nature portfolio

Peer Review File

Elevated intracellular copper induces CTR1 monomerization and prevents copper uptake

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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)
The authors investigate the molecular mechanism of how the transporter CTR1 regulates copper uptake into a cell. In a first part, the thermodynamic stability of wild-type CTR1 and an endocytosis-deficient mutant, CTR1(M150L), was calculated using molecular dynamics simulations. They then constructed a fusion of CTR1 with the photoactivatable fluorescent protein mEos4b (CTR1_mE), which was found to be fully functional. With this fusion, PALM experiments and quantitative analyses of the oligomeric state of CTR1_mE were performed at endocytic sites identified by Rab5. The authors found that high copper levels induce the de-oligomerization of CTR1 from a functional homotrimer to monomers. Together with the surface depletion of CTR1 under copper stress, the authors derive a comprehensive molecular model of copper uptake regulation by CTR1.

A key to quantification methods are molecular controls. For that purpose, the authors chose the Tac antigen, which is present in the cells in a monomer-dimer equilibrium. Tac should be insensitive to high copper concentrations, but the observed shift in monomers and dimers is large, e.g. from 0.48 to 0.26 – these are very similar values to CTR1 (Figure 3). I understand that in case of Tac, that was found to be not significant (statistical analysis), whereas it was found significant for CTR1. More information and clarification is needed on this important point:
- The authors used a t-Test. Does the data meet the criteria (normality)? Otherwise, Mann-Whitney would be an alternative.
- Please show all data points (Figure 3, Ext Fig 4) instead of only bar plots, e.g. in a violin plot or in dots next to bars.
- How do the authors interpret the discrepancy in significance? That would of course also depend on the type of test that should match the type of distribution and sample size in each scenario.
- Given the large error (e.g., 0.48 and 0.26 being not distinguishable for Tac; in addition, the authors rate their method of quantification to have an accuracy of around 10%): how accurate is the estimate of the dimer population in Figure 3c and 3h? This is a general question, and unrelated to the multi-step model.
- Why did the authors choose a reference protein with a mixed oligomeric state, rather than a pure dimer or trimer?

The quantification of protein oligomers from SMLM data has become increasingly important in recent years. In addition to the method chosen by the authors, an alternative approach that analyzes the kinetics of fluorescence emission to report protein oligomers (qPALM) exists and has been shown to be useful (e.g. PMIDs 38226794 and 38457493). I am not asking the authors to analyze their data using this method as well (although this should be directly possible and it would be interesting to compare the two methods); nevertheless, I would like to ask the authors to briefly mention and discuss both approaches in the discussion section, especially with regard to the points raised above. This will be helpful for the reader.

One limitation of the study is that the mechanism of de-trimerization remains unclear. The discussion here remains somewhat vague and points to an unknown “sensor protein”.

Minor comments

Extended Data Fig. 2, I find it difficult to see the internalization of CTR1 at high copper levels from the one cell shown. Could the authors rather show a larger panel of many cells (tens or more)?

Extended Data Fig. 3, it is pretty much accepted in the field that an evanescent light field penetrates to around 100 nm into a cell; the authors made an extra effort to show that here, which I appreciate.

The abbreviation ‘mE’, for mEos4b, is a bit confusing to read and maybe unusual.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The study tests an interesting and novel idea that the de-oligomerization of the copper transporter CTR1 is an initial step in CTR1 endocytosis, which effectively stops copper (Cu) uptake. While the concept is very intriguing, the evidence of de-oligomerization is not very strong (or not convincingly explained), and the mechanism detailing how a stable trimer dissociates into monomer has not yet been investigated. The work appears to be at the early stage.

Specific comments:
1. The authors seem to assume that copper binding at M150 of CTR1 triggers endocytosis. There is little basis for such assumption. If this was the case, then each time Cu binds to CTR1 the protein would endocytose, i.e. CTR1 will act as transferrin receptor rather than a transporter. Furthermore, the C-terminal mutant that retains M150 but has the C-terminal HCH motif mutated does not endocytose, which suggests that the response to elevated copper comes from the cytosol. The alternative explanation for M150 mutant not being able to endocytose is that the mutation inhibits Cu uptake and eliminate cytosolic Cu sensing by CTR1. If the authors believe that their model is correct, this alternative scenario needs to be eliminated

2. “The increased stability of the trimer in the mutant raises the possibility that the ability of CTR1 to alter its oligomeric state plays a critical role in its regulation of copper uptake” – Can the authors calculate the energetic cost of de-oligomerization?

3. How does the insertion of 3 GFP-FLAG into a small and tightly packed trimeric protein, like CTR1 affect protein stability? AlphaFold3 analysis of the wild type and CTR1-GFP-Flag trimers suggests a significant destabilization of the trimer by the fusion. The authors should provide evidence that the stability of the wild-type and fusion CTR1 are similar. This can be done either computationally or experimentally (for example, by analysis of thermostability/urea sensitivity on nondenaturing gels or by correlation spectroscopy)

4. Presence of monomers, dimers, and trimers at the plasma membrane at steady state needs to be verified independently because it may be a result of overexpression and/or instability of the fusion protein (see above). In addition to surface biotinylation experiments that convincingly show Cu induced endocytosis, the authors should perform blue native gel analysis for endogenous CTR1 to ensure that the fusion construct behaves identically, when it comes to monomers, dimers, and trimers.

5. Can the authors exclude that the increased activity of CTR1mE is caused by CTR1mE homotrimer rather than heterotrimers with the endogenous CTR1? To better illustrate functionality of CTR1mE – the comparison should be made with the wild-type CTR1 expressed at the same level as CTR1mE.

6. It is not clear how method with 10 nm resolution can resolve individual CTR1 subunits, which have a cross-section in the membrane of 5 nm. This needs to be explained better.

7. Related, if the method does resolve CTR1 monomer from trimers then the individual CTR1 signals within Rab5 puncta should be markedly different from CTR1-signals outside Rab5 puncta, where functional CTR1 is uniformly trimer. Is this what the authors observe? Figure 2e (the CTR1 map) is difficult to interpret

8. The smND assay is a new and very intriguing methodology and it has to be explained better. For example, can the authors exclude that the “de-oligomerization” is simply an increase in the distance between fluorophores due to copper-induced conformational changes in CTR1. It was demonstrated that CTR1 undergoes conformational changes upon Cu binding (PMID: 36409899). The authors should formally rule out this possibility.

(Remarks on code availability)
Not applicable

Reviewer #3

(Remarks to the Author)
The Cu uptake transporter CTR1 undergoes endocytosis in response to excess Cu to maintain cellular Cu homeostasis. By employing single-molecule localization microscopy (SMLM) and single-molecule neighbor density (smND) assays, the authors demonstrate that excess Cu induces rapid monomerization of the trimeric CTR1 prior to endocytosis. This process inhibits Cu uptake and is impaired in the endocytosis-deficient CTR1 (M150L) mutant. Thus, authors conclude that de-oligomerization of CTR1, linked with endocytosis as a rapid mechanism to prevent excess Cu entry. While the study is

interesting and important, a major limitation is the absence of data showing functional consequences of de-oligomerization of CTR1 in response to excess Cu, both in vitro and in vivo. For instance, experiments demonstrating Cu-induced cell death (cuproptosis) using CTR1 wild type and mutants are needed. Moreover, it is essential to perform live cell imaging of WT or mutant CTR1 with Rab5 in COS7 cells. Additional experiments are therefore required to increase the quality of this manuscript.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The article titled "Human Transporter De-oligomerization Regulates Copper Uptake into Cells" presents a combined computational and experimental study. The authors demonstrate that an excess of copper induces the monomerization of CTR1, which typically transports copper as a trimer. This monomerization process is suggested to prevent copper overload.

The article is intriguing as it provides new insights into the still unclear aspects of copper internalization. However, several issues need to be addressed before the paper can be considered for publication.

In the introduction, where the mechanism of copper uptake is discussed, the authors should reference recent papers that have investigated the detailed mechanism of copper transport. For example, see Janos et al. https://doi.org/10.1017/qrd.2022.2 and Aupic et al. https://doi.org/10.1073/pnas.2214602119.

The authors claim in the first paragraph that the M150L mutant significantly stabilizes the trimeric form by decreasing the ΔΔG. However, examining the structure of CTR1, it appears that the selectivity filter of the CTR1 trimer may be affected by introducing a residue larger than methionine. Previous MD simulation studies have shown that methionine is already quite bulky. Have the authors performed actual MD simulations to verify the structural impact of this mutation on CTR1 stability?

Another aspect that needs clarification is that M150L is the bottom methionine triad in the structure, which means that the upper methionine triad M154 can still bind copper and stabilize the trimer in this manner. The authors should also consider the other M150L variant to ensure copper is not bound to the upper triad of Met in selectivity filter and therefore it is not stabilizing the trimer structure in this manner.

As far as I understand, MD simulations are performed in the absence and presence of copper. However, no reference to their parameterization is provided in the methods section. It is well known that this parameterization is not trivial and may require QM-based simulations.

Additionally, RMSF values are reported on page 3 with several decimal digits. Have the authors checked how the computed RMSF depends on the simulation length? The observed difference is probably smaller than the error and not significant. This is particularly relevant when the authors try to spot differences based on the RMSF.

Furthermore, the experimental structure of CTR1 lacks residues from the N-terminus and part of the C-terminus. According to the methods, these parts have been added to the CTR1 model. If this is the case, since they are both disordered, how is their conformation modeled?

The results on the structure and copper-binding abilities of CTR1 are affected by the presence and conformation of the N-term and C-term residues see Aupic et al. https://doi.org/10.1073/pnas.2214602119 and Walke et al., Biophysical Journal, Volume 121, 1194-1204. Thus understanding their influence is a relevant point.

These parts contain several copper-binding residues, thus the authors should also create and test the oligomerization properties of these truncated variants (truncanted N-term and/or truncated C-term) in response to copper stress.

Some contradictory statements are provided in the text. In the discussion, the authors mention the YxxM motif of the intracellular loops (please state exactly the residues you are considering). The authors mention that copper binding at these methionine sites stabilizes the trimer, preventing its dissociation. How does this occur? Can they better argument this point?

In this respect, can the authors increase the intracellular concentration of copper, without relying on CTR1 transport, and check whether it is really the intracellular increase of copper that is affecting the oligomerization status?

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for addressing my comments.

There are a few points – minor, yet I see them as mandatory – that need further attention:

(1) The authors integrated the qPALM method in the discussion (lines 390ff), which I think is very helpful for the reader. Yet, references to further direct the interested reader are missing. Some original work should be integrated, e.g. PMID 27466316 and PMID 31937565. Along these lines: 'blinking' is not an artefact in PALM, it is rather valuable kinetic information that reports on molecule numbers (in qPALM, qPAINT etc.).
(2) I politely disagree in pointing the reader towards not using dimeric proteins as a reference. There are various dimeric reference proteins available (CD28 being a a very robust one; CTLA4 reported as well); another option are single and dual-labelled monomeric proteins. I propose to rephrase this section and recommend to the reader to implement appropriate controls.
(3) Please mention the origin and the exact name for the CTLA4 plasmids.
(4) How does incomplete labelling affect the results of the method the authors use? The detection efficiency of mEos is around 50% (this value varies in different reports). I do understand the approach the authors use is based on reference data generated from molecules of known oligomers; in my view, one or two sentences in the discussion that point at this would suffice, and it will help the interested reader.
(5) I appreciate that the authors added imaging data in Extended Figure 2. However, it is not possible to see single receptor clusters even in the zoom images (that have a scale of 10 µm). I wonder whether there are isolated clusters at all, and what the molecular density of this receptor is – as compared to the physiological density of CTR1.

All of the above points can be addressed with minor editing.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This reviewer appreciates the authours' thoughtful and constructive response to the critique. The revised manuscript is significantly improved: it is clearer, it includes new and useful controls, and it is, overall, more convincing. I have no further concerns

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In response to this reviewer's comments, authors responded that their major focus is to establish that CTR1 undergoes a structural change (de-oligomerization) that precedes endocytosis in elevated copper, which prevents excess Cu entry. While study is important in terms of showing relationship between structural change and endocytosis, it is essential to show the functional consequence of preventing endocytosis, such as excess Cu entry and Cu-induced cell death at least in vitro. Thus, authors should perform additional experiments to show the functional significance of de-oligomerization of CTR1, which is not difficult to do, to increase the quality of this paper.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
I believe the authors' discovery of CTR1 deoligomerization importantly contributes to understanding CTR1 function and regulation, and merits publication in Nature Communications. However, I am not fully convinced that their computational modelling supports the claim that the M150L mutation stabilizes the trimeric structure of Ctr1. Their experiments with Cu ionophore clearly show that deoligomerization is caused by an increase in intracellular Cu concentration and is not primarily driven by intrinsic thermodynamic stability of the CTR1 trimeric transmembrane domain or how the latter is affected by copper binding at M150 or M154. This suggests that the M150L mutation, as the authors also state, primarily prevents deoligomerization by preventing copper internalization and not by stabilizing the trimeric assembly. Therefore, I think that the estimation of the effect of the M150L mutation on CTR1 oligomerization affinity is not really relevant for the paper's main outcome and due its relative speculative nature perhaps detracts more than it adds to the scientific quality of the paper. In any way, while the authors address many points previously raised, some further improvements are needed before publication.

• Authors write on line 95:

However, in the absence of copper, the RMSF data showed increased flexibility in M150L (1.84 Å compared to the wild type 1.75 Å), especially within the intracellular loop (residues 98 to 136) and cytosolic tail (residues 176 to 190, Fig. 1f).

Firstly, while the authors now show that the average RMSF stabilizes after 900 ns, no error estimate is provided, therefore it is hard to estimate whether the relatively small 0.09 Å difference is significant. Moreover, panel 1f appears to show that in subunit A CTR1 is in fact more flexible than CTR1 (M150L). Additionally, in the transmembrane part that should be most relevant for the oligomerization, the differences seem particularly negligible and in any case do not suggest greater flexibility of the wt variant. I suggest the authors at a minimum include error estimates, either by employing the block average method on the final 500 ns of trajectories or by treating subunits as simulation replicas.

• The most convincing evidence about the effect of M150L mutation on the thermodynamic stability of the CTR1 trimer comes from computational mutagenesis. Can the authors add a brief explanation regarding the scoring function employed by the Eris algorithm for calculating the free energy and how it treats the Met-Cu(I) interaction. Does the DMD optimization significantly affect the geometry of the metal binding site?

Minor points:

• In figure 1d, the grid lines and the y-axis tics do not match.

• In the following sentence, starting on line 75, the word ‘change’ is unnecessary and leads to confusion.

In the absence of copper, the leucine substitution at M150 (M150L) reduced the free energy change (ΔΔG) by –5.11 kcal/mol compared to the wild type.

• Authors state on line 81:

Although the M154 site in the M150L mutant still coordinates copper, it is unlikely to be the dominant stabilizing factor, as the Cu(I)–M154 bond lengths in the mutant (average 2.7 Å) are slightly longer than those in the wild-type (2.4 Å), suggesting weaker coordination (Fig. 1a).

Please clarify if these distances stem from MD simulations.

• In the method section, line 641, the authors state:

We utilized the Amber force field, which includes well-validated parameters for Cu(I) coordination.60

I assume they employed the non-bonded copper model, however the reference they give (Schwartz R, et al. Protein Science. 2022;31(12):e4464) describes the development of parameters for a bonded copper model that are not provided directly provided with Amber/AmberTools. If they have indeed used the non-bonded model the reference should be removed. Additionally, the authors should state explicitly which protein force field (ff12SB/ff14SB,…), water model (TIP3P/OPC,…) and ion parameters (Li-Merz/Young-Cheatham,…) they employed.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for responding to and addressing all my comments. The revised manuscript has significantly improved, and there are no more open points from my side.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Although the reviewer requested that the authors should demonstrate the functional consequences of CTR1 de-oligomerization in response to intracellular Cu elevation such as Cu-induced cell death using both CTR1 wild-type and the endocytosis-deficient CTR1-M150L mutant, this experiment was not included in the revised manuscript. Instead, the authors only assessed the effect of the endocytosis inhibitor Dynasore on $CuCl_2$-induced cell death. Therefore, the revision does not adequately address the original concern.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
I am content with the authors' response to my previous comments. I have no additional concerns regarding publication.

(Remarks on code availability)

Point-by-point response to reviewers

[Reply to all reviewers] Thank you for your critical and constructive comments. Following your suggestion, we have revised the manuscript accordingly. Below, we respond (in red) to each question/comment (in black *italic*), with our additions to the text indicated (in blue).

Reviewer #1

The authors investigate the molecular mechanism of how the transporter CTR1 regulates copper uptake into a cell. In a first part, the thermodynamic stability of wild-type CTR1 and an endocytosis-deficient mutant, CTR1(M150L), was calculated using molecular dynamics simulations. They then constructed a fusion of CTR1 with the photoactivatable fluorescent protein mEos4b (CTR1_mE), which was found to be fully functional. With this fusion, PALM experiments and quantitative analyses of the oligomeric state of CTR1_mE were performed at endocytic sites identified by Rab5. The authors found that high copper levels induce the de-oligomerization of CTR1 from a functional homotrimer to monomers. Together with the surface depletion of CTR1 under copper stress, the authors derive a comprehensive molecular model of copper uptake regulation by CTR1.

1. *A key to quantification methods are molecular controls. For that purpose, the authors chose the Tac antigen, which is present in the cells in a monomer-dimer equilibrium. Tac should be insensitive to high copper concentrations, but the observed shift in monomers and dimers is large, e.g. from 0.48 to 0.26 – these are very similar values to CTR1 (Figure 3). I understand that in case of Tac, that was found to be not significant (statistical analysis), whereas it was found significant for CTR1. More information and clarification is needed on this important point:*
   1.1 *The authors used a t-Test. Does the data meet the criteria (normality)? Otherwise, Mann-Whitney would be an alternative.*

   [**Reply 1.1**] We sincerely appreciate the reviewer for pointing out the importance of ensuring that the statistical test used is appropriate for our dataset. The reviewer correctly noted that a t-test assumes normality, which may not always be valid. To ensure the robustness of our statistical approach, we reexamined our data by performing a normality test (Shapiro-Wilk) on the datasets. Our analysis revealed that only the Cu-treated condition passed the normality test, indicating that the assumptions required for a parametric t-test are not fully met. Given this, we reanalyzed the data using the Mann-Whitney U test, a non-parametric alternative that does not assume normal distribution. This reanalysis yielded the same conclusion as our original approach: CTR1 undergoes a statistically significant shift in oligomeric state under high copper conditions, whereas the population distribution of Tac^{mEos} remains unchanged, confirming that our findings are robust and not artifacts of the statistical method employed.

   We have included details of the Shapiro-Wilk normality test and the Mann-Whitney U test in the *Methods* and clearly indicated the statistical methods used in the legend of Figure 3 and Extended Data Figure 4. These changes ensure transparency and reinforce the validity of our conclusions.

   [**Changes in the Main text**] To ensure robust statistical interpretation, the Shapiro-Wilk normality test was initially performed to assess data distribution. Due to deviation from normality in some datasets, statistical comparisons between experimental conditions were conducted using the non-parametric Mann-Whitney U test rather than a parametric t-test. Additionally, data points are explicitly shown using dots overlayed on bar graphs, enhancing data transparency and readability.

1.2 *Please show all data points (Figure 3, Ext Fig 4) instead of only bar plots, e.g. in a violin plot or in dots next to bars.*

[**Reply 1.2**] To provide greater clarity on our data processing pipeline, we outline the sequence of data transformation leading to the reported results. First, SMLM and fluorescence microscopy was used to detect individual CTR1 molecules and Rab5$^+$ area, respectively. In each Rab5$^+$ area, local protein concentration was determined by quantifying CTR1 molecules relative to the area. Rab5$^+$ area with similar protein concentrations were grouped for subsequent analysis. *ND* values at individual concentrations were then computed and compiled into *ND* distributions. The mean values and sem of data crossing different concentrations for each experimental condition were computed to generate Fig. 3b, 3g and Extended Data Fig. 4d (right). Note that *ND* distributions reflect a mixture of monomers, dimers, and trimers and are influenced by local concentration. Thus, direct interpretation of raw *ND* data does not yield precise oligomeric composition. With data grouped into defined concentration ranges, a theoretical model was applied to fit the *ND* distributions. This process enabled the extraction of the relative fractions of different oligomers under each condition. Quantitative results were derived from the fitted data. Both the weighted average oligomeric state ($\bar{N}$; e.g., Fig. 3d and 3i, Extended Data Fig. 4c and 4e right) and the oligomer population (Fig. 3e and 3j, Extended Data Fig. 4b and 4e left) were generated from these fitted results, where the mean and sem were calculated from the fitted results.

To further clarify the link between the *ND* distributions and the extracted oligomeric populations, we have included raw data points in Fig. 3 and Extended data Fig. 4.

[Changes in the Main text]

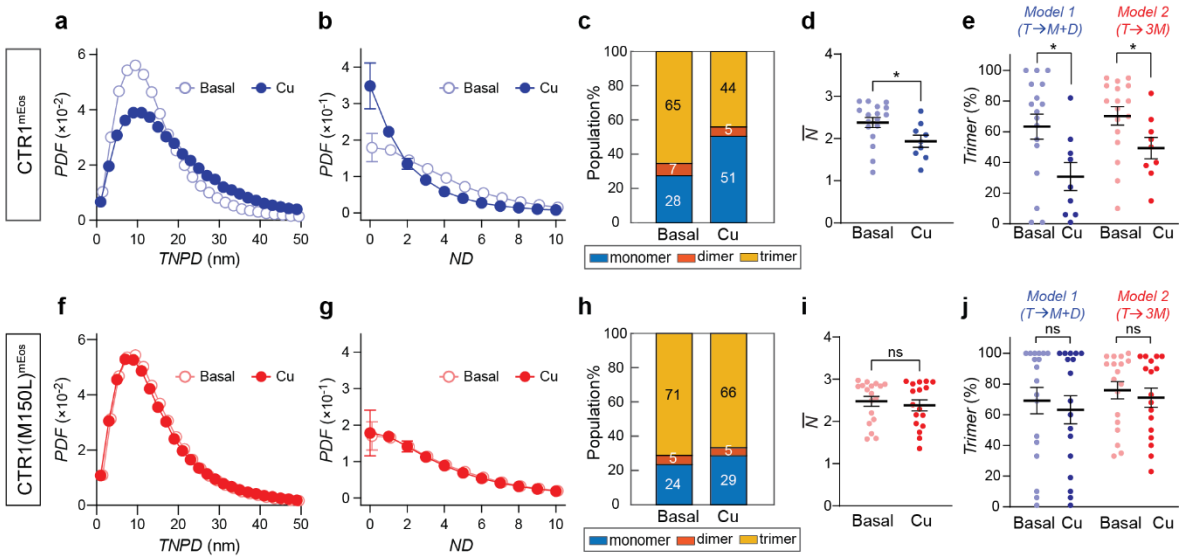
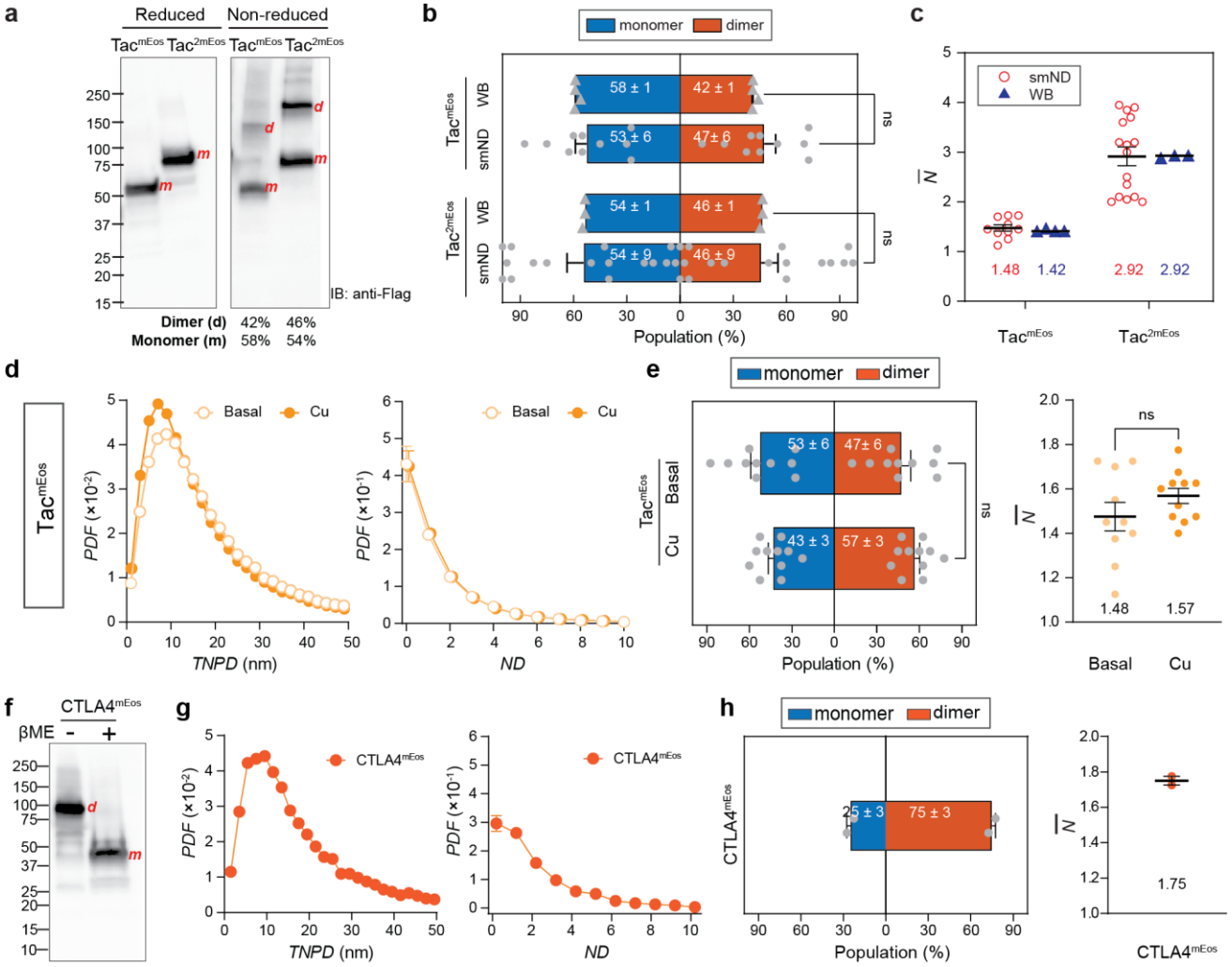


Fig. 3 | Copper-induced changes in CTR1 oligomeric states assessed by TNPD and smND Assays. a, **b**, Probability density functions of two nearest pairwise distance (*TNPD*, **a**) and neighbor density (*ND*, **b**) for CTR1^{mEos} under basal (open circles) and Cu-stressed (filled circles) conditions. Copper treatment increases *TNPD* and reduces *ND*, indicating larger separation between CTR1 units and de-trimerization of CTR1, respectively. **c**, **d**, Extracted oligomer populations (**c**) and weighted average number of subunits ($\bar{N}$, **d**) for CTR1^{mEos} show that copper significantly modulated oligomer populations and $\bar{N}$ in CTR1^{mEos}. **e**, CTR1 trimer percentages extracted from smND with two dissociation scenarios: Model 1 (T → M + D) and Model 2 (T → 3M). Model 1 and Model 2 provide the lower and upper limits of trimer populations, respectively. In both models, copper induced significant trimer populations decrease. (**f**–**j**) Similar to (**a**–**e**) but for CTR1(M150L) ^{mEos}. No significant change in *TNPD* distributions (**f**) nor *ND* distributions (**g**). Copper does not affect the extracted oligomer populations (**h**), $\bar{N}$ (**i**), nor trimer population (**j**) in rab5$^+$ areas, confirming that the M150L mutation locks CTR1 in a trimeric state. Data are summarized from three independent experiments. Graphs (**b, d, e, g, i,** and **j**) are shown as mean ± SEM. *Mann-Whitney* test. *$p < 0.05$. See also Supplementary Table 3.



Extended Data Fig. 4 | Validation of smND assay and $\bar{N}$ as a metric for evaluating oligomeric state composition using membrane-anchored Tac^{mEos} and Tac2^{mEos}. **a**, Non-reduced immunoblotting analysis of Tac^{mEos} and Tac^{2mEos} overexpressed in COS7, showing their oligomeric populations as 59% monomers and 41% dimers for Tac^{mEos}, and 54% monomers and 46% dimers for Tac^{2mEos} with no significant cleavage. Equal amounts of the total protein were resolved in both non-reduced and reduced SDS-PAGE, and then the full-length proteins were detected by using anti-Flag antibody. The corresponding percentages of the monomer (*m*) and dimer (*d*) populations within each fusion protein were quantified by the band intensities from the non-reduced gel. **b**, Comparison of oligomer populations of Tac-fusion proteins extracted from immunoblotting and smND analysis. The immunoblotting and the smND data of Tac^{mEos} and Tac^{2mEos} were collected under basal conditions, showing no significant (ns) differences in Tac oligomeric state distributions. **c**, Comparison of $\bar{N}$ of Tac^{mEos} and Tac^{2mEos} extracted from the oligomer populations in immunoblotting (triangle) and smND analysis (circle). Values are closely matched across methods. **d**, Probability density functions of *TNPD* (left) and *ND* (right) results of Tac^{mEos} under basal (open circle) and Cu-stressed (closed circle) conditions. No significant changes are observed, supporting the copper insensitivity of the Tac construct. **e**, Extracted dimer populations and the corresponding $\bar{N}$ values of Tac^{mEos} remained statistically unchanged between basal and Cu-treated conditions, confirming that the oligomeric state of the mEos tag is not affected by Cu treatment. **f**, Immunoblotting analysis of CTLA4^{mEos} resolved in non-reducing (without βME) and reducing (with βME) SDS-PAGE showed over 90% of the CTLA4^{mEos} as dimer. **g,** Probability density functions of *TNPD* (left) and *ND* (right) results of CTLA4^{mEos} under basal condition. **h.** Extracted dimer populations and the corresponding $\bar{N}$ values of CTLA4^{mEos} showed 75% dimer population *in situ*. Panels **b**, **c**, **e** and **h** are shown as mean ± SEM.

1.3 How do the authors interpret the discrepancy in significance? That would of course also depend on the type of test that should match the type of distribution and sample size in each scenario.

[**Reply 1.3**] The reviewer raised a question regarding the discrepancy in statistical significance between CTR1 and Tac^{mEos} despite similar population shifts. This discrepancy most likely stems

from differences in sample size and data saturation, which affect statistical power. In our initial experiments, our Tac^{mEos} dataset had a smaller sample size compared to CTR1, meaning that the data were less saturated and exhibited greater variability between individual measurements. This resulted in broader confidence intervals, reducing the ability of statistical tests to detect significant differences between basal and Cu-treated conditions. To address this, we conducted additional smND experiments on Tac^{mEos} under both basal and Cu-treated conditions, increasing the sample size comparable to CTR1 to enhance statistical precision.

With this expanded dataset, we now observe that the Tac^{mEos} population shift (10%) in response to Cu treatment is much smaller than previously estimated (22%). The updated analysis (Extended Data Fig. 4e) shows that Tac^{mEos} exhibits smaller changes, and statistical testing consistently confirms no significant difference between basal and Cu-treated conditions. In contrast, CTR1 shows a significant population shift under the same conditions. This reinforces that copper-induced de-oligomerization is a specific feature of CTR1 and does not occur in Tac^{mEos}. Therefore, the difference in statistical significance is not due to an artifact of statistical testing but rather reflects the fact that Tac^{mEos} does not respond to copper in the same way as CTR1.

We have incorporated these additional experiments and the corresponding updated analyses into *Extended Data Figure 4e* in **Reply 1.2**. The main text and figure legends have also been revised to clearly reflect the increased sample size and improved statistical precision, emphasizing the specificity of copper-induced oligomerization shifts in CTR1 compared to Tac^{mEos}.

[**Changes in the Main text**] To assess whether copper treatment affects the oligomeric state of a protein unrelated to copper homeostasis, we further analyzed Tac^{mEos} under both basal and Cu-treated conditions using a sample size comparable to that for CTR1. Comparing Tac^{mEos} under these two conditions revealed no significant difference in $\bar{N}$, indicating that Cu treatment does not alter the oligomeric state of the mEos tag itself (Extended Data Fig. 4d,e).

1.4 *Given the large error (e.g., 0.48 and 0.26 being not distinguishable for Tac; in addition, the authors rate their method of quantification to have an accuracy of around 10%): how accurate is the estimate of the dimer population in Figure 3c and 3h? This is a general question, and unrelated to the multi-step model.*

[**Reply 1.4**] We acknowledge the reviewer's concern regarding the accuracy of dimer population estimates, particularly in relation to our reported 10% error. Our previous theoretical model (Analytical Chemistry, 2023 PMC10842889) estimated that the smND approach has an intrinsic accuracy of approximately 10% when extracting protein oligomeric states of proteins with multiple oligomeric states under a saturated dataset. The Tac^{mEos} data under basal and copper condition (see **Reply 1.3**) align well with this theoretical prediction, providing experimental validation that fluctuations within this range reflect the inherent variability of single-molecule detection and analysis rather than systematic errors.

Given this accuracy threshold, population shifts close to 10% must be interpreted with caution, as they may fall within measurement uncertainty. Both $CTR1^{mEos}$ and $CTR1(M150)^{mEos}$ exhibit dimer populations below 10%, meaning that any detected dimer populations are near the resolution limit of our method and should be interpreted cautiously. However, the overall population shift for CTR1 remains robust (>20%) and well above the 10% uncertainty threshold,

reinforcing that copper-induced de-oligomerization is a genuine effect for CTR1. The observed dimer population variability does not affect this conclusion.

Thus, while the smND approach has inherent limitations, the estimates of dimer populations in Fig. 3c and 3h should be interpreted with caution due to their low abundance, but remain within the expected resolution of this method, and the key conclusions remain valid: CTR1 undergoes significant oligomeric changes in response to Cu.

We have included clarifications in the *Discussion* section. These modifications explicitly note the caution required when interpreting low-abundance dimer populations, thereby providing transparent context regarding our method's inherent accuracy and reinforcing the robustness of our core conclusions regarding CTR1 oligomeric changes.

[**Changes in the Main text**] The smND assay inherently provides an oligomeric state quantification accuracy within approximately 10%. Thus, observed dimer populations below or near this threshold must be interpreted cautiously, reflecting the assay's resolution limits rather than definitive biological variability.

1.5 *Why did the authors choose a reference protein with a mixed oligomeric state, rather than a pure dimer or trimer?*

[**Reply 1.5**] In principle, relying on a strictly dimeric protein would seem ideal for validating smND, but in practice even "known" dimers tend to occupy multiple states in cells. Proteins are synthesized as monomers and then assemble into their higher-order forms, typically creating an equilibrium of monomeric and oligomeric species rather than a single uniform structure. For example, despite non-reduced blots classifying CTLA4 as a stable dimer, our smND data still detect a substantial (about 25%) monomeric fraction in cells, indicating that prior biochemical designations may not fully reflect in vivo equilibria (Extended Data Fig. 4f-h in **Reply 1.3**). To circumvent this ambiguity, we employed two constructs engineered to bypass potential re-equilibration steps: Tac^{mEos}, containing a single mEos, and Tac^{2mEos}, which fuses two mEos4b units in tandem (see details in **Reply 1.3**). Both are anchored at the plasma membrane via the Tac antigen, ensuring consistent membrane localization. In Tac^{2mEos}, the tandem arrangement physically locks two fluorophores within a single polypeptide chain, acting as a "built-in dimer" reference, whereas Tac^{mEos} remains monomeric. This design sidesteps the variable assembly pathways seen in ordinary proteins and provides a more definitive test platform for smND under well-defined single- vs. dual-fluorophore conditions.

To clearly articulate our rationale for selecting these reference constructs and address the reviewer's concern, we have included an explicit description of our validation strategy using Tac^{mEos} and Tac^{2mEos} in the *Methods* sections. Additionally, *Extended Data Fig. 4f-h* has been updated to explicitly illustrate the presence of mixed oligomeric states in typical reference proteins, thereby clarifying the necessity and utility of our engineered reference constructs

[**Changes in the Main text**] Although one might prefer a purely dimeric control, many biochemically designated "dimers" still exhibit partial monomeric states in cells (Extended Data Fig. 4f–h). To sidestep this ambiguity, we engineered Tac^{mEos} (single mEos4b) and Tac^{2mEos} (tandem mEos4b) as references with effectively 'locked' single- versus dual-chromophore stoichiometries anchored to the membrane via Tac-antigen. These constructs allowed benchmarking of smND performance under well-defined oligomeric conditions, free from confounding self-assembly pathways.

2. *The quantification of protein oligomers from SMLM data has become increasingly important in recent years. In addition to the method chosen by the authors, an alternative approach that analyzes the kinetics of fluorescence emission to report protein oligomers (qPALM) exists and has been shown to be useful (e.g. PMIDs 38226794 and 38457493). I am not asking the authors to analyze their data using this method as well (although this should be directly possible and it would be interesting to compare the two methods); nevertheless, I would like to ask the authors to briefly mention and discuss both approaches in the discussion section, especially with regard to the points raised above. This will be helpful for the reader.*

   [**Reply 2**] We thank the reviewer for pointing out the relevant qPALM approach. Indeed, both qPALM and smND use single-molecule localization microscopy to quantify protein oligomerization inside cells, yet they differ in how they collect and interpret data. qPALM counts all fluorescence bursts from photoactivated fluorescent proteins within each cluster—using known monomeric (e.g., β1AR-mEos3.2 or CD86-mEos3.2) and dimeric (e.g., CTLA-4–mEos3.2 or CD28-mEos3.2) references to calibrate two common sources of error: (i) blinking, whereby a single fluorophore produces multiple emission events, and (ii) incomplete labeling, whereby some fraction of the tagged subunits never becomes fluorescent. This approach yields direct stoichiometry provided cluster densities are low enough to spatially separate each emission cluster (e.g., tens to a few hundreds of molecules per μm²) rather than having them overlap. smND, by contrast, calculates the number of neighbors within a fixed radius around each localization, building a neighbor density histogram that is fitted with theoretical or simulated curves for monomers, dimers, or higher-order oligomers (e.g., trimers, tetramers, or beyond). Both methods provide super-resolution detail in intact cells and yield quantitative measures of oligomerization.

   However, qPALM works best when well-characterized monomer/dimer references exist and when the expression (hence cluster density) remains sufficiently low to track all emission bursts distinctly. smND is particularly advantageous for mapping diverse equilibrium mixtures or larger complexes (i.e., oligomeric states above dimer) but depends on selecting an appropriate “neighbor radius” and on having sparse enough labeling to avoid excessive random clustering.

   We have now included a brief comparison of the qPALM and smND methodologies in the *Discussion* section of our manuscript. This addition highlights their respective strengths, limitations, and suitable applications, thereby providing readers with essential context for interpreting our results and understanding the broader utility of each approach.

   [**Changes in the Main text**] In addition to our smND assay, alternative single-molecule methodologies, such as qPALM, offer complementary approaches by counting fluorescence bursts per cluster, accounting for blinking and incomplete labeling via calibration with well-characterized monomeric or dimeric references. While qPALM can yield direct stoichiometry, it often requires low expression levels to separate clusters spatially, and accurate calibration controls for blinking behavior. By contrast, smND infers oligomeric states from local neighbor densities, enabling analysis of multi-state protein complexes (e.g., monomers, dimers, trimers) even at moderate expression levels. Both approaches leverage single-molecule resolution in intact cells and can be complementary in dissecting complex equilibrium mixtures.

3. *One limitation of the study is that the mechanism of de-trimerization remains unclear. The discussion here remains somewhat vague and points to an unknown "sensor protein".*

   [**Reply 3**] We agree that the detailed molecular mechanism of CTR1 de-oligomerization is not fully elucidated. We currently hypothesize an as-yet-unidentified intracellular sensor or adaptor that binds CTR1's cytosolic loop upon local Cu accumulation. However, fully identifying that sensor would require far more extensive biochemical and proteomic exploration. As our core finding is that CTR1 subunits dissociate under Cu stress, and that endocytosis is coupled to this de-oligomerization, regardless of the precise sensor involved. Providing detailed mechanism of de-trimerization is thus beyond our scope.

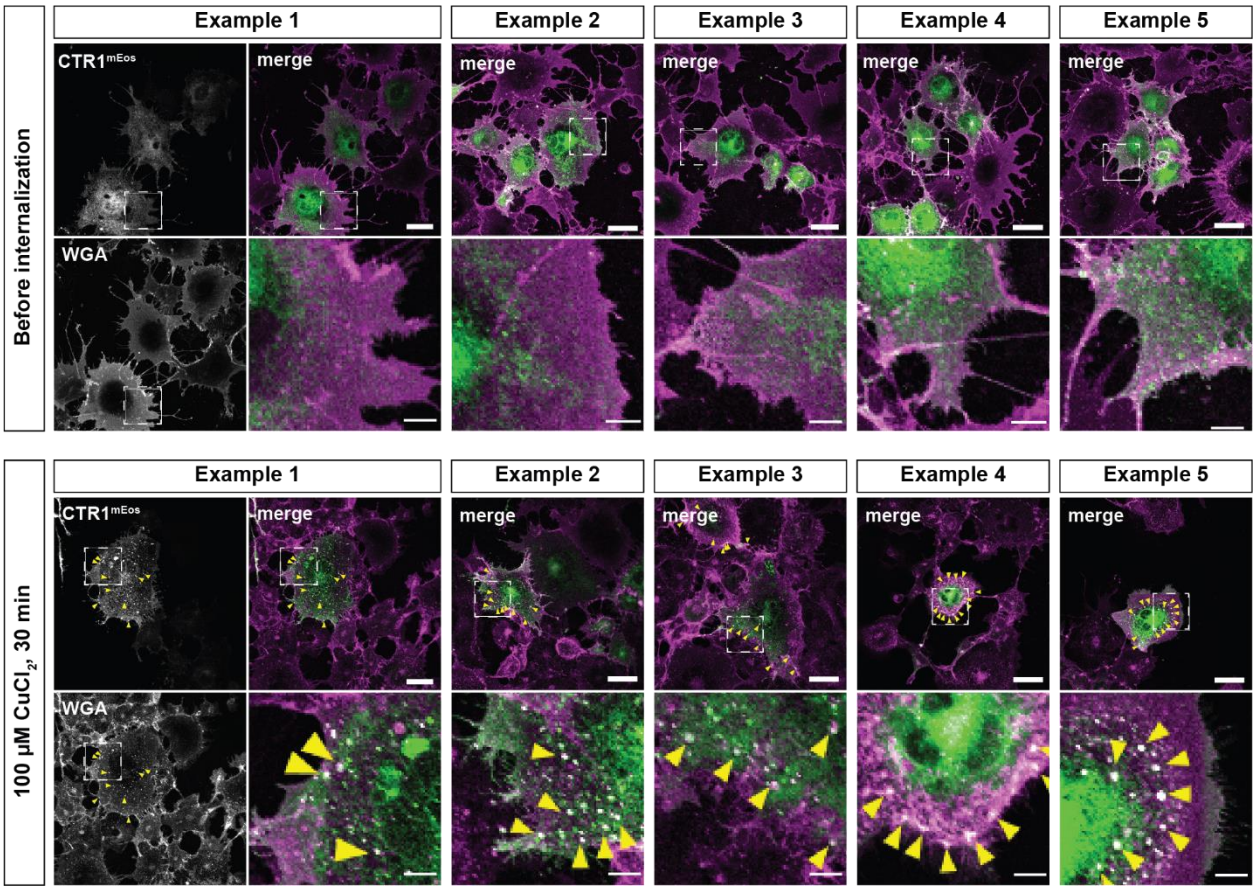
   We have explicitly addressed this limitation in the *Discussion* section of the revised manuscript by clearly acknowledging the unidentified nature of the proposed intracellular sensor or adaptor. Additionally, we suggest potential future experimental strategies, such as CRISPR-based screens or pull-down mass spectrometry, that could help identify these important components in subsequent studies.

   [**Changes in the Main text**] Although our study reveals significant copper-induced de-oligomerization of CTR1 linked to its endocytosis, the precise molecular players facilitating this structural transition remain unidentified. We hypothesize the involvement of intracellular sensors or adaptor proteins interacting with CTR1's cytosolic domain in response to elevated copper levels. Identifying these components is beyond the current study's scope and will require future proteomic or genetic screening approaches, such as CRISPR-based identification or affinity pull-down mass spectrometry.

4. *(minor comments): Extended Data Fig. 2, I find it difficult to see the internalization of CTR1 at high copper levels from the one cell shown. Could the authors rather show a larger panel of many cells (tens or more)?*

   [**Reply 4**] We appreciate the reviewer's suggestion and have taken additional images to better illustrate the internalization behavior of $CTR1^{mEos}$ upon copper stimulation. We have expanded *Extended Data Fig. 2* to include more representative cells under basal and Cu-stressed conditions. Due to the small size of endocytic vesicles, we further annotate colocalized puncta with arrows and provide enlarged images to improve discernibility. This revision demonstrates the reproducibility of CTR1 internalization upon copper treatment.

   [**Changes in the Main text**]



Extended Data Fig. 2 | WGA-surface labeling tracks Cu-induced endocytosis of CTR1. The confocal images of COS7 cells overexpressing CTR1^{mEos} before (top Examples 1-5) and after (bottom Examples 1-5) treatment with 100 μM $CuCl_2$ for 30 minutes. Cells were labeled with Alexa647-WGA to visualize membrane trafficking. The Example 1 images depict the distributions of CTR1^{mEos} and Alexa647-WGA (left two panels). The right two panels show a merged image of CTR1^{mEos} (green) and WGA (magenta), and the selected zoom in area (dash line box). Examples 2-5 show another four cells showing similar behaviors. After Cu stimulation, plasma membrane-derived internalized CTR1^{mEos} and WGA were imaged simultaneously. Arrows indicate colocalized puncta (white spots), indicating the endocytosis of CTR1 through endocytic vesicles. The enlarged images of these vesicles are provided to facilitate clear identification. The increase of WGA intracellular puncta and colocalization with CTR1^{mEos} indicates effective copper-induced endocytosis. Scale bar: 50 μm; zoom in area scale bar: 10 μm.

5. *(minor comments): Extended Data Fig. 3, it is pretty much accepted in the field that an evanescent light field penetrates to around 100 nm into a cell; the authors made an extra effort to show that here, which I appreciate.*

   [**Reply 5**] We thank the reviewer for the compliment regarding Extended Data Fig. 3.

6. *(minor comments): The abbreviation 'mE', for mEos4b, is a bit confusing to read and maybe unusual.*

   [**Reply 6**] We agree that the abbreviation "mE" may be confusing and have revised it as "mEos" for clarity.

Reviewer #2

The study tests an interesting and novel idea that the de-oligomerization of the copper transporter CTR1 is an initial step in CTR1 endocytosis, which effectively stops copper (Cu) uptake. While the concept is very intriguing, the evidence of de-oligomerization is not very strong (or not convincingly explained), and the mechanism detailing how a stable trimer dissociates into monomer has not yet been investigated. The work appears to be at the early stage. Specific comments:

[**Reply**] We appreciate the reviewer's interest in the mechanistic aspects.

7. *The authors seem to assume that copper binding at M150 of CTR1 triggers endocytosis. There is little basis for such assumption. If this was the case, then each time Cu binds to CTR1 the protein would endocytose, i.e. CTR1 will act as transferrin receptor rather than a transporter. Furthermore, the C-terminal mutant that retains M150 but has the C-terminal HCH motif mutated does not endocytose, which suggests that the response to elevated copper comes from the cytosol. The alternative explanation for M150 mutant not being able to endocytose is that the mutation inhibits Cu uptake and eliminate cytosolic Cu sensing by CTR1. If the authors believe that their model is correct, this alternative scenario needs to be eliminated.*

   [**Reply 7**] We appreciate the reviewer's thoughtful comment and acknowledge that our original phrasing might have led to misunderstanding. To clarify, we do not propose that copper binding at Met150 of CTR1 directly triggers endocytosis upon each binding event. Rather, our model, supported by previous studies (Guo et al., JBC 2004; Petris et al., JBC 2003) and our own recent findings, emphasizes that functional copper transport into the cytosol is critical for subsequent CTR1 endocytosis. In other words, copper must effectively pass through CTR1 into the cell to activate intracellular signaling pathways that recruit endocytic machinery to CTR1.

   To further test this hypothesis and address the alternative scenario proposed by the reviewer—that the lack of endocytosis in the CTR1(M150L) mutant might simply result from impaired copper uptake—we conducted additional biotinylation experiments using a copper ionophore (Cu-elesclomol) to artificially elevate intracellular copper. Notably, when cells expressing CTR1(M150L)^{mEos} were treated with 2 μM of the membrane-permeable ionophore Cu-elesclomol (CuES), mutant CTR1 exhibited clear copper-induced internalization. This result compellingly indicates that elevated intracellular copper, rather than extracellular copper binding to the Met150 site alone, is the key factor driving CTR1 endocytosis. These results explicitly eliminate the alternative scenario posed by the reviewer. The mutation at M150 does indeed impair copper uptake, but the essential trigger for CTR1 endocytosis originates intracellularly, after copper has traversed the plasma membrane via CTR1, rather than from extracellular copper binding alone. We have explicitly updated the *Discussion* section of our manuscript to clarify this critical point. Specifically, we have now clearly described that functional intracellular copper transport, rather than extracellular copper binding alone, is essential for initiating CTR1 endocytosis. Moreover, we have included details of the new ionophore-based experiments, emphasizing that intracellular copper accumulation is a key regulator of CTR1 oligomerization and endocytosis.

   [**Changes in the Main text**] Our smND results (Fig. 3c–e) demonstrate that CTR1 exists in a dynamic equilibrium of different oligomeric states and shifts toward the monomeric state in response to elevated copper levels, which trigger endocytosis. While molecular dynamics simulations suggested that both the M150 mutation and copper binding at specific sites (M150 and M154) stabilize CTR1 trimers, experimental observations revealed a contrasting

phenomenon: copper induces de-trimerization of wild-type CTR1 at early endocytic areas, a response blocked in the M150L mutant. This raises a key question: is endocytosis triggered directly by copper binding at Met150, or does the M150L mutant fail to internalize simply because it blocks copper uptake and prevents the intracellular signal required for endocytosis?

To distinguish between structural stabilization and cytosolic signaling as the cause of impaired endocytosis in the M150L mutant, we conducted additional experiments using a membrane-permeable copper ionophores, Cu-elesclomol (CuES).[34,35] These reagents bypass CTR1-mediated uptake and directly elevate intracellular copper levels. The biotinylation assay shows that, in contrast to $CuCl_2$ fails to trigger internalization of CTR1(M150L), cells treated with CuES robustly demonstrated decrease of surface CTR1 populations. This indicates a restoration of the mutant's ability to internalize (Fig. 5a). Computational energy calculations reveal substantial intrinsic stability of the CTR1 trimer (dissociation energy 34.56 kcal/mol plus ΔΔG 9.62 kcal/mol), indicating that physiological dissociation under copper stress likely involves additional intracellular effectors or mechanical forces supplied by the recruited endocytic machinery. Thus, the key role of Met150 lies in enabling copper permeation, not in acting as a sensor or trigger itself.

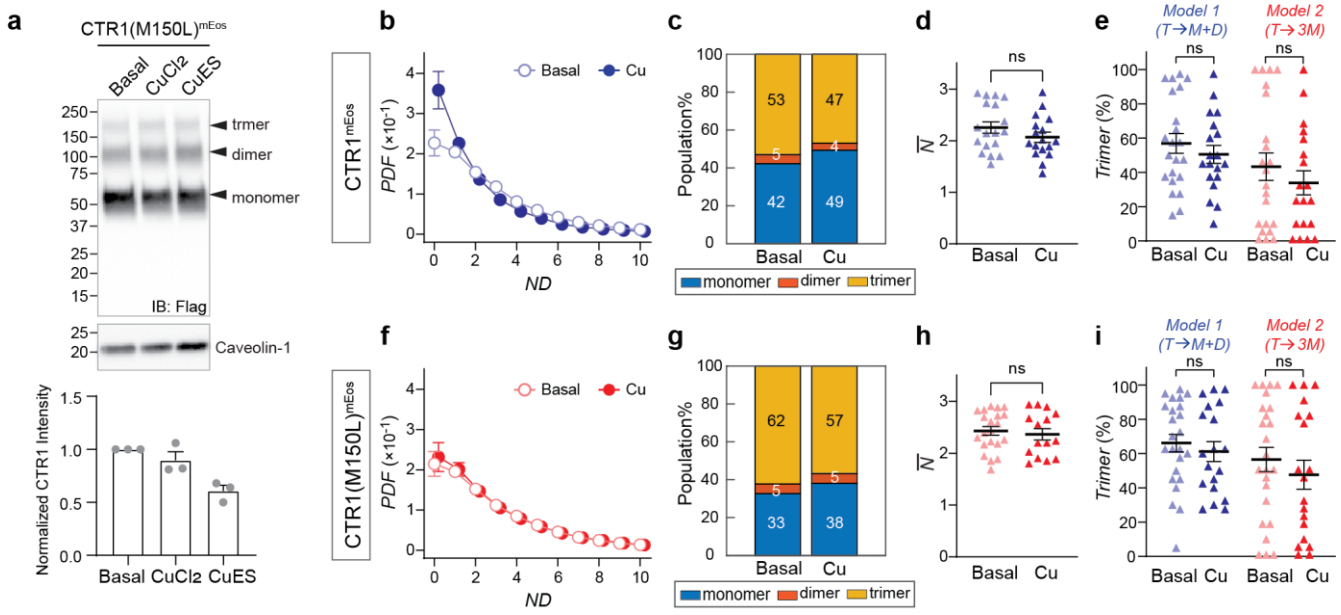


Fig. 5 | Intracellular copper elevation triggers CTR1 dissociation primarily in Rab5⁺, not Rab5⁻, compartments. a, Biotinylation assay comparing the surface abundance of the CTR1(M150L)^{mEos} under different copper treatments. Treatment with 100 μM $CuCl_2$ alone fails to trigger its internalization, whereas exposure to 2 μM of the membrane-permeable CuES ionophore restores endocytosis. This confirms that elevated intracellular copper, rather than extracellular copper binding, drives CTR1 internalization. Representative immunoblots show total and biotinylated surface CTR1(M150L)^{mEos} normalized to the loading control Caveolin-1. (**b–i**), smND analysis of CTR1 oligomerization in Rab5⁻ compartments. Under basal conditions, wild-type CTR1^{mEos} (**b–e**) shows minimal changes in trimer populations upon copper stress, shifting from approximately 53% to 47%, within the uncertainty of smND. Similarly, the endocytosis-deficient CTR1(M150L)^{mEos} mutant (**f–i**) demonstrates negligible oligomeric shifts (~62% to 57%) under copper treatment in Rab5⁻ areas, confirming that significant copper-induced dissociation of CTR1 occurs specifically in Rab5-positive compartments. Data represent mean ± SEM from three independent experiments.

8. *"The increased stability of the trimer in the mutant raises the possibility that the ability of CTR1 to alter its oligomeric state plays a critical role in its regulation of copper uptake" – Can the authors calculate the energetic cost of de-oligomerization?*

[**Reply 8**] We appreciate the reviewer's insightful suggestion regarding the energetic analysis of CTR1 de-oligomerization. We performed computational energy calculations using FoldX, a widely used protein modeling tool that combines empirical force fields with statistical potentials to predict binding energies and mutational effects on stability. For the CTR1 trimer, FoldX estimated the binding energies between subunits (chains A-B, B-C, and A-C) as −11.19 kcal/mol, −11.29 kcal/mol, and −12.08 kcal/mol, respectively. Summing these pairwise interactions suggests a total energetic cost of ~34.56 kcal/mol for complete trimer dissociation.

Importantly, our experimental data clearly demonstrate that despite the energetic stabilization of the trimer in the presence of copper, even mutant CTR1 readily undergoes de-oligomerization when intracellular copper levels are sufficiently elevated. This apparent contradiction between computational stabilization and observed dissociation can be reconciled by our proposed model: intracellular copper accumulation activates endocytic machinery, providing an additional mechanical or energetic input required for trimer dissociation. Thus, the calculated energetic values represent intrinsic thermodynamic stabilities of CTR1 trimers, whereas the physiological de-oligomerization involves additional intracellular factors beyond simple thermodynamic equilibrium.

We have explicitly incorporated these energetic calculations and clarified their interpretation in the *Results* and *Methods* sections (see **Reply 7**) section of our manuscript. Specifically, we now clearly indicate that intrinsic thermodynamic stability calculated by FoldX contrasts with the experimentally observed physiological dissociation, emphasizing the necessity of additional intracellular factors for CTR1 de-oligomerization under copper stress.

[**Changes in the Main text**] To evaluate the thermodynamic stability of wild-type CTR1, we used FoldX to estimate the dissociation energy of its trimeric structure.[20] FoldX calculated the binding energies between subunit pairs, namely A-B, B-C, and A-C, as −11.19 kcal/mol, −11.29 kcal/mol, and −12.08 kcal/mol, respectively. The total dissociation energy, obtained by summing these pairwise interactions, was approximately 34.56 kcal/mol, representing the energetic cost required to fully dissociate the trimer into individual subunits.

9. *How does the insertion of 3 GFP-FLAG into a small and tightly packed trimeric protein, like CTR1 affect protein stability? AlphaFold3 analysis of the wild type and CTR1-GFP-Flag trimers suggests a significant destabilization of the trimer by the fusion. The authors should provide evidence that the stability of the wild-type and fusion CTR1 are similar. This can be done either computationally or experimentally (for example, by analysis of thermostability/urea sensitivity on nondenaturing gels or by correlation spectroscopy).*

[**Reply 9**] The reviewer raises an important question regarding whether the insertion of mEos4b-FLAG destabilizes the trimeric structure of CTR1. To address this, we directly compared the functional activity of wild-type CTR1 and CTR1^{mEos} by measuring intracellular copper levels under basal and high-copper conditions. If the fusion construct were significantly destabilized or impaired in trimer formation, we would expect a decrease in copper uptake efficiency, as only the trimeric form of CTR1 is competent for copper transport. However, cells expressing wild-type CTR1 and those expressing CTR1^{mEos} exhibit similar intracellular copper levels under both basal

and 50 μM $CuCl_2$ conditions, indicating that the fusion does not significantly disrupt the trimeric state or function of CTR1.

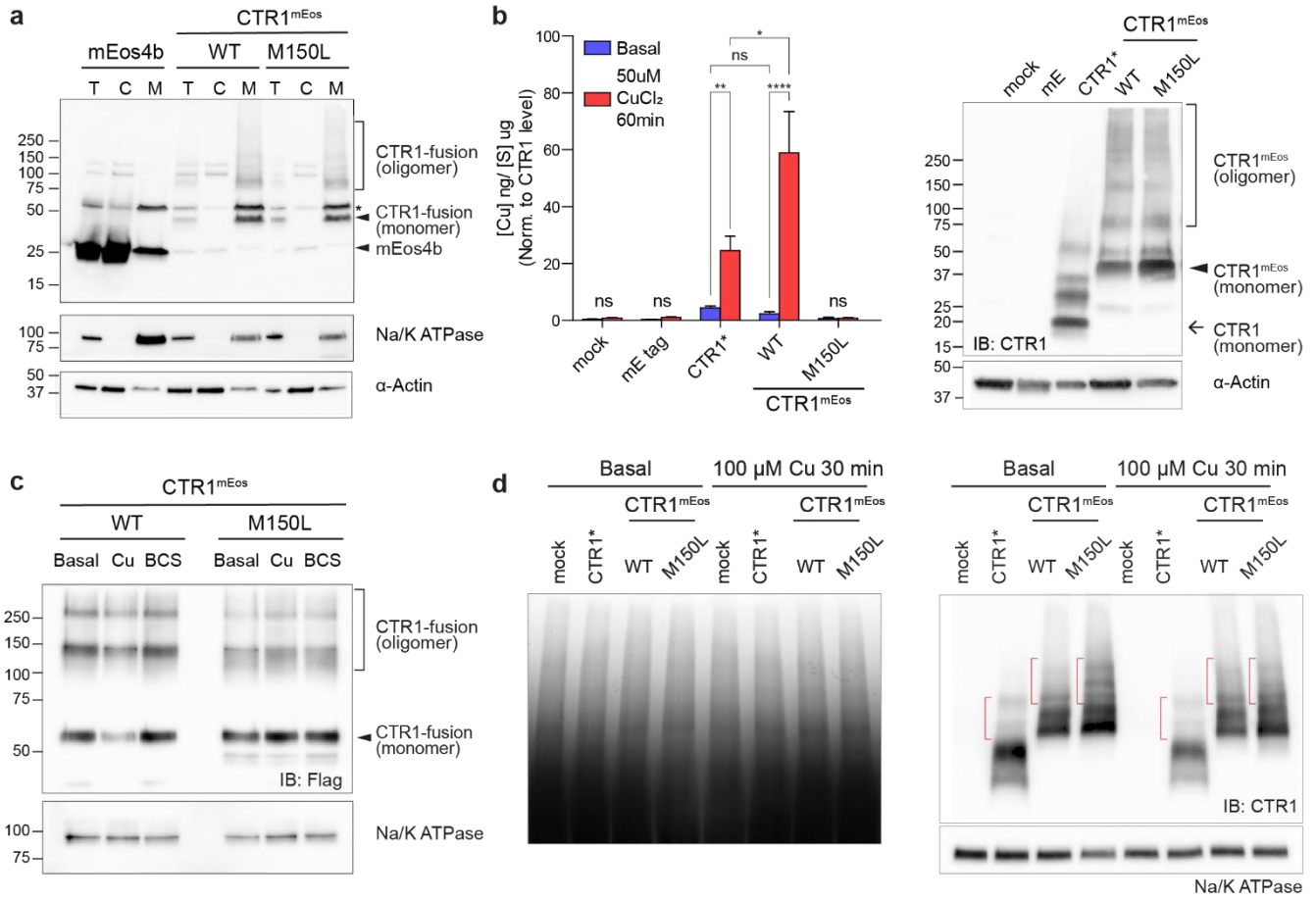
In addition to these functional assays, our previous membrane fractionation and immunoblotting experiments (Extended Data Fig. 1a) show that $CTR1^{mEos}$ exhibits similar membrane localization patterns to wild-type CTR1, reinforcing that the fusion does not alter the stability or trafficking behavior of the protein.

To further validate that $CTR1^{mEos}$ maintains trimeric stability, we also performed blue native PAGE as reviewer suggested in the later question to assess its oligomeric state under native conditions (Extended Data Fig. 1d). Both wild-type CTR1 and $CTR1^{mEos}$ exhibit the same banding pattern, with four distinct bands observed, corresponding to different oligomeric states. While the exact relationship between band number and oligomeric state remains uncertain, the presence of the same four-band pattern in both constructs suggests that $CTR1^{mEos}$ maintains a similar oligomeric distribution to wild-type CTR1. The relative distribution of these bands remains comparable between wild-type CTR1 and $CTR1^{mEos}$, indicating that the fusion does not significantly alter the oligomeric equilibrium.

Together, these results collectively indicate that the mEos4b-FLAG fusion does not destabilize CTR1's trimeric structure or impair its copper transport function. While AlphaFold3 predictions suggest possible structural perturbations, our experimental functional data demonstrate that $CTR1^{mEos}$ remains fully capable of mediating copper uptake and retains its native oligomeric state, supporting the validity of using this construct to study CTR1 oligomerization dynamics.

We have incorporated the experimental validation data into Extended Data Fig. 1, explicitly describing these stability and oligomeric analyses in both the figure legend and the Methods section. Additionally, the manuscript now clearly emphasizes the agreement between our functional assays, membrane localization, and native PAGE experiments, collectively reinforcing the conclusion that the mEos4b-FLAG fusion does not significantly affect CTR1 stability or its functional oligomeric state.

[**Changes in the Main text**] The generated CTR1 fusion protein, $CTR1^{mEos}$, showed the expected full-length product (Extended Data Fig. 1a) and retained both copper uptake activity and Cu-responsive endocytosis, as validated through direct Cu measurement using ICP-MS and surface biotinylation. Specifically, ICP-MS quantification showed comparable intracellular copper levels between $CTR1^{mEos}$ and wild-type CTR1 under identical conditions, confirming that the fusion protein maintains effective copper transport activity (Extended Data Fig. 1b). Cells that overexpressed $CTR1^{mEos}$ showed reduced surface $CTR1^{mEos}$ abundance in the presence of high copper (Extended Data Fig. 1c; Extended Data Fig. 2), indicating effective Cu-induced endocytosis. Additionally, blue-native PAGE analyses demonstrated that $CTR1^{mEos}$ exhibits oligomeric patterns identical to wild-type CTR1 at the plasma membrane, further validating the structural integrity and native behavior of the fusion construct. Due to extremely low endogenous CTR1 expression, heterotrimer formation between endogenous CTR1 and $CTR1^{mEos}$ is negligible (Extended Data Fig. 1d), affirming that observed oligomeric transitions primarily reflect the functional homotrimers of the exogenous protein rather than artifacts of overexpression or instability.



Extended Data Fig. 1 | Characterization of CTR1 fusion proteins. a, Immunoblotting of COS7 cells expressing mEos4b-Flag (mEos4b), CTR1^{mEos}, or CTR1(M150L)^{mEos} proteins. Cells were fractionated into total (T), cytosolic (C), and membrane (M) fractions. Equal amounts of protein from each fraction were probed with an anti-Flag antibody. A duplicate membrane was probed with Na/K ATPase and α-Actin to serve as markers for the plasma membrane and cytosol, respectively. The asterisk (*) denotes a non-specific band. **b**, Intracellular copper levels in HEK293 cells expressing mEos4b-Flag, non-tagged CTR1 (CTR1*), CTR1^{mEos} or CTR1(M150L)^{mEos}, measured by ICP-MS (top). The copper levels were normalized to exogenous CTR1 expression detected by immunoblotting (bottom). Both CTR1* and CTR1^{mEos} exhibit increased copper uptake under copper-treated conditions, confirming that the mEos4b-Flag tag does not impair CTR1's transport function. **c**, Immunoblot of surface biotinylated CTR1^{mEos} and CTR1(M150L)^{mEos} under basal, copper-stressed (Cu), and copper-deficient (BCS) conditions. Reduced surface CTR1^{mEos} levels upon copper treatment indicate effective endocytosis, which is absent in CTR1(M150L)^{mEos}. **d,** Blue-native PAGE (BN-PAGE) of plasma membrane fractions isolated from COS7 cells transfected with various CTR1 constructs as indicated or mock control. Due to the low abundance of CTR1 protein which cannot be directly visualized on the PAGE, the PAGE was further transfer to PVDF blot and subject to immunoblotting using ant-CTR1 antibody to detect all the CTR1. Multiple high molecular weight bands (brackets) are observed for all constructs, indicating the presence of high-order oligomers. Notably, endogenous CTR1 is not detectable under our experimental conditions, as shown by the absence of signal in the mock lane. An equal proportion of membrane extracts were resolved in parallel under standard SDS-PAGE and immunoblotting probed with Na/K ATPase served as a loading control.

10. *Presence of monomers, dimers, and trimers at the plasma membrane at steady state needs to be verified independently because it may be a result of overexpression and/or instability of the fusion protein (see above). In addition to surface biotinylation experiments that convincingly show Cu induced endocytosis, the authors should perform blue native gel analysis for endogenous CTR1 to ensure that the fusion construct behaves identically, when it comes to monomers, dimers, and trimers.*

[**Reply 10**] The reviewer raises an important concern regarding whether the observed monomer, dimer, and trimer populations might be artifacts of overexpression and/or instability of the fusion protein rather than reflecting the true biological behavior of CTR1.

We first exclude the possibility of instability of the fusion protein. Extended Data Fig. 1a showing both wild-type and mutant CTR1 fusion proteins are enriched in membrane fraction, supporting that they are indeed membrane-anchored. In addition, no obvious cleavage fragments corresponding to soluble mEos4b, which was performed in parallel serving as reference on the blot, were detected in the cytosol fractions. It supports the stability of these CTR1 fusion proteins in our expression system.

To further confirm that the observed oligomeric states are not artifacts of overexpression, we performed blue native PAGE using plasma membrane fractions from COS7 cells to assess the oligomeric distribution of $CTR1^{mEos}$ under native conditions (Extended Data Fig. 1d). The results show that wild-type CTR1 and $CTR1^{mEos}$ exhibit the same banding pattern, with multiple distinct bands corresponding to different oligomeric states. Importantly, the presence of these bands in plasma membrane-enriched fractions confirms that these oligomeric species exist at the plasma membrane, rather than being a consequence of overexpression artifacts or cytosolic aggregation. The overall distribution of oligomeric species remains comparable between wild-type CTR1 and $CTR1^{mEos}$, further supporting that the fusion construct does not alter the oligomeric behavior of CTR1 at the plasma membrane.

To comprehensively address overexpression issue, we consider the possible ways in which overexpression could hypothetically affect oligomeric distribution and demonstrate that none of these scenarios account for our observed results.

Scenario 1: Overexpression leads to a higher population of monomers

If overexpression were responsible for the observed oligomeric shift, we would expect a substantial fraction of monomers even under basal conditions. However, our smND analysis shows that CTR1 remains predominantly trimeric at steady state, and monomeric and dimeric species only increase upon copper exposure, directly contradicting the hypothesis that overexpression passively drives monomer formation.

Scenario 2: Overexpression leads to a higher population of trimers

Another possible overexpression-driven artifact could be the stabilization of trimer formation, as higher protein abundance increases the likelihood of trimeric interactions. In this case, we would expect that upon copper exposure, an even greater fraction of CTR1 molecules would remain in the trimeric state due to mass action favoring oligomerization. However, our smND analysis reveals the opposite effect, where the $\bar{N}$ decreases in the copper rather than increases in response to copper. If overexpression were passively driving trimerization, the population of monomers should remain low regardless of copper levels, but our data demonstrate a controlled shift toward monomers upon copper treatment.

Scenario 3: Overexpression causes a random distribution of oligomeric States

Another possibility is that overexpression disrupts CTR1's regulated oligomeric equilibrium, leading to a broad and unstructured distribution of monomers, dimers, and trimers. If this were the case, we would expect the ND distribution to be highly variable, lacking a defined oligomeric preference. However, our data show that CTR1 follows a structured, condition-dependent transition: under basal conditions, trimers predominate, whereas copper exposure results in a controlled increase in monomers. The specificity of this transition is inconsistent with a random overexpression effect and instead indicates a tightly regulated mechanism.

Additional evidence supporting a regulated oligomeric transition

To further exclude overexpression artifacts, we tested whether CTR1^{mEos} functions identically to wild-type CTR1 by measuring intracellular copper levels under basal and high-copper conditions. Since only trimeric CTR1 can efficiently mediate copper transport, if overexpression disrupted its native oligomeric state, we would expect a measurable difference in copper uptake. However, our results show that cells expressing CTR1^{mEos} and wild-type CTR1 accumulate intracellular copper at similar levels, confirming that the fusion construct retains normal trimeric function. This further supports that CTR1^{mEos} faithfully recapitulates the native behavior of wild-type CTR1 and that its oligomeric transition is not an artifact of overexpression.

By considering all possible overexpression artifacts (whether favoring monomers, trimers, or a random distribution), we demonstrate that none can explain the observed results. Instead, our data consistently show that CTR1 undergoes a regulated, biologically driven transition from trimer to monomer upon copper exposure, regardless of expression level. Importantly, our smND analysis of CTR1 expression levels directly shows that higher CTR1 concentrations favor trimer formation rather than monomer formation, further confirming that the monomeric shift we observe in the presence of copper is not an artifact of overexpression but a controlled regulatory event.

We acknowledge the reviewer's request for a blue-native gel analysis of endogenous CTR1 to confirm physiological oligomeric distributions. Due to the low endogenous CTR1 abundance in our COS7/HEK293 lines and difficulties in maintaining complex integrity under native conditions, we have not yet obtained robust BN-PAGE data for endogenous CTR1. Nonetheless, our functional assays (unchanged copper uptake, stable membrane localization, and regulated trimer-to-monomer shift under Cu stress) collectively indicate that the fusion construct mirrors the native behavior. We plan to explore BN-PAGE or other native analyses (e.g., crosslinking, correlation spectroscopy) in future work to further corroborate these findings.

We have explicitly incorporated the blue native PAGE results into *Extended Data Fig. 1* and updated the corresponding figure legends and Methods section to highlight these additional experiments (see **Reply 9**). Furthermore, we included a discussion in the manuscript addressing potential artifacts from overexpression, outlining the scenarios discussed above, and explicitly clarifying why our experimental evidence strongly supports the physiological relevance of the observed oligomeric distributions.

11. *Can the authors exclude that the increased activity of CTR1mE is caused by CTR1mE homotrimer rather than heterotrimers with the endogenous CTR1? To better illustrate functionality of CTR1mE – the comparison should be made with the wild-type CTR1 expressed at the same level as CTR1mE.*

[**Reply 11**] While it is theoretically possible for heterotrimers to form, our experimental evidence suggests that this scenario is highly unlikely. Our blue native PAGE analysis using plasma membrane fractions from COS7 cells (*Extended Data Fig. 1d*, **Reply 9**) reveals that endogenous CTR1 is not detectable under our experimental setup (mock lane). This indicates that the endogenous CTR1 expression level is extremely low in our system, making the formation of heterotrimers with CTR1^{mEos} highly improbable. Given the overexpression of CTR1^{mEos}, the vast majority of CTR1 complexes at the plasma membrane are expected to be composed of the exogenous protein rather than mixed with undetectable levels of endogenous CTR1.

Additionally, we acknowledge the reviewer's request for a comparison with wild-type CTR1 expressed at the same level as $CTR1^{mEos}$. While absolute expression matching can be challenging, our functional assays (e.g., intracellular copper accumulation) show that $CTR1^{mEos}$ exhibits comparable activity to wild-type CTR1 under identical experimental conditions. This further supports that the increased activity of $CTR1^{mEos}$ arises from its functional homotrimers rather than potential interactions with endogenous CTR1.

Based on these findings, we conclude that while heterotrimer formation is highly unlikely due to the absence of detectable endogenous CTR1 in our system. The functional activity of $CTR1^{mEos}$ is thus primarily attributed to its homotrimeric state, reinforcing the validity of using this construct to study CTR1 oligomerization and function.

We have clearly described the blue native PAGE analysis and clarified the low expression levels of endogenous CTR1 in the updated *Extended Data Fig. 1d*. Additionally, we have explicitly addressed the unlikely formation of heterotrimers in the Discussion section, emphasizing that the functional activity observed for $CTR1^{mEos}$ reflects its homotrimeric state. These revisions strengthen the manuscript by transparently addressing the potential concern regarding heterotrimer formation.

12. *It is not clear how method with 10 nm resolution can resolve individual CTR1 subunits, which have a cross-section in the membrane of 5 nm. This needs to be explained better.*

[**Reply 12**] We fully agree that smND does not directly resolve the 5 nm structure of a single complex. Instead, it captures the 2D coordinates of each fluorescent subunit and uses the spatial distribution (i.e., how close or far subunits appear) to distinguish oligomeric states. Smaller expansions (~1–2 nm) wouldn't register as "separate spots" if they remain within the same ~40 nm region of interest. So truly "split" subunits are typically more than 20–40 nm apart. We have clarified this point in the Methods ("Single-molecule neighbor density assay") to avoid confusion. We have provided more detailed working principles of smND in **Reply 2**, and emphasized how one can resolve oligomeric states with a resolution lower than the protein complex itself in *Discussion*.

[**Changes in the Main text**] Although the method does not directly resolve proteins smaller than its ~10 nm resolution limit, it effectively discriminates oligomeric states through spatial distribution patterns, and reliably distinguishing monomers from higher-order oligomers like trimers.

13. *Related, if the method does resolve CTR1 monomer from trimers then the individual CTR1 signals within Rab5 puncta should be markedly different from CTR1-signals outside Rab5 puncta, where functional CTR1 is uniformly trimer. Is this what the authors observe? Figure 2e (the CTR1 map) is difficult to interpret*

[**Reply 13**] In response to the reviewer's suggestion, we examined whether our method distinguishes CTR1's oligomeric state inside ($Rab5^+$) and outside ($Rab5^-$) Rab5-labeled compartments, we performed a detailed comparison of $Rab5^+$ and $Rab5^-$ regions. To identify $Rab5^-$ areas, we first excluded $Rab5^+$ compartments and then selected random regions displaying comparable CTR1 locations and signal intensities for both the M150L mutant and WT CTR1 under the same treatment conditions. Here, intensity refers to the height of the CTR1

signal relative to the coverslip, ensuring that the chosen Rab5$^-$ regions closely matched the Rab5$^+$ areas in protein distribution.

Several molecular mechanisms could explain how CTR1 detrimerizes under copper stress. One possibility is that Rab5$^+$ endosomes serve as the primary site for trimer reduction, leading to a more pronounced loss of trimers in these compartments. Another possibility is that Rab5$^-$ regions play an equally or more effective role in detrimerization, producing comparable or greater changes outside Rab5$^+$ areas. A third scenario is that CTR1 detrimerizes uniformly throughout the membrane, making no specific compartment the main site. Our experimental data help us evaluate which pathway dominates.

Our data revealed a clear compartment-specific difference: WT CTR1 exhibited a marked decrease in trimer content within Rab5$^+$ compartments under copper stress (from ~65% to ~44%). In comparison, the trimeric population in Rab5$^-$ regions showed only minor changes under copper stress (from ~53% to ~47%). These results suggest the model where Rab5$^+$ endosomes serve as primary sites for copper-induced CTR1 detrimerization. We interpret the consistently lower trimer content and limited copper-induced changes observed in Rab5$^-$ areas as potentially reflecting lateral diffusion of CTR1 subunits from their primary dissociation sites at Rab5$^+$ compartments, combined with the inherently heterogeneous membrane environment of Rab5$^-$ regions. Rab5$^-$ areas, lacking specialized factors that stabilize trimeric CTR1, may represent an equilibrated mixture of oligomeric states, influenced by transient interactions and diffusional processes. Furthermore, the M150L mutant showed minimal responsiveness in both Rab5$^+$ (from ~71% to ~66%) and Rab5$^-$ areas (from ~62% to ~57%) under copper stress, indicating that functional copper transport and subsequent intracellular signaling are necessary to trigger CTR1 dissociation effectively. Collectively, these findings strengthen the conclusion that Rab5$^+$ compartments predominantly catalyze copper-induced CTR1 dissociation, with Rab5$^-$ areas reflecting secondary or diffusive processes rather than primary regulatory events.

Past studies have shown that CTR1 primarily undergoes endocytosis via CME, involving regions eventually marked by Rab5 as early endosomes. Thus, the observed trimer dissociation predominantly in Rab5$^+$ areas could align well with CME-derived endocytic vesicle formation, suggesting that dissociation may occur shortly before or during the initial internalization steps mediated by clathrin-coated vesicles. Although our current data alone do not conclusively determine the precise spatial-temporal relationship between CTR1 dissociation and CME, the significant oligomeric shift observed in Rab5$^+$ compartments is consistent with the notion that these areas could represent hotspots for dissociation events linked to established CME mechanisms. The modest changes seen in Rab5$^-$ compartments might reflect subsequent diffusion of dissociated CTR1 subunits away from primary dissociation sites or local heterogeneities in membrane environments rather than active copper-induced dissociation. Further detailed studies will be needed to fully validate and clarify this integrated mechanism.

We have incorporated these comparative analyses of CTR1 oligomeric states in Rab5$^+$ and Rab5$^-$ compartments into the revised *Results* section and added *Fig. 5* (**Reply 7**) to highlight these findings, clearly emphasizing Rab5$^+$ compartments as primary sites for copper-induced CTR1 detrimerization. Analysis Details were provided in the *Supplementary Method* section. On the other hand, we also update *Fig. 2e* to improve the readability of the CTR1 map.

[**Changes in the Main text**] To further delineate compartment-specific differences in CTR1 oligomerization upon copper stress, we compared CTR1 trimer populations in $Rab5^+$ and $Rab5^-$ regions. WT CTR1 demonstrated a substantial and statistically significant reduction in trimer content within $Rab5^+$ compartments (from ~65% to ~44%, Fig. 3c–e). Conversely, $Rab5^-$ areas exhibited only minor oligomeric shifts (from ~53% to ~47%, within smND uncertainty, Fig. 5b–e). These data imply that CTR1 dissociation primarily occurs at or near $Rab5^+$ compartments, with $Rab5^-$ areas likely reflecting secondary equilibria or diffusional redistribution. In contrast, the endocytosis-deficient M150L mutant showed minimal changes (~5%) in trimer populations in both $Rab5^+$ (71% to 66%, Fig. 3h) and $Rab5^-$ (62% to 57%, Fig. 5g) compartments, indicating impaired responsiveness to copper-induced intracellular signals required for CTR1 dissociation.

[Changes in Supplementary Methods] ***CTR1 population analysis of Rab5$^-$ area.*** To analyze the oligomeric states of CTR1 localized outside Rab5 compartments ($Rab5^-$ areas), a systematic approach involving several sequential processing steps was followed: defining $Rab5^-$ regions, excluding unsuitable regions, compensating single-molecule background noise, accurately calculating local concentrations, and performing theoretical PND analysis. $Rab5^-$ regions were defined by selecting areas outside these identified $Rab5^+$ compartments. To ensure reliable analysis, regions too close to gold fiducial markers, cell edges, or imaging frame boundaries were excluded. Single-molecule background noise was compensated by sampling molecule density in regions outside the cellular boundaries and subsequently subtracting a corresponding number of randomly selected mEos4b coordinates from each $Rab5^-$ region.

Accurate calculation of local CTR1 concentrations was critical for theoretical PND modeling. We calculated the concentration within each $Rab5^-$ region using polygons precisely constructed around grouped mEos4b molecule locations. Subsequently, regions were sorted based on their CTR1 concentration. To ensure statistical reliability and saturation, concentration groups with fewer than five distinct $Rab5^-$ regions were excluded from subsequent analysis.

Finally, measured PND distributions were computed from mEos4b coordinates, and theoretical PND distributions were calculated using parameters such as the total number of molecules, area density, and fluorophore activation efficiency. These theoretical distributions were used to extract oligomeric subpopulations from experimentally measured PND distributions.

[Change of Fig. 2e]

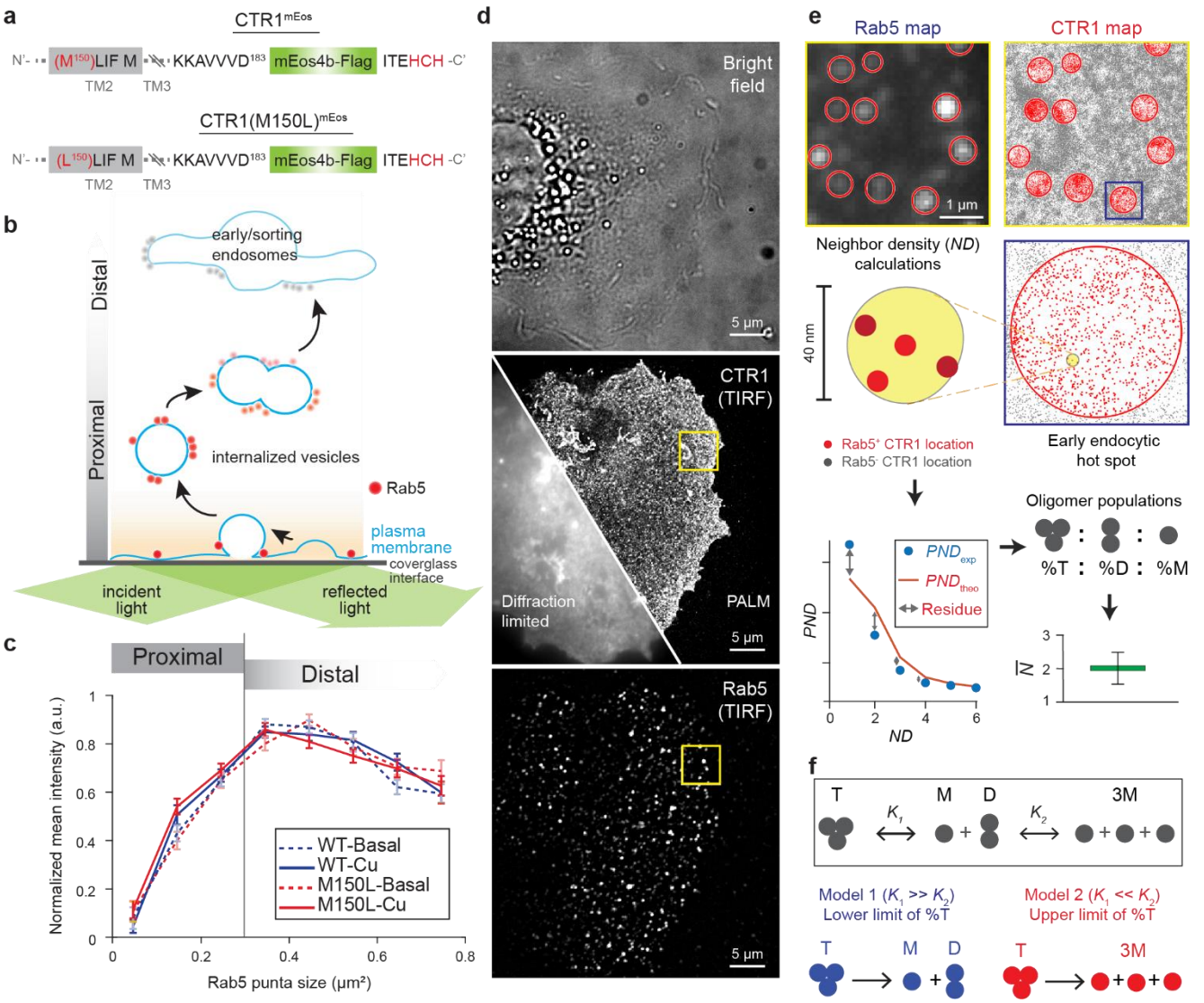


Fig. 2 | Quantification of CTR1 oligomeric states in endocytic vesicles using TIRF imaging and single-molecule localization microscopy. a, Schematic representation of wild-type (CTR1^{mEos}) and mutant (CTR1(M150L) ^{mEos}) constructs. **b**, Illustration of the relationship between Rab5 intensity and endocytic vesicle positions under TIRF illumination. Rab5 marks early/sorting endosomes, with higher intensity observed in smaller, newly formed vesicles near the plasma membrane and decreasing signal as vesicles mature and move deeper into the cell. **c**, Rab5 intensity plotted against vesicle size in COS7 cells expressing wild-type CTR1^{mEos} (WT) and CTR1(M150L)^{mEos} (M150L) under basal and Cu-stressed conditions. All conditions show similar profiles: Rab5 intensity increases with puncta size up to 0.4 μm² and then decreases, indicating comparable endocytic progression across samples. Data represent mean ± SEM; two-way ANOVA (see Supplementary Table 3). **d**, Representative images of COS7 cells showing the transmission (top), CTR1^{mEos} TIRF (middle), and Rab5-mCherry TIRF (bottom) signals. **e**, Workflow for the smND assay. Neighboring CTR1 molecules within a 40 nm radius were counted to generate a ND histogram. Experimental *ND* distributions (PND_{exp}) were fitted with theoretical distributions (PND_{theo}) to obtain CTR1 oligomer populations and $\bar{N}$. **f**, Two dissociation models used to estimate the lower (Model 1) and upper (Model 2) bounds of CTR1 trimer populations from smND data.

14. *The smND assay is a new and very intriguing methodology and it has to be explained better. For example, can the authors exclude that the "de-oligomerization" is simply an increase in the distance between fluorophores due to copper-induced conformational changes in CTR1. It was demonstrated*

that CTR1 undergoes conformational changes upon Cu binding (PMID: 36409899). The authors should formally rule out this possibility.

[**Reply 14**] We understand the concern. Indeed, if CTR1's transmembrane helices partially splay apart, it might appear as a "distance increase." However, our smND analysis relies on each fluorescent subunit's location being separated by >40 nm to be counted as "different neighbors." A small splay of 1–2 nm would not cause subunits to appear ~40 nm apart. Moreover, even if the trimer partially expands, we would not expect a large fraction of subunits to lose their immediate neighbors altogether. The best explanation for losing neighbors in a 40 nm radius is genuine subunit separation (true dissociation). We will add a short discussion acknowledging that small conformational changes are possible but would not alone explain a substantial fraction of "monomer-like" distributions in the *Result* section.

[**Changes in the Main text**] Furthermore, while small conformational changes upon copper binding could theoretically alter inter-subunit distances, the extent of neighbor separation (>40 nm) required by smND strongly suggests genuine dissociation rather than minor structural adjustments. Thus, the observed copper-induced changes are consistent with authentic CTR1 trimer-to-monomer transitions rather than artifacts arising from conformational expansions alone.

Reviewer #3

15. *The Cu uptake transporter CTR1 undergoes endocytosis in response to excess Cu to maintain cellular Cu homeostasis. By employing single-molecule localization microscopy (SMLM) and single-molecule neighbor density (smND) assays, the authors demonstrate that excess Cu induces rapid monomerization of the trimeric CTR1 prior to endocytosis. This process inhibits Cu uptake and is impaired in the endocytosis-deficient CTR1 (M150L) mutant. Thus, authors conclude that de-oligomerization of CTR1, linked with endocytosis as a rapid mechanism to prevent excess Cu entry. While the study is interesting and important, a major limitation is the absence of data showing functional consequences of de-oligomerization of CTR1 in response to excess Cu, both in vitro and in vivo. For instance, experiments demonstrating Cu-induced cell death (cuproptosis) using CTR1 wild type and mutants are needed. Moreover, it is essential to perform live cell imaging of WT or mutant CTR1 with Rab5 in COS7 cells. Additional experiments are therefore required to increase the quality of this manuscript.*

[**Reply 15**] We agree that live-cell imaging of CTR1 dynamics and more in-depth physiological readouts (e.g., cuproptosis) would be valuable. However, these would significantly extend the scope of our current study. Our major focus here is to establish that CTR1 undergoes a structural change (de-oligomerization) that precedes endocytosis in elevated copper. We do plan future work to explore real-time CTR1 de-oligomerization, as well as the specific cytotoxic endpoints (cell viability or cell-death pathways). We have mentioned these follow-up directions in the *Discussion*. (Future directions: more related to membrane-trafficking related questions, such as the choice of trafficking routes or the fate of internalized CTR1).

We have updated the *Discussion* section of our manuscript to clearly outline these limitations and specify our planned future research directions (see **Reply 3**). Specifically, we now highlight our intention to pursue real-time imaging experiments and physiological assays such as

cuproptosis in subsequent studies, thus transparently addressing the reviewer's suggestions while maintaining clarity regarding the scope and focus of our current findings.

Reviewer #4

The article titled "Human Transporter De-oligomerization Regulates Copper Uptake into Cells" presents a combined computational and experimental study. The authors demonstrate that an excess of copper induces the monomerization of CTR1, which typically transports copper as a trimer. This monomerization process is suggested to prevent copper overload.The article is intriguing as it provides new insights into the still unclear aspects of copper internalization. However, several issues need to be addressed before the paper can be considered for publication.

16. *In the introduction, where the mechanism of copper uptake is discussed, the authors should reference recent papers that have investigated the detailed mechanism of copper transport. For example, see Janos et al. https://doi.org/10.1017/qrd.2022.2 and Aupic et al. https://doi.org/10.1073/pnas.2214602119.*

    [**Reply 16**] We appreciate the reviewer's suggestion to incorporate recent studies that have investigated the molecular mechanism of copper transport. The work by Janos et al. (*QRB Discovery*, 2022) and Aupič et al. (*PNAS*, 2022) significantly contributes to our understanding of CTR1 function at the molecular level. Janos et al. utilized molecular dynamics (MD) and quantum mechanics/molecular mechanics (QM/MM) simulations to reveal the conformational plasticity of methionine triads in CTR1's selectivity filter, demonstrating that these residues regulate Cu(I) transport efficiency. Aupič et al. combined MD simulations with circular dichroism to show that intrinsically disordered regions (IDRs) at the N-terminal domain of CTR1 modulate copper uptake by affecting the selectivity filter's conformational state.

    While these studies have provided crucial insights into CTR1-mediated copper permeation, they do not directly address the relationship between CTR1's oligomeric state and endocytosis in regulating copper uptake. Our study uniquely integrates SMLM with smND analysis to uncover how excess copper induces CTR1 de-trimerization, a structural transition that precedes endocytosis. Unlike previous work, which focuses on the structural determinants of copper entry through CTR1, our study reveals a regulatory mechanism linking transporter oligomerization to endocytic trafficking. This distinction is critical, as it establishes a new paradigm wherein CTR1 structural transitions actively contribute to controlling cellular copper levels beyond mere permeation through the transporter.

    To address the reviewer's concern, we have revised the Introduction to integrate these references appropriately. We explicitly highlight how prior studies have contributed to our understanding of CTR1 selectivity and structural flexibility while emphasizing that our work provides novel insights into how copper-induced de-oligomerization serves as a regulatory mechanism coupling transporter function with cellular trafficking processes.

    [**Changes in the Main text**] CTR1 exists as a homotrimer. Recent studies have provided structural insights into its copper transport mechanism. The selectivity filter methionine triads exhibit conformational plasticity, with Cu(I) binding modulating their orientation to regulate transport efficiency.[12] Additionally, the intrinsically disordered N-terminal domain influences selectivity filter conformation, further fine-tuning copper uptake.[13,14] While these findings have

advanced our understanding of CTR1's molecular architecture and transport function, they do not fully explain how cells dynamically regulate CTR1 to prevent overload.

17. *The authors claim in the first paragraph that the M150L mutant significantly stabilizes the trimeric form by decreasing the ΔΔG. However, examining the structure of CTR1, it appears that the selectivity filter of the CTR1 trimer may be affected by introducing a residue larger than methionine. Previous MD simulation studies have shown that methionine is already quite bulky. Have the authors performed actual MD simulations to verify the structural impact of this mutation on CTR1 stability?*

[**Reply 17**] Yes, as stated in the manuscript, "We further explored CTR1's structural dynamics through molecular dynamics simulations in membrane environments". These simulations confirm that the M150L substitution does not disrupt the overall stability of the trimeric structure but instead reinforces inter-subunit interactions.

We have now detailed these molecular dynamics simulations and their outcomes in the *Methods* and *Results* sections of the manuscript. Specifically, the revised manuscript explicitly states that the MD simulations confirm the M150L substitution enhances inter-subunit interactions without disturbing the global trimeric structure (see **Reply 8**).

18. *Another aspect that needs clarification is that M150L is the bottom methionine triad in the structure, which means that the upper methionine triad M154 can still bind copper and stabilize the trimer in this manner. The authors should also consider the other M150L variant to ensure copper is not bound to the upper triad of Met in selectivity filter and therefore it is not stabilizing the trimer structure in this manner.*

[**Reply 18**] In the wild-type structure, copper interacts with all three M154 residues with bond lengths of approximately 2.4 Å, consistent with typical Cu(I)-S(Met) coordination distances (~2.2-2.4 Å). In the M150L mutant, copper exhibits slightly longer bond lengths with M154 residues (2.9 Å, 2.6 Å, and 2.7 Å), suggesting a weaker interaction than in the wild type. This analysis indicates that the trimer structure is unlikely to be stabilized via copper-mediated interactions at the upper triad in the M150L variant.

We have incorporated this structural analysis into the *Results* section of our revised manuscript, clearly specifying the altered copper-binding geometry in the M150L variant. The update explicitly reflects these bond-length measurements, clarifying that the reduced capacity of the upper triad stabilizes the trimer structure via copper interactions in the M150L mutant.

[**Changes in the Main text**] Although the M154 site in the M150L mutant still coordinates copper, it is unlikely to be the dominant stabilizing factor, as the Cu(I)–M154 bond lengths in the mutant (average 2.7 Å) are slightly longer than those in the wild-type (2.4 Å), suggesting weaker coordination (Fig. 1a).

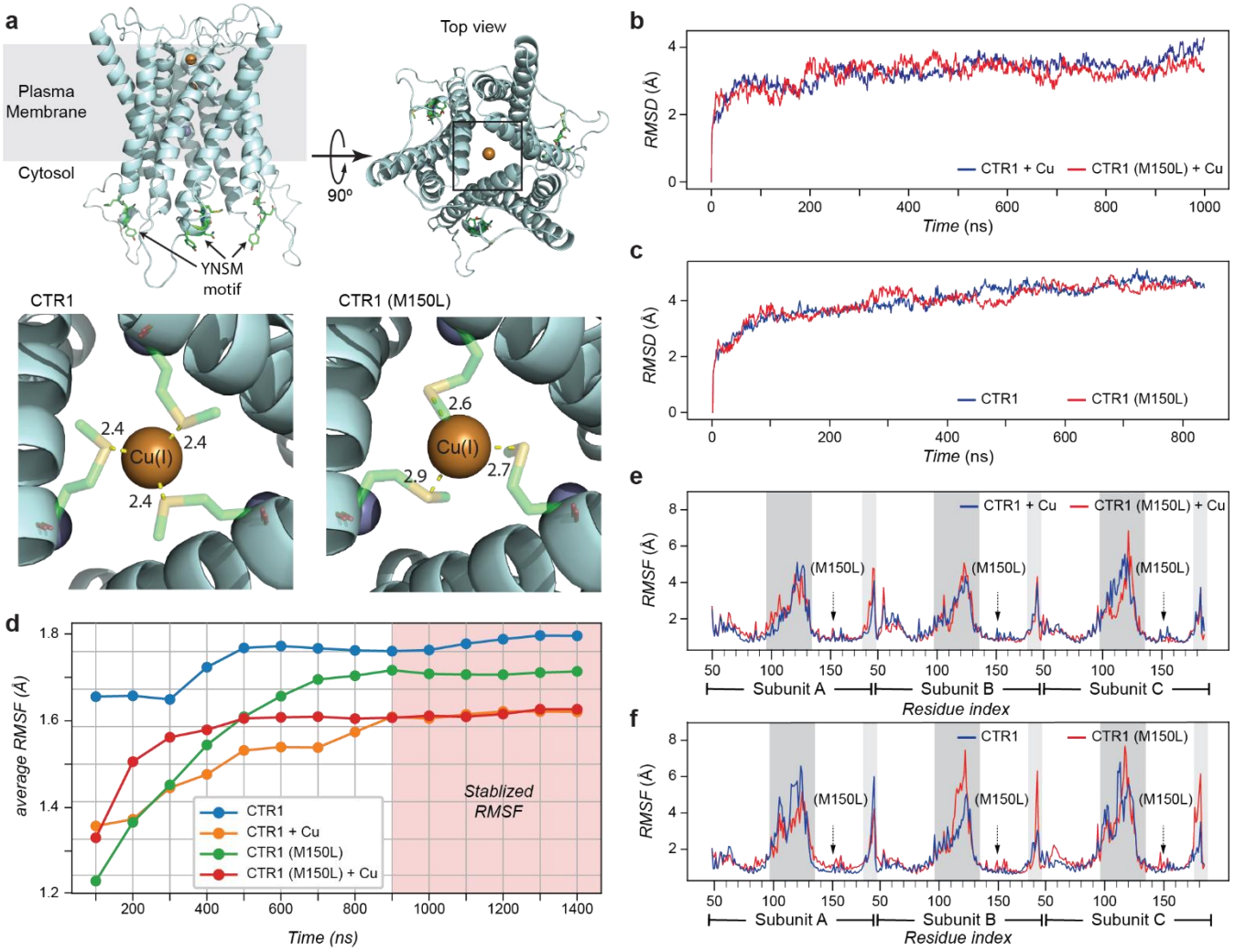


Fig. 1 | Computational modeling reveals increased stability of the CTR1(M150L) trimer. a, Top: structural models of CTR1 under side (left) and top views (right). Bottom: zoom in area showing Cu(I) coordination in CTR1 (left) and the M150L mutant (right) coordinating two Cu(I) ions at Met150 and Met154 sites. The Cu(I)–Met154 bond is longer in the mutant (~2.7 Å) than in the wild type (~2.4 Å), indicating weaker interaction. **b**, **c**, Root-mean-square deviation (RMSD) of wild-type CTR1 (blue) and CTR1(M150L) (red) in the presence (**b**) and absence (**c**) of copper over 800–1000 ns. Both constructs maintain overall structural stability under all conditions. **d**, Time evolution of root-mean-square fluctuation (RMSF) averaged across all residues shows stabilization after 900 ns. **e**, RMSF of wild-type CTR1 and CTR1(M150L) in the presence of copper shows similar patterns. **f**, RMSF of wild-type CTR1 and CTR1(M150L) in the absence of copper highlights increased flexibility in the M150L mutant, particularly in the intracellular loop (dark gray area) and cytosolic tail regions (light gray area).

19. *As far as I understand, MD simulations are performed in the absence and presence of copper. However, no reference to their parameterization is provided in the methods section. It is well known that this parameterization is not trivial and may require QM-based simulations.*

[**Reply 19**] Thank you for your comment. We utilized the Amber force field, which includes well-validated parameters for Cu(I) coordination. While QM-based simulations provide an alternative approach, prior studies (Schwartz R, et al. Protein Science. 2022;31(12):e4464) have demonstrated that Amber's force field parameters for Cu(I) yield reliable results for metalloproteins. The study by Schwartz et al. (2022) that simulated Cu bond distances (~2.3-2.4) matched experimental X-ray crystallography data.

We have now explicitly referenced our parameterization approach in the *Methods* section for clarity.

[**Changes in the Main text**] We utilized the Amber force field, which includes well-validated parameters for Cu(I) coordination.[55]

20. *Additionally, RMSF values are reported on page 3 with several decimal digits. Have the authors checked how the computed RMSF depends on the simulation length? The observed difference is probably smaller than the error and not significant. This is particularly relevant when the authors try to spot differences based on the RMSF.*

[**Reply 20**] We have investigated how the RMSF values stabilize over increasing simulation time as reviewer suggested, confirming that our reported differences are consistent throughout the trajectory and not artifacts of sampling duration. We have also reduced the number of decimal digits for clarity, and the key observation remains that the M150L mutant shows greater flexibility in the absence of copper (1.84 Å) compared to wild-type (1.75 Å), while their copper-bound forms remain similar (1.64 Å vs 1.65 Å).

We have incorporated the new RMSF stability analysis into the revised manuscript as an additional figure (*Fig. 1d* in **Reply 18**), indicating that the reported RMSF differences are robust and consistent over simulation time.

[**Changes in the Main text**] After confirming the root-mean-square fluctuation (RMSF) stabilized after 900 ns (Fig. 1d), we further check the RMSF data of both the wild type and the M150L mutant are comparable in the presence of copper (1.64 Å and 1.65 Å for wild-type and M150L CTR1, respectively, Fig. 1e).

21. *Furthermore, the experimental structure of CTR1 lacks residues from the N-terminus and part of the C-terminus. According to the methods, these parts have been added to the CTR1 model. If this is the case, since they are both disordered, how is their conformation modeled?*

[**Reply 21**] Thank you for your comment. The missing N- and C-terminal residues were modeled using MODELLER. While these regions lack well-defined secondary structure, MODELLER generated plausible conformations with physical energy potentials. This initial model was subsequently refined through MD simulations.

We have updated the *Methods* section to describe the use of MODELLER for generating initial conformations of the missing N- and C-terminal residues, as well as detailing their subsequent refinement via MD simulations.

[**Changes in the Main text**] To accurately represent the human CTR1 structure, we removed the extra polypeptide of BRIL and added back the missing residues in the crystal structure using Modeller.[53]

22. *The results on the structure and copper-binding abilities of CTR1 are affected by the presence and conformation of the N-term and C-term residues see Aupic et al.* https://doi.org/10.1073/pnas.2214602119 *and Walke et al., Biophysical Journal, Volume 121, 1194-1204. Thus understanding their influence is a relevant point. These parts contain several copper-binding residues, thus the authors should also create and test the oligomerization properties of these truncated variants (truncanted N-term and/or truncated C-term) in response to copper stress.*

[**Reply 22**] We appreciate the reviewer's suggestion to examine the influence of the N-terminal (N-term) and C-terminal (C-term) regions on CTR1's structure and function, particularly in light of previous studies (Aupič et al., *PNAS*, 2022; Walke et al., *Biophys. J.*, 2022). These studies provide valuable insights into how the N-term and C-term contribute to copper binding and conformational changes in CTR1, highlighting their potential regulatory roles in Cu transport. However, neither of these studies specifically investigates CTR1's oligomeric state or how its de-oligomerization is linked to endocytosis, which is the central focus of our work.

Our study directly addresses how CTR1 de-oligomerization serves as a regulatory mechanism for copper uptake and endocytosis, providing a new perspective on how CTR1 abundance at the plasma membrane is dynamically controlled. Using SMLM and smND analysis, we demonstrate that high copper levels induce CTR1 de-trimerization, a key step preceding endocytosis. This mechanism is distinct from the copper-binding dynamics of the N-term and C-term, as it directly regulates transporter internalization rather than modulating Cu permeation through the selectivity filter.

While it is possible that the N-term and C-term influence CTR1's overall structure, the core regulatory mechanism we describe, copper-induced de-oligomerization leading to endocytosis, is driven by structural transitions in the transmembrane domain of CTR1, where oligomerization is primarily controlled. Thus, the suggestion to generate and test truncated variants (lacking the N-term and/or C-term) in response to copper stress, while interesting, is beyond the scope of the present study. Future work could investigate whether N-term or C-term truncations modulate CTR1 de-oligomerization or affect its interaction with endocytic machinery, but our current data strongly support a model in which CTR1 de-trimerization is the primary regulatory step linking copper sensing to transporter removal from the membrane.

We have acknowledged this important consideration and updated the *Introduction* section (see **Reply 16**) of our manuscript to address the potential roles of N-terminal and C-terminal regions, referencing recent studies. Additionally, we highlight this topic as a relevant direction for future research to explore their influence on CTR1 oligomerization and endocytosis.

23. *Some contradictory statements are provided in the text. In the discussion, the authors mention the YxxM motif of the intracellular loops (please state exactly the residues you are considering). The authors mention that copper binding at these methionine sites stabilizes the trimer, preventing its dissociation. How does this occur? Can they better argument this point?*

[**Reply 23**] We appreciate the reviewer's careful reading of our discussion and the opportunity to clarify this point. The YNSM motif was referenced in the context of endocytic regulation, not as a Cu-binding site. The key Cu-binding residues in CTR1 are M150 and M154, located within the selectivity filter of the transmembrane domain, where they directly coordinate Cu(I) and facilitate transport. Our data show that Cu binding at these sites increases the stability of the trimer, reinforcing its structural integrity. However, at high cellular Cu concentrations, CTR1 undergoes a regulated transition from a trimeric to a monomeric state, which precedes its endocytosis. This suggests that Cu coordination initially stabilizes the transporter, but excessive Cu exposure promotes structural changes that shift CTR1 toward an endocytic fate. In contrast, the YNSM motif is relevant to endocytic regulation, likely serving as a recognition site for intracellular trafficking machinery. To prevent any misinterpretation, we have revised the

discussion to explicitly state that Cu binding occurs at M150 and M154, ensuring that the role of the YNSM motif is only discussed in the context of CTR1 internalization.

We have revised the *Discussion* section to clearly specify that copper binding occurs at the M150 and M154 residues and explicitly state that the YNSM motif is relevant solely for endocytic regulation. These clarifications ensure the accurate interpretation of the roles of different regions within CTR1.

[**Changes in the Main text**] Building on our results, a plausible model is that intracellular copper, transported through CTR1, is sensed by a downstream factor that recruits endocytic components to the intracellular region of CTR1. The YNSM motif, located in the cytosolic loop of CTR1 (residues 103–106, Fig. 1a), has been proposed as a potential docking site for endocytic machinery.[36] This recruitment may generate the mechanical force or conformational change necessary to destabilize the trimer and promote the shift toward the monomeric state. In this model, copper binding at Met150 and Met154, located within the transmembrane domain, serves to coordinate Cu^{+} and facilitate transport. Binding at these sites stabilizes the trimer structure under basal conditions, consistent with our MD simulations. However, when intracellular copper rises to a threshold, endocytic recruitment is activated, leading to regulated trimer dissociation and internalization. This framework explains how CTR1 can remain trimeric during normal transport yet undergo de-trimerization once copper homeostasis is threatened. In the M150L mutant, copper coordination and transport are disrupted, so intracellular copper does not rise, and the endocytic machinery is not engaged—leaving the trimer structurally intact and endocytosis blocked.

24. *In this respect, can the authors increase the intracellular concentration of copper, without relying on CTR1 transport, and check whether it is really the intracellular increase of copper that is affecting the oligomerization status?*

[**Reply 24**] We appreciate the reviewer's suggestion and fully agree that it is the intracellular copper that triggers the structural (de-oligomerization) changes in CTR1. As detailed in our **Reply 7**, we conducted experiments using the copper ionophore Cu-elesclomol, which increases intracellular copper independently of CTR1-mediated transport. These results confirm that the elevation of intracellular copper alone is sufficient to reduce surface CTR1 level, suggesting as the outcome of changes in CTR1 oligomerization and subsequent endocytosis. We refer the reviewer to **Reply 7** for a detailed discussion of these findings.

Point-by-point response to reviewers

[Reply to all reviewers] Thank you for your critical and constructive comments. Following your suggestion, we have revised the manuscript accordingly. Below, we respond (in red) to each question/comment (in black *italic*), with our additions to the text indicated (in blue).

Reviewer #1 (Remarks to the Author):
I thank the authors for addressing my comments.
There are a few points – minor, yet I see them as mandatory – that need further attention:

1. *The authors integrated the qPALM method in the discussion (lines 390ff), which I think is very helpful for the reader. Yet, references to further direct the interested reader are missing. Some original work should be integrated, e.g. PMID 27466316 and PMID 31937565. Along these lines: 'blinking' is not an artefact in PALM, it is rather valuable kinetic information that reports on molecule numbers (in qPALM, qPAINT etc.).*

   [Reply 1-1] We thank the reviewer for these important suggestions. We have now included the recommended citations (PMID: 27466316 and 31937565) to provide the reader with key original works on the qPALM method. We also agree that "blinking" is a valuable photophysical property exploited by these quantitative methods and not simply an artifact. We have revised the text to reflect this more accurate description.

   [Changes in Discussion: *Limitations of the study*] qPALM leverages the stochastic photophysical behavior of fluorescent proteins, such as blinking and photoactivation, to determine stoichiometry, accounting for factors like incomplete labeling via calibration with well-characterized monomeric or dimeric references.[45,46]

2. *I politely disagree in pointing the reader towards not using dimeric proteins as a reference. There are various dimeric reference proteins available (CD28 being a a very robust one; CTLA4 reported as well); another option are single and dual-labelled monomeric proteins. I propose to rephrase this section and recommend to the reader to implement appropriate controls.*

   [Reply 1-2] We thank the reviewer for this valuable clarification, and we agree completely. Our intention was not to discourage the use of standard dimeric controls, but to highlight that *in vivo* equilibria can be complex. We have rephrased this section to remove any ambiguity. We now explicitly acknowledge the value of well-established dimeric references like CD28 or CTLA4 and frame our engineered Tac-tandem constructs as a complementary strategy that provides an unambiguous ground truth for fluorophore number, thereby serving as a robust control for the smND quantification itself.

   [Changes in Methods: *Single-molecule neighbor density (smND) assay*] To benchmark smND performance, it is crucial to use appropriate oligomeric controls, for which there are two primary strategies. The first strategy uses well-characterized proteins that are known to self-assemble into defined oligomeric states *in vivo*, such as CD28 or CTLA4. A complementary strategy is to engineer constructs with a precisely controlled number of fluorophores per molecule. This approach provides an unambiguous ground truth for fluorophore stoichiometry, thereby directly testing the quantification capabilities of the smND model, independent of biological assembly equilibria. In this study, we leveraged both approaches for comprehensive validation. We employed CTLA4 as an expected homodimeric biological standard. However, we measured a steady-state population of ~75% dimers, reflecting its dynamic equilibrium *in situ* and confirming the assay's sensitivity to mixed biological states (Extended Data Fig. 4f–h). We then used Tac^{mEos} (single mEos4b) and Tac^{2mEos} (tandem mEos4b) as references with effectively 'locked' single- versus dual-chromophore stoichiometries

anchored to the membrane via Tac-antigen. These constructs allowed benchmarking of smND performance under well-defined oligomeric conditions.

3. *Please mention the origin and the exact name for the CTLA4 plasmids.*

   [Reply 1-3] We apologize for this omission. We have now added the specific details for the CTLA4 plasmid to the Methods section.

   [Changes in Methods: *DNA plasmid construction*] The plasmid expressing CTLA4^{mEos} was constructed by replacing the mEos2 fluorescent protein in the original plasmid pirespuro2-CTLA4-mEos2, a kind gift from Mike Heilemann (Addgene plasmid # 98285; RRID:Addgene_98285),[47] with mEos4b between the AgeI and NotI restriction sites for experimental consistency with our other constructs.

4. *How does incomplete labelling affect the results of the method the authors use? The detection efficiency of mEos is around 50% (this value varies in different reports). I do understand the approach the authors use is based on reference data generated from molecules of known oligomers; in my view, one or two sentences in the discussion that point at this would suffice, and it will help the interested reader.*

   [Reply 1-4] This is a critical point, and we thank the reviewer for prompting us to clarify it. Our smND theoretical model explicitly accounts for incomplete labeling and detection through the photo-activation efficiency (PE) parameter, which is a cornerstone of the PND_{theo} calculation. For example, a trimer where only two of the three mEos4b tags are detected (due to stochastic photoactivation) will contribute to the experimental neighbor density distribution in a predictable way. The model calculates the probability of such events using a binomial distribution based on the PE value and incorporates this into the theoretical fitting curves for each oligomeric state. This makes the method robust to incomplete detection, as it statistically models the expected distribution of observed neighbors for a given true oligomeric state, rather than assuming 100% detection. We have added a sentence to the Discussion to clarify this.

   [Changes in Discussion: *Limitations of the study*] A key strength of the smND method is that its theoretical framework explicitly accounts for the incomplete detection of fluorophores (e.g., a photo-activation efficiency for mEos proteins of ~40-50%).[44] By modeling the probable number of detected subunits for each oligomeric state, the method can robustly determine oligomeric populations even when not all subunits within a complex are localized.

5. *I appreciate that the authors added imaging data in Extended Figure 2. However, it is not possible to see single receptor clusters even in the zoom images (that have a scale of 10 µm). I wonder whether there are isolated clusters at all, and what the molecular density of this receptor is – as compared to the physiological density of CTR1.*

   [Reply 1-5] We thank the reviewer for this observation, which allows us to clarify the purpose of Extended Figure 2. The reviewer is correct that single receptor clusters are not resolved in these images. These confocal images were intended solely to demonstrate the general biological function of copper-induced endocytosis of our CTR1mEos construct, by showing the co-internalization of the cell-surface marker (WGA) and CTR1^{mEos} into intracellular puncta upon copper treatment. This validates that the tagged protein traffics correctly. All quantitative analysis of oligomeric state was performed using SMLM under TIRF illumination (presented in Figure 3), which provides the necessary single-molecule resolution. We have revised the figure caption of Extended Figure 2 to make its purpose clear and avoid any implication that these images are used for single-molecule analysis.

   [Changes in *Extended Figure 2*] Note that these confocal images are intended to demonstrate the copper-induced co-internalization of surface-labeled CTR1^{mEos} and the membrane dye WGA,

confirming the endocytic competence of the fusion protein. They are not used for single-molecule or oligomeric state analysis, which is performed using SMLM as shown in Figure 3.

All of the above points can be addressed with minor editing.

[Reply] We thank the reviewer for their constructive feedback. We agree, and we appreciate that these minor edits have helped improve the quality and clarity of our work.

Reviewer #2 (Remarks to the Author):

This reviewer appreciates the authours' thoughtful and constructive response to the critique. The revised manuscript is significantly improved: it is clearer, it includes new and useful controls, and it is, overall, more convincing. I have no further concerns.

[Reply] We thank the reviewer for their positive feedback and support of our manuscript.

Reviewer #3 (Remarks to the Author):

1. *In response to this reviewer's comments, authors responded that their major focus is to establish that CTR1 undergoes a structural change (de-oligomerization) that precedes endocytosis in elevated copper, which prevents excess Cu entry. While study is important in terms of showing relationship between structural change and endocytosis, it is essential to show the functional consequence of preventing endocytosis, such as excess Cu entry and Cu-induced cell death at least in vitro. Thus, authors should perform additional experiments to show the functional significance of de-oligomerization of CTR1, which is not difficult to do, to increase the quality of this paper.*

   [Reply 3-1] We thank the reviewer for their persistence on this critical point. Their suggestion prompted us to perform additional functional experiments to directly test the importance of the complete CTR1 regulatory pathway for cell survival. We sought to test the hypothesis that de-trimerization and endocytosis are two cooperative and equally essential components of the cellular defense against copper toxicity.

   To test the hypothesis that endocytosis provides a necessary protective mechanism, we used the dynamin inhibitor Dynasore to specifically block the internalization step. Our new results show a strong synergistic lethality between endocytosis inhibition and copper stress. While high copper or 80 μM Dynasore alone caused only moderate effects, blocking endocytosis *before* adding high copper resulted in massive cell death within 24 hours. This finding demonstrates that de-trimerization alone is an insufficient defense over time. It strongly supports our model that endocytosis plays a crucial, non-redundant role. As noted in our discussion, we propose that this second step is essential to clear the dissociated, inactive subunits from the plasma membrane, thereby preventing their potential re-assembly into functional, copper-transporting trimers.

   We have included these important findings in Figure 5j and have added a new paragraph to the Results and updated the Discussion. We are grateful for the reviewer's suggestion, as these new experiments further strengthened the functional significance and overall impact of our manuscript.

[Changes in the Results: *Intracellular Cu triggers compartment-specific CTR1 dissociation for cell survival*] Finally, to directly test the functional importance of endocytosis as an essential protective pathway, we inhibited internalization using the dynamin inhibitor Dynasore during copper stress. Our results show a strong synergistic cytotoxicity of copper stress and endocytic blockage. As shown in Fig. 5j, after 24 hours, treatment with either 100 μM $CuCl_2$ or 80 μM Dynasore alone had a moderate impact on cell viability. In contrast, blocking endocytosis with Dynasore prior to copper exposure led to a profound loss of cell viability, with cells appearing rounded and detached. These results demonstrate that a functional endocytic pathway is also critical for protecting cells from acute copper toxicity.

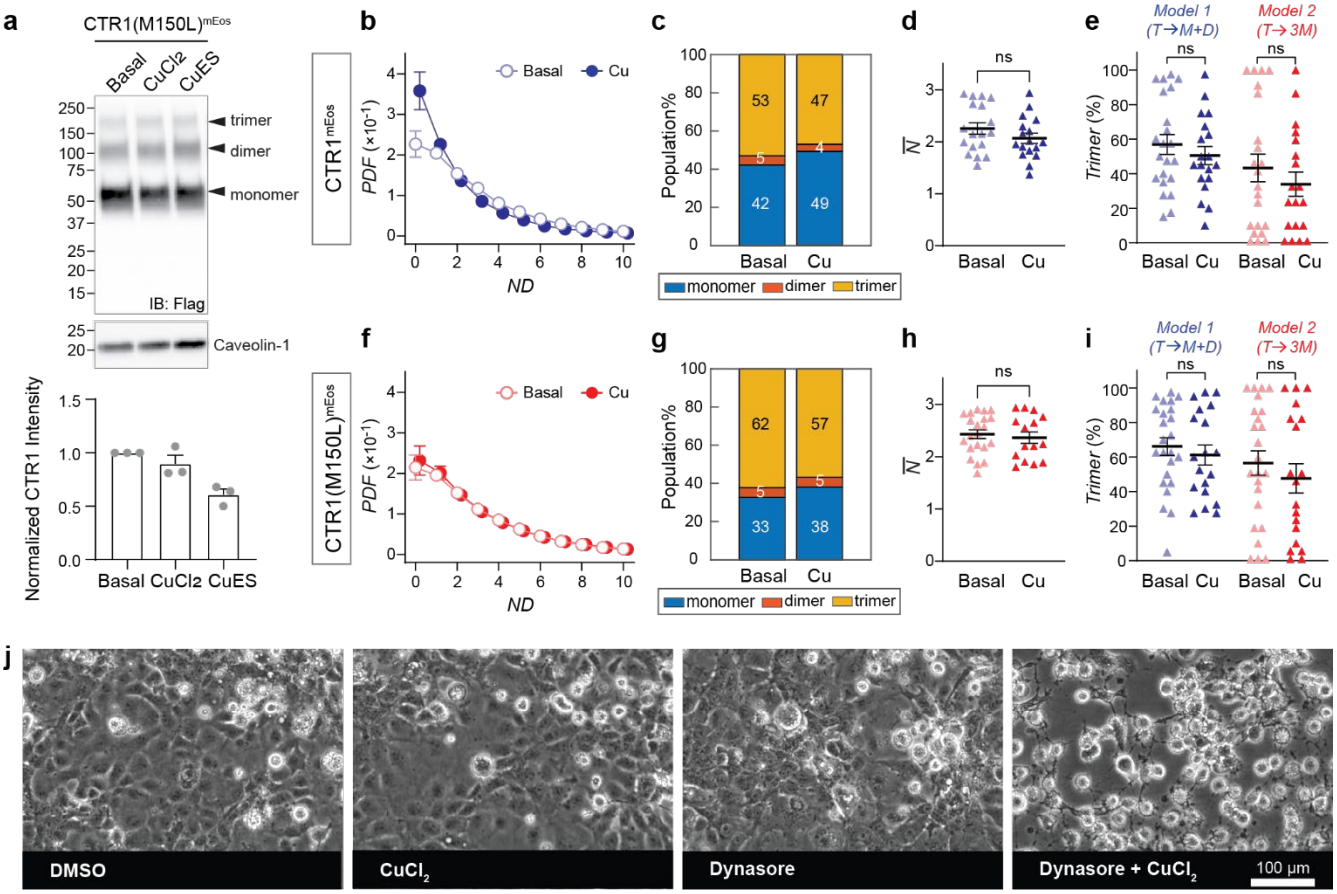


Fig. 5 | Intracellular copper elevation triggers CTR1 dissociation primarily in Rab5⁺, not Rab5⁻, compartments. a, Biotinylation assay comparing the surface abundance of the CTR1(M150L)^{mEos} under different copper treatments (top). Normalized CTR1 intensity plot (normalized to the loading control Caveolin-1, bottom) shows that treatment with 100 μM $CuCl_2$ alone fails to trigger its internalization, whereas exposure to 2 μM of the membrane-permeable CuES ionophore restores endocytosis. This confirms that elevated intracellular copper, rather than extracellular copper binding, drives CTR1 internalization. (**b–i**), smND analysis of CTR1 oligomerization in Rab5⁻ compartments. Under basal conditions, wild-type CTR1^{mEos} (**b–e**) shows minimal changes in trimer populations upon copper stress, shifting from approximately 53% to 47%, within the uncertainty of smND. The endocytosis-deficient CTR1(M150L)^{mEos} mutant (**f–i**) demonstrates negligible oligomeric shifts (~62% to 57%) under copper treatment in Rab5⁻ areas, confirming that significant copper-induced dissociation of CTR1 occurs specifically in Rab5⁺ compartments. Data represent mean ± SEM from three independent experiments. **j,** Representative bright-field images of COS7 cells after 24 hours of treatment with vehicle (DMSO), 100 μM $CuCl_2$, 80 μM Dynasore, or Dynasore and $CuCl_2$ combined. A profound loss of cell viability (i.e., cells appearing rounded and detached) is observed only in the combined treatment, demonstrating that blocking endocytosis sensitizes cells to copper-induced death.

[Changes in Discussion: *Benefits of CTR1 de-trimerization in copper regulation*] However, if these monomers were to persist on the plasma membrane, they could potentially re-assemble into functional trimers, counteracting the initial protective effect. Our functional data support this model,

showing that when endocytosis is blocked with Dynasore, cells become hypersensitive to copper toxicity (Fig. 5j). This highlights the cooperative nature of the defense: de-trimerization provides the rapid stopgap, while endocytosis provides the essential follow-through by clearing the dissociated subunits from the membrane to ensure a permanent shutdown of copper uptake and promote cell survival.

[Changes in Methods: *Cell viability assay*] To assess cell viability under copper stress with endocytosis inhibition, COS7 cells were seeded in multi-well plates and cultured in DMEM medium containing 10% FBS overnight to approximately 70% confluency. The Cells were then serum-starved for 2 hours before being treated with either 80 μM Dynasore (Calbiochem 324410) or an equivalent volume of the vehicle, DMSO (final 0.2%, VWR 97063-136) for an additional 30 minutes. Subsequently, copper stress was induced by adding $CuCl_2$ to a final concentration of 100 μM in the medium containing 1% FBS. Cell viability was assessed after 24 hours by bright-field microscopy. Adherent cells were considered viable, whereas non-adherent, rounded cells were classified as non-viable.

Reviewer #4 (Remarks to the Author):

I believe the authors' discovery of CTR1 deoligomerization importantly contributes to understanding CTR1 function and regulation, and merits publication in Nature Communications.

[Reply] We thank the reviewer for their positive assessment of our work's contribution and their support for its publication in *Nature Communications*!

1. *However, I am not fully convinced that their computational modelling supports the claim that the M150L mutation stabilizes the trimeric structure of Ctr1. Their experiments with Cu ionophore clearly show that deoligomerization is caused by an increase in intracellular Cu concentration and is not primarily driven by intrinsic thermodynamic stability of the CTR1 trimeric transmembrane domain or how the latter is affected by copper binding at M150 or M154. This suggests that the M150L mutation, as the authors also state, primarily prevents deoligomerization by preventing copper internalization and not by stabilizing the trimeric assembly. Therefore, I think that the estimation of the effect of the M150L mutation on CTR1 oligomerization affinity is not really relevant for the paper's main outcome and due its relative speculative nature perhaps detracts more than it adds to the scientific quality of the paper. In any way, while the authors address many points previously raised, some further improvements are needed before publication.*

   [Reply 4-1] We are very grateful to the reviewer for this insightful and constructive critique. We agree that our experimental data, particularly the Cu-ionophore experiment, provide the most direct and compelling evidence that CTR1 de-oligomerization is an active process triggered by intracellular copper elevation, rather than a passive process governed by the intrinsic thermodynamic stability of the transmembrane domains. The reviewer's point that computational modeling may detract from this stronger, clearer experimental narrative is well-taken.

   Following the reviewer's suggestion to reconsider the role of this section, we have re-framed the section to present it not as a definitive conclusion about stability, but as an exploration of a plausible, hypothesis-generating idea that required direct experimental testing. We believe this new structure strengthens the paper's narrative. The modeling now serves to pose a key question: is the M150L phenotype driven by intrinsic structural stability or by its failure to transport copper? This framing helps to "pave the way" for our subsequent experimental results, highlighting how the ionophore experiment, in particular, was crucial for distinguishing between these two possibilities. We have

rewritten the first section of the Results to reflect this new, hypothesis-driven approach and hope the reviewer finds that this revision fully addresses their concern by better integrating the computational work into the paper's core logical flow.

2. *Authors write on line 95:*
"However, in the absence of copper, the RMSF data showed increased flexibility in M150L (1.84 Å compared to the wild type 1.75 Å), especially within the intracellular loop (residues 98 to 136) and cytosolic tail (residues 176 to 190, Fig. 1f)."
Firstly, while the authors now show that the average RMSF stabilizes after 900 ns, no error estimate is provided, therefore it is hard to estimate whether the relatively small 0.09 Å difference is significant. Moreover, panel 1f appears to show that in subunit A CTR1 is in fact more flexible than CTR1 (M150L). Additionally, in the transmembrane part that should be most relevant for the oligomerization, the differences seem particularly negligible and in any case do not suggest greater flexibility of the wt variant. I suggest the authors at a minimum include error estimates, either by employing the block average method on the final 500 ns of trajectories or by treating subunits as simulation replicas.

   [Reply 4-2] We thank the reviewer for these insightful comments on the RMSF analysis. As suggested, we have now included standard error estimates for the RMSF values, calculated using a block averaging method over 100 ns intervals from the simulation trajectories. These error bars have been added to the revised version of Fig. 1f. We also appreciate the reviewer's sharp observation regarding the flexibility of subunit A. We agree this is an interesting point and have added a sentence to the manuscript discussing this potential asymmetry and what it might suggest about the mutation's localized effects.

   [Changes in the Results: *Computational modeling suggests a possible link between trimer stability and endocytosis*] In the absence of copper, the RMSF data increased, with the M150L (1.84 Å ± 0.07) demonstrating higher overall flexibility than that of the wild type (1.75 ± 0.05 Å), especially within the intracellular loop (residues 98 to 136) and cytosolic tail (residues 176 to 190, Fig. 1f). Of note, this average RMSF masks some regional difference. For example, subunit A of the wild type appears more flexible than in the M150L mutant. It suggests a potential asymmetry in how the mutation affects local flexibility.

3. *The most convincing evidence about the effect of M150L mutation on the thermodynamic stability of the CTR1 trimer comes from computational mutagenesis. Can the authors add a brief explanation regarding the scoring function employed by the Eris algorithm for calculating the free energy and how it treats the Met-Cu(I) interaction. Does the DMD optimization significantly affect the geometry of the metal binding site?*

   [Reply 4-3] Thank you for the suggestion. We have now added a detailed description of the Eris algorithm's scoring function to the Methods section. To summarize, Eris uses the Medusa force field, a physics-based energy function that accounts for van der Waals forces, solvation, and hydrogen bonding, with parameters from the cited Yin et al. 2007 paper. Regarding the second point, the DMD optimization has a negligible impact on the metal binding site's geometry. Eris primarily uses DMD to relieve any atomic clashes after side-chain repacking. Upon inspection of the resulting structures, we confirmed that the backbone of the binding site remained essentially unchanged, with deviations of less than 0.1 Å.

   [Changes in the Method: *Evaluating CTR1 stability through computational mutagenesis*] Eris employs the Medusa force field, a physics-based energy function that accounts for key molecular interactions. These include both attractive and repulsive van der Waals forces, solvation energies, and hydrogen bonding—within the backbone, between side chains, and between side chains and the backbone. The force field also incorporates side-chain rotamer preferences based on backbone

φ and ψ dihedral angles. The Met–Cu(I) interaction is modeled within the Medusa framework. The parameterization of the force field was described previously.[62]

Minor points:

4. In figure 1d, the grid lines and the y-axis tics do not match.

[Reply 4-4] We have corrected the axis ticks in the figure.

5. In the following sentence, starting on line 75, the word 'change' is unnecessary and leads to confusion.
In the absence of copper, the leucine substitution at M150 (M150L) reduced the free energy change (ΔΔG) by −5.11 kcal/mol compared to the wild type.

[Reply 4-5] We have removed the redundant word "change" from the sentence describing the free energy calculation.

6. Authors state on line 81:
"Although the M154 site in the M150L mutant still coordinates copper, it is unlikely to be the dominant stabilizing factor, as the Cu(I)–M154 bond lengths in the mutant (average 2.7 Å) are slightly longer than those in the wild-type (2.4 Å), suggesting weaker coordination (Fig. 1a)."
Please clarify if these distances stem from MD simulations.

[Reply 4-6] We have clarified that the Cu(I)–Met154 bond lengths were averaged from the MD simulation trajectories.

[Changes in Results: *Computational modeling suggests a possible link between trimer stability and endocytosis*] Although the M154 site in the M150L mutant still coordinates copper, it is unlikely to be the dominant stabilizing factor, as the Cu(I)–M154 bond lengths, averaged from our MD simulations, are slightly longer (average 2.7 Å) than those in the wild-type (2.4 Å), suggesting weaker coordination (Fig. 1a, right).

7. In the method section, line 641, the authors state:
"We utilized the Amber force field, which includes well-validated parameters for Cu(I) coordination.60"
I assume they employed the non-bonded copper model, however the reference they give (Schwartz R, et al. Protein Science. 2022;31(12):e4464) describes the development of parameters for a bonded copper model that are not provided directly provided with Amber/AmberTools. If they have indeed used the non-bonded model the reference should be removed. Additionally, the authors should state explicitly which protein force field (ff12SB/ff14SB,…), water model (TIP3P/OPC,…) and ion parameters (Li-Merz/Young-Cheatham,…) they employed.

[Reply 4-7] We sincerely thank the reviewer for identifying this important error in our methods section. The reviewer is correct; we used a non-bonded model for Cu(I), and the citation to Schwartz et al. was inappropriate and has been removed to avoid confusion. We have now explicitly stated the complete force field and parameter sets used in our simulations: the ff14SB protein force field, the TIP3P water model, and the Li–Merz parameters for monovalent metal ions, including Cu(I). The Methods section has been updated accordingly.

[Changes in the Method: *Molecular Dynamics Simulations*] The protein was parameterized using the ff14SB force field, with the TIP3P water model and Li–Merz parameters for monovalent transition metal cations.

Point-by-point response to reviewers

[Reply to all reviewers] Thank you for your critical and constructive comments. Below, we respond (in red) to each question/comment (in black *italic*), with our additions to the text indicated (in blue).

Reviewer #1 (Remarks to the Author):

I thank the authors for responding to and addressing all my comments. The revised manuscript has significantly improved, and there are no more open points from my side.

[Reply 1] We sincerely thank the reviewer for the careful evaluation and supportive feedback. We are grateful for the constructive suggestions provided in earlier rounds, which helped strengthen the manuscript.

Reviewer #3 (Remarks to the Author):

1. *Although the reviewer requested that the authors should demonstrate the functional consequences of CTR1 de-oligomerization in response to intracellular Cu elevation such as Cu-induced cell death using both CTR1 wild-type and the endocytosis-deficient CTR1-M150L mutant, this experiment was not included in the revised manuscript. Instead, the authors only assessed the effect of the endocytosis inhibitor Dynasore on $CuCl_2$-induced cell death. Therefore, the revision does not adequately address the original concern.*

   [Reply 3-1] We appreciate the reviewer's emphasis on functional consequences. We agree that assessing cell viability under copper stress is an important orthogonal readout. However, a direct WT vs. CTR1(M150L) cell-death comparison would be intrinsically confounded: CTR1(M150L) is copper-transport deficient and does not accumulate intracellular Cu to the same extent under challenge. Any survival difference between WT and M150L would therefore primarily reflect impaired Cu entry in the mutant rather than the specific contributions of CTR1 oligomeric state or endocytosis. For this reason, we did not pursue the WT vs. M150L viability experiment. To make this rationale explicit for readers, we have added a short paragraph in the Discussion explaining why we did not pursue a WT–CTR1(M150L) cell-death assay.

   Instead, our revised manuscript provides three convergent lines of evidence that together connect CTR1 structural state, trafficking, and a cell-level outcome—without relying on a transport-deficient mutant comparison:

   1. Temporal ordering (structure before trafficking): Using TNPD/smND analyses within $Rab5^+$ early endocytic compartments under TIRF, we observe that elevating intracellular Cu is associated with a shift toward lower-order/monomeric CTR1 prior to endocytosis (Results; Fig. 3a–e). This establishes sequence (monomerization precedes internalization) while avoiding a causal overclaim.
   2. Trigger specificity (intracellular Cu is sufficient): Using a membrane-permeable ionophore (Cu-elesclomol) to raise intracellular Cu, we observe robust CTR1 internalization by surface biotinylation (Results; Fig. 5a). This indicates that intracellular Cu—not extracellular binding alone—is sufficient to induce the trafficking response.
   3. Orthogonal functional outcome (cell viability under stress): Dynamin inhibition (Dynasore) plus Cu produces a pronounced loss of viability at 24 h, whereas either treatment alone has only modest effects (Results; Fig. 5j). This is consistent with a protective role for endocytosis during Cu overload and provides the requested functional readout at the cell level.

   Taken together, these data support a temporally ordered and mechanistically plausible model in which elevated intracellular Cu is associated with early CTR1 de-oligomerization in $Rab5^+$

compartments and coincides with endocytosis that limits further Cu entry; blocking endocytosis sensitizes cells to Cu toxicity at the population level.

[Changes in the METHOD DETAILS: *Cell viability assay*] To avoid transport-deficiency confounding, we did not pursue a WT versus CTR1(M150L) cell-death comparison, because CTR1(M150L) fails to accumulate intracellular copper under challenge and any survival difference would be dominated by reduced Cu entry rather than oligomerization or endocytosis per se.

Reviewer #5 (Remarks to the Author):

I am content with the authors' response to my previous comments. I have no additional concerns regarding publication.

[Reply] We sincerely thank the reviewer for the careful evaluation and supportive feedback. We are grateful for the constructive suggestions provided in earlier rounds, which helped strengthen the manuscript.