nature portfolio

Peer Review File

Condensation of MATCAP promotes CENP-E-dependent chromosome congression in mitosis

Corresponding Author: Dr Liangyu Zhang

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)
In this very interesting manuscript, Yang and colleagues investigate the molecular mechanism underlying CENP-E motor guidance by detyrosinated microtubules. By a thorough combination of in cell and in vitro biochemical/biophysical assays, they provide a convincing case in which an Intrinsically Disordered Region (IDR) on the microtubule detyrosinase MATCAP is required for liquid-liquid phase separation (LLPS) and is involved in microtubule binding. More specifically, the authors show that MATCAP distinctively localizes to spindle microtubules and centrosomes (as compared to vasohibins) and this association is exacerbated upon its overexpression. Critically, the IDR is required for MATCAP association with spindle microtubules and its absence strongly compromises chromosome congression. Mechanistically, the authors show that MATCAP interacts with CENP-E motor domain and this interaction depends on MATCAP's IDR. Crucially, this appears to be independent of tubulin detyrosination, suggesting that MATCAP serves a dual role: the local recruitment of CENP-E to microtubules, while promoting its processivity by detyrosinating tubulin on the lattice. Overall, this work offers important mechanistic insight into how microtubule detyrosination and its machinery spatially orchestrates chromosome congression, while highlighting a key role of MATCAP (and less so of vasohibins) in the process. There are however a few points that require attention as detailed below.

Major issues:

1- One confounding point in the proposed model concerns the recruitment of tubulin dimers to MATCAP condensates (Extended Data Fig. 5i,g). Previous work by the Brummelkamp lab has shown that MATCAP has a preference for polymerized tubulin compared to tubulin dimers. Yet, in the present paper, the authors evoque the local recruitment of tubulin dimmers as a means to facilitate tubulin detyrosination. The need to locally concentrate tubulin dimers to potentiate microtubule detyrosination requires clarification.

2- Another confounding point in the proposed model is whether MATCAP condensates recruit CENP-E, independently of its detyrosinase activity. Some in vitro experiments provided in this work suggest so (e.g. Fig. 2d and Fig. 6f,g), but it is possible that CENP-E was recruited to microtubules after MATCAP-enhanced detyrosination (in vitro and/or in cells). Indeed, purified CENP-E motor domain prefers detyrosinated microtubules, regardless of MATCAP (PMID: 25908662). One possible experiment that would allow the clarification of this issue would be to investigate whether a catalytic dead version of MATCAP containing the IDR is able to associate with CENP-E motor domain and whether this affects CENP-E recruitment to microtubules (in vitro and/or spindles).

3- A decisive experiment supporting the role of MATCAP LLPS-dependent microtubule detyrosination in guiding CENP-E-mediated chromosome congression is the deletion of IDR, which results in chromosome congression defects upon knockdown of the endogenous MATCAP (Fig. 6a). However, since deletion of IDR from exogenously expressed MATCAP (in the presence of endogenous MATCAP) is sufficient to reduce detyrosinated tubulin on spindles and CENP-E spindle association, it would be enlightening to test whether this also results on chromosome congression problems.

4- Defective kinetochore-microtubule attachments reported upon preventing MATCAP to form condensates are not convincing (Fig 1g and Fig 6c). All the authors show is chromosome mispositioning. Attachment status should be investigated with appropriate markers (e.g. Mad1/Mad2 or Astrin).

5- The authors generate a new home-made anti-MATCAP antibody, which is critical for the present study, but insufficiently characterized and described in the methods. Full details, such as immunization antigen, purification, specificity, etc, should be provided.

Minor issues:

1- FRAP experiments. The authors refer to the local exchange of MATCAP molecules within droplets. However, it seems that only about 20-30% of MATCAP molecules are mobile. The author should also provide information on the respective mobile and immobile fractions and how they interpret the latter in light of their electrostatic and hydrophobic interactions model.

2- One interesting link offered by this manuscript that would be worth discussing is the possibility that BuGZ, recently shown (PMID: 40425854) to be a target of parthenolide (a known inhibitor of microtubule detyrosination), that also forms “condensates” on microtubules, might somehow limit the activity of MATCAP.

3- The authors perform most of their in cell studies in U2OS cells. However, at some point they switch to HeLa and Cos-7 cells, for no obvious reason. Related to this, the Cos-7 cell permeabilization single-molecule experiments are very interesting and convincing, but in the Methods section the authors refer using U2OS cells (line 579). Please clarify.

4- Could the authors clarify whether all shMATCAP constructs result in chromosome congression defects and which one or more were used in the functional studies in cells?

5- Methods: please clarify whether sum projections were used for quantification of immunofluorescence images (line 600).

Helder Maiato

Reviewer #2

(Remarks to the Author)
This study proposes that MATCAP, a microtubule-associated tyrosine carboxypeptidase, facilitates mitotic chromosome alignment by forming dynamic condensates on microtubules via its N-terminal intrinsically disordered region (IDR). The authors suggest that, due to this liquid–liquid phase separation (LLPS) ability, MATCAP facilitates tubulin detyrosination and recruits the motor protein CENP-E. Although the study provides a deeper insight into the role of tubulin detyrosination and associated enzymes in chromosome movement during mitosis, several key conclusions are insufficiently supported, particularly regarding the role of MATCAP in chromosome congression and the functional relevance of LLPS. The LLPS behavior of MATCAP is primarily demonstrated using overexpression systems and most of the results that are used as evidence for the role of MATCAP LLPS can be alternatively interpreted.

Major points:

1. Although the contribution of tubulin detyrosination to CENP-E-mediated chromosome congression has been previously reported, the involvement of MATCAP in this process remained unclear. This study provides convincing evidence that MATCAP localizes to mitotic spindle and contributes to accurate chromosome congression in mitosis, which is in line with its role in microtubule detyrosination. The authors also propose that in addition to its role as a tubulin tyrosine carboxypeptidase, MATCAP condensates load CENP-E to microtubules. However, it remains unclear whether and to which extent MATCAP itself or MATCAP-mediated detyrosination modulates CENP-E activity. The interaction between MATCAP and CENP-E was shown only by overexpressing the two proteins. Many studies have already reported various CENP-E interactors by this way – has any of these found MATCAP as an interactor? The interaction between endogenous MATCAP and CENP-E should be validated to confirm physiological relevance beyond overexpression systems used in this study. For instance, endogenous CENP-E could be immunoprecipitated and the interaction with MATCAP could be tested by immunoblotting MATCAP, as well as by performing mass-spectrometry-based proteomics analysis. Also, although claimed in line 121, the direct interaction between MATCAP and CENP-E in an entirely in vitro conditions has not been shown.

2. The phenotypic differences between shMATCAP and shVASH1/2 (Fig. 1f) require further explanation. Does the modest difference in CENP-E localization (Fig. 1c) fully account for the disparity in congression defects? Quantitative comparison of the individual and joint impact of shMATCAP and shVASH on detyrosination levels and chromosome congression is lacking. shMATCAP, shVASH1/2, and shMATCAP+shVASH1/2 conditions should be compared. This may help to determine whether the observed phenotypes are due to reduced detyrosination per se or specific to MATCAP function.

3. Data in Fig. 2g–i show no significant difference in CENP-E motility between MATCAP and VASH1/2, contradicting the stronger phenotype observed with MATCAP depletion. The functional relevance of the reported direct interaction between MATCAP and CENP-E is unclear, especially since both MATCAP and VASH1/2 seem to influence CENP-E localization and activity.

4. The single molecule motility assay used to study the effect of MATCAP on CENP-E function is neither fully in vitro nor in cellulo. Instead, classical in vitro motility assays using purified components would be more appropriate. The authors have already used these assays in other experiments and have also shown the ability to purify CENP-E motor.

5. While the proposed LLPS of MATCAP is novel and interesting, it is unclear if this is an artifact of the protein overexpression. The extent of MATCAP overexpression relative to endogenous levels is not reported nor quantified. This information is critical to assess physiological relevance and potential artifacts.

6. The formation of LLPS should be studied under cytomimetic buffer conditions to better reflect physiological environments.

7. It would be of great importance to prove the formation of endogenous MATCAP LLPS using techniques such as CRISPR-based knock-in of e.g. GFP-MATCAP WT and GFP-MATCAP ΔIDR.

8. The use of 1,6 Hexanediol would also affect LLPS of other established mitotic proteins and is therefore insufficient to claim its specific effect on the formation of endogenous MATCAP LLPS. This should be properly discussed in the text.

9. Light induced promotion of MATCAP LLPS using the Cry2 system is shown to enhance the localization of MATCAP on microtubules. Does this also increase detyrosination levels? A corelative microscopy-based imaging would be informative to understand the localization and function of these chimeric constructs.

10. The N-terminal IDR of MATCAP was shown to act as a microtubule-binding domain (PMID: 35482892), thus the MATCAPΔIDR constructs are not expected to localize to the microtubules nor to be able to detyrosinate them and rescue the mitotic phenotype. The results relying on chimeric constructs of MATCAP with MATCAP IDR swapped with other domains are interesting but raise several key concerns. While the authors interpret these data as that the IDR-mediated LLPS is critical for MATCAP's activity, the results could also be interpreted as that the IDR domain simply mediates the interaction with microtubules and thereby affects its localization and function independent of the proposed LLPS. HEC1-CH and EB1-CH domains bind specifically to microtubule tips, while KIF5B motor walks along the microtubule. Thus, the chimeras containing these three domains bind differently to microtubules compared to the IDR-based microtubule-binding domains and cannot be expected to rescue the MATCAP activity in a similar way. The constructs that rescue MATCAP activity contain positively charged IDRs that are known to bind microtubules (LEM2, Tau and EB1 IDRs). The loss of localization and function of both MATCAPΔIDR-EB1-KR6Q IDR and MATCAP-8A mutants could be attributed to the disruption of the positively charged residues, thereby interfering with microtubule binding independent of LLPS. Thus, all effects of MATCAP IDR truncations and mutants could be interpreted by their inability to bind and detyrosinate microtubules, thereby affecting CENP-E activity and its role in chromosome congression during mitosis.

Minor points:

1. Line 40: MCAK is a kinesin-13. If describing two different kinesin 13 family members, both should be named.

2. Line 49-50: As most α-tubulin isotypes are genetically encoded with a tyrosine residue at their C-terminal tails, the act of removing the tyrosine by TCPs (VASHs and MATCAP) happens first and hence could be interpreted as writing the code, while adding the tyrosine back by TTL could be considered as erasing.

3. Quantitative validation of MATCAP knockdown via western blot and immunofluorescence is missing. Inclusion of these data is essential to confirm the efficiency and specificity of the shRNA-mediated depletion.

4. Four shRNAs are listed in the Methods, but only three are shown in Extended Data Fig. 1d. It is unclear which shRNA was used predominantly in functional assays. This should be clarified.

5. Lines 93–94: shVASH1/VASH2 knockdown resulting in ~40% reduction in CENP-E localization is termed "slight attenuation" while ~60% reduction from shMATCAP is termed "abolished".

6. Videos of the time-lapse imaging of mitosis should be provided in addition to the still images.

7. Line 109: Typographical error (extra period).

8. Lines 117-119: The colocalization of MATCAP and CENP-E is only partial, and the interpretation should reflect the same.

9. In Figure 2e, VASH proteins should be co-transfected with SVBP to ensure proper complex formation and enzymatic activity during co-immunoprecipitation.

10. Details on the GFP-CENP-E construct used in Fig2f-i is missing in the Methods section.

11. Details about MATCAP-RDI is missing in the Methods section.

12. The significance of co-condensation of MATCAP with soluble tubulin is unclear. Given MATCAP's preference for polymerized microtubules for its activity, what would its interaction with soluble tubulin dimer reflect in a physiological context?

13. Extended Data Fig. 3l shows highly impure VASH preparations with low SVBP ratio, which may compromise the interpretation of LLPS assays. Improved purification and quantification are recommended.

14. The rescue experiments in Fig4e, f should be accompanied with corresponding rescue of detyrosination levels.

15. Fig. 6c results should be quantified.

16. It is unclear why the CENP-E motor domain fails to bind microtubules independently in Fig. 6f–g. This should be addressed.

17. Line 320: Typographical error (“dimension”).

18. Line 404: “…is essential in control of in mitotic spindle plasticity control...” should be rewritten.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done an impressive work addressing the issues raised by the reviewers and I am now comfortable in recommending publication in Nature Communications after clarifying a couple of remaining minor issues:

1- Although the authors make a strong convincing point that MATCAP can also detyrosinate tubulin dimers, the rationale of evoking this role to compensate the highly dynamic state of microtubules in mitosis is hard to reconcile with the fact that most detyrosinated microtubules in the spindle are the ones that are more stably attached to kinetochores, which incorporate new tubulin subunits through flux from their kinetochore-associated microtubule plus-ends where detyrosination levels are lower. Henceforth, unless the authors envision some alternative mechanism, I really cannot see how this could work and I recommend toning down this claim. The fact that it can bind to tubulin dimers does not necessarily imply that it binds under normal in cellulo conditions where microtubules will dominate the interaction with MATCAP.

2- The cell depicted in Fig. 6c for the MATCAP-8A mutant is in early anaphase (sister chromatids have already separated). For the sake of consistency, I would recommend replacing by a representative cell in metaphase to make it more comparable to the other conditions.

Reviewer #2

(Remarks to the Author)
I would like to congratulate the authors for their careful and thorough revision of the manuscript. The revised version reflects substantial improvement in clarity, organization, and scientific rigor, and I recommend it for publication in Nature Communications.

Remaining minor comments:
- Extended Data Fig. 5a and b seem to be referenced incorrectly in the text.
- Check whether the shRNA sequences are correctly provided in the revised manuscript, as it seems that the most potent sequence from the original submission has been excluded now (original sequence shMATCAP #2: 5’-AGTCCCATTACACCTACAATC-3’).

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all pendiding issues and I can now recommend publication of this very nice work in Nature Communications.

Reviewer #2

(Remarks to the Author)
The authors have properly addressed all additional minor issues raised after the revision. I recommend the revised manuscript for publication.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-by-point response to the reviewers' comments on NCOMMS-25-52606-T

Reviewer #1 (Remarks to the Author):

In this very interesting manuscript, Yang and colleagues investigate the molecular mechanism underlying CENP-E motor guidance by detyrosinated microtubules. By a thorough combination of in cell and in vitro biochemical/biophysical assays, they provide a convincing case in which an Intrinsically Disordered Region (IDR) on the microtubule detyrosinase MATCAP is required for liquid-liquid phase separation (LLPS) and is involved in microtubule binding. More specifically, the authors show that MATCAP distinctively localizes to spindle microtubules and centrosomes (as compared to vasohibins) and this association is exacerbated upon its overexpression. Critically, the IDR is required for MATCAP association with spindle microtubules and its absence strongly compromises chromosome congression. Mechanistically, the authors show that MATCAP interacts with CENP-E motor domain and this interaction depends on MATCAP's IDR. Crucially, this appears to be independent of tubulin detyrosination, suggesting that MATCAP serves a dual role: the local recruitment of CENP-E to microtubules, while promoting its processivity by detyrosinating tubulin on the lattice. Overall, this work offers important mechanistic insight into how microtubule detyrosination and its machinery spatially orchestrates chromosome congression, while highlighting a key role of MATCAP (and less so of vasohibins) in the process. There are however a few points that require attention as detailed below.

We greatly appreciate the reviewer's enthusiasm on our study and his/her insightful suggestions extended to our revision. Listed below are our point-by-point responses to concerns raised by the reviewer.

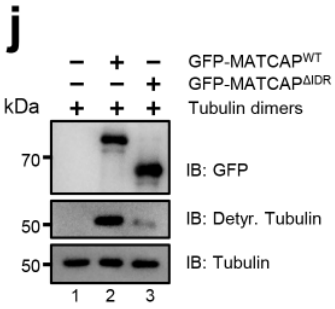
Major issues:

1- One confounding point in the proposed model concerns the recruitment of tubulin dimers to MATCAP condensates (Extended Data Fig. 5i,g). Previous work by the Brummelkamp lab has shown that MATCAP has a preference for polymerized tubulin compared to tubulin dimers. Yet, in the present paper, the authors evoque the local recruitment of tubulin dimmers as a means to facilitate tubulin detyrosination. The need to locally concentrate tubulin dimers to potentiate microtubule detyrosination requires clarification.

We thank the reviewer for raising this important point regarding the recruitment of tubulin dimers to MATCAP condensates. We also noticed the reports from the Brummelkamp lab and the Krzysztof lab, which demonstrate that MATCAP preferentially interacts with polymerized tubulin rather than tubulin dimers. Accordingly, we have cited these valuable studies in our revised manuscript. Importantly, both their findings and our experimental outcomes indicate that MATCAP (also referred to TMCP1) is capable of detyrosinating tubulin dimers as well (PMID: 35482892, 37703372; Extended Data Fig. 5j).

Our results do not contradict previous studies but revealed new dimension of MATCAP's function. While MATCAP possesses high efficiency of detyrosination on polymerized tubulin, it also exhibits considerable activity towards tubulin dimers; the co-condensation of MATCAP with tubulin dimers promotes its ability to detyrosinate these tubulin dimers. We reason that this property is particularly important during mitosis as microtubules are highly dynamic during this process, where rapid polymerization and depolymerization cycles may possess limited access for MATCAP to modify stable microtubules. By efficiently modifying tubulin dimers in phase-separated compartments, MATCAP could help maintain pools of detyrosinated tubulin efficiently and in a spatiotemporally controlled manner, facilitating rapid adaptation during cytoskeletal remodeling. We have clarified this point in the revised text (Page10, Line 255-261).

(Redaction)



Extended Data Fig. 5j. MATCAP condensates enhanced detyrosination activity

j, *In vitro* detyrosination assays using GFP-MATCAP^{WT} or GFP-MATCAP$^{\Delta IDR}$ proteins toward tubulin heterodimers.

Newly Added References:

1. Landskron, L. et al., Posttranslational modification of microtubules by the MATCAP detyrosinase. *Science* **376**, 815-+ (2022).
2. Nicot, S. et al., A family of carboxypeptidases catalyzing α- and β-tubulin tail processing and deglutamylation. *Science Advances* **9**(2023).

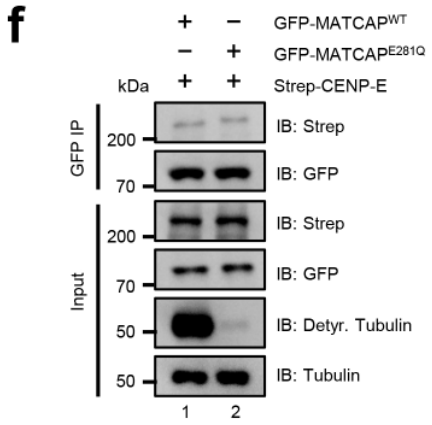
2- Another confounding point in the proposed model is whether MATCAP condensates recruit CENP-E, independently of its detyrosinase activity. Some in vitro experiments provided in this work suggest so (e.g. Fig. 2d and Fig. 6f,g), but it is possible that CENP-E was recruited to microtubules after MATCAP-enhanced detyrosination (in vitro and/or in cells). Indeed, purified CENP-E motor domain prefers detyrosinated microtubules, regardless of MATCAP (PMID: 25908662). One possible experiment that would allow the clarification of this issue would be to investigate whether a catalytic dead version of MATCAP containing the IDR is able to associate with CENP-E motor domain and whether this affects CENP-E recruitment to microtubules (in vitro and/or spindles).

We thank the reviewer for reiterating whether MATCAP condensates recruit CENP-E independently of its detyrosinase activity. To address this concern, we have carried out two lines of experiments during revision as suggested by the reviewer:

First, we have generated a catalytically dead point mutant of MATCAP^{E281Q} which retains its phase separation activity but lost enzymatic activity. Co-immunoprecipitation assays revealed that the mutant MATCAP^{E281Q} interacts with the motor domain of CENP-E, similar to that of wild-type MATCAP (Extended Data Fig. 3f).

Second, using an *in vitro* reconstitution assay, we found that the mutant MATCAP^{E281Q} condensates efficiently recruit the CENP-E motor domain both on microtubules or in solution

(Fig.2h, i). Thus, the aforementioned evidence rules out the possibility that recruitment occurs solely through microtubule detyrosination. Thus, our newly added data firmly support our newly proposed working model in which MATCAP condensates recruit CENP-E independently of its detyrosinase activity.



Extended Data Fig. 3f. The association of CENP-E with catalytically dead MATCAP mutant
GFP-MATCAP or GFP-MATCAP^{E281Q} was co-transfected with Strep-CENP-E in HEK293T cells, and their interactions were tested by co-immunoprecipitation.

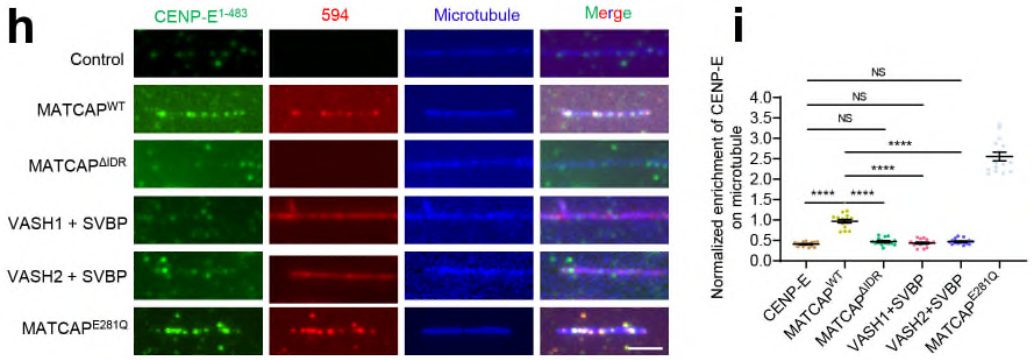


Fig. 2h-i. Recruitment of CENP-E motor by catalytically dead MATCAP condensates *in vitro*
h, TIRF images of Alexa Fluor 594-labeled MATCAP, VASH1, or VASH2 (10 nM; red) and GFP-CENP-E$^{1-483}$ (100 nM; green) localization to a GMPCPP-stabilized Cy5-labeled microtubule seed (blue). Scale bars, 2 μm.
i, Quantification of CENP-E fluorescence intensity, normalized to microtubule signal as shown in **h.** For each group, *n* = 15 microtubules, pooled from three independent experiments. Data represent mean ± s.e.m., Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates $P > 0.05$, ****$P < 0.0001$.

3- A decisive experiment supporting the role of MATCAP LLPS-dependent microtubule detyrosination in guiding CENP-E-mediated chromosome congression is the deletion of IDR, which results in chromosome congression defects upon knockdown of the endogenous MATCAP (Fig. 6a). However, since deletion of IDR from exogenously expressed MATCAP (in the presence of endogenous MATCAP) is sufficient to reduce detyrosinated tubulin on spindles and CENP-E spindle association, it would be enlightening to test whether this also results on chromosome congression problems.

We thank the reviewer for raising this important point regarding the potential dominant-negative effect of the MATCAP$^{\Delta IDR}$ mutant in the presence of endogenous protein. We agree that this is a critical consideration for interpreting our complementation experiments.

In our last submission, the assessment of CENP-E localization to spindle microtubules was performed under conditions where endogenous MATCAP was knocked down. Under these conditions, re-expression of the MATCAP$^{\Delta IDR}$ mutant failed to restore CENP-E localization (Fig. 6e).

Importantly, in the presence of endogenous MATCAP, expression of the MATCAP$^{\Delta IDR}$ mutant did not reduce CENP-E association with spindle microtubules (see blew). Furthermore, at the western blot level, expression of MATCAP$^{\Delta IDR}$ in cells with intact endogenous MATCAP did not alter global microtubule detyrosination levels compared to control cells (Extended Data Fig. 6d, 7b, 9c).

These results indicate that the MATCAP$^{\Delta IDR}$ mutant does not exert a dominant-negative effect over the endogenous, wild-type protein. The observed functional defects—including reduced spindle CENP-E localization and chromosome congression errors—are only apparent when the mutant is expressed in the absence of functional endogenous MATCAP. This supports the conclusion that the IDR is essential for MATCAP's role in supporting CENP-E function and chromosome congression.

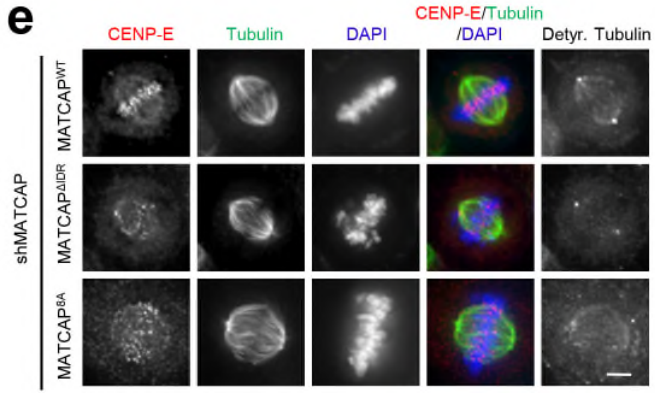


Fig. 6e. Re-expression of MATCAP$^{\Delta IDR}$ mutant failed to restore CENP-E localization and microtubule detyrosination in the absence of endogenous MATCAP

Immunofluorescence staining of CENP-E and detyrosinated tubulin in U2OS cells expressing FLAG-MATCAP or its mutants in the absence of endogenous MATCAP. Cells were fixed with methanol, and stained with CENP-E antibody, detyrosinated α-tubulin antibody, and α-tubulin antibody. DNA was stained with DAPI. Scale bar, 5 μm.

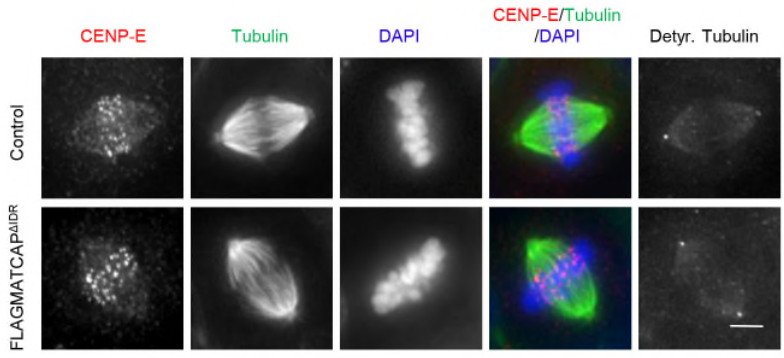
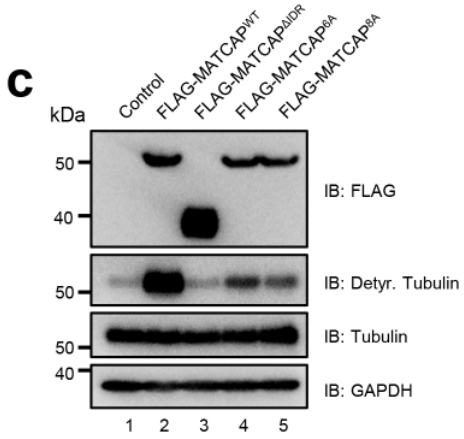


Fig. x. MATCAP$^{\Delta IDR}$ did not reduce CENP-E spindle association in the presence of endogenous MATCAP

Immunofluorescence staining of CENP-E and detyrosinated tubulin in U2OS cells expressed FLAG-MATCAP$^{\Delta IDR}$. Cells expressing wild type FLAG-MATCAP and FLAG-MATCAP$^{\Delta IDR}$ were fixed with methanol, and triply stained with CENP-E antibody and detyrosinated α-tubulin antibody and α-tubulin antibody. DNA was stained with DAPI. Scale bar, 5 μm.



Extended Data Fig. 9c. MATCAP$^{\Delta IDR}$ did not alter tubulin detyrosination in the presence of endogenous MATCAP

Effect of engineered MATCAP chimeric variants overexpression on detyrosinated tubulin levels. Immunoblot of cell lysates from HEK293T cells transfected with plasmids expressing engineered FLAG-MATCAP variants (MATCAP^{WT}, MATCAP$^{\Delta IDR}$, MATCAP^{6A}, MATCAP^{8A}). The expression of FLAG-MATCAP was observed using anti-FLAG antibodies. The levels of detyrosinated and total α-tubulin were assessed using the respective antibodies, with GAPDH serving as the loading control.

4- Defective kinetochore-microtubule attachments reported upon preventing MATCAP to form condensates are not convincing (Fig 1g and Fig 6c). All the authors show is chromosome mispositioning. Attachment status should be investigated with appropriate markers (e.g. Mad1/Mad2 or Astrin).

We thank the reviewer for highlighting the need for more direct evidence of kinetochore-microtubule attachment defects. In response, we have examined attachment status using Astrin, a well-established marker for mature, stable kinetochore-microtubule attachments.

Our newly added immunofluorescence analysis reveals reveals that upon MATCAP knockdown, Astrin localization at kinetochores is markedly diminished (Fig. 1h, i). Critically, MATCAP mutants (MATCAP$^{\Delta IDR}$ and MATCAP^{8A}) fail to restore Astrin's localization at kinetochores in the absence of endogenous MATCAP (Fig 6c). The loss of the Astrin signals at kinetochores under these conditions provides direct molecular evidence of defects in the kinetochore-microtubule attachments in MATCAP condensation-deficient cells. We have now included these new data in the revised manuscript as revised Fig. 1h and Fig. 1i.

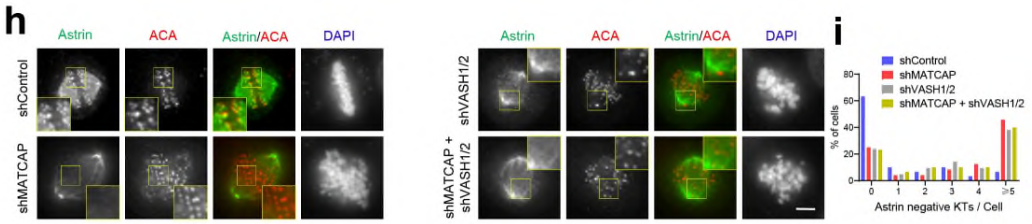


Fig. 1h-i. Knockdown of MATCAP diminishes the localization of Astrin at kinetochores

h, Immunofluorescence staining of Astrin and centromere (ACA) in U2OS cells transfected with Control, MATCAP or VASH1/2 shRNA. Mitotic cells were stained with Astrin antibody and centromeres (ACA), respectively. DNA was counterstained with DAPI. Scale bar, 5 µm.

i, Quantification of the percentage of cells with Astrin-negative kinetochores (KTs) per cell in **h.** For Control, *n* = 60; shMATCAP, *n* =72; shVASH1/2, *n* = 63; shMATCAP + shVASH1/2, *n* = 60, *n* represents the number of cells pooled from three independent experiments.

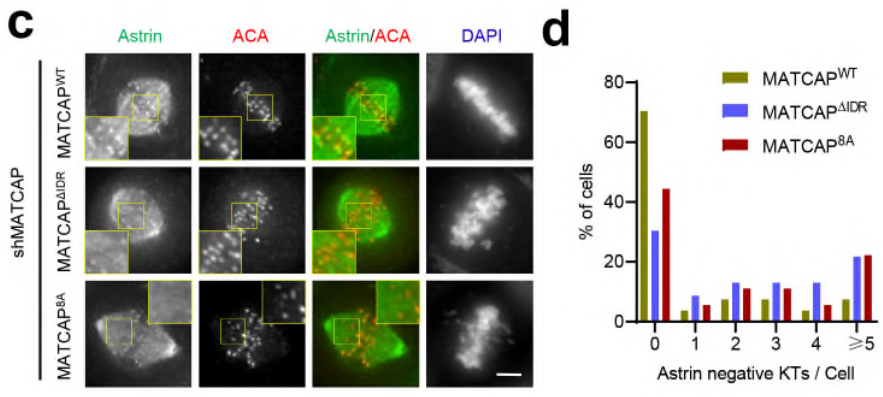


Fig. 6c-d. LLPS-deficient mutant of MATCAP fails to restore the kinetochore localization of Astrin

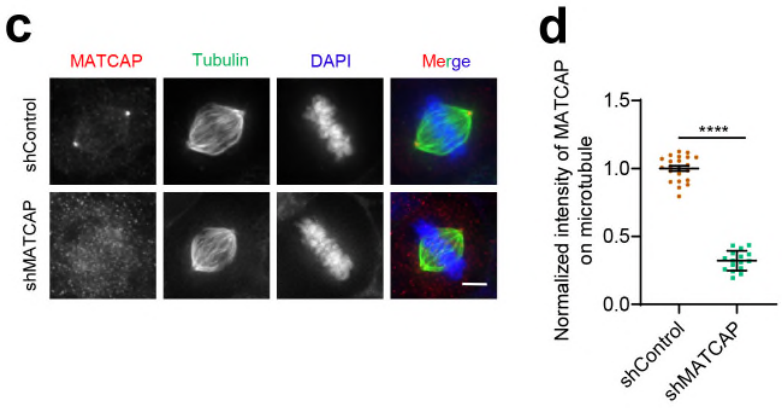
c, Immunofluorescence staining of Astrin and centromeres (ACA) in U2OS cells expressed FLAG-MATCAP or its mutants in the absence of endogenous MATCAP. Mitotic cells stained with Astrin antibody and centromeres (ACA), respectively. DNA was counterstained with DAPI. Scale bar, 5 μm.
d, Quantification of the percentage of cells with Astrin negative KTs per cell in **c.** For MATCAP, *n* =27; MATCAP$^{\Delta IDR}$, *n* =23; MATCAP^{8A}, *n* = 18, *n* represents the number of cells pooled from three independent experiments.

5- The authors generate a new home-made anti-MATCAP antibody, which is critical for the present study, but insufficiently characterized and described in the methods. Full details, such as immunization antigen, purification, specificity, etc, should be provided.

We thank the reviewer for this important suggestion, and have now included comprehensive details about the antibody, including immunization antigen, immunization and purification protocols, as well as specificity characterization in the Methods section of the revised manuscript (Page 18, Line 525-532).

The revised text now reads: "To generate anti-MATCAP antibody, BALB/c mice were immunized subcutaneously with 20 μg of purified His-tagged MATCAP$^{\Delta IDR}$ recombinant protein (amino acids 138–471) emulsified in an equal volume of Complete Freund's Adjuvant (Sigma, F5881). Subsequent booster immunizations (20 μg antigen in Incomplete Freund's Adjuvant) were given at two-week intervals. Terminal or non-terminal blood collections were performed after the final boost by cardiac puncture or orbital bleeding, respectively. The collected blood was allowed to clot at 37 °C for 10 min. Following coagulation, the samples were maintained at 4 °C overnight to facilitate serum separation. Serum was separated by centrifugation at 1,000 g for 10 minutes, recovered under sterile conditions, aliquoted, and stored at or below -20 °C until use."

For antibody specificity characterization, despite extensive tests, we were unable to reliably detect endogenous MATCAP using our home-made anti-MATCAP antibody in Western blot assays; we hence used a commercial MATCAP antibody for Western blot analyses throughout the manuscript. However, in immunofluorescence, MATCAP was robustly detected by our home-made antibody; signals on the centrosome and spindle pools were largely undetectable in shRNA-positively transfected cells, indicating a high specificity of the antibody in immunostaining. We have now included these new data in the revised manuscript as Extended Data Fig. 1c, d.



Extended Data Fig. 1c-d. Specificity characterization of the home-made anti-MATCAP antibody
c, Immunofluorescence staining of MATCAP and tubulin in metaphase HeLa cells expressing control or MATCAP shRNA. Cells were fixed with methanol, and stained with MATCAP serum antibody and α-tubulin antibody. DNA was stained with DAPI. Scale bars, 5 μm.
d, Quantification of shRNA knockdown efficiency in **c.** For Control, n = 22, shMATCAP, n = 16; n represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by two-sided unpaired Student's t-test. ****P < 0.0001.

Minor issues:

1- FRAP experiments. The authors refer to the local exchange of MATCAP molecules within droplets. However, it seems that only about 20-30% of MATCAP molecules are mobile. The author should also provide information on the respective mobile and immobile fractions and how they interpret the latter in light of their electrostatic and hydrophobic interactions model.

We thank the reviewer for this insightful comment regarding the dynamics of MATCAP molecules within condensates. As suggested, we have now performed a detailed quantitative analysis of MATCAP dynamics in the mobile and immobile fractions using FRAP assays.

In our FRAP assays, the pre-bleach fluorescence intensity (I_i) drops to a minimum (I_0) upon bleaching and recovers over time to a plateau (I_∞). The mobile fraction (Mf) was calculated as $Mf = (I_\infty - I_0)/(I_i - I_0)$, and the immobile fraction (IMf) as $1 - Mf$. Our measurements reveal a mobile fraction of approximately 33.5%, indicating that a substantial proportion of MATCAP molecules exhibit restricted mobility within the condensates.

Due to the nature of FRAP, the recovery dynamics may underestimate the mobility of molecules within the condensates. We agree that a significant immobile fraction might exist in these condensates, as is commonly observed in many condensates (PMID: 34636323, 31937751, 32494070). We interpret this immobile fraction as part of the natural maturation process, involving increasing interactions between molecules within the condensate. As the condensate becomes more crowded inside, both electrostatic and hydrophobic interactions among the molecules can also increase (PMID: 29961577, 27392146). The most dynamic fraction likely corresponds to molecules that are peripherally or transiently associated with the condensate interface.

We have updated the Methods with the calculation formula and a summary of mobile/immobile fractions in the revised manuscript (Page 20, Lines 584-586).

References:
1, Song, X.Y. et al. Phase separation of EB1 guides microtubule plus-end dynamics. *Nature Cell Biology* **25**, 79-91 (2023). PMID: 36536176
2, King, M.R. & Petry, S. Phase separation of TPX2 enhances and spatially coordinates microtubule nucleation. *Nature Communications* **11**(2020). PMID: 31937751
3, von Appen, A. et al. LEM2 phase separation promotes ESCRT-mediated nuclear envelope reformation. *Nature* **582**, 115-118 (2020). PMID: 32494070
4, Wang, J. et al. A Molecular Grammar Governing the Driving Forces for Phase Separation of

Prion-like RNA Binding Proteins. *Cell* **174**, 688-699 (2018). PMID: 29961577
5, Pak, C.W. et al. Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. *Molecular Cell* **63**, 72-85 (2016). PMID: 27392146

2- One interesting link offered by this manuscript that would be worth discussing is the possibility that BuGZ, recently shown (PMID: 40425854) to be a target of parthenolide (a known inhibitor of microtubule detyrosination), that also forms "condensates" on microtubules, might somehow limit the activity of MATCAP.

We agree that the potential functional interplay between BuGZ and MATCAP represents an interesting and timely direction for discussion. BuGZ was showed to possess temperature-dependent liquid-liquid phase separation driven by its intrinsically disordered region. This activity is essential for concentrating tubulin and promoting microtubule bundling and polymerization (PMID: 26388440).

Per the reviewer's suggestion, we have now discussed that the PTL-mediated inhibition of tubulin detyrosination may be an indirect effect of BuGZ on MATCAP (Page 14, Line 408-414) and added related literature into the revised manuscript (PMID: 26388440, 40425854, 17409447).

References:
1, Jiang, H. et al. Phase transition of spindle-associated protein regulate spindle apparatus assembly. *Cell* **163**, 108-122 (2015). PMID: 26388440
2, Eibes, S. et al. Parthenolide disrupts mitosis by inhibiting ZNF207/BUGZ-promoted kinetochore-microtubule attachment. *Embo Journal* **44**, 3764-3793 (2025). PMID: 40425854
3, Fonrose, X. et al. Parthenolide inhibits tubulin carboxypeptidase activity. *Cancer Research* **67**, 3371-3378 (2007). PMID: 17409447

3- The authors perform most of their in cell studies in U2OS cells. However, at some point they switch to HeLa and Cos-7 cells, for no obvious reason. Related to this, the Cos-7 cell permeabilization single-molecule experiments are very interesting and convincing, but in the Methods section the authors refer using U2OS cells (line 579). Please clarify.

To ensure our conclusion on MATCAP is not cell line-biased, we validated key findings across several commonly used cell systems, including U2OS, HeLa, and Cos-7. In some cases, different cell types were employed to optimize conditions for imaging or biochemical analysis.

For the single-molecule imaging experiments, both Cos-7 and U2OS cells were used as they possessed flat morphology and high adherent activity, which facilitates high-resolution imaging. The data presented in the main figures were acquired in U2OS cells. We have now clarified this distinction in the figure legend and the Methods section in the revised manuscript to avoid any confusion.

4- Could the authors clarify whether all shMATCAP constructs result in chromosome congression defects and which one or more were used in the functional studies in cells?

In response, we tested three distinct shRNAs targeting different regions of MATCAP. Based on immunoblot analysis in Extended Data Fig. 1d, we found shMATCAP #2 and #3 exhibited higher and similar knockdown efficiency. shMATCAP #2 targets the 5'UTR region of the mRNA, facilitating subsequent rescue experiments. Therefore, this shRNA construct was used for all subsequent functional experiments in cells. We have now elaborated this in the revised manuscript.

5- Methods: please clarify whether sum projections were used for quantification of immunofluorescence images (line 600).

In response, for the purpose of visualization, all representative immunofluorescence images shown in the figures are presented as maximum intensity projections. For the quantification of fluorescence signals, the levels of microtubule detyrosination and CENP-E at the spindle were measured using sum projections of the raw image data with Fiji/ImageJ. We have clarified this

in the Methods section of the revised manuscript (Page 23, Lines 706-707).

Reviewer #2 (Remarks to the Author):

This study proposes that MATCAP, a microtubule-associated tyrosine carboxypeptidase, facilitates mitotic chromosome alignment by forming dynamic condensates on microtubules via its N-terminal intrinsically disordered region (IDR). The authors suggest that, due to this liquid–liquid phase separation (LLPS) ability, MATCAP facilitates tubulin detyrosination and recruits the motor protein CENP-E. Although the study provides a deeper insight into the role of tubulin detyrosination and associated enzymes in chromosome movement during mitosis, several key conclusions are insufficiently supported, particularly regarding the role of MATCAP in chromosome congression and the functional relevance of LLPS. The LLPS behavior of MATCAP is primarily demonstrated using overexpression systems and most of the results that are used as evidence for the role of MATCAP LLPS can be alternatively interpreted.

We greatly appreciate the reviewer's enthusiasm on our study and his/her insightful suggestions extended to our revision to strengthen our study. Listed below are our point-by-point responses to concerns raised by the reviewer. We hope that our diligent work and fine revision satisfies the reviewer's critiques.

Major points:

1a. Although the contribution of tubulin detyrosination to CENP-E-mediated chromosome congression has been previously reported, the involvement of MATCAP in this process remained unclear. This study provides convincing evidence that MATCAP localizes to mitotic spindle and contributes to accurate chromosome congression in mitosis, which is in line with its role in microtubule detyrosination. The authors also propose that in addition to its role as a tubulin tyrosine carboxypeptidase, MATCAP condensates load CENP-E to microtubules. However, it remains unclear whether and to which extent MATCAP itself or MATCAP-mediated detyrosination modulates CENP-E activity.

As annotated to reviewer #1's critique, MATCAP condensates recruit CENP-E independently of its detyrosination activity. We kindly ask the reviewer to refer our detailed response to reviewer #1 (point #2). In summary, the catalytically dead mutant of MATCAP^{E281Q} remains associating with CENP-E judged by an immunoprecipitation assay. MATCAP^{E281Q} also recruits CENP-E to the condensates that were formed on *in vitro* reconstituted microtubules or in solution (Fig. 2h, i; Extended Data Fig. 3f, g). These results support the notion that MATCAP recruits CENP-E to microtubules independent of its detyrosinase activity.

Beyond recruitment, MATCAP promotes the processive motility of CENP-E along microtubules by detyrosinating microtubules, similar to those of VASH1 and VASH2 (Fig. 2j, k; Extended Data Fig. 3d, e). We have tried diligently to distinguish MATCAP's detyrosination activity from its phase separation-driven recruitment of CENP-E using catalytically inactive MATCAP^{E281Q}. However, due to a strong binding of MATCAP^{E281Q} to microtubules, common among MT-associated enzymes, CENP-E was absorbed by MATCAP^{E281Q} on microtubules, resulting in a chronic block of CENP-E motility on microtubules (Fig. 2j, bottom).

Given the fact that both wild-type and catalytically inactive mutant MATCAP^{E281Q} recruit CENP-E onto the *in vitro* reconstituted microtubules, we reason that MATCAP condensation promotes CENP-E loading while MTACAP-mediate detyrosination facilitates CENP-E motility. We have now elaborated this notion in the revised manuscript and hope that the reviewer satisfies our revision.

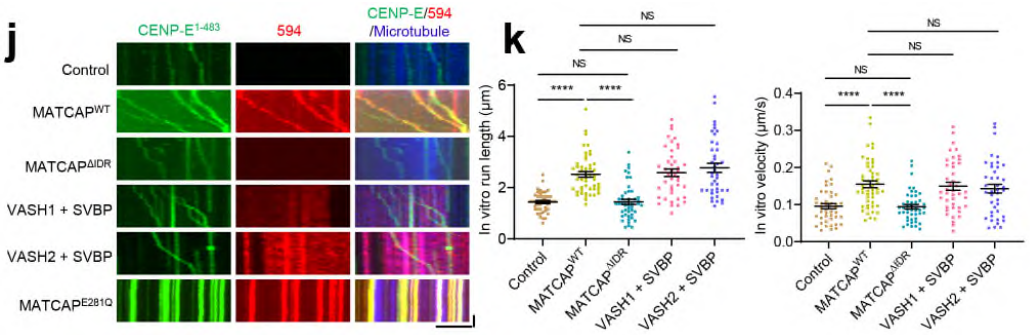


Fig. 2j-k. The movement of CENP-E motor along microtubules in the presence of detyrosination enzymes or their mutants

j, Fluorescence kymograph showing CENP-E$^{1-483}$ motion along microtubule lattice. Horizontal scale bar, 2 μm; vertical scale bar, 10 s. Both MATCAP and VASH1/2-mediated microtubule detyrosination increase CENP-E motility, but only MATCAP (via its IDR) enhances initial CENP-E binding to microtubules., revealing two distinct mechanisms for regulating motor processivity.

k, Mean velocity and characteristic length of CENP-E$^{1-483}$ motility on microtubules as shown in **j**. For Control, n = 44; MATCAP, n = 50; MATCAP$^{\Delta IDR}$, n = 46; VASH1, n = 42; VASH2, n = 41, n represents CENP-E molecules pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates $P > 0.05$, ****$P < 0.0001$.

1b. The interaction between MATCAP and CENP-E was shown only by overexpressing the two proteins. Many studies have already reported various CENP-E interactors by this way – has any of these found MATCAP as an interactor? The interaction between endogenous MATCAP and CENP-E should be validated to confirm physiological relevance beyond overexpression systems used in this study. For instance, endogenous CENP-E could be immunoprecipitated and the interaction with MATCAP could be tested by immunoblotting MATCAP, as well as by performing mass-spectrometry-based proteomics analysis.

We have surveyed existing literature and public interaction databases for CENP-E interactors identified via mass spectrometry (e.g., PMID: 37934467, 30655516). To our knowledge, MATCAP has not been reported as a CENP-E interactor before this study. Thus, our study discovered a previously uncharacterized interaction between MATCAP and CENP-E.

To further confirm the interaction of endogenous proteins, we have performed immunoprecipitation assays using an antibody against endogenous CENP-E from mitotic cell lysates, followed by mass spectrometry and immunoblotting analyses. Our proteomic analysis successfully identified MATCAP as a bona fide interacting protein of CENP-E. Furthermore, immunoblotting of the immunoprecipitated endogenous CENP-E confirmed the presence of MATCAP (see below and Fig. 2b). These results provide direct evidence for a functional association between endogenous MATCAP and CENP-E. The newly performed immunoprecipitation data have been included in the revised manuscript.

Bait	Prey	Coverage (%)	Peptides
	CENP-E	15	35
	CENP-F	7	17
CENP-E	MATCAP	19	7
	CLASP1	2	2
	Aurora A	2	1

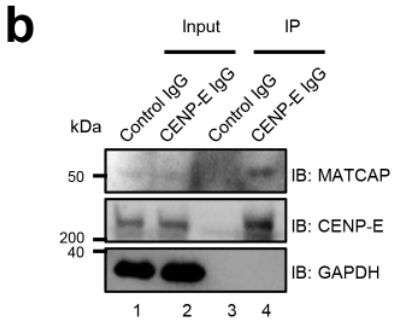


Fig. 2b. The interaction between endogenous MATCAP and CENP-E analyzed by mass-spectrometry and Western blotting

Left. List of CENP-E-interacting proteins. Endogenous CENP-E was immunoprecipitated, and its interacting proteins were identified by mass spectrometric analysis.
b, Endogenous immunoprecipitation from mitotic cell lysates using control IgG or rabbit anti-CENP-E (HpX) antibody. Immunoprecipitation samples were resolved by Western blotting using mouse anti-CENP-E antibody and rabbit anti-MATCAP antibody to confirm the interaction between MATCAP and CENP-E.

References:
1, Wu, M. et al. LUBAC controls chromosome alignment by targeting CENP-E to attached kinetochores. *Nature Communications* **10**(2019). PMID: 30655516
2, Wu, J.C. et al. A farnesyl-dependent structural role for CENP-E in expansion of the fibrous corona. *Journal of Cell Biology* **223**(2023). PMID: 37934467

1c. Also, although claimed in line 121, the direct interaction between MATCAP and CENP-E in an entirely in vitro conditions has not been shown.

We had attempted to characterize a direct interaction between MATCAP and CENP-E using bacterially expressed recombinant proteins. However, production of recombinant full-length CENP-E from bacteria is challenging, given its length and the complexity of its structure. We then turn to engineer recombinant Strep-CENP-E from 293T cells for the in vitro binding assays. To minimize co-purifying host proteins, we used a stringent purification buffer containing 300 mM KCl and 1% Triton X-100. The *in vitro* pull-down experiment confirms an intrinsically disordered region (IDR)-dependent binding between MATCAP and CENP-E under defined *in vitro* conditions (Fig. 2f). We have now included the newly obtained data and elaborated this line of experimentation in the revised manuscript.

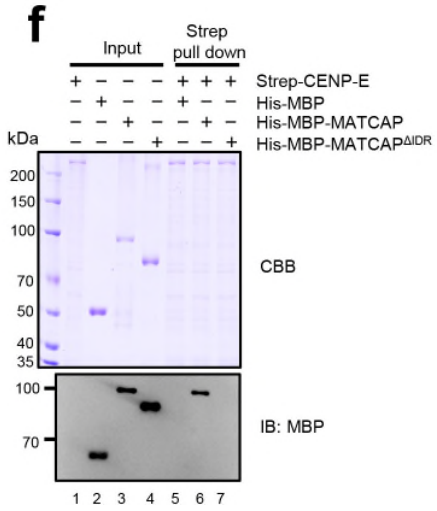


Fig. 2f. MATCAP interacted with CENP-E via its N-terminal IDR

Recombinant Strep-CENP-E were purified and used as affinity matrices to isolate bacterially expressed His-MBP, His-MBP-MATCAP or His-MBP-MATCAP$^{\Delta IDR}$. The binding fraction was analyzed by immunoblotting and protein levels were analyzed with SDS-PAGE gel stained by CBB.

2. The phenotypic differences between shMATCAP and shVASH1/2 (Fig. 1f) require further explanation. Does the modest difference in CENP-E localization (Fig. 1c) fully account for the disparity in congression defects? Quantitative comparison of the individual and joint impact of shMATCAP and shVASH on detyrosination levels and chromosome congression is lacking. shMATCAP, shVASH1/2, and shMATCAP+shVASH1/2 conditions should be compared. This may help to determine whether the observed phenotypes are due to reduced detyrosination per se or specific to MATCAP function.

We agree with the reviewer that a systematic comparison of the individual and combined effects of MATCAP and VASH1/2 depletion is essential to decipher their respective contributions to CENP-E localization and chromosome congression.

In response, we have now carried out new set of experiments by comparing detyrosination levels and chromosome congression in cells expressing control shRNA, shMATCAP, shVASH1/2, or shMATCAP + shVASH1/2. Western blot analysis revealed that the expression of shMATCAP, shVASH1/2, or shMATCAP + shVASH1/2 reduced detyrosinated tubulin levels to 58%, 43%, and 12%, respectively (Fig. 1e). Expression of shMATCAP, shVASH1/2, or shMATCAP + shVASH1/2 reduced CENP-E localization on spindle microtubules by 63.5%, 38.2%, and 64%, respectively (Fig.1 b, c). Additionally, live cell imaging demonstrated that depletion of MATCAP, VASH1/2, or MATCAP + VASH1/2 increased the percentage of metaphase-like cells with misaligned chromosomes to 39.5%, 16.0%, and 40.5%, respectively (Fig. 1d, g).

Based on these findings, we reason that MATCAP recruits CENP-E in two ways: through detyrosination of spindle microtubules, similar to VASH1/2, and through unique direct recruiting. The data also indicates that when detyrosinated tubulin levels decreased to 58%, further reduction in tubulin detyrosination did not lead to additive defects in CENP-E binding or chromosome congression. This suggests that the role of detyrosination in spindle microtubules regarding chromosome congression is dose-dependent. Moreover, the difference in chromosome congression defects caused by MATCAP and VASH1/2 depletions is more pronounced than the difference observed in detyrosinated tubulin levels. We speculate that the abundance of CENP-E recruited specifically by MATCAP facilitates chromosome congression in a manner that cannot be compensated for by tubulin detyrosination alone. Together, these data underscore the significance of MATCAP-mediated recruitment of CENP-E over the sole role of VASH1/2 in microtubule detyrosination during mitosis. We have included this new data and interpretation in the revised manuscript (Fig. 1b-g; Page 5).

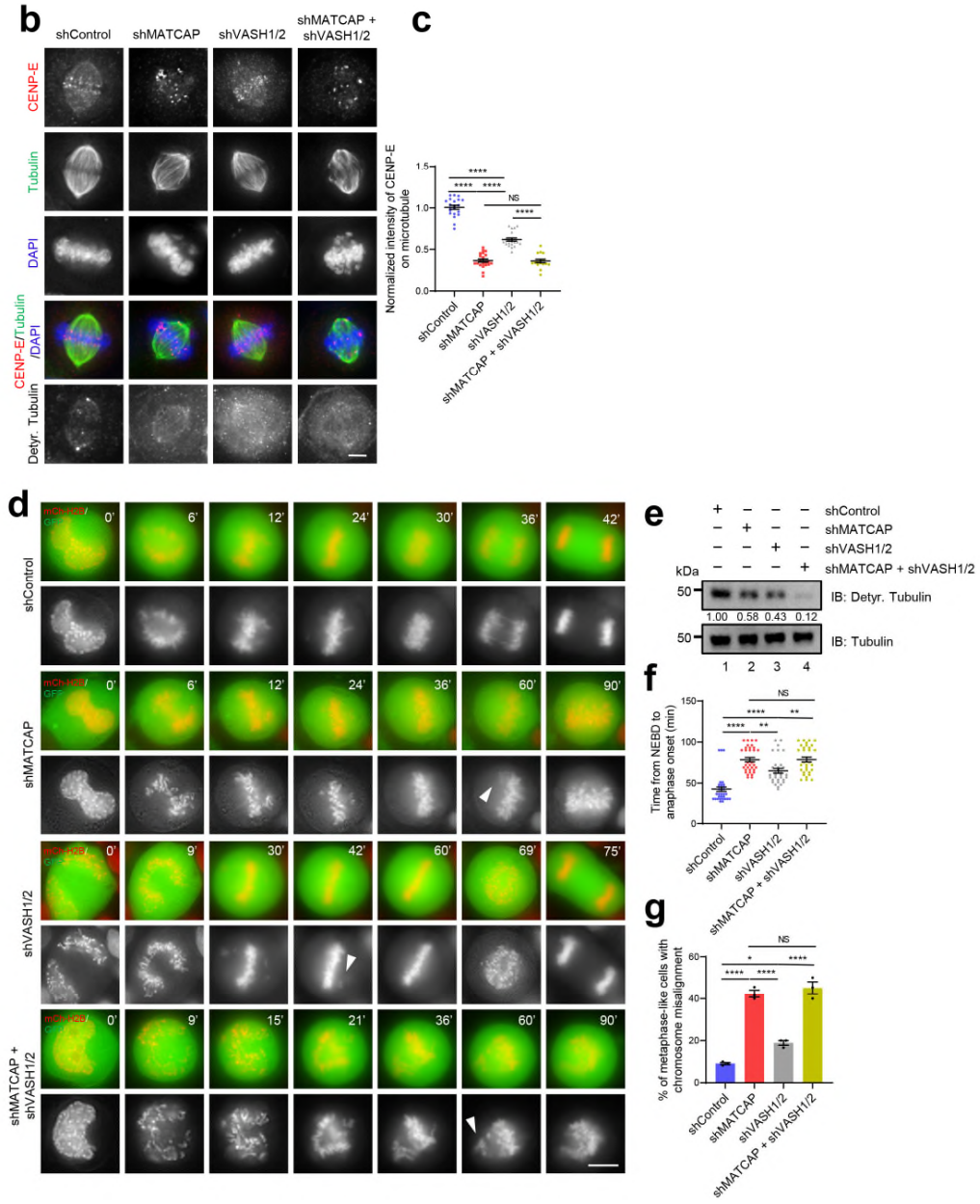


Fig. 1. MATCAP orchestrates CENP-E-dependent chromosome congression

b, Immunofluorescence staining of CENP-E and detyrosinated tubulin in U2OS cells following individual or combined knockdown of MATCAP and VASH1/2 using shRNA. Cells were fixed with methanol, and stained with CENP-E antibody and detyrosinated α-tubulin antibody and α-tubulin antibody. DNA was stained with DAPI. Scale bar, 5 μm.

c, Statistical analyses of the intensity of CENP-E on the spindle in **b.** For Control, $n = 21$; shMATCAP, $n = 21$; shVASH1/2, $n = 21$; shMATCAP + shVASH1/2, $n = 16$, n represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. ****$P < 0.0001$.

d, Representative real-time imaging showing chromosome movements in U2OS cells stably expressing mCherry-H2B and transfected with the indicated PLKO-GFP constructs (Control, shMATCAP or shVASH1/2). Time in minutes. White arrowheads point to misaligned chromosomes. Scale bar, 10 μm.

e, Immunoblot analysis of tubulin detyrosination in U2OS cells following individual or combined knockdown of MATCAP and VASH1/2 using shRNA.

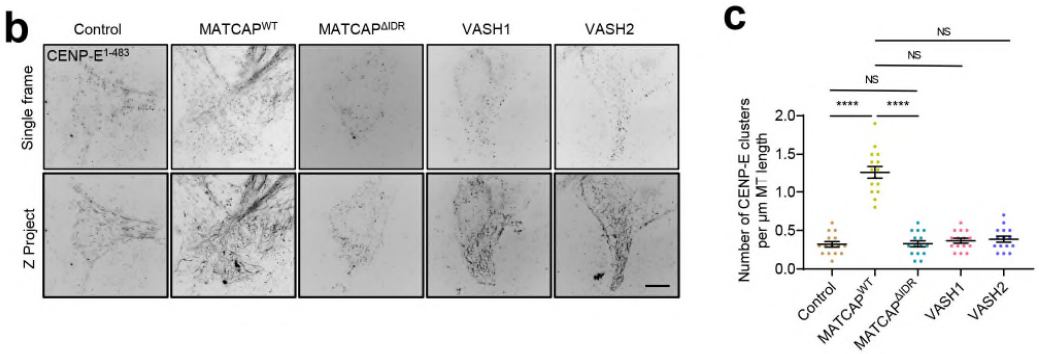
f, Statistical analysis of time from nuclear envelope breakdown (NEBD) to anaphase onset (or imaging

duration) as described in **d.** For Control, *n* = 32; shMATCAP, *n* = 33; shVASH1/2, *n* = 32; shMATCAP + shVASH1/2, *n* = 31, *n* represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates P > 0.05, ****P < 0.0001, shMATCAP vs. shVASH1/2, **P = 0.0066; shVASH1/2 vs. shMATCAP + shVASH1/2, **P = 0.007.
g, Statistical analysis of the percentage of cells with mitotic progression arrest as described in **d.** For Control, *n* = 32; shMATCAP, *n* = 33; shVASH1/2, *n* = 32; shMATCAP + shVASH1/2, *n* = 31, n represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates P > 0.05, ****P < 0.0001, and *P = 0.0187.

3. Data in Fig. 2g–i show no significant difference in CENP-E motility between MATCAP and VASH1/2, contradicting the stronger phenotype observed with MATCAP depletion. The functional relevance of the reported direct interaction between MATCAP and CENP-E is unclear, especially since both MATCAP and VASH1/2 seem to influence CENP-E localization and activity.

In response, we have carried out additional experiments to demonstrate the different contributions of MATCAP and VASH1/2 to CENP-E activity in mitosis (please see our responses to point #2 of reviewer #1 and points #1 and #2 of reviewer #2). Briefly, we provided both in vitro and in vivo evidence supporting that MATCAP regulates CENP-E in two ways: the phase separation-driven recruitment of CENP-E and catalysis-elicited tubulin detyrosination. In contrast, VASH1/2 solely influences CENP-E through tubulin detyrosination. The specific recruitment of CENP-E by MATCAP to spindle microtubules facilitates chromosome congression in a manner that was not compensated by VASH1/2.

The observations in the referred experiment (Fig. 2g-i in the previous submission) do not contradict but align with our main conclusions. As noted by the reviewer, in this experiment, MATCAP and VASH1/2 similarly influence CENP-E mobility via tubulin detyrosination. coincident with the fact that both types of enzymes possess similar detyrosination ability (Fig. 1e). Our new quantification also revealed that MATCAP facilitates CENP-E recruitment to microtubules, as reflected by more CENP-E on microtubules in MATCAP-expression cells (Extended Data Fig. 3b, c). Therefore, it is the recruitment of CENP-E by MATCAP that accounts for the stronger phenotype observed with MATCAP depletion. We have now included this new quantification data and a clear interpretation in the revised manuscript.



Extended Data Fig. 3b-c. Recruitment of CENP-E motor by MATCAP in cells

b, Representative images of CENP-E$^{1-483}$ proteins binding on microtubules in fixed U2OS cells expressing relevant detyrosination enzymes or mutants. Scale bar, 10 μm.
c, Quantification of GFP-CENP-E$^{1-483}$ clusters bound to microtubules as described in **b**. The number of CENP-E clusters was quantified and normalized to the length of randomly selected microtubule segments (10 μm in length). For each group, *n* = 15 microtubules pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates P > 0.05, and ****P < 0.0001.

4. The single molecule motility assay used to study the effect of MATCAP on CENP-E function is neither fully in vitro nor in cellulo. Instead, classical in vitro motility assays using purified components would be more appropriate. The authors have already used these assays in other experiments and have also shown the ability to purify CENP-E motor.

We agree with the reviewer that a classical *in vitro* motility assay using purified components would provide the most direct and unambiguous evidence for assessing the effect of MATCAP on CENP-E motility. For the referred single-molecule motility assay, we chose to perform the single-molecule tracking in permeabilized cells as described previously (PMID: 36893247) for a specific reason: this approach allows us to observe CENP-E motor dynamics on its native, cellular microtubule network, which retains essential features such as post-translational modifications and associated regulatory proteins. This provides a more physiologically relevant context than a pure *in vitro* reconstituted system.

In response to the reviewer's suggestion, we have carried out an in vitro assay to assess CENP-E motility as recommended by this reviewer. We have incorporated the new data in the revised manuscript and would like to invite the reviewer to examine our responses to the second comment of the reviewer #1 and the first comment from reviewer #2 for details. Briefly, the newly obtained data provide additional support to our early conclusion.

Reference:

1, Deguchi, T. *et al*., Direct observation of motor protein stepping in living cells using MINFLUX. *Science* **379**, 1010-1015 (2023). (PMID: 36893247)

5. While the proposed LLPS of MATCAP is novel and interesting, it is unclear if this is an artifact of the protein overexpression. The extent of MATCAP overexpression relative to endogenous levels is not reported nor quantified. This information is critical to assess physiological relevance and potential artifacts.

We thank the reviewer for raising this critical point regarding the physiological relevance of MATCAP condensation and the potential for overexpression artifacts. We agree with the reviewer that assessing expression levels is essential for interpreting phase separation phenomena.

The optogenetic (opto) experiments presented in the previous submission were performed in COS-7 cells (derived from African green monkeys) to facilitate observation. However, due to the significant sequence divergence between human and monkey MATCAP orthologs, the antibodies available against human MATCAP protein do not recognize the endogenous MATCAP protein in COS-7 cells. This limitation precluded us from conducting a direct quantitative comparison between the level of exogenously expressed human MATCAP-Cry2 fusion protein and the endogenous monkey MATCAP using Western blot.

To overcome this limitation, we have now carried out additional optogenetic experiments in U2OS cells with the endogenous MATCAP knocked down. Western blot analyses revealed that the protein level of exogenous Cry2-mCherry MATCAP was comparable to that of the endogenous MATCAP. Given that the transfection efficiency was appropriate 70%, we speculate the expression level of exogenous Cry2-mCherry MATCAP was within 2-fold higher than the endogenous MATCAP protein, providing a reliable system for assessing the condensation properties of MATCAP near its endogenous concentration.

Live-cell imaging revealed that in the absence of blue-light activation, the new optoMATCAP fusion exhibited a mixed distribution in U2OS cells, with both microtubule-bound and diffuse cytoplasmic localization. Strikingly, blue-light illumination triggered a rapid redistribution of MATCAP, with a significant reduction in the diffuse pool while an increase in the microtubule-bound fraction. Notably, in nocodazole-treated cells where microtubules were depolymerized, light-dependent formation of discrete cytoplasmic droplets was readily detected (Fig. 3a & Extended Data Fig. 4b, c), indicating that MATCAP possesses a previously uncharacterized condensation property. In contrast, an IDR-deleted mutant exhibited diffuse cytoplasmic localization and failed to form droplets and to enrich on microtubules, regardless of blue-light on or off.

To further address concerns about physiological relevance, additional strategies were used

to ensure our conclusions are not driven by non-physiological overexpression: All our *in vitro* phase separation assays were conducted below the reported endogenous cellular concentration of MATCAP, which is ~30 nM as quantified by the OpenCell database (PMID: 35271311, https://opencell.sf.czbiohub.org/gene/ENSG00000196123). In fact, most of our experiments were done at a concentration of 1 nM. Besides, we have performed key *in vitro* phase separation assays in a buffer containing 10 mg/mL cell lysate to mimic the physiological conditions (PMID: 38614097). In these near-physiological conditions, TIRF microscopy revealed that MATCAP forms dynamic condensates on microtubules at as low as 100 pM and exhibits obvious liquid-like behavior, including fusion between neighboring condensates (Extended Data Fig. 5b, c). Thus, these observations demonstrate that MATCAP undergoes condensation at concentrations below its endogenous level, strongly supporting the physiological relevance of this phenomenon. Please also see our detailed response to the next comment.

Together, these data indicate that MATCAP phase separation is not an artifact of overexpression but represents a true property observable at physiologically relevant concentrations in live cells.

References:

1, Cho, N.H. et al., OpenCell: Endogenous tagging for the cartography of human cellular organization. *Science* 375(6585):eabi6983 (2022). PMID: 35271311

2, Hedtfeld, M., Dammers, A., Koerner, C. & Musacchio, A. A validation strategy to assess the role of phase separation as a determinant of macromolecular localization. *Molecular Cell* **84** (2024). PMID: 38614097

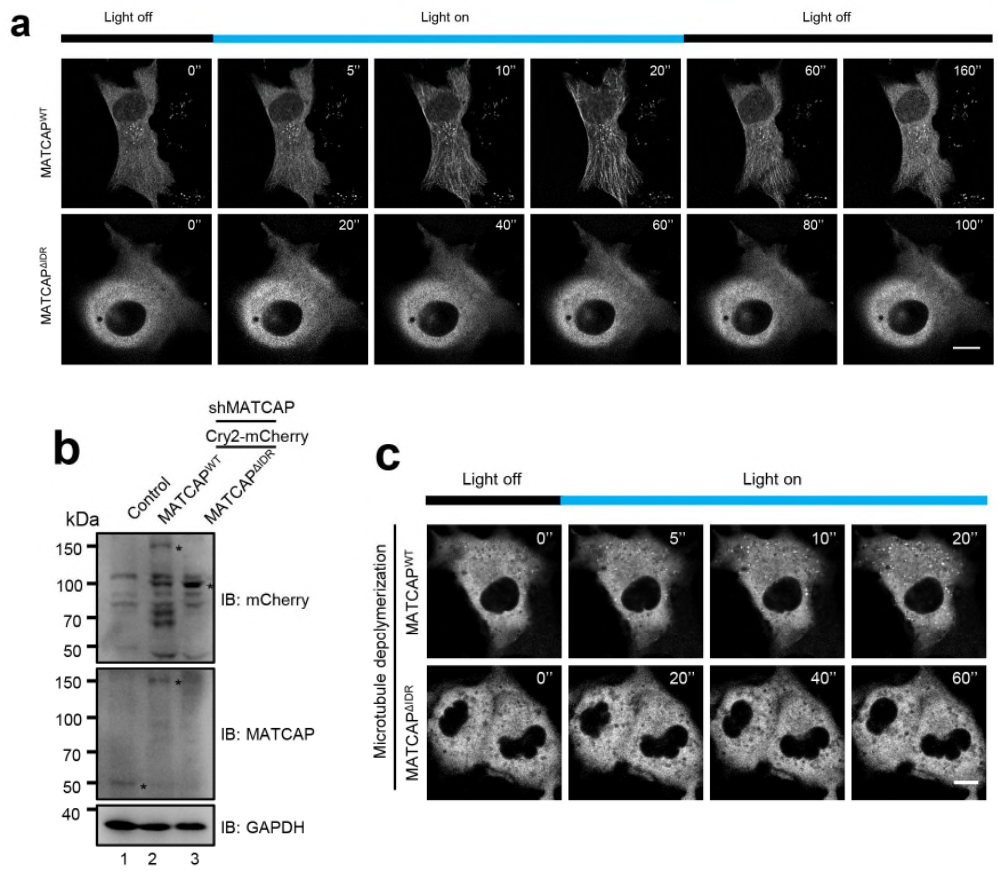


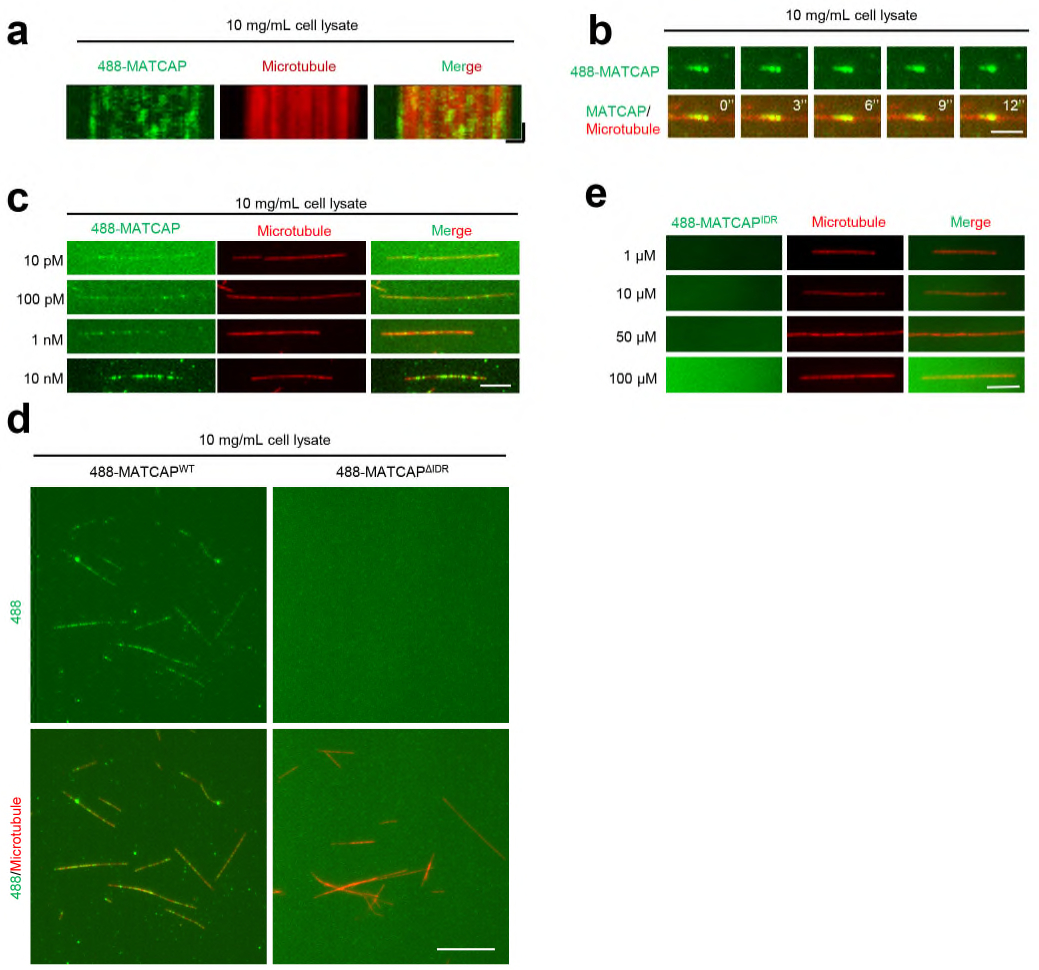
Fig. 3a & Extended Data Fig. 4b-c. Optogenetic experiments showing that MATCAP undergoes condensation in cells

a, Time-lapse images of U2OS cells depleted of endogenous MATCAP and expressing Cry2olig-MATCAP or Cry2olig-MATCAP$^{\Delta IDR}$, before and after blue-light activation, Blue light activation induced the enrichment of Cry2olig-MATCAP but not Cry2olig-MATCAP$^{\Delta IDR}$ on microtubules. Top: schematic

design of blue-light-induced droplet reporter. Bottom: time-lapse analysis of OptoMATCAP in U2OS cells upon blue light on or off. Scale bar,10 μm.

b, Western blot showing protein levels of Cry2-mCherry-MATCAP, Cry2-mCherry-MATCAP$^{\Delta IDR}$, relative to that of the endogenous MATCAP in U2OS cells. The asterisks in the upper panel indicate Cry2-mCherry-tagged MATCAP and its mutant, while the asterisks in the middle panel indicate the endogenous MATCAP and Cry2-mCherry-MATCAP, respectively. Cry2-mCherry-MATCAP$^{\Delta IDR}$ could not be recognized by the MATCAP antibody due to the lack of antigen.

c, Time-lapse images of U2OS cells depleted of endogenous MATCAP and expressing Cry2olig-MATCAP or Cry2olig-MATCAP$^{\Delta IDR}$, before and after blue-light activation. Cells were treated with nocodazole to disassemble microtubule. Scale bar,10 μm.



Extended Data Fig. 5. Condensation of MATCAP on MTs via its N-terminal IDR

a, Kymograph of TIRF images showing the formation and dissolution of Alexa Fluor 488-labeled MATCAP condensate on microtubules in presence of 10 mg/mL whole-cell lysate, 100 mM KCl, and MRB80 buffer. The protein concentration was 1 nM. Horizontal scale bar, 2 μm; vertical scale bar, 20 s.

b, Representative images of Alexa Fluor 488-labeled MATCAP condensate fusion on microtubule in presence of 10 mg/mL whole-cell lysate, 100 mM KCl, and MRB80 buffer. The protein concentration was 10 nM. Scale bars, 5 μm.

c, TIRF microscopy showing condensate formation by different concentrations of Alexa Fluor 488-labeled MATCAP on microtubules in presence of 10 mg/mL whole-cell lysate, 100 mM KCl, and MRB80 buffer. Scale bars, 5 μm.

d, Large field of view of a TIRF image showing the formation of condensates by 1 nM Alexa Fluor 488-labeled MATCAP along microtubules in presence of 10 mg/mL whole-cell lysate, 100 mM KCl, and MRB80 buffer. The protein concentration was 1 nM. Scale bars, 5 μm.

e, TIRF images showing that Alexa Fluor 488-labeled MATCAP-IDR does not localize to microtubules. Scale bars, 5 μm.

6. The formation of LLPS should be studied under cytomimetic buffer conditions to better reflect physiological environments.

We thank the reviewer for this important suggestion. In our last submission, all phase separation experiments *in vitro* were conducted in a buffer designed to mimic the intracellular environment, containing 150 mM KCl, 50 mM HEPES (pH 7.4) to approximate physiological ionic strength and reducing potential—a system widely adopted in biomolecular condensate research (PMID: 39824183, 40412392, 31937751). We agree that this condition cannot fully mimic the cytoplasmic conditions and therefore employed whole-cell lysates (10 mg/mL), which has been previously used for testing protein LLPS in a near physiological buffer (PMID: 38614097), to validate some of our key in vitro LLPS experiments. The newly obtained images were shown in the response to comment #5 of reviewer #2.

Under these conditions, real-time imaging revealed that MATCAP molecules initially associated diffusely with the microtubule lattice, followed by nucleation and progressive expansion, indicative of condensate formation. These condensates were dynamic, exhibiting dissolution, locomotion along microtubules, and fusion between neighboring condensates. Concentration-dependence analysis revealed that the condensates were detectable when MATCAP concentrations dropped to as low as 100 pM, below which only diffuse distributions were observed (Extended Data Fig. 5a-c). Upon deletion of the IDR, MATCAP$^{\Delta IDR}$ diffused in the solution and displayed minimal binding to microtubules (Extended Data Fig. 5d). Moreover, the IDR of MATCAP did not localize to microtubules even at a concentration of 100 μM (Extended Data Fig. 5e).

These results confirm that MATCAP undergoes phase separation under cytomimetic buffer conditions and within a crowded macromolecular environment, and that the IDR is required for its condensation, supporting the notion that MATCAP phase separation possesses physiological relevance.

References:

1, Ding, M.R. et al. A biophysical basis for the spreading behavior and limited diffusion of Xist. *Cell* **188**, 978-997 (2025). PMID: 39824183

2, Yan, X. et al. Intra-condensate demixing of TDP-43 inside stress granules generates pathological aggregates. *Cell* **188**, 4123-4140 (2025). PMID: 40412392

3, King, M.R. & Petry, S. Phase separation of TPX2 enhances and spatially coordinates microtubule nucleation. *Nature Communications* **11** (2020). PMID: 31937751

4, Hedtfeld, M., Dammers, A., Koerner, C. & Musacchio, A. A validation strategy to assess the role of phase separation as a determinant of macromolecular localization. *Molecular Cell* **84** (2024). PMID: 38614097

7. It would be of great importance to prove the formation of endogenous MATCAP LLPS using techniques such as CRISPR-based knock-in of e.g. GFP-MATCAP WT and GFP-MATCAP $^{\Delta IDR}$.

We fully agree with the reviewer that using CRISPR-based knock-in cell lines to demonstrate the LLPS of MATCAP would provide gold-standard and compelling evidence for its LLPS in cells. However, despite extensive efforts, in-house and commercial service, to generate CRISPR-based knock-in cell lines of GFP-MATCAP WT and GFP-MATCAP$^{\Delta IDR}$ in multiple pursuits, we failed to obtain accurate knock-in cell lines. The technique challenges were due mainly to the lack of an ideal sequence around the genomic locus for sgRNA targeting and/or the low accessibility of the targeting locus for cutting and repair.

Given our inability to establish CRISPR-based knock-in cell lines up to date, we made specific efforts to investigate the LLPS of MATCAP at concentrations near or even below endogenous levels. For instance, in our Opto experiments, Western blot analysis confirmed that the expression level of exogenous Cry2-mCherry MATCAP was comparable to that of endogenous MATCAP (Fig. 3a & Extended Data Fig. 4b, c). In our *in vitro* assays, MATCAP undergoes LLPS at concentrations as low as 1 nM (Fig. 3i & Extended Data Fig. 5a-d) in both

classic buffer and in the cell lysate buffer. Together, these findings suggest that the LLPS of MATCAP is not merely an artifact of overexpression but true physiochemical property of MATCAP.

8. The use of 1,6 Hexanediol would also affect LLPS of other established mitotic proteins and is therefore insufficient to claim its specific effect on the formation of endogenous MATCAP LLPS. This should be properly discussed in the text.

We agree with the reviewer that 1,6-HD is a non-specific agent known to disrupt a wide range of biomolecular condensates, including those formed by various mitotic regulators. As such, its effects cannot be attributed solely to MATCAP condensate disruption.

In order to minimize off-target and cytotoxic effects, we strictly followed a short-term, low-concentration treatment protocol (1.5%, 2 minutes) established in prior work (PMID: 34404453), which has been shown to preferentially disrupt dynamic condensates while preserving cell viability. Under these carefully controlled conditions, we observed a significant and rapid reduction in the microtubule-associated localization of endogenous MATCAP. Since we have also used multiple other orthogonal approaches throughout the study—including deletion of the IDR and *in vitro* reconstitution—to assess the contribution of hydrophobic interactions to MATCAP's LLPS, we reason that the reduction in MATCAP's microtubule binding seen in hexanediol treatment reflects MATCAP's response to hexanediol treatment.

However, we could not completely exclude the possibility of indirect effects. As suggested by the reviewer, we have now included a discussion in the revised manuscript explicitly noting the broad activity of 1,6-HD as below (Page 8, line 190-195): "We then employed 1,6-Hexanediol (1,6-HD), a non-specific agent, to disrupt weak hydrophobic interactions that contribute to the formation of many cellular biomolecular condensates. We treated the U2OS cells with 1.5% 1,6-HD for 2 min to minimize the cytotoxicity and indirect effects (PMID: 34404453). Upon treatment, the endogenous MATCAP exhibited a significant reduction in microtubule localization (Fig. 3b, c). Although we could not fully rule out the possibility of indirect effects, these results suggest a role of hydrophobic interactions in MATCAP condensation on microtubules."

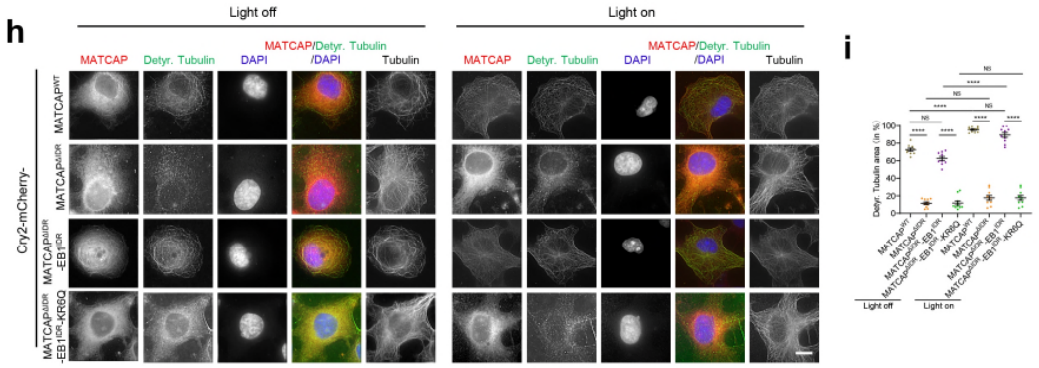
References:

Liu, X.Y. et al. Time-dependent effect of 1,6-hexanediol on biomolecular condensates and 3D chromatin organization. Genome Biology 22(2021). PMID: 34404453

9. Light induced promotion of MATCAP LLPS using the Cry2 system is shown to enhance the localization of MATCAP on microtubules. Does this also increase detyrosination levels? A corelative microscopy-based imaging would be informative to understand the localization and function of these chimeric constructs.

We thank the reviewer for this insightful suggestion. We have now included correlative images that illustrate the localization of Cry2-MATCAP under light alongside tubulin detyrosination levels. The Cry2 system-induced oligomerization enhanced the microtubule localization of wild-type MATCAP and MATCAP$^{\Delta IDR}$-EB1^{IDR} (where MATCAP IDR was replaced by EB1 IDR), but did not enhance the localization of MATCAP$^{\Delta IDR}$ and MATCAP$^{\Delta IDR}$-EB1^{IDR}-KR6Q (where MATCAP IDR was replaced by EB1 KR6Q IDR, which lacks the multivalent interactions). Consistently, the tubulin detyrosination was also enhanced in Cry2-MATCAP and Cry2-MATCAP$^{\Delta IDR}$-EB1^{IDR} under light, while no increase was observed in Cry2-MATCAP$^{\Delta IDR}$ and Cry2-MATCAP$^{\Delta IDR}$-EB1^{IDR}-KR6Q under the same conditions.

Thus, the tubulin detyrosination level increased when MATCAP LLPS was promoted by the Cry2 system. We have included the new data in the revised manuscript as Extended Data Fig 7h and i.



Extended Data Fig 7h-i. Condensation of MATCAP on microtubule promotes detyrosination

h, Representative immunofluorescence images of COS-7 cells expressing the indicated optoMATCAP constructs under blue light off and blue light activation conditions. Cells were immunostained with antibodies against detyrosinated and total α-tubulin. DNA was counterstained with DAPI. Scale bar, 10 μm.

i, Quantification of detyrosinated areas of microtubules in COS-7 cells under blue light off and blue light activation conditions as in **h**. For each group, n = 10 cells, pooled from three independent experiments. Data represent mean ± s.e.m., Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates $P > 0.05$, ****$P < 0.0001$.

10. The N-terminal IDR of MATCAP was shown to act as a microtubule-binding domain (PMID: 35482892), thus the MATCAP$^{\Delta IDR}$ constructs are not expected to localize to the microtubules nor to be able to detyrosinate them and rescue the mitotic phenotype. The results relying on chimeric constructs of MATCAP with MATCAP IDR swapped with other domains are interesting but raise several key concerns. While the authors interpret these data as that the IDR-mediated LLPS is critical for MATCAP's activity, the results could also be interpreted as that the IDR domain simply mediates the interaction with microtubules and thereby affects its localization and function independent of the proposed LLPS. HEC1-CH and EB1-CH domains bind specifically to microtubule tips, while KIF5B motor walks along the microtubule. Thus, the chimeras containing these three domains bind differently to microtubules compared to the IDR-based microtubule-binding domains and cannot be expected to rescue the MATCAP activity in a similar way. The constructs that rescue MATCAP activity contain positively charged IDRs that are known to bind microtubules (LEM2, Tau and EB1 IDRs). The loss of localization and function of both MATCAP$^{\Delta IDR}$-EB1-KR6Q IDR and MATCAP-8A mutants could be attributed to the disruption of the positively charged residues, thereby interfering with microtubule binding independent of LLPS. Thus, all effects of MATCAP IDR truncations and mutants could be interpreted by their inability to bind and detyrosinate microtubules, thereby affecting CENP-E activity and its role in chromosome congression during mitosis.

We agree with the reviewer that the IDR replacement could also be alternatively interpreted as a loss of specific microtubule lattice binding activity rather than purely a loss of LLPS activity. To further investigate the proposal, we have spent extensive efforts to conducted additional experiments to separate these two potential functions of the IDR.

Analysis of the cryo-EM structure of the MATCAP^{E281Q} mutant bound to microtubules published by Brummelkamp's lab, revealed a well-defined interface involving only the carboxypeptidase domain, with no discernible density for the IDR, suggesting that IDR does not strongly bind to microtubules (PMID: 35482892). To further validate this, we assessed the microtubule binding activity of MATCAP IDR both in cells and in vitro. Immunofluorescence and TIRF microscopy showed no detectable binding between the IDR and microtubules (Extended Data Fig 2d, Extended Data Fig 5e). Obviously, we recognize that these data do not exclude the possibility that the IDR facilitates MATCAP's microtubule binding through weak or transient interactions.

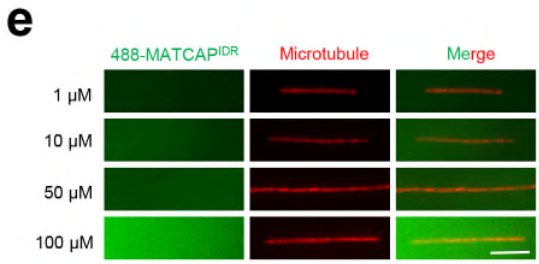
We agree with the reviewer that our previous IDR mutation analyses cannot definitively distinguish between disrupted phase separation and impaired microtubule binding, as these

functions may be intertwined. Given that hexanediol disrupts the LLPS of MATCAP, we reasoned that hydrophobic interactions contribute to MATCAP's LLPS. We think hydrophobic interactions may be less important for microtubule binding and thus tried to mutate these residues. Through bioinformatic analysis, we identified four clusters of hydrophobic residues within the IDR. We mutated these hydrophobic amino acids to Alanine and generated a series of Patch mutants. The mutants displayed various decreases (10%-35%) in microtubule binding; however, quantification of the detyrosinated tubulin revealed that the decreases in detyrosination activity are proportionate to the reductions in microtubule binding activity for each mutant (see below). These observations indicate that we could not separate the microtubule-binding activity from LLPS.

Consequently, we sought more appropriate IDRs for IDR replacement to distinguish between microtubule binding activity and the LLPS activity of MATCAP. We acknowledge the reviewer's concern that the domains (HEC1-CH, EB1-CH, KIF5B) used in the last submission which engage microtubule binding in distinct manners—tip-tracking or motor activity—which may not mimic the native IDR-mediated binding of MATCAP. Thus, it is not surprising that those chimers could not rescue the MATCAP activity in the same way like wild type MATCAP.

To address this issue, we tested alternative microtubule-binding modules, including the microtubule-binding domain (MTBD) of MAP7 (87–139 aa; PMID: 35050657) and the calponin homology (CH) domain of Plasmodium EB1 (PyEB1), which loses plus-end tracking but retains lattice binding affinity (PMID: 37208365), to better mimic the microtubule-binding characteristics of MATCAP IDR. The CH domain of PyEB1 and the minimal MTBD of MAP7 were selected because they are well-structured and are not expected to contribute to phase separation, but have been validated to functionally interact with microtubules lattice. Additionally, we used the IDR of FUS, which undergoes LLPS but does not contribute to microtubule binding, as a control. Our data reveal that MATCAP$^{\Delta IDR}$-PyEB1^{CH} and MATCAP$^{\Delta IDR}$-MAP7^{MTBD} associate with microtubules similar to MATCAP WT, but do not exhibit any phase separation ability, and hence failed to rescue the microtubule detyrosination activity (Extended Data Fig. 6a-d), suggesting that both phase separation and microtubule engagement are critical for MATCAP's detyrosination activity.

Taken together with our previous data that only chimeras containing IDRs from LEM2, Tau, or EB1—which confer both LLPS and weak microtubule binding—successfully rescued the detyrosination activity of MATCAP, we reason that LLPS is critical for MATCAP's detyrosination activity. Although our data support this working model, we agree that alternative interpretations are still possible, we have now elaborated the current limitation in the revised Discussion section.



Extended Data Fig. 5e. The IDR domain of MATCAP does not exhibit binding to microtubules
TIRF images of Alexa Fluor 488-labeled MATCAP^{IDR} at various concentrations with microtubules. Scale bars, 5 μm.

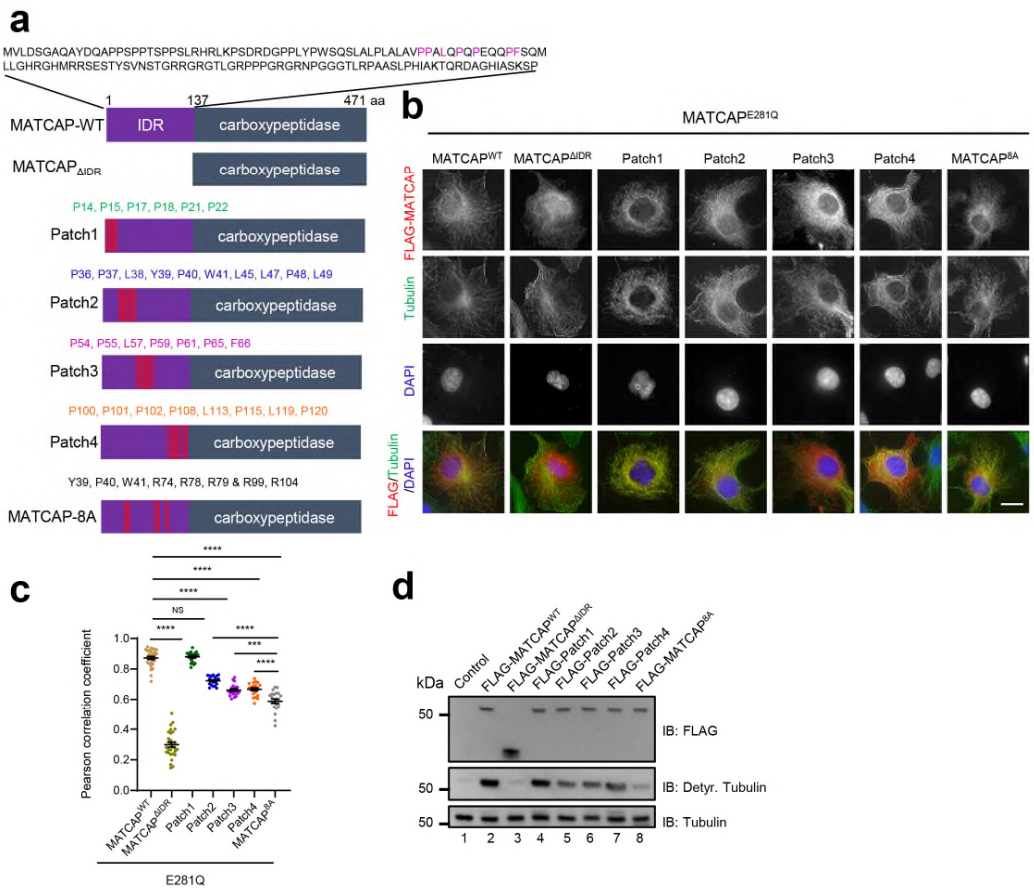


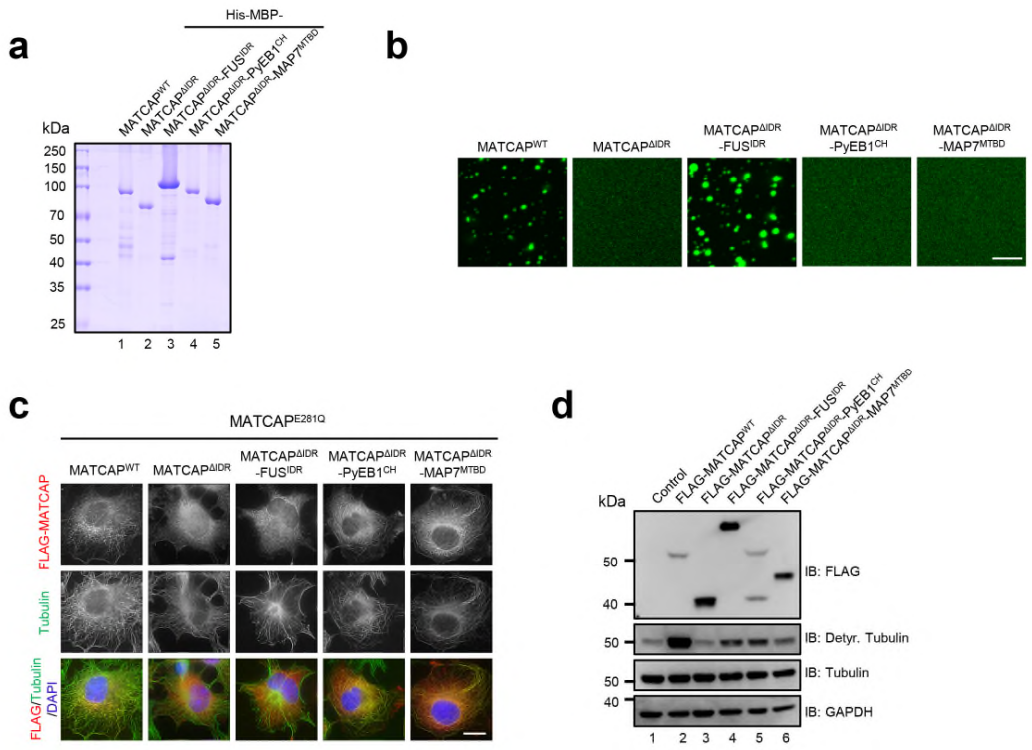
Fig. x. Mutagenesis of hydrophobic patches within the IDR revealed that these regions are essential for function

a, Schematic annotation for MATCAP-WT and the clusters of hydrophobic residues within the IDR. The clusters of hydrophobic residues within the IDR were mutated to Alanine residues.

b, Immunofluorescence images of COS-7 cells expressing engineered FLAG-MATCAP^{E281Q} chimeric variants. DNA was stained with DAPI, FLAG-MATCAP was stained with an anti-FLAG antibody and microtubules were stained with α-tubulin antibody. Scale bar, 10 µm.

c, Quantification of the colocalization of MATCAP mutants and microtubules from immunofluorescent stainings in **e.** For MATCAP, n = 29; MATCAP$^{\Delta IDR}$, n = 29; Patch1, n = 22; Patch2, n = 20; Patch3, n = 20; Patch4, n = 20; and MATCAP^{8A}, n = 22; n represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance. NS (not significant) indicates $P > 0.05$, ****$P < 0.0001$.

d, Effect of engineered MATCAP chimeric variants overexpression on detyrosinated tubulin levels. Immunoblot of cell lysates from HEK293T cells transfected with plasmids expressing engineered FLAG-MATCAP variants. The expression of FLAG-MATCAP was observed using anti-FLAG antibodies. The levels of detyrosinated and total α-tubulin were assessed using the respective antibodies.



Extended Data Fig. 6. Phase separation and microtubule engagement synergistically promote the detyrosination activity of MATCAP

a, Coomassie staining of engineered His-MBP-MATCAP chimeric proteins (MATCAP, MATCAP$^{\Delta IDR}$, MATCAP$^{\Delta IDR}$-FUS^{IDR}, MATCAP$^{\Delta IDR}$-PyEB1^{CH}, or MATCAP$^{\Delta IDR}$-MAP7^{MTBD}) used in vitro phase separation assay.

b, *In vitro* phase separation of 5 μM Alexa Fluor 488-labeled His-MBP-MATCAP variants (MATCAP, MATCAP$^{\Delta IDR}$, MATCAP$^{\Delta IDR}$-FUS^{IDR}, MATCAP$^{\Delta IDR}$-PyEB1^{CH}, or MATCAP$^{\Delta IDR}$-MAP7^{MTBD}) upon PreScission cleavage of His-MBP tag. Scale bars, 10 μm.

c, Immunofluorescence images of COS-7 cells expressing engineered FLAG-MATCAP^{E281Q} chimeric variants (MATCAP, MATCAP$^{\Delta IDR}$, MATCAP$^{\Delta IDR}$-FUS^{IDR}, MATCAP$^{\Delta IDR}$-PyEB1^{CH}, or MATCAP$^{\Delta IDR}$-MAP7^{MTBD}). DNA was stained with DAPI, FLAG-MATCAP was stained with an anti-FLAG antibody and microtubules were stained with α-tubulin antibody. Scale bar, 10 μm.

d, Effect of engineered MATCAP chimeric variants overexpression on detyrosinated tubulin levels. Immunoblot of cell lysates from HEK293T cells transfected with plasmids expressing engineered FLAG-MATCAP chimeric variants (MATCAP, MATCAP$^{\Delta IDR}$, MATCAP$^{\Delta IDR}$-FUS^{IDR}, MATCAP$^{\Delta IDR}$-PyEB1^{CH}, or MATCAP$^{\Delta IDR}$-MAP7^{MTBD}). The expression of FLAG-MATCAP was observed using anti-FLAG antibodies. The levels of detyrosinated and total α-tubulin were assessed using the respective antibodies, with GAPDH serving as the loading control.

Minor points:

1. Line 40: MCAK is a kinesin-13. If describing two different kinesin 13 family members, both should be named.

We apologize for the lack of clarity in our original description, and have now replaced “kinesin-13” with “MCAK” in the revised manuscript.

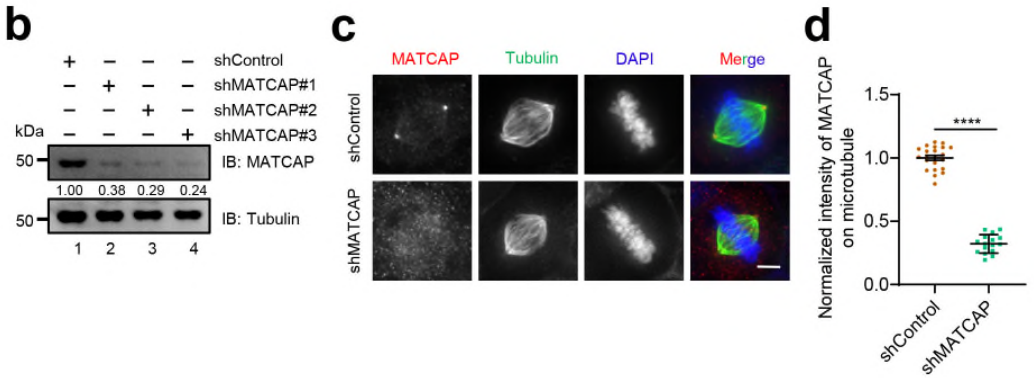
2. Line 49-50: As most α-tubulin isotypes are genetically encoded with a tyrosine residue at their C-terminal tails, the act of removing the tyrosine by TCPs (VASHs and MATCAP) happens first and hence could be interpreted as writing the code, while adding the tyrosine back by TTL

could be considered as erasing.

We agree with the reviewer and recognize that the interpretation of "writer" and "eraser" is context dependent. To avoid any potential confusion, we have removed this metaphorical comparison from the revised manuscript.

3. Quantitative validation of MATCAP knockdown via western blot and immunofluorescence is missing. Inclusion of these data is essential to confirm the efficiency and specificity of the shRNA-mediated depletion.

In response, we have now included the quantitative validation of MATCAP knockdown using both Western blot and immunofluorescence analyses. Western blot analysis revealed that the three tested MATCAP shRNAs all exhibited efficient depletion. Since shMATCAP #2 targets the 5′ UTR region of the mRNA, it facilitates the following rescue experiments. Therefore, this shRNA was used for all functional experiments in cells. Immunofluorescence demonstrated that MATCAP was robustly detected on the spindle and spindle poles in control cells, while these signals were largely undetectable in shRNA #2-positive transfected cells, indicating high specificity of the antibody in immunofluorescence and confirming the high knockdown efficiency of the MATCAP shRNA. Quantification of MATCAP fluorescence intensity revealed an approximately 70% reduction in the shRNA-positive transfected cells. We have now included these new data in the revised manuscript as Extended Data Fig. 1b-d.



Extended Data Fig. 1b-d. Endogenous MATCAP localization and shRNA knockdown efficiency
b, Knockdown efficiency of shMATCAP. Lysates of cells expressing control or MATCAP shRNA were probed with MATCAP and Tubulin antibodies.
c, Immunofluorescence staining of MATCAP and tubulin in metaphase HeLa cells expressing control or MATCAP shRNA. Cells were fixed with methanol, and stained with MATCAP serum antibody and α-tubulin antibody. DNA was stained with DAPI. Scale bars, 5 μm.
d, Quantification of shRNA knockdown efficiency in **c**. For Control, n = 22, shMATCAP, n = 16; n represents the number of cells pooled from three independent experiments. Data represent mean ± s.e.m. Statistical significance was assessed by two-sided unpaired Student's t-test. ****$P < 0.0001$.

4. Four shRNAs are listed in the Methods, but only three are shown in Extended Data Fig. 1d. It is unclear which shRNA was used predominantly in functional assays. This should be clarified.

We thank the reviewer for pointing out this oversight and apologize for any confusion caused by the discrepancy in the previous version of the manuscript.

We had initially generated four shRNAs; however, the fourth shRNA was excluded from subsequent experiments, including the knockdown efficiency testing as shown in Extended Data Fig. 1b, as it failed to efficiently knock down MATCAP. Among the three effective shRNAs, the second shRNA (shMATCAP#2) targets the 5′ UTR of the mRNA, facilitating the following rescue experiments. Therefore, it was used for all functional assays.

We have now removed the sequence of the fourth shRNA from the revised Methods section

and clearly indicated in the legend of Extended Data Fig. 1b that the second shRNA was used for all the subsequent functional assays, to avoid any potential confusion.

5. Lines 93–94: shVASH1/VASH2 knockdown resulting in ~40% reduction in CENP-E localization is termed "slight attenuation" while ~60% reduction from shMATCAP is termed "abolished".

We have rephrased the text to reflect the knockdown data more precisely. The revised text now reads: "VASH1/2 depletion caused an appropriate 40% reduction of CENP-E on the spindle, whereas MATCAP knockdown produced a much more pronounced decrease (~ 60%)." We hope this revision provides a clearer and more accurate description of the data.

6. Videos of the time-lapse imaging of mitosis should be provided in addition to the still images.

We thank the reviewer for the suggestion. We have provided the time-lapse videos of mitotic progression as supplementary materials.

7. Line 109: Typographical error (extra period).

We thank the reviewer for catching this typographical error. We have removed the extra period in the revised manuscript.

8. Lines 117-119: The colocalization of MATCAP and CENP-E is only partial, and the interpretation should reflect the same.

We thank the reviewer for this important clarification. We have now rephrased the description as: "During mitosis, in addition to kinetochore localization, CENP-E also co-localizes with MATCAP at centrosomes and spindle microtubules from prophase through metaphase (Fig. 2a), consistent with a potential recruitment mechanism." to reflect our observation more accurately.

9. In Figure 2e, VASH proteins should be co-transfected with SVBP to ensure proper complex formation and enzymatic activity during co-immunoprecipitation.

We thank the reviewer for raising this important point. In our revised experiments, we co-transfected VASH proteins with SVBP to ensure proper complex formation and enzymatic activity during the co-immunoprecipitation assays. This new data does not alter our conclusions. We have included the new data in the revised manuscript as (Fig. 2c).

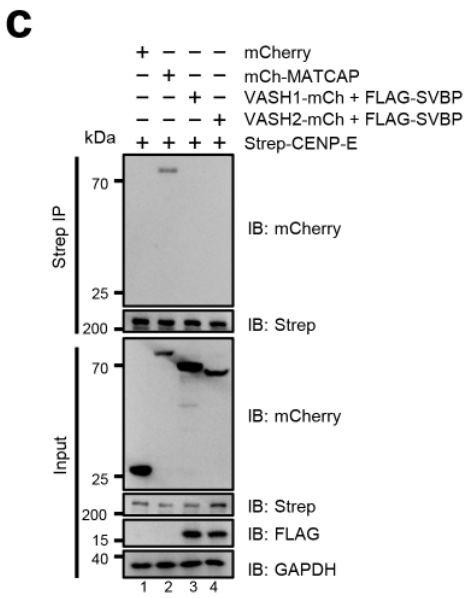


Fig. 2c. Strep-CENP-E co-precipitated mCherry-MATCAP, but not VASH1/2
mCherry, mCherry-MATCAP, VASH1-mCherry + FLAG-SVBP or mCherry-VASH2-mCherry + FLAG-SVBP were co-transfected with Strep-CENP-E in HEK293T cells, and their interactions were tested by co-immunoprecipitation.

10. Details on the GFP-CENP-E construct used in Fig2f-i is missing in the Methods section.

We thank the reviewer for this suggestion. We have included a detailed description of the GFP-CENP-E construct in the Methods section of the revised manuscript. The text has been revised to: To generate Strep-CENP-E$^{1-483}$-GFP, CENP-E$^{1-483}$-GFP was amplified and inserted into pTT5-Strep vector with AgeI and XbaI digestion.

11. Details about MATCAP-RDI is missing in the Methods section.

We thank the reviewer for this suggestion. We have included a detailed description of the MATCAP-RDI construct in the Methods section of the revised manuscript. The text has been revised to: For MATCAP IDR reversed plasmids, a mutant plasmid encoding the N-terminal domain (residues 1-137) in reverse order (137-1) was generated by gene synthesis. The synthesized DNA fragment, was subsequently cloned into the corresponding backbone vector.

12. The significance of co-condensation of MATCAP with soluble tubulin is unclear. Given MATCAP's preference for polymerized microtubules for its activity, what would its interaction with soluble tubulin dimer reflect in a physiological context?

We thank the reviewer for raising this important point regarding the recruitment of tubulin dimers to MATCAP condensates. Briefly, while MATCAP demonstrates high detyrosination efficiency on polymerized tubulin, it also retains significant activity toward tubulin dimers. The co-condensation of MATCAP with tubulin dimers likely facilitates its detyrosination of these tubulin dimers. We reason that this property is particularly important during mitosis, when microtubules are highly dynamic. The rapid polymerization and depolymerization cycles during this process may limit the window of opportunity for MATCAP to modify stable microtubules effectively. We have now elaborated this in the revised discussion session (Page 10, Lines 255-261).

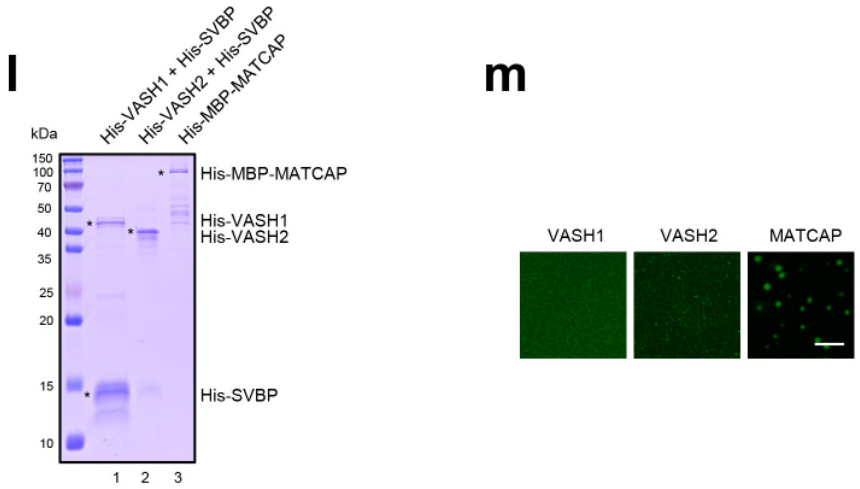
We kindly invite the reviewer to examine our detailed response to the first comment of the reviewer #1.

13. Extended Data Fig. 3l shows highly impure VASH preparations with low SVBP ratio, which may compromise the interpretation of LLPS assays. Improved purification and quantification are recommended.

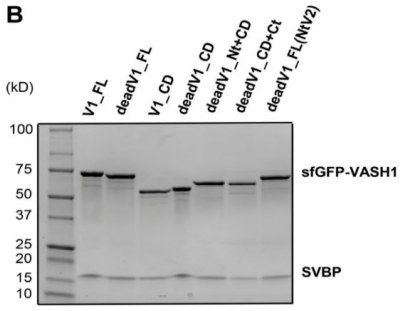
We thank the reviewer for raising this valid concern regarding the purity of the VASH preparations. In response, we have optimized our purification protocol by incorporating additional steps for purifying the VASH-SVBP complex, including size-exclusion chromatography and ion-exchange, which significantly improved the purity of the complex (Extended Data Fig. 4l). However, Coomassie blue staining still indicates a dimmer band for SVBP relative to VASH. This observation aligns with previous publications, such as those by Ramirez-Rios et al., which also reported a dimmer Coomassie blue staining band for SVBP compared to VASH in the purified complex. We speculate that this may be attributed to the chemical property of SVBP, which is less stable and more prone to degradation during purification.

Using the newly purified VASH-SVBP complex, we have further carried out the TIRFM and LLPS assays. The newly purified recombinant SVBP-VASH1 proteins still failed to form condensates in vitro, while SVBP-VASH2 exhibited weak LLPS properties as described in the last submission. Although we cannot completely rule out the potential impact of complex quality on LLPS, we believe that our evidence regarding the LLPS properties of the VASH-SVBP complex remains strong. We have updated the new purification protocol in the Methods section and included representative images in the revised manuscript as Extended Data Fig. 4l, m.

Therefore, we used these newly purified VASH-SVBP complex in our TIRFM and LLPS assays. LLPS assays exhibited that recombinant SVBP-VASH1 proteins failed to form condensate *in vitro*, whereas SVBP-VASH2 exhibited weak liquid-liquid phase separation (LLPS) propensity. We have updated the Methods section accordingly and included a representative gel and image in the revised manuscript to document the improved purification.



Extended Data Fig. 4l-m. Purified chimeric proteins and *in vitro* phase separation.
l, Coomassie staining of engineered His-VASH1/2+His-SVBP chimeric proteins and His-MBP-MATCAP.
m, Representative images of *in vitro* phase separation of Alexa Fluor 488-labeled His-MBP-MATCAP, His-VASH1/2+His-SVBP (50 μM) after PreScission cleavage. Scale bars, 10 μm.



Ramirez-Rios *et al*., 2022: SDS-PAGE of a complete set of VASH1–SVBP protein preparations.

Reference:
Ramirez-Rios, S. et al. VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules. *Journal of Cell Biology* **222**(2022).

14. The rescue experiments in Fig4e, f should be accompanied with corresponding rescue of detyrosination levels.

In response, we have now included Western blot analysis showing the microtubule detyrosination levels in the rescue experiments presented in Fig. 4e. The results confirm that the rescue of chromosome congression by MATCAP WT and MATCAP$^{\Delta IDR}$-EB1$_{IDR}$ was accompanied by a restoration of microtubule detyrosination levels (Fig. 4f). As predicted, phase separation-deficient MATCAP$^{\Delta IDR}$-EB1^{IDR}-KR6Q failed to rescue chromosome congression and coincided with defects in microtubule detyrosination. We have included the Western blot data in the revised manuscript to project the correlation of MATCAP phase separation to its function in chromosome segregation

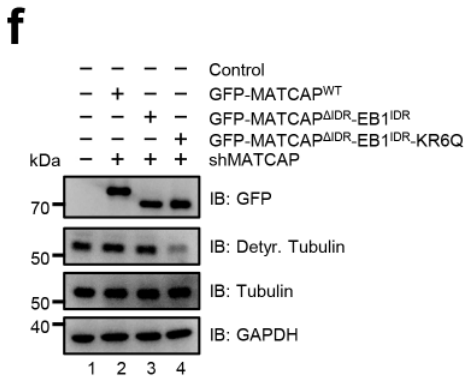


Fig. 4f. Restoration of microtubule detyrosination levels by various MATCAP chimeric variants
Characterization of detyrosinated tubulin expression levels of GFP-MATCAP (MATCAP, MATCAP$^{\Delta IDR}$-EB1^{IDR}, MATCAP$^{\Delta IDR}$-EB1^{IDR}-KR6Q) in endogenous MATCAP-depleted cells.

15. Fig. 6c results should be quantified.

In response, we have now quantified the results as revised Fig. 6d and added this new data to the revised manuscript. We would like to invite the reviewer to examine our response to the fourth critiques from the reviewer #1.

16. It is unclear why the CENP-E motor domain fails to bind microtubules independently in Fig. 6f–g. This should be addressed.

The failure of the CENP-E motor to bind to microtubules by itself in our initial experiments

was a true reflection of its ATPase activity (Fig. 6f-g). Given that CENP-E binds to and moves along microtubules in an ATP-dependent manner, we had omitted these factors in our initial assays to specifically evaluate MATCAP's role in recruiting CENP-E to microtubules in the absence of an ATP-dependent microtubule-binding activity of CENP-E.

In response to the reviewer's comment, we have now added the experimental outcome in the presence of ATP and Mg^{2+}. The new TIRF microscopy data show that the CENP-E motor domain alone exhibits microtubule binding and velocity along microtubules (MATCAP$^{\Delta IDR}$ and MATCAP^{8A}), while wild-type MATCAP robustly promotes both recruitment and processive motility of CENP-E on microtubules (Fig. 6g-i). Thus, the newly added evidence further supports our conclusion that MATCAP facilitates CENP-E recruitment and motility on microtubules, which reinforces our previous findings.

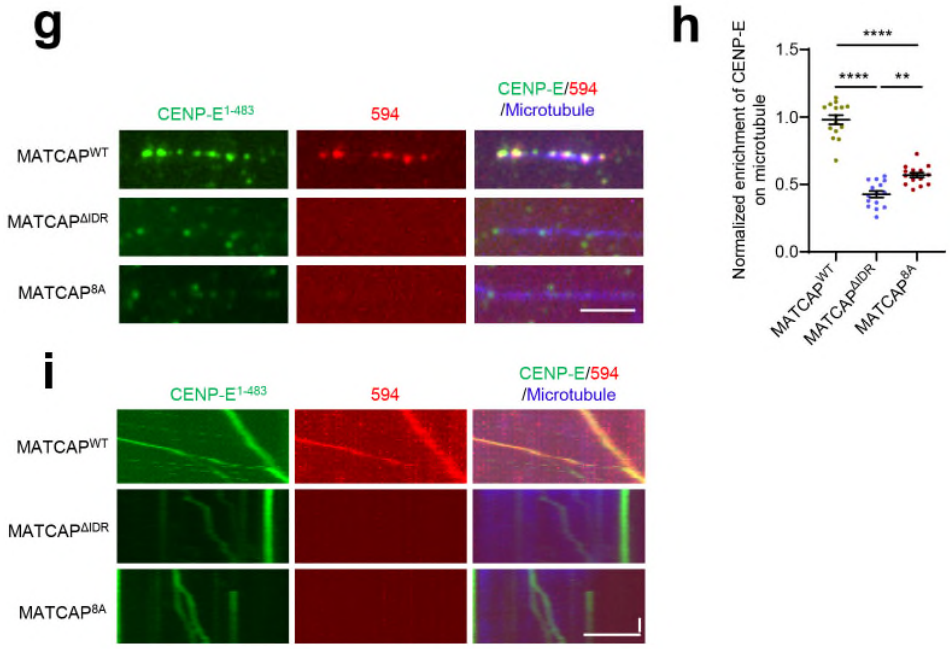


Fig. 6g-i. *In vitro* microtubule binding and motility of CENP-E motor in the presence of various MATCAP mutants

g. TIRF images of Alexa Fluor 594-MATCAP (10 nM; red) and GFP-CENP-E$^{1-483}$ (100 nM; green) localization to a GMPCPP-stabilized Cy5-labeled microtubule seed (blue). Scale bars, 2 μm.

h. Quantification of CENP-E fluorescence intensity, normalized to microtubule signal as shown in **g**. For each group, n = 15 microtubules, pooled from three independent experiments. Data represent mean ± s.e.m., Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS (not significant) indicates $P > 0.05$, ****$P < 0.0001$. **$P = 0.0012$.

i. Fluorescence kymograph showing CENP-E$^{1-483}$ motion along microtubule lattice in the presence of indicated proteins. Horizontal scale bar, 2 μm; vertical scale bar, 10 s.

17. Line 320: Typographical error ("dimension").

We apologize for this oversight and thank the reviewer for pointing out this error. We have corrected this typo in the revised manuscript.

18. Line 404: "…is essential in control of in mitotic spindle plasticity control..." should be rewritten.

Thanks for your careful checks. We are sorry for our carelessness. we have revised the sentence to: "LLPS-driven compartmentalization is increasingly recognized as essential for controlling mitotic spindle plasticity during cell division."

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for coaching the review process and for all the constructive suggestions provided, which have significantly improved our manuscript. We hope that our revision has properly addressed all the concerns and satisfies the reviewer.