyses and this was explained in the figure caption to avoid confusion to the reader. Finally, we increased the legend font size in Figures 2B and 2C, added system labels to Figures 2D and 2E, and included a legend for Figure 2F.

• *Figure 3 (& Figure S2 and S3): Caption and corresponding main text (line 200-216) are not clear. E.g. - What do the control values show? Which MD simulation replicates were used? What do the error bars show?*

We thank the reviewer for pointing this out. We have revised Figures S2 and S3, and updated their captions as well as the caption for Figure 3 to include a clearer explanation of the relative solvent-accessible surface area (SASA) analysis. The updated captions now specify the systems analyzed, clarify the nature and purpose of the control simulations, and explain the meaning of the error bars.

• *Figure 4: caption of Figure 4 is missing the description for partial figures E-F. Descriptions for 4 B-D do not seem to match the depicted figures. Not clear which plot represents which variant in B and C.*

We appreciate the reviewer's feedback. The caption for Figure 4 was from an earlier version of the manuscript, and we have now updated it to match the current figure. Additionally, we have revised Figure 4B–D by adding labels to indicate which of the simulations is represented in the plot, ensuring clarity and avoiding confusion.

• *Figure 5: B-D – individual plots should be labelled with the corresponding complex variants. The plot in 5C appears to be cropped on the bottom.*

We thank the reviewer for their feedback. We added a label to clearly indicate which system is represented in Figure 5B-D and fixed the issue with the cropped x axis label of Figure 5C.

• *Figure S1: Missing label. Like 2D&E, Figure S1 should clearly indicate which plot represents which model.*

We added the label regarding the system shown in the plot for Figure S1.

2. Figure 1 shows and corresponding results section (line 121-138) show an overview of a published NMR complex structure and Alphafold database models but no new data. The text section appears redundant as more or less the same information is given in the methods section (line 600-619). Figure 1 would be more appropriate as supplemental material.

We thank the reviewer for their feedback. We would like to clarify that the structure shown in Figure 1 is not an NMR structure but an AlphaFold model. To enhance clarity, we have made the following modifications to Figure 1: we removed the AlphaFold

model from panel A, and in panel C, we color-coded the AlphaFold- Multimer model based on its pLDDT score and included the PAE matrix. We believe that Figure 1 is essential for providing a comprehensive overview of the OPTN–LC3B complex, including the AlphaFold multimer model selected for our simulations. This context is crucial for understanding the rationale behind our computational approach. We have revised the corresponding results section at page 4 and the Figure 1 caption to ensure consistency and clarity.

3. In Figure 2D and E (& Figure S1), the authors investigate secondary structure content of the Optineurin LIR variants in complex with LC3B. They claim increased β-strand propensity in the LIR binding pocket. This heading is somewhat misleading as it suggests structural changes of LC3B, even though they investigated these changes in Optineurin LIR when bound to LC3B.

We have modified the text to avoid this confusion both in the figure 2 caption and in the main text at page 6

Additionally, while β-strand occurrence indeed slightly increases for F178, it decreases for I181 and R182. Strikingly, the changes in β-strand appear rather insignificant compared to other changes (e.g. α-helix content) between the Optineurin (un-)phosphorylated LIR variants. The authors should reconsider the text corresponding to these figures in the results (line 177-198) and discussion (563-565) sections.

We acknowledge that the β-strand content at I181 indeed decreases already in the mono-phosphorylated variant, while R182 shows a slight increase. In the penta-phosphorylated form, both residues exhibit reduced β-strand propensity. We have now revised the corresponding text in both the Results section at page 6 and in the Discussion sections at page 18 to more accurately reflect these observations. Furthermore, as the reviewer rightly points out, changes in β-strand propensity are relatively subtle. We have emphasized that the most significant structural rearrangements upon multiple phosphorylation are observed in α-helical content: a clear decrease in helicity in the N-terminal region preceding the core LIR (residues 160–168) and a pronounced increase between residues 172–176. These shifts likely contribute more substantially to the altered binding behavior of the penta phosphorylated variant. We appreciate the opportunity to clarify these aspects and have updated the manuscript accordingly.

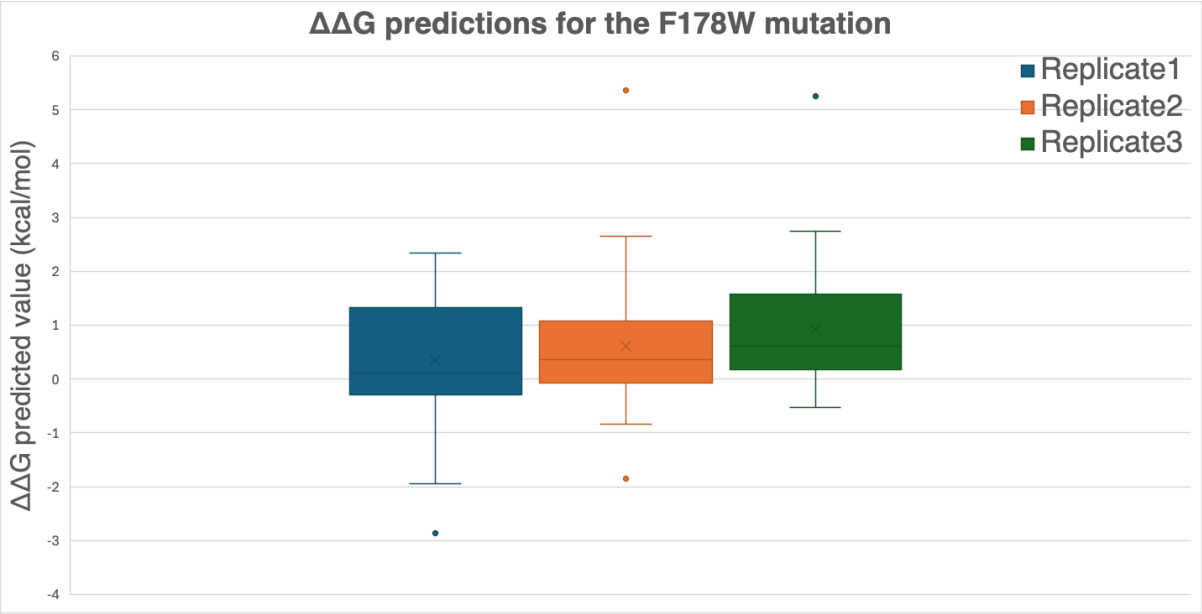
4. Figure 5A and Figure 6A-D as well as the corresponding paragraphs in the main text show the same data. This should be changed.

We have designed the figures like this on purpose to help the reader in understanding the different sections and the integration between the experimental and computational data. We felt during the writing that we needed this kind of visual support ourselves

and the strategy was also validated by other colleagues in our group so we would rather prefer to keep it as it stands now.

5. In line 349-356 the F178W mutation is discussed. An influence of this mutation has been experimentally shown, both in this manuscript and previously (Rogov et al., 2013). The authors should discuss why only structures from one MD replicate show an effect on binding free energy.

We thank the reviewer for the helpful comment. Upon re-evaluating the data, we found that only two structures from replicate 1 and one from replicate 2 exhibited a predicted ΔΔG < -1 kcal/mol for the F178W mutation (see plot at the end of this paragraph; the plot and the data represented in it are available on the OSF repository here: https://osf.io/6rmeb/files/osfstorage# in the folder analysis_F178W). Therefore, the original sentence suggesting that "some structures from replicate 2" showed a stabilizing effect was imprecise and has been corrected in the manuscript (page 11). We believe that the absence of a consistent stabilizing signal across the ensemble may be due to limitations of the FoldX energy function, which is known to be less accurate in predicting stabilizing mutations (see for example Buß et al, 2018 doi: 10.1186/s12859-023-05537-0).



6. Some of the in vitro is not well fitted – making the reported Kd values somewhat unreliable (E.g. Fig. 6E, R2: 0.76). While it is good that they mention the limitations of the data in the text, they should also consider resorting to other methods for affinity measurements (e.g. BLI) to generate more reproducible data.

We appreciate the reviewer's comment regarding the quality of the binding curve in Fig. 6E (F178W variant, $R^2 = 0.76$). We agree that the data for this particular variant show a less ideal fit. However, we would like to clarify that this dataset was not cherry-picked—it was in fact the very first peptide we tested using MDS, and we have retained it in the figure for completeness and transparency. The F178W variant serves primarily as a control in our study, as its enhanced affinity has been thoroughly characterized in previous work by ITC (as cited in the main text, Rogov 2013 10.1042/BJ20121907). The K_d we observe is in line with these earlier findings, supporting the overall consistency of our data even if the individual fit is not perfect. We believe that in this case, the existing literature provides a strong foundation for interpreting the binding affinity of this variant, and thus additional biophysical measurements such as BLI are not strictly necessary for this specific control.

7. In the discussion (line 553-554), the authors claim to be the first to show kinetic measurements of natural ATG8-LIR interactions by SPR. SPR has been employed by others, some showing on- and off-rates and others Kd from steady-state analysis (e.g. Kwon et al., 2016, DOI: 10.1080/15548627.2016.1243199, Schwarten et al., 2009, DOI: 10.4161/auto.5.5.8494). Considering the limitations of the method mentioned by the authors (line 501-503) as well as the reproducibility issues with only one replicate shown (line 531), this claim appears inaccurate. Additionally, the sentence referring to SPR in the abstract (line 57-58) should be rephrased accordingly.

We thank the reviewer for pointing out these earlier studies. We have now revised the relevant sections in the Results and Discussion Sections at pages 16 and 18, to appropriately acknowledge the prior work, with specific emphasis on Kwon et al., 2016. While Schwarten et al., 2009 indeed reports steady-state K_d values derived from SPR, we note that it does not include kinetic rate constants (on- and off-rates).

Minor comments:
1. Abbreviations are not consistently introduced when first mentioned (e.g. SASA, RMSF, MDS)

We have revised the manuscript so that the abbreviations are introduced the first time that they appear in the main text

2. Hydrophobic pockets 1 and 2 (HP1 and HP2) are often referred to as HP1 pocket. 'Pocket' is already part of the abbreviation and simply referring to HP1 and HP2 would be correct.
We revised it in the text

3. Referring to the F178W OPTN LIR as a p62 mimicking LIR (e.g. line 350 & 446) seems outdated, as there are various W-type LIRs. Mutating F178 to W results in a

WVEI core LIR, which resembles other LIRs (e.g. the NIX/BNIP3 LIR WVEL) more than the p62 LIR (WTHL).

We have revised in the text and we no longer refer to F178W as a p62 mimicking peptide

4. The WT Kon value in Figure 7A (0.52 (M-1s-1)/104) does not match the value in the main text (line 509, 0.55 (M-1s-1)/104).

As mentioned in the answer to the previous reviewers and above we have now corrected this in the Figure 7A

5. Figure 2A and Table 1 compare MD simulations with published NMR data, thereby validating the applied method rather than showing new results. While this supports the presented data, it might be more appropriate to move this data to the appendix to avoid taking the focus away from new results.

We appreciate the reviewer's comments; however, we believe this section remains essential in the main text. In our view, these results are crucial in demonstrating the quality of the collected simulations and justifying further analysis.

6. In line 206, the authors refer to F178 and I181 of the Optineurin LIR as HP1 and HP2 residues. This is misleading and should be rephrased – e.g. to "residues occupying HP1 and HP2 respectively".

We revised the text accordingly.

7. Check References. Information referring to reference #63 in line 273-275 could not be found in the publication.

We thank the reviewer for pointing this out. We acknowledge that the original sentence unintentionally implied that reference #63 supported a specific interaction between LC3B and the p62 LIR motif. Our intention, however, was to refer more generally to electrostatic interactions observed between lysine residues in LC3B and acidic residues within LIR peptides. Specifically, reference #63 reports molecular dynamics simulations showing a stable electrostatic interaction between K51 of LC3B and D1021 in the LIR motif of AMBRA1. To better reflect this general mechanism and avoid confusion with p62, we have revised the sentence at page 9 accordingly.

8. Units should be included in supplementary tables where applicable.

We thank the reviewer for bringing this to our attention. We carefully reviewed all the Supplementary Tables and have updated Table S2, Table S3, and Table S4 to include

the missing units (% occurrence), which indicate the percentage of frames in a given simulation where the interaction was present.

9. Line 88: "components of the autophagy machinery" should be changed to ATG8 or LC3/GABARAP as these are the components the authors refer to.

We have revised this part.

10. Line 90-91 suggest that the core LIR residues do not contribute to paralog specificity. This is not true, see for example Rogov et al., 2017, DOI: 10.15252/embr.201643587.

We acknowledge that our original phrasing may have been misleading and have revised the text at page 3 to clarify our intended meaning.

11. Line 98-100: "…mechanism in which LC3 proteins recruit autophagy factors to drive the autophagosome growth and amplify selective autophagy in an LC3-dependent feedback loop". The second part of the sentence appears redundant.

We have revised the statement at page 3 to avoid redundancy.

12. Line 238-239: "It also identifies L166 as another site in the N-terminal flanking region of OPTN that is switched on by phosphorylation". This sounds like L166 is phosphorylated. Should be rephrased accordingly.

We have rephrased this statement at page 8 of the manuscript.

13. Line 455-456: "Our findings suggest that the MDS binding assay can identify and validate predicted binding affinity changes exceeding 2 kcal/mol." Is this true? Clear difference between WT OPTN and F178W OPTN can be seen in the MDS (Fig 6 A,E,I). Fig 5B and Table S5 suggest lower values. This should be clarified.

We would like to clarify that the conclusion is related to the fact that since with MDS we cannot see estimate clear differences in Kd for the pS177 and the pS177-M183A where we predict changes in binding free energies with our computational approach around 2 kcal/mol, as detailed in the text. F178W results have nothing to do with this part since for that mutation the computational approach can be sensitive and provides higher ΔΔG values.

Dear Professor Papaleo,

Your manuscript entitled "Decoding Phospho-Regulation and Flanking Regions in Autophagy-Associated Short Linear Motifs: A Case Study of Optineurin-LC3B Interaction" has now been seen by 3 referees, whose comments are appended below. You will see from their comments below that while they find your work of considerable interest, some important points are raised. We are interested in the possibility of publishing your study in Communications Biology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise and resubmit your manuscript, taking into account the points raised. In particular, please address the last comments from Reviewer #4 regarding toning down some statements, adding some references, and modifying some of the figures, as well as the last minor points from Reviewer #2.

We would like to thank the Editor and Reviewer for the useful and attentive criticisms. Our answers are reported below point by point for sake of clarity and the page numbers refer to the document with track changes.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have properly addressed all reviewer comments in their revised manuscript and have clearly responded to all requests. They have provided additional experimental data, clarified previous ambiguities, and corrected critical errors. I recommend the revised manuscript for publication in its present form.

Reviewer #3 (Remarks to the Author):

With the additional analysis and experiments, as well as changes to the text and figures, the authors have done a good job addressing my concerns.

A few remaining, very minor points:
1) line 258: Figure S4A-C -> Figure S4A-D, since the text refers to all 4 residues

We fixed this in the text at page 8.

2) lines 412-415: Data supporting the claim that R37 only tolerates mutation to K seems to be missing. Or are the statements inferred from the data in Fig. 4? This should be made more clear.

We appreciate the reviewer’s comment. In response, we have added a new Supplementary Figure (now Figure S6), which presents heatmaps from the in silico saturation mutagenesis scan of selected LC3B residues, including those discussed in the manuscript. Additionally, we have revised the text on

page 12 to accurately reflect the mutational effects observed across different peptide phosphorylation states.

3) Line 539, Fig. 7A: Order of magnitude of k_on is 10^3 in the text but 10^-3 in the figure. For k_off, orders agree between text and figure.

We thank the reviewer for bringing this to our attention. The correct k_on magnitude is indeed 10^3 and we corrected it in Figure 7A.

4) Supplementary figures 1-3 still (and new figure S4) seem to be missing captions. Or are they in a different file?

We apologize for the inconvenience. The previously uploaded files were incorrect. The updated Supplementary Figures now include both the figures and their corresponding captions.

Reviewer #4 (Remarks to the Author):

The revised manuscript is much clearer, and the authors addressed most of my remarks. However, I still have several questions/suggestions regarding main text and figures.

Main Text:
• While the authors have slightly modified the wording regarding their use of experimental methods (SPR and MDS) to determine affinity and kinetics, describing it as novel seems inadequate to me (at least for SPR). It is true that many studies focus on steady state, due to rapid on-off kinetics that the authors of this study also faced, however, applying SPR to study LIR-LDS interaction is not uncommon and there are also examples of selective autophagy receptor LIRs with ATG8 proteins (e.g. Stephani et al., 2020, https://doi.org/10.7554/eLife.58396 & Fumagalli et al., 2016, https://doi.org/10.1038/ncb3423).

“While direct kinetic measurements of LIR interactions remain limited due to their rapid on-off kinetics, some SPR-based studies81–83 have explored both steady-state and kinetic parameters for LC3B interactions - though none specifically focused on autophagy receptors.”
“Moreover, we have generated novel structural 18 insights including binding affinity and size measurements by Microfluidic Diffusional Sizing and kinetic measurements by Surface Plasmon Resonance, both first in the context of natural autophagy receptor LIR-ATG8 interactions.”

We thank the reviewer for the insightful comment and agree that SPR has been previously employed to study LIR–ATG8 interactions, including with selective autophagy receptors, as shown in the cited studies. We have revised the relevant statements in the manuscript to reflect this, specifically clarifying that while SPR has been used in this context, our work extends prior characterizations and that the application of Microfluidic Diffusional Sizing (MDS) represents a novel aspect of our study. The revised text can be found on pages 16 and 18, and the suggested references (Stephani et al., 2020; Fumagalli et al., 2016) have been added accordingly.

• I am missing a reference for the following sentence:

“In contrast, the OPTN LIR on-rate aligns with the one measured for the natural cysteine protease ATG4B LIR’s interaction with LC3B, while the six-fold lower affinity of the autophagy receptor OPTN is largely owing to its increased off-rate.”

We thank the reviewer for pointing out the missing citation. The original sentence incorrectly implied similar on-rates and a higher off-rate for OPTN compared to ATG4B. We have corrected this in the manuscript at page 16 to reflect that ATG4B’s much higher on-rate—rather than differences in dissociation—drives its stronger affinity, while the off-rates of both peptides are comparable. We have also added the appropriate reference (Tang et al., 2022: doi: 10.1021/acs.biochem.2c00482) to support this discussion.

• The text referring to Figure S5 is not consistent/clear. The following sentences should be rephrased accordingly.
- “Multiple phosphorylations do not decrease the flipping of F178”
- “..five phosphorylation can decrease the flipping of F178 outside the HP1 of LC3B”
- ”…the multiple phosphorylation cannot rescue the flipping movement of the OPTN phenylalanine residue in the HP1 of LC3B”

We thank the reviewer and agree that the text referring to Figure S5 was inconsistent. We have therefore revised the caption title of Figure S5, as well as the corresponding text in the Results section (page 10) and the Discussion section (page 18) to ensure clarity and consistency.

Figure 1:
• I find the visualization of the AlphaFold model not ideal as the cartoon representation is simply overlaying the surface, implying the LC3B cartoon to be in front of the optineurin surface. I believe removing the Optineurin surface and making the LC3B surface semi-transparent would make the figure more coherent.

We agree with the reviewer and we modified Figure1 panel C accordingly.

• Caption should clarify that depicted model is trimmed while PAE matrix refers to model of full-length proteins

We have updated the figure caption to explicitly state that the structure displayed is the truncated construct used for simulations ($LC3B_{1\text{-}120}$-$OPTN_{158\text{-}191}$), whereas the accompanying PAE matrix is derived from the full-length protein models.

Figure 3:
• I am missing an explanation on why the control behaves differently than the actual runs (especially evident for D48 accessibility with phospho-variants)

We thank the reviewer for pointing this out. The differences in solvent accessibility—especially for D48—between the simulations based on AlphaFold (AF) models and the one derived from the experimental LC3B–OPTN complex likely arise from differences in the modeled peptide length. The AF models include a longer OPTN fragment (residues 158–191), while the experimental structure

contains a shorter peptide (residues 169–185). Upon inspecting the simulation trajectories, we observed that the extended flanking regions in the AF models occasionally move to cover parts of the LC3B surface, partially shielding residues such as D48 and K51. This dynamic masking appears to reduce their solvent exposure, particularly in the phosphorylated variants, and likely explains the observed differences in SASA. The “movies” of the different simulations are available at the paper OSF repository https://osf.io/6rmeb in the folder “simulations_data”. We added a paragraph in the section methods at page 23. In light of this, we have also updated Figure 3, Figures S2 and S3 (and their captions) to more accurately describe the simulation based on the experimental structure, avoiding the term "control" to prevent potential misinterpretation, since the constructs differ in sequence length and are not directly equivalent.

Figure 4/S4:

• Depiction of the atomic contacts with the respective frequencies could be clearer. Due to different number of contacts for each peptide (why are additional ones with 0 occurrence shown?), the residues are not depicted next to each other in the same row. This makes it harder to see differences between the peptides.

We appreciate the reviewer’s suggestion to improve clarity in Figure 4/S4. However, each panel in the figure represents the local environment around a specific residue in a given simulation, so the set of contacting residues necessarily differs between peptides. Aligning all panels to show identical residue lists would risk omitting genuine contacts in some simulations or “cherry-picking” data. Regarding the appearance of contacts with “zero” occurrence: we do not include residues with truly zero contacts. Some contacts with very low—but nonzero—frequencies can appear indistinguishable from zero in the heatmap. We have verified that all shown residues register at least one contact event, and those with frequencies below 1% simply appear nearly yellow on the color scale. Regarding this last point, we added a sentence explaining this in the corresponding methods section at page 24 to avoid confusion.

Figure 5/6:

• While I understand that keeping the MDS measurements together in Figure 6 is helpful, restructuring the text in a more logical rather than chronological order as mentioned by reviewer 1, would be preferable over showing the same panel twice.

We thank the reviewer for the helpful suggestion. Following the comment, we have removed the duplicated panels from the original Figure 6 to avoid redundancy with Figure 5. We also updated the references to the correct Figures in the text at pages 11, 14 and 15 and the figure caption accordingly to reflect this change.

Figure S5:

• X-axis should be in Angstrom, as the main text refers to the distances in Angstrom.

We have corrected the X-axis in panels A and B.

Minor remarks

• “However, the multiple phosphorylation cannot rescue the flipping movement of the OPTN phenylalanine residue in the HP1 of LC3B.” à should be ‘multiple phosphorylations’
• “Phosphorylation of OPTN is important to regulate its cellular function, and the structural changes it induces should are integral to understanding the..” à The ‘should’ is too much.

We have corrected these in the text on pages 18 and 19, respectively.