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

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Abstract

Accurate segregation of mitotic chromosomes requires pre-anaphase alignment driven by microtubule-based motor proteins. Tubulin detyrosination is essential to guide CENP-E-driven chromosome motility in mitosis. However, the mechanisms of action and physiochemical properties of the detyrosinases for decoding CENP-E motility remain elusive. Here we show that microtubule-associated tyrosine carboxypeptidase (MATCAP) forms molecular condensates on microtubules through liquid-liquid phase separation (LLPS) to constitute the tubulin detyrosination machinery. MATCAP undergoes IDR-dependent phase separation to form biomolecular condensates that enrich tubulin and CENP-E, thereby stabilizing kinetochore-microtubule attachments. Real-time imaging of cells expressing LLPS-deficient MATCAP mutants reveals the importance of MATCAP LLPS dynamics in mitotic chromosome alignment. Mechanistically, phase separation of MATCAP spatiotemporally consolidates tubulin decoding with CENP-E motility to ensure a robust metaphase alignment during mitosis. These findings delineate a signaling cascade that integrates phase separation and tubulin detyrosination with mitotic progression for maintenance of genomic stability.

KEY WORDS

Mitosis, biomolecular condensate, microtubule detyrosination, MATCAP, CENP-E

Introduction

Microtubules (MTs) are essential components of the cytoskeleton composed of polymerized α - and β -tubulin. They participate in a wide range of cellular events, including cell motility¹, cargo transport, cellular organization, and cell division²⁻⁴. Human cells express multiple tubulin isotypes and many post-translational modifications (PTMs) have been found to occur on tubulin, including acetylation, detyrosination/tyrosination, glycylation, and polyglutamylation, etc. A variety of differential expression of α -tubulin and β -tubulin isotypes, together with their post-translational modifications, constitute the known “tubulin code”, which accounts for some of the diverse cellular functions of microtubules⁵. A prominent tubulin PTM is the reversible removal and readdition of the C-terminal tyrosine residue of α -tubulin; this evolutionarily conserved cycle is termed the “detyrosination/tyrosination cycle”⁶.

The detyrosination/tyrosination cycle regulates microtubule-associated proteins (MAPs), motors—including kinesins (Kif5B, CENP-E, MCAK)⁷⁻¹¹ and CAP-Gly domain proteins (CLIP170, dynein-dynactin’s p150^{Glued})¹². This dynamic modification cycle has been implicated in the development and function of the brain¹³⁻¹⁶ and in the skeletal muscle contraction of the heart¹⁷⁻²⁰. α -tubulin detyrosination stabilizes kinetochore-microtubule attachments and establishes spatial gradients to guide chromosome alignment by enhancing CENP-E motor activity while suppressing MCAK-mediated depolymerization^{9, 21}. This modification fine-tunes microtubule dynamics and attachment plasticity—excessive detyrosination impairs error correction, whereas its deficiency disrupts spindle integrity and delays mitotic progression^{22, 23}. However, how the spatial gradient of detyrosination is established in the spindle remains elusive.

The detyrosination/tyrosination cycle involves TTL, the VASH1/VASH2-SVBP complexes^{13, 24}, and MATCAP (also described as TMCP1^{25, 26}). VASH1 and VASH2, which share >50% sequence identity, belong to the transglutaminase-like cysteine protease superfamily and contain a conserved catalytic Cys–His–Leu triad; their crystal structures reveal a globular catalytic domain that binds SVBP^{22, 27-31}. In contrast, the catalytic domain of MATCAP belongs to the gluzincin metallopeptidase clan, which is characterized by a Zinc ion (Zn^{2+}), bearing a degenerate version of the family signature motif (HExxxH instead of HExxH)³². Despite these structural insights, the regulatory mechanisms controlling the “detyrosination/tyrosination cycle” remain largely unknown.

MATCAP contains a positively charged N-terminal intrinsically disordered region (IDR) that

lacks structural information. The enzymatic mechanism underlying MATCAP-mediated α -tubulin detyrosination remains incompletely characterized due to the low identity of its N-terminal sequence. Progressive truncation of MATCAP IDR reduces the protein's localization to microtubules in cells²⁵. That evokes the question of how the IDR contributes to the function of MATCAP.

IDRs have been implicated in driving biomolecular condensation and compartmentalization of chemical reactions in cells³³⁻³⁵. Here we show that MATCAP undergoes phase separation on microtubules via its N-terminal IDR to facilitate tubulin detyrosination. MATCAP condensates on microtubules enrich tubulin and CENP-E and LLPS-deficient MATCAP mutation disrupts local enrichment of CENP-E and tubulin detyrosination, leading to chromosome congression defects in mitosis. These results reveal a molecular mechanism by which MATCAP LLPS-dependent recruitment and detyrosination regulate chromosome congression.

Results

MATCAP-elicited α -tubulin detyrosination enables CENP-E function during mitosis

During mitosis, the α -tubulin detyrosination code has been proposed to function as a navigation system that coordinates bipolar chromosome congression by regulating the kinetochore motor CENP-E activities⁹. However, the properties of detyrosinases contributing to the precise regulation of CENP-E remain unclear. To investigate how detyrosinases regulate CENP-E, we first examined the subcellular distribution of detyrosinases during mitosis. Overexpression of VASH1-SVBP, VASH2-SVBP or MATCAP increased α -tubulin detyrosination on metaphase spindles; strikingly, their subcellular localizations diverged: VASH1-SVBP and VASH2-SVBP were diffusely cytoplasmic, whereas MATCAP was enriched on spindle microtubules (**Fig. 1a**). To determine the localization of endogenous MATCAP during mitosis, we generated MATCAP-specific mouse antibodies and conducted immunofluorescence. MATCAP localized to spindle microtubules and centrosomes during mitosis (**Supplementary Fig. 1a**). shRNA-mediated knockdown of MATCAP reduced the corresponding bands in Western blots and decreased the spindle localization in immunofluorescence, confirming the specificity of shRNAs and antibodies (**Supplementary Fig. 1b-d**). In addition, MATCAP co-localized with detyrosinated spindle microtubules, and its depletion reduced microtubule detyrosination (**Supplementary Fig. 1e-g**). Thus, we conclude that MATCAP is a bona fide spindle microtubule-binding detyrosinase, which is consistent with findings from prior research^{25, 26}.

To determine whether α -tubulin detyrosination modulates CENP-E activity, we first analyzed CENP-E localization after depletion of VASH1 and VASH2 (hereafter VASH1/2), MATCAP, or both. VASH1/2 depletion caused an appropriate 40% reduction of CENP-E on the spindle, whereas MATCAP knockdown produced a much more pronounced decrease (~ 60%). Notably, combined knockdown of VASH1/2 and MATCAP did not yield an additive effect (**Fig. 1b, c**), suggesting that CENP-E recruitment relies on a threshold level of microtubule detyrosination, beyond which further reduction does not yield additional defects.

We next assessed the functional impact of detyrosination on mitosis by time-lapse imaging of chromosome dynamics. U2OS cells depleted of MATCAP or VASH1/2 exhibited delayed mitotic progression and chromosome alignment defects (**Fig. 1d-g**). Although both knockdowns caused delays, MATCAP depletion more frequently led to metaphase arrest with persistent chromosome misalignment, whereas VASH1/2-depleted cells often eventually progressed through mitosis. The

phenotype of double-knockdown cells was no more severe than that of MATCAP single-knockdown cells (**Fig. 1d-g & Supplementary Movie 1-4**), further supporting a dominant role for MATCAP-mediated microtubule detyrosination in chromosome congression and CENP-E recruitment. Notably, the difference in chromosome congression defects between MATCAP and VASH1/2 depletion was greater than the difference in overall detyrosinated tubulin levels (**Fig. 1e-g**), suggesting that MATCAP may facilitate chromosome congression by additional, as-yet-uncharacterized mechanisms.

We next asked whether the mitotic defects in MATCAP-depleted cells reflect aberrant kinetochore-microtubule attachments. We used the Astrin-SKAP complex as a marker of aligned, bi-oriented kinetochores^{36, 37}. Immunofluorescence revealed that depletion of either MATCAP or VASH1/2 significantly increased the fraction of metaphase cells with one or more Astrin-negative kinetochores (**Fig. 1h, i**). Given that α -tubulin detyrosination fine-tunes kinetochore-microtubule attachments²¹, these findings indicate that the mitotic defects after MATCAP or VASH1/2-depletion are at least partially due to impaired kinetochore-microtubule attachments.

MATCAP interacts with CENP-E via its N-terminal IDR

Because MATCAP localizes to spindle microtubules and its depletion causes a larger reduction of spindle-associated CENP-E than VASH1/2, we hypothesized that MATCAP facilitates CENP-E spindle localization via a direct interaction. 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. Immunoprecipitation of endogenous CENP-E from mitotic cells followed by immunoblotting confirmed association with MATCAP (**Fig. 2b**). To test specificity, mCherry-MATCAP, VASH1, or VASH2 were co-expressed with Strep-tagged CENP-E in HEK293T cells and subjected to Streptactin affinity isolation. Strep-CENP-E specifically co-purified mCherry-MATCAP but not VASH1 and VASH2 (**Fig. 2c**). Streptavidin pull-downs with purified proteins further confirmed a direct interaction between MATCAP and CENP-E and showed that this interaction depends on the N-terminal intrinsically disordered region (IDR) of MATCAP (**Fig. 2d-f**). Domain-mapping pull-downs indicated that MATCAP binds predominantly to the CENP-E motor domain (CENP-E¹⁻⁴⁸³), with only weak interaction with its C-terminal region (**Fig. 2e, g**). Together, these data reveal a physiological interaction mediated by the CENP-E motor domain and the MATCAP N-terminal

IDR.

The N-terminal IDR of MATCAP has been implicated in microtubule association²⁵. However, Cryo-EM structural determination of the MATCAP-microtubule complex revealed a defined interface involving only the carboxypeptidase domain bound to the C-terminal tail of α -tubulin, with no discernible density for the IDR²⁵, suggesting that the IDR does not strongly bind to microtubules. To determine the role of IDR, we generated truncation mutants. Immunofluorescence demonstrated that wild-type MATCAP associated with spindle microtubules, whereas the truncation mutant lacking IDR (MATCAP ^{Δ IDR}; Δ aa1–137) failed to localize to the spindle microtubules and exhibited a cytoplasmic diffuse distribution, despite their comparable expression levels in cells (**Supplementary Fig. 2a-c**). Moreover, the IDR alone (MATCAP^{IDR}) did not localize to spindle microtubules (**Supplementary Fig. 2d**). These results indicate that both the carboxypeptidase domain and the IDR are necessary but individually insufficient for microtubule targeting.

Consistently, MATCAP lacking IDR exhibited significantly reduced tubulin detyrosination activity in cells compared with wild-type GFP-MATCAP (**Supplementary Fig. 2e-g**), indicating that the IDR is essential for both the subcellular localization and the catalytic function. By contrast, deletion of disordered regions in VASH1 and VASH2 did not affect their detyrosination activity at all (**Supplementary Fig. 2h, i**), suggesting that the IDR-mediated regulation of MATCAP is unique among detyrosinases.

To determine the functional specificity of VASH1/2 and MATCAP in regulating CENP-E activity, we established a reconstituted CENP-E motility assay as described previously³⁸. Briefly, U2OS cells expressing the proteins of interest were permeabilized, gently fixed to preserve microtubule architecture, and then incubated with purified GFP-tagged CENP-E motor domain (GFP-CENP-E¹⁻⁴⁸³). The movement of GFP-CENP-E¹⁻⁴⁸³ toward microtubule plus-ends was then tracked (**Supplementary Fig. 3a**). Lattice-SIM analysis revealed that MATCAP overexpression enhanced both microtubule localization and motility of CENP-E, whereas VASH1/2 overexpression increased CENP-E motility to a similar extent, but did not enhance its recruitment to microtubules (**Supplementary Fig. 3b-e**). Thus, recruitment of CENP-E is a unique property of MATCAP among tubulin detyrosinases. In addition, overexpression of MATCAP ^{Δ IDR} did not alter CENP-E recruitment or motility compared with vector control (**Supplementary Fig. 3b-e**), further strengthening that the IDR is required for MATCAP-mediated CENP-E recruitment and

for microtubule detyrosination that promotes CENP-E movement.

To directly examine how MATCAP regulates CENP-E, we performed in vitro single-molecule motility assays using total internal reflection fluorescence (TIRF) microscopy. Recombinant wild type MATCAP significantly increased CENP-E recruitment to microtubules as co-condensates (**Fig. 2h, i**). Interestingly, this enhancement was independent of MATCAP's enzymatic activity as the catalytically dead mutant (MATCAP^{E281Q}) remained robustly interactive with CENP-E and promoted its recruitment to microtubules in vitro (**Fig. 2h, i & Supplementary Fig. 3f**). This line of evidence indicates that MATCAP's interaction with CENP-E is separated from its enzymatic activity. Beyond recruitment, MATCAP significantly enhanced CENP-E processivity along microtubules (**Fig. 2j-k**). However, catalytically dead mutant MATCAP^{E281Q} attenuated CENP-E dynamics within condensates although MATCAP^{E281Q} promoted the initial recruitment of CENP-E, which suggests that initial CENP-E loading onto microtubules requires MATCAP-condensation independent of detyrosinase activity and further motility of CENP-E on microtubule requires active MATCAP detyrosinase. Consistent with this notion, deletion of the IDR (MATCAP^{ΔIDR}) abolished MATCAP condensation and dissipated CENP-E motility from co-condensation with MATCAP (**Fig. 2h-k**). Compared to MATCAP, VASH1/2 similarly enhanced CENP-E motility via tubulin detyrosination, but did not increase CENP-E recruitment to microtubules (**Fig. 2h-k & Supplementary Fig. 3g**), reinforcing the conclusion from the Lattice-SIM analysis in the fixed cells. Thus, we conclude that both MATCAP condensation and enzymatic activity are essential for CENP-E motility and processivity on microtubules.

MATCAP forms condensates via its N-terminal IDR in cells and in vitro

IDRs have been implicated in the biomolecular condensate formation via liquid–liquid phase separation (LLPS)^{34,39}. To test whether MATCAP forms condensates in cells, we implemented an optogenetic strategy using light-activated optoDroplet technology⁴⁰. Specifically, we engineered an optoMATCAP sensor (Cry2-mCherry-MATCAP) as a tool to visualize condensation in cells. The optoMATCAP conferred tunable, light-induced polymerization of Cry2 and possessed multivalent interactions of MATCAP (**Supplementary Fig. 4a**). We expressed the fusion protein in cells at low levels. Western blotting revealed that the expression of Cry2-mCherry MATCAP was comparable to that of the endogenous MATCAP (**Supplementary Fig. 4b**). Given that the transfection efficiency was appropriate 70%, we estimate the expression of exogenous Cry2-

mCherry MATCAP was within 2-fold of the endogenous MATCAP level, providing a reliable system for assessing the condensation properties of MATCAP near physiological concentration.

Live-cell imaging revealed that, without blue-light activation, Cry2olig-mCherry-MATCAP displayed a mixed distribution, with both microtubule-bound and diffuse cytoplasmic pools. Strikingly, blue-light illumination triggered a rapid redistribution: the diffuse pool significantly decreased while microtubule-associated condensates increased. This relocalization was reversible—the microtubule-associated condensates progressively disassembled after blue light was turned off (**Fig. 3a**). To determine whether microtubule binding was a prerequisite for condensation, we depolymerized microtubules with nocodazole. Strikingly, light-induced formation of discrete cytoplasmic droplets was readily observed (**Supplementary Fig. 4b, c**). These data indicate that MATCAP possesses a previously uncharacterized condensation property. By contrast, the IDR-deleted MATCAP mutant retained diffuse cytoplasmic localization and failed to form condensates, regardless of blue-light on or off (**Fig. 3a & Supplementary Fig. 4c**), indicating that the IDR is necessary for the condensation of MATCAP.

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 effects⁴¹. 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.

To biochemically characterize the condensation capacity of MATCAP, we established an in vitro reconstitution system. Recombinant MATCAP and its mutants were expressed as N-terminal 6xHis-maltose-binding protein (His-MBP) fusions with a PreScission protease cleavage site between the MBP tag and MATCAP (**Fig. 3d**). After purification and Alexa Fluor 488 labeling, the His-MBP tag was removed. In an in vitro phase separation assay, MATCAP underwent spontaneous LLPS to form spherical condensates at room temperature and this condensation was further promoted by the addition of the macromolecular crowding agent PEG5000 (PEG) (**Fig. 3d & Supplementary Fig. 4d**). Time-lapse imaging showed fusion of MATCAP condensates over time (**Fig. 3e**). Fluorescence recovery after photobleaching (FRAP) analysis revealed rapid dynamics within these condensates ($t_{1/2} = 12.5$ s) with a mobile fraction of $\approx 33.5\%$ (**Fig. 3f**).

MATCAP formed micrometre-scale condensates in a concentration-dependent manner: higher protein concentration yielded larger and more numerous condensates (**Supplementary Fig. 4e, f**). The condensates were sensitive to both high salt concentration and 1,6-hexanediol (**Supplementary Fig. 4g, h & Fig. 3g, h**), indicating contributions from both electrostatic and hydrophobic interactions to MATCAP condensation.

To test whether the IDR of MATCAP mediates condensation, we examined three engineered variants: the isolated IDR (MATCAP^{IDR}), the IDR deletion mutant (MATCAP^{ΔIDR}), and an IDR-sequence-reversed mutant (MATCAP^{RDI}). In vitro phase separation assay revealed that both MATCAP^{IDR} and MATCAP^{ΔIDR} required PEG-induced crowding to trigger even marginal condensate formation, failing to phase separate spontaneously on their own (**Fig. 3d**). MATCAP^{RDI} retained in vitro condensation capacity but showed partially reduced spindle microtubule localization in cells, and its tubulin detyrosination activity is slightly reduced compared with wild type (**Fig. 3d & Supplementary Fig. 4i, j**), reflecting the critical role of MATCAP condensation in tubulin detyrosination.

To further characterize the condensation properties of MATCAP, we quantified MATCAP phase behavior by constructing phase diagrams across protein and salt concentrations. Turbidimetric measurements ($\lambda = 600$ nm) of in vitro assemblies provided a robust biophysical readout, with optical density thresholds defining the phase boundary between soluble and condensed states. MATCAP underwent phase separation at ~ 1 nM at physiological salt concentration (**Fig. 3i & Supplementary Fig. 4k**), well below the cellular concentration of ~ 30 nM as measured by OpenCell⁴², suggesting a true condensation ability of MATCAP at physiological condition. MATCAP^{ΔIDR} showed no detectable condensation under the same conditions (**Fig. 3j**), confirming the IDR is essential for MATCAP condensate formation.

Because MATCAP and vasohibins are evolutionarily distinct detyrosinases with different microtubule recognition modes and mitotic localizations (**Fig. 1a**), we compared their condensation propensities. Purified SVBP-VASH1 did not undergo phase separation in vitro, whereas SVBP-VASH2 formed few small condensates only at a concentration of 50 μ M—far above its reported cellular concentration (~ 0.77 nM, measured by OpenCell⁴²) (**Supplementary Fig. 4l, m**). These data suggest that, unlike MATCAP, SVBP-VASH complexes are unlikely to undergo physiologically relevant condensation in cells.

MATCAP co-condenses with tubulin on microtubules via its N-terminal IDR

Although our aforementioned phase separation assays were conducted under physiological salt concentration⁴³⁻⁴⁵, we sought to confirm these observations under more cytomimetic conditions. We therefore employed whole-cell lysates (10 mg/mL), which have been previously used to examine protein phase separation under near-physiological conditions⁴⁶. Real-time TIRF imaging revealed that MATCAP molecules initially associated diffusely with the microtubule lattice, then nucleated and progressively expanded into condensates (**Supplementary Fig. 5a**), reminiscent of behaviors reported for TPX2 on single microtubules^{44, 47} and Tau, BuGZ, and LEM2 on microtubule bundles⁴⁸⁻⁵². Furthermore, MATCAP condensates exhibited dynamic behaviors, including movement along microtubules and fusion with neighboring condensates (**Supplementary Fig. 5b**). We next determined the concentration dependence of condensation. As shown in **Supplementary Fig. 5c**, MATCAP condensates appeared at concentrations ≥ 100 pM, which defined a critical concentration for phase separation under our experimental conditions and its relevance in cellular biology. Deletion of the IDR (MATCAP ^{Δ IDR}) abolished condensate formation, resulting in diffused distribution and minimal microtubule binding (**Supplementary Fig. 5d**), suggesting the importance of the IDR in MATCAP function. Interestingly, the IDR alone (MATCAP^{IDR}) failed to localize onto microtubules even at 100 μ M (**Supplementary Fig. 5e**), demonstrating that the IDR is necessary but not sufficient for condensate formation on microtubules and must act in the context of the full-length MATCAP protein.

We next tested whether MATCAP condensates concentrate tubulin dimers. In MRB80 buffer with 100 mM KCl, MATCAP condensates efficiently recruited rhodamine-labeled tubulin dimers (**Supplementary Fig. 5f, g**). Importantly, recruitment of soluble tubulin dimers to the microtubule lattice required MATCAP condensation; MATCAP ^{Δ IDR}, which is unable to form condensates, failed to recruit tubulin dimers (**Supplementary Fig. 5h**). Consistently, MATCAP condensation was essential for detyrosination of soluble tubulin dimers, as MATCAP ^{Δ IDR} exhibited minimal detyrosination activity (**Supplementary Fig. 5i, j**). Therefore, IDR-mediated MATCAP condensation creates a catalytic compartment by spatially concentrating both the enzyme and its substrate, thereby enhancing reaction efficiency.

Although MATCAP preferentially interacts with polymerized tubulin over tubulin dimers^{25, 26}, both our data and others' work indicate that MATCAP is capable of detyrosinating tubulin dimers as well. Under normal *in cellulo* conditions, microtubules dominate the interaction with MATCAP.

However, the spatial confinement and high local concentrations in MATCAP condensates might facilitate its detyrosination activity towards tubulin dimers. This ability to modify dimers within specific condensates provides a localized mechanism to fine-tune tubulin detyrosination levels, potentially facilitating rapid adaptation during cytoskeletal remodeling. Thus, we reason that MATCAP condensation provides a spatial regulation of tubulin detyrosination.

MATCAP condensation facilitates its detyrosination activity

To determine the functional contributions of the N-terminal IDR and clarify the relationship between MATCAP condensation, microtubule binding, and detyrosination activity, we generated a series of chimeric MATCAP proteins in which the native IDR was replaced with heterologous regions possessing distinct properties (**Fig. 4a**). First, we substituted MATCAP IDR with the well-characterized, phase-separation-prone IDR of fused in sarcoma (FUS)⁵³. The FUS IDR has a negative isoelectric point (pI) and is not expected to bind microtubules. Conversely, we replaced MATCAP IDR with structured microtubule-binding domains—the calponin homology (CH) domain from *Plasmodium* EB1 (PyEB1-CH) and the microtubule-binding domain (MTBD) from MAP7^{54, 55}—which are known to bind the microtubule lattice but are not expected to drive phase separation. MATCAP^{ΔIDR}-FUS^{IDR} retained phase separation capacity but failed to bind microtubules, and showed reduced detyrosination activity (**Supplementary Fig. 6a-d**). By contrast, MATCAP^{ΔIDR}-PyEB1^{CH} and MATCAP^{ΔIDR}-MAP7^{MTBD} bound microtubules via their structured domains but did not undergo efficient phase separation and hence showed low detyrosination activity (**Supplementary Fig. 6a-d**). These results indicate that both phase-separation capability and microtubule engagement are essential for the detyrosination activity of MATCAP.

Next, we generated three additional chimeric proteins in which the MATCAP IDR was replaced with IDRs from LEM2, Tau (proline-rich domain, PRD) and EB1—regions selected for their phase-separation propensity and positive predicted pI^{52, 56, 57}. Remarkably, MATCAP^{ΔIDR}-LEM2^{IDR}, MATCAP^{ΔIDR}-Tau^{PRD} and MATCAP^{ΔIDR}-EB1^{IDR} all showed phase separation capability comparable to wild-type MATCAP in vitro (**Fig. 4a, b & Supplementary Fig. 7a**). TIRF microscopy revealed that each chimera associated with microtubules normally (**Supplementary Fig. 7b, c**), and immunofluorescence confirmed restoration of microtubule localization to levels comparable to wild type (**Fig. 4c**). Consistently, their cellular detyrosination activities were similar

to those of wild-type MATCAP (**Fig. 4c, d & Supplementary Fig. 7d**). By contrast, MATCAP^{ΔIDR}, which lacks phase separation and microtubule engagement capacities, failed to detyrosinate tubulin (**Fig. 4a-d & Supplementary Fig. 7a-d**). Therefore, the MATCAP IDR confer two essential features—phase separation propensity and weak electrostatic microtubule-binding—both required for detyrosination activity.

We next determined whether these three chimeric MATCAP retained comparable detyrosination activity on tubulin dimers. In vitro detyrosination assays using purified chimeras and tubulin dimers showed each chimera restored activity to near-wild-type levels, consistent with cellular data (**Supplementary Fig 7e, f**). As controls, MATCAP^{ΔIDR}, which lacks phase-separation and tubulin-enrichment abilities, exhibited minimal detyrosination of tubulin dimers, and His-MBP-MATCAP, which fails to condense due to the N-terminal tag (**Fig. 3d**), also did not detyrosinate tubulin dimers (**Supplementary Fig. 7g**).

To directly test the requirement of phase separation for detyrosination activity, we exploited a phase-separation-deficient EB1 IDR variant (EB1^{IDR}-KR6Q), in which six conserved lysine/arginine residues are substituted with glutamine to reduce positive charge⁵⁷. As expected, the chimera (MATCAP^{ΔIDR}-EB1^{IDR}-KR6Q) showed attenuated activities in phase separation, microtubule binding, and tubulin detyrosination, both in vitro and in cells, comparable to MATCAP^{ΔIDR} (**Fig. 4a-d & Supplementary Fig. 7a-d**). These data further indicate that the IDR-mediated phase separation is essential for MATCAP detyrosination activity.

To further confirm the direct contribution of condensation to MATCAP detyrosination activity, we employed the optogenetic system described earlier. Blue-light-induced condensation of optoMATCAP on the microtubule lattice produced a concomitant increase in microtubule detyrosination (**Supplementary Fig. 7h, i**). This blue light-triggered condensation and functional enhancement were strictly dependent on the IDR phase-separation potential: optoMATCAP^{ΔIDR} and optoMATCAP^{ΔIDR}-EB1^{IDR}-KR6Q mutants failed to enrich on microtubules and to detyrosinate tubulin, regardless of illumination. These experiments provide direct evidence that enhanced condensation of MATCAP contributes to the increase in its detyrosination activity, likely in part by promoting microtubule binding.

To determine whether the phase separation property of MATCAP is required for its function in mitosis, we expressed MATCAP mutants in cells depleted of endogenous MATCAP by shRNA and monitored chromosome alignment by live cell imaging. Wild-type MATCAP restored

chromosome alignment, and MATCAP^{ΔIDR}-EB1^{IDR} restored chromosome alignment to a lesser extent (**Fig. 4e-g**). In contrast, the LLPS-deficient MATCAP^{ΔIDR}-EB1^{IDR}-KR6Q mutant, which has reduced microtubule detyrosination activity, failed to restore chromosome alignment (**Fig. 4e-g & Supplementary Movie 5-7**). Together, these findings indicate that MATCAP phase separation is essential for its mitotic role in promoting chromosome alignment.

Multivalent weak interactions among different MATCAP regions account for the MATCAP LLPS

To further investigate the phase separation mechanism, we explored the molecular interactions between MATCAP molecules using nuclear magnetic resonance (NMR) spectroscopy. Initially, the ¹H-¹⁵N HSQC spectrum of the N-terminal region displayed a narrow peak distribution (<1ppm) in the ¹H dimension, suggesting that the N-terminus is disordered. Backbone assignment was performed using standard triple-resonance experiments, covering all non-proline residues. Secondary chemical shifts calculations confirmed that the N-terminus exhibited typical intrinsic disorder characteristics (**Supplementary Fig. 8a, b**). Given that recent studies on phase separation have primarily focused on IDRs, we first probed the intermolecular interactions between N-terminal IDRs. We prepared two samples: a reference sample containing 0.1 mM ¹⁵N-labeled IDR and another containing 0.7 mM ¹⁵N-labeled IDR. Molecular interactions were detected by comparing the spectral peak changes at different concentrations. We observed the attenuation of signal intensity at multiple sites (**Fig. 5a**), after calibrating with protein concentration, but no chemical shift perturbations (CSPs), suggesting weak interactions between the N-terminal IDR. Notably, in addition to the IDR region, the carboxypeptidase domain also contributes to the formation of MATCAP condensates (**Fig. 3d**). Therefore, we further studied the interactions between the IDR and carboxypeptidase domain. Compared to the reference sample, when 0.2 mM unlabeled carboxypeptidase domain was added to the 0.1 mM ¹⁵N-labeled IDR sample, the signal intensity attenuation was enhanced, and significant CSPs occurred (**Fig. 5b, Supplementary Fig. 8c**), particularly in segments 39YPW41, 69MLLGHRGHMRR79 (**Fig. 5b**).

The segments include some hydrophobic and positively charged amino acids, implicating a combination of hydrophobic and electrostatic interactions in condensate formation. Consistent with this, MATCAP condensates are sensitive to 1,6-hexanediol treatment or high salt concentration (**Fig. 3g-i**), and the two additional arginines (R99, R104) also contribute to

condensation (**Supplementary Fig. 8d, e**). To test the functional importance of the identified interface, we mutated Y39, P40, W41, R74, R78, and R79, identified by NMR to alanine (MATCAP^{6A}) and combined these with MATCAP^{2A} (R99A, R104A) to generate MATCAP^{8A} (**Fig. 5c**). In vitro phase separation assays revealed that the phase separation ability of MATCAP^{8A} was much lower than that of MATCAP^{WT} (**Fig. 5d, e**), indicating that these eight residues are critical for MATCAP condensate formation.

To complement biochemical experiments and systematically evaluate the effects of 8A mutant on MATCAP condensate formation, we performed ultra-coarse-grained computational modelling using the crystal structures of its carboxypeptidase domain (**Supplementary Fig. 8f**). This ultra-coarse-grained model simplifies the carboxypeptidase domain into a rigid body matching its crystal structure unit with proper charges. For IDR, we utilized the residue-based resolution with one bead of $r_{aa} = 0.19$ nm representing an amino acid as commonly applied in studies of intrinsically disordered proteins^{58, 59}. MATCAP clustered quickly and formed condensates like those in the in vitro assays (**Supplementary Fig. 8g**). The MATCAP^{8A} cluster size and formation rate were more extensively perturbed compared with those for MATCAP^{WT} (**Fig. 5f**); the phase diagram revealed phase separation perturbation in MATCAP^{8A} (**Fig. 5g**). Assessment of MATCAP molecular interaction frequency suggested that the 8A mutations reduced both intramolecular and intermolecular interactions (**Fig. 5h**). Thus, we conclude that the eight amino acid residues are essential for MATCAP phase separation in vitro.

Consistent with our proposed model, wherein the N-terminal IDR of MATCAP drives LLPS to potentiate microtubule binding and detyrosination activity, TIRF microscopy revealed significantly attenuated microtubule-binding capacity in MATCAP^{8A} in vitro (**Supplementary Fig. 9a, b**) and Western blotting analysis showed a dramatic reduction in detyrosination activity in cells, compared to wild-type (**Supplementary Fig. 9c**). Therefore, key residues within the IDR mediate phase separation and detyrosination activity of MATCAP.

MATCAP condensation is required for chromosome congression

As previously demonstrated, MATCAP plays an important role in chromosome alignment during mitosis (**Fig. 1h**). To test the functional relevance of MATCAP LLPS in chromosome congression, we re-expressed GFP-tagged MATCAP^{WT}, MATCAP^{ΔIDR}, or MATCAP^{8A} mutant into U2OS cells depleted of endogenous MATCAP by shRNA. Live-cell imaging showed that

GFP-MATCAP^{WT} restored mitotic progression, whereas the LLPS-deficient mutants (MATCAP^{ΔIDR} or MATCAP^{8A}) failed to rescue chromosome alignment (**Fig. 6a, b & Supplementary Movie 8-10**). These data indicate that MATCAP phase separation is essential for accurate chromosome congression during mitosis.

To determine whether chromosome congression defects reflect aberrant microtubule–kinetochore interactions, we scored Astrin, a marker of bi-oriented kinetochores. Re-expression of MATCAP^{ΔIDR} or MATCAP^{8A} mutants significantly increased the percentage of metaphase cells with misaligned chromosomes (**Fig. 6c, d**), consistent with defective attachments. Correspondingly, immunofluorescence labeling showed reduced CENP-E recruitment to spindle microtubules in cells re-expressing the LLPS-deficient mutants compared with wild-type MATCAP (**Fig. 6e, f**).

Because the CENP-E recruitment was impaired in MATCAP^{8A} cells, we tested whether MATCAP^{8A} fails to interact with CENP-E. Co-immunoprecipitation analyses revealed that MATCAP^{ΔIDR} did not bind CENP-E¹⁻⁴⁸³, while MATCAP^{8A} displayed a weakened interaction relative to wild type (**Supplementary Fig. 9d**). In co-condensation assays, purified GFP-CENP-E¹⁻⁴⁸³ and Alexa Fluor 594-labeled MATCAP efficiently formed co-condensates, whereas MATCAP^{ΔIDR} and MATCAP^{8A} failed to co-condense with CENP-E¹⁻⁴⁸³ (**Supplementary Fig. 9e**). Consistently, both mutants failed to form condensates on microtubules and did not recruit CENP-E to microtubules (**Fig. 6g, h**). Given their reduced dephosphorylation activity (**Supplementary Fig. 9c**), CENP-E motility on microtubules was less processive in the presence of MATCAP^{ΔIDR} and MATCAP^{8A} than with that of MATCAP^{WT} (**Fig. 6i**).

Taken together, these data indicate that MATCAP condensation enhances its microtubule and tubulin dimer association and, collectively with a direct mechanism, promotes dephosphorylation activity, which locally enriches CENP-E to spindle microtubules to facilitate chromosome congression (**Fig. 7**). These combined effects of MATCAP's catalytic and scaffolding roles, both dependent on LLPS, support accurate chromosome congression mediated by mitotic motor CENP-E during mitosis.

Discussion

LLPS-driven compartmentalization is increasingly recognized as an essential spatiotemporal regulation for controlling mitotic spindle plasticity during cell division^{33, 57}. Our study reveals a

previously unrecognized spatial regulatory mechanism of the tubulin detyrosination/tyrosination cycle⁶⁰⁻⁶³. We show that MATCAP forms phase-separated condensates on microtubules via its N-terminal IDR. This condensate formation facilitates the detyrosination activity of MATCAP in part by increasing microtubule association and creating localized enzymatic compartments that concentrate tubulin. The highly ordered polymeric architecture of microtubule lattices facilitates recruitment of microtubule-associated proteins MAPs through specific interactions involving folded domains and/or IDRs. Therefore, many MAPs form condensates on microtubules. These MAPs collectively establish multivalent interaction networks that nucleate surface-bound biomolecular condensates via phase separation. Importantly, these assemblies are rarely observed in the cytosol and instead depend on spatial confinement provided by the microtubule lattice or growing plus-ends^{44, 48, 49, 57, 64, 65}.

Among MAPs, BuGZ is compelling functional partner for MATCAP. BuGZ undergoes temperature-dependent LLPS, driven by its IDR; this transition concentrates tubulin and promotes microtubule bundling and polymerization^{51, 66}. Recent work shows that parthenolide (PTL), a known inhibitor of tubulin detyrosination, primarily targets BuGZ during mitosis^{67, 68}. This suggests that BuGZ likely synergizes with MATCAP in association with stable microtubules. Alternatively, PTL's effect on detyrosination could be indirect, arising from the loss of BuGZ-dependent microtubule stability that is required for efficient MATCAP activity. Thus, it would be of great interest to ascertain the spatiotemporal interaction of MATCAP-BuGZ relative to MATCAP-CENP-E compartmentalization down the road.

Cryo-EM structural analysis revealed that the carboxypeptidase domain of MATCAP directly engages the C-terminal tail of α -tubulin via a defined interface²⁵. Notably, although MATCAP ^{Δ IDR} retains an intact catalytic domain, its microtubule-binding affinity and detyrosination activity were severely diminished. The N-terminal IDR necessitates a bifunctional integration of phase separation propensity and low-affinity electrostatic interactions with MTs to facilitate spatiotemporally regulated MT engagement^{44, 48, 49}. This dual mechanism establishes multivalent adhesive scaffolds through phase-separated condensate formation, synergizing with the carboxypeptidase domain to enhance multivalent binding avidity while maintaining dynamic MT association, a prerequisite for executing enzymatic compartmentalization on cytoskeletal networks.

Our data support that MATCAP phase separation facilitates detyrosination, partly through enhancing its microtubule/tubulin association. For instance, light-induced enhancement of

optoMATCAP phase separation increases its MT association and detyrosination activity, and replacing the endogenous IDR with the FUS IDR partially restores activity compared with IDR deletion. However, we recognize that the two functions of phase separation and microtubule binding of MATCAP are mechanistically intertwined: mutations that perturb phase separation commonly alter microtubule binding, so reduced detyrosination in IDR variants may reflect changes in both properties. Despite extensive effort, true separation-of-function mutants — retaining the native microtubule-binding motif but lacking phase-separation capacity — remain to be identified and will be important for future mechanistic dissection.

CENP-E is a mitotic kinesin essential for chromosome congression and anaphase central spindle organization. MATCAP also co-condensates with and locally concentrates CENP-E on microtubules (**Fig. 7**). Functionally, MATCAP orchestrates mitotic progression through an integrated mechanism: IDR-mediated condensation enables the initial recruitment and local enrichment of CENP-E on microtubules, while its detyrosination activity, which is also regulated by the phase separation, promotes the subsequent processive motility of the recruited motors by generating detyrosinated microtubule tracks. This model is supported by the functional deficiency observed in LLPS-deficient MATCAP mutants that fail to rescue chromosome congression defects. In these MATCAP phase separation-deficient mutant-expressing cells, both CENP-E recruitment and processive mobility are impaired, which is consistent with a model in which MATCAP-generated detyrosinated microtubule tracks facilitate CENP-E-dependent transport of chromosomes toward the spindle equator^{6,9}.

Previous studies show that vasohibin deficiency leads to mitotic spindle misorientation/shortening, astral microtubule loss, and delayed chromosome segregation—phenotypes resembling those caused by MCAK overexpression. Detyrosinated α -tubulin selectively stabilizes correct kinetochore-microtubule attachments, enabling centromeric depolymerases to distinguish it from defective attachments, whereas persistent detyrosination of kinetochore microtubules disrupts error correction without altering their stability^{21-23,69}. MATCAP may overlap with vasohibin in global spindle microtubule detyrosination, but MATCAP phase-separation behavior provides an additional regulatory layer for spindle microtubule detyrosination and CENP-E-dependent chromosome congression.

These findings advance our understanding of how biomolecular condensation organizes enzymatic processes⁷⁰. LLPS not only concentrates enzymes and substrates but also couples

tubulin post-translational modification to motor activity, ensuring spatiotemporal precision during mitosis. This mechanism may extend to other PTM-catalyzing enzymes, suggesting a broader paradigm for compartmentalized catalysis⁷¹. Therapeutically, selectively targeting mitosis-prominent LLPS (e.g., MATCAP) in hyperproliferative cells could offer strategies to harness malignant cell proliferation without affecting housekeeping gene (e.g., vasohibin⁷²) functions in normal cells. Future work should be directed to delineate the regulatory mechanisms underlying MATCAP condensation (e.g., PTMs) and its crosstalk with other mitotic PTMs^{73, 74} and test these mechanisms in vivo to validate pathophysiological relevance. Our study also raises questions about the evolutionary conservation of LLPS-dependent detyrosination and its role in non-mitotic contexts, such as neuronal transport^{14, 26, 32, 75, 76}.

In sum, this study reveals MATCAP as a phase-separation-dependent orchestrator of mitotic fidelity that bridges tubulin biochemistry, mitotic kinesin CENP-E loading, and chromosome segregation. LLPS-driven condensation emerges as a common regulatory strategy to spatially and temporally tune microtubule dynamics, the tubulin code, and motor functions, thereby ensuring accurate mitotic division²⁹.

Methods

Plasmids

To generate Flag-tagged full-length MATCAP and its deletion mutants, PCR-amplified MATCAP cDNA was cloned into p3×FLAG-Myc-CMV-24 vector (Sigma) using HindIII and BamHI. GFP-MATCAP and its deletion mutants were constructed by inserting amplified MATCAP cDNA into pEGFP-C1 vector via HindIII and SalI. Plasmid Cry2-mCherry-MATCAP was constructed by inserting MATCAP cDNA into the linearized Cry2-mCherry backbone (Addgene, 60032) C-terminal to mCherry using Vazyme (C212). For bacterial expression, His-MBP-HRV3C-MATCAP was generated by inserting amplified MATCAP cDNA into the modified pET-28a expression vector encoding an N-terminal 6×His tag followed by an MBP solubility tag and a PreScission cleavage site.

For MATCAP IDR replacement plasmids, FUS¹⁻²¹⁴/PyEB1¹⁻¹⁷⁷/MAP7⁸⁷⁻¹³⁹/LEM2⁴³⁻²⁰²/Tau_2N4R¹⁵¹⁻²⁵⁴/EB1¹²⁰⁻¹⁹⁰ were synthesized and inserted into the FLAG-MATCAP, GFP-MATCAP or His-MBP-HRV3C-MATCAP plasmid to replace MATCAP IDR. To generate the plasmid MATCAP RDI (IDR reversed), the amino-acid sequence of the IDR domain (residues 1-137) of MATCAP was reversed in order (137-1) and synthesized as a DNA fragment, and subsequently cloned into the corresponding backbone vector.

FLAG-tagged CENP-E truncations of CENP-E¹⁻⁴⁸³, CENP-E⁴⁸⁴⁻²⁰⁷⁰, and CENP-E²⁰⁷¹⁻²⁷⁰¹ were generated by inserting the corresponding PCR-amplified fragments into p3×FLAG-Myc-CMV-24 vector using HindIII and BamHI. Strep-tagged full-length CENP-E and Strep-CENP-E¹⁻⁴⁸³-GFP were cloned into pTT5-Strep vector (Addgene) using AgeI and XbaI. SVBP and VASH1 or VASH2 coding sequences were cloned into the upstream and downstream multiple cloning sites of a modified pCDFDuet-1(Novagen) to enable co-expression of vasohibin-SVBP protein complex in *Escherichia coli*. For mammalian expression, FLAG-SVBP-T2A-vasohibin-mCherry was cloned into the pcDNA3.1 vector (Addgene).

All mutations were generated by Mut Express II Fast Mutagenesis Kits (Vazyme Biotech) according to the manufacturer's instructions. All constructs and mutations were verified by sequencing (Tsingke Biotechnology).

Cell culture and transfection

HEK293T, COS-7, RPE1, HeLa and U2OS cells were purchased from the American Type Culture

Collection (ATCC) and maintained as monolayers in advanced DMEM (Invitrogen) with 10% fetal calf serum (FCS, HyClone) and 100 units/mL penicillin plus 100 µg/mL streptomycin (Invitrogen) at 37 °C in 5% CO₂. Plasmid transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

RNA interference

The lentivirus-based vectors PLKO.1 or PLKO.1-GFP, along with pSPAX2 and pMD2.G, were ordered from Addgene. shRNA oligonucleotides were annealed and cloned into pLKO.1 or pLKO.1-GFP, and lentivirus was produced by co-transfection of HEK293T cells with pSPAX2 and pMD2.G. The following targeting sequences were used: shMATCAP#1, 5'-CATCGCCAGACCATCGATTTC-3'; shMATCAP#2, 5'-CTTCTTGGAAGGCACCAATTT-3'; shMATCAP#3, 5'-ATGGTCGATTGTGCGCAAATA-3' (shMATCAP#2 was targeted to 3'-UTR and used in all functional studies in cells). VASH1 shRNAs: 5'-CCTGGGAATTTACCTCACCAA-3' (#1) and 5'-CACAGGACATAGTGGTGCTTT-3' (#2). VASH2 shRNAs: 5'-TTTGACTTTGAGGACTCTTAC-3' (#1) and 5'-GAAGGTCAAGATTGGGCTGTA-3' (#2). To enhance knockdown efficiency, pools of two shRNAs targeting different regions of VASH1 or VASH2 were used. The virus-containing medium was collected and used to infect target cells for gene knockdown.

Antibodies

Primary antibodies and dilutions used were: mouse anti- α -tubulin (Sigma, T9026, 1:1,000 for IF and 1:5,000 for WB); mouse anti- α -tubulin-FITC (Sigma, F2168, 1:500 for IF); anti-ACA antibody (Immunovision, HCT-0100, 1:800 for IF); rabbit anti-FLAG (Sigma, F7425, 1:200 for IF; F1804, 1:2,000 for WB); rabbit anti-GFP (Proteintech, 50430-2-AP, 1:2,000 for WB); rabbit anti-mCherry (Proteintech, 26765-1-AP, 1:2,000 for WB); mouse anti-GAPDH (Proteintech, 60004-1-Ig, 1:50,000 for WB); rabbit anti-detyrosinated α -tubulin (Abcam, ab48389, 1:200 for IF and 1:1,000 for WB); mouse anti-CENP-E (Abcam, ab5093, 1:200 for IF, 1:1,000 for WB); rabbit anti-Astrin (Proteintech, 14726-1-AP, 1:200 for IF); rabbit anti-Strep Tag II (Proteintech, 85798-1-RR, 1:5,000 for WB); mouse anti-MBP (Cell Signaling Technology, 8G1, #2396, 1:1,000 for WB); rabbit anti-MATCAP (Sigma, ZRB2972, 1:1,000 for WB); mouse anti-MATCAP (home-made, 1:50 for IF); rabbit anti-CENP-E (HpX, 1:200 for IF) was generated as previously^{77, 78}. All

secondary antibodies were purchased from Jackson ImmunoResearch, except the HRP-conjugated mouse anti-rabbit IgG (Light-Chain Specific) (Cell Signaling Technology, 93702S).

To generate anti-MATCAP antibody, BALB/c mice were immunized subcutaneously with 20 µg of purified His-tagged MATCAP^{AIDR} 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.

Protein expression and purification from *E. coli*

E. coli BL21 cells were transformed with expression plasmids and cultured in LB supplemented with the appropriate antibiotic. Protein expression were overexpressed at 16 °C for 20 h. Protein expression was induced at an OD₆₀₀ of 0.6–0.8 with 0.5 mM IPTG and carried out at 16 °C for 20 h. Cells were harvested by centrifugation at 3,600 × g, 4 °C, for min, and then resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM TCEP) containing protease inhibitor cocktails (Amresco). Cells were lysed by sonication and lysates were clarified by centrifugation at 18,000× g, 4 °C for 30 min. Recombinant His-MBP-MATCAP was purified using fast flow Ni-NTA column (GE healthcare) and eluted in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 200 mM imidazole, 1 mM TCEP. The elution fractions containing proteins were then concentrated and buffer-exchanged into 50 mM HEPES, pH 7.4, 500 mM KCl, 1 mM TCEP using 30-kDa Amicon centrifugal filter units (Amicon Ultra-4 Merck Millipore).

For vasohibin-SVBP protein complex, following Ni-NTA capture, the complex was further purified by size-exclusion chromatography on HiLoad 16/600 superdex 75 pg column (GE healthcare) in suspension buffer. Fractions containing the SVBP/VASH1 complex were collected and dialyzed into ion exchange buffer I (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT) and applied to a HiTrap SP HP (1 mL) column (GE Healthcare). Ion exchange chromatography was performed using buffer I and buffer II (20 mM Tris-HCl, pH7.4, 1 M NaCl, 1 mM DTT) as

appropriate.

Protein expression and purification from HEK293T cells

To purify Strep-tagged CENP-E or Strep-GFP-tagged CENP-E¹⁻⁴⁸³, HEK293T cells seeded in 15-cm dishes were transfected with 20–30 µg plasmid DNA per dish using polyethylenimine (PEI). At 36 h post-transfection, the medium was removed from the dishes, and cells were quickly collected with cold PBS (10 ml for each 15-cm dish) into 15-ml tubes. Then, cells were centrifuged at 1,000× rpm, 4 °C for 5 min to remove the supernatant, and the total pellets were resuspended in 3 mL lysis buffer (80 mM PIPES, pH 6.8, 300 mM KCl, 2 mM MgCl₂, 2 mM ATP, 1 mM EGTA, 1% Triton-X-100) supplemented with protease inhibitors (Roche). Cells were lysed by sonication, and lysates were clarified by centrifugation at 14,000× rpm, 4 °C for 20 min. The supernatant was collected and incubated with 60 ~ 100 µL StrepTactin beads (GE Healthcare) at 4 °C for 45 min. After centrifuging at 3,000× rpm, 4 °C for 1 min, the supernatant was removed and the beads were washed four times with 1 ml lysis buffer and twice with 1 ml wash buffer (80 mM PIPES, pH 6.8, 100 mM KCl, 2 mM MgCl₂, 2 mM ATP, 1 mM EGTA). Proteins were eluted with 60 ~ 100 µL elution buffer (80 mM PIPES, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 2 mM ATP, 1 mM EGTA, 2.5 mM D-Desthiobiotin).

Protein fluorescence labeling

Purified proteins were prepared in 50 mM HEPES, pH 7.4, 500 mM KCl, 1 mM TCEP. Alexa Fluor 488 (ThermoFisher) or Alexa Fluor 594 (ThermoFisher) were dissolved in DMSO and incubated with the corresponding protein at a 1:1 molar ratio for 1 h at room temperature. Reactions were quenched with 200 mM Tris, pH 8.3. The free dye and other small molecules were removed from the proteins by passing the reaction mixture through a Hitrap desalting column with label buffer. Fluorescence labeling efficiency was measured by Nanodrop 2000 (ThermoFisher). For imaging, labeled proteins were further diluted into unlabeled protein in the same buffer; typically, the final labeled: unlabeled ratio was 1:100.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed on a Zeiss LSM 880 confocal microscope equipped with a Plan-Apochromat 63×/1.4 oil immersion objective. MATCAP was bleached using the 488 nm laser

line. For each experiment, two pre-bleach images were acquired, followed by photobleaching of a defined region with 50 iterations of the 488 nm laser at 100% power. Time-lapse images were then recorded every 2 s for 5 min. Fluorescence intensity within the bleached region was normalized to its pre-bleach intensity. To correct for overall photobleaching during imaging, the recovery curves were also normalized to a non-bleached region in the same field.

The mobile and immobile fractions were calculated as follows⁷⁹, mobile fractions:

$$Mf = (I_{\infty} - I_0)/(I_i - I_0) \quad (1)$$

where I_i is the pre-bleach fluorescence intensity, I_0 is the intensity immediately after bleaching, and I_{∞} is the plateau intensity during recovery; immobile fraction:

$$(Imf) = 1 - Mf \quad (2)$$

Immunoprecipitation and pull-down assays

For Strep-tagged and GFP-tagged protein immunoprecipitations, transfected cells were collected and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) supplemented with protease inhibitor cocktail (Sigma). Cell lysates were clarified by centrifugation at $16,000 \times g$ for 20 min at 4 °C and incubated with StrepTactin beads (GE Healthcare) or Anti-GFP nanobody agarose beads (AlpaLifeBio, KTSM1301) at 4 °C with gentle rotation for 2 h. Beads were washed three times with lysis buffer and bound material was eluted by boiling in 100 μ L of 2 $\times$ SDS-PAGE loading buffer prior to SDS-PAGE and western blotting.

For MBP pull-down assays, HEK293T cells seeded in six-well plates were transfected with 1 μ g FLAG-tagged-CENP-E construct using PEI. At 36h post-transfection, cells were collected with cold PBS, pelleted, and then resuspended in 500 μ L lysis buffer (20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.01% Triton X-100). Cells were lysed by sonication, and lysates were clarified by centrifugation at 14,000 $\times$ rpm, 4°C for 20 min. The supernatant was collected and incubated with purified MBP-tagged proteins immobilized on agarose beads at 4 °C for 2 h. Beads were subsequently washed three times with 1 mL of lysis buffer each time. Bound proteins were boiled in SDS-PAGE loading buffer. The samples were separated by SDS-PAGE and subjected to Western blot.

For endogenous immunoprecipitation, RPE-1 cells were synchronized at the G1/S boundary using a double-thymidine block protocol. Briefly, cells were treated with 2 mM thymidine (Sigma) for 16 hours, released into fresh medium for 8 hours, and subjected to a second 16-hour thymidine

treatment. After the final release into thymidine-free medium, 20 μ M MG132 (Sigma) was added 8 hours post-release to enrich for mitotic cells. Cells were harvested 3 hours after MG132 treatment. Cells were washed in ice-cold PBS and lysed in lysis buffer (20 mM HEPES-KOH, pH 6.8, 50 mM KCl, 2 mM EGTA, and 2 mM MgCl₂) supplemented with protease inhibitor cocktail (Sigma). Cell lysates were clarified by centrifugation at 16,000 $\times$ g for 20 minutes at 4 °C. For immunoprecipitation, 20 μ L of a 50% slurry of Pierce™ Protein A/G Agarose beads (Thermo Scientific) were pre-coupled to 3 μ g of CENP-E antibody (HpX) or rabbit IgG (Sino Biological) by rotating for 3 hours at 4 °C in lysis buffer. The beads were washed twice with lysis buffer to remove unbound antibodies. Clarified lysates were then incubated with the antibody-bound beads overnight at 4°C with rotation. Beads were washed three times with 1 mL lysis buffer each time. One-third of the immunoprecipitate was analyzed by SDS-PAGE/western blot, and the remaining sample was reserved for mass spectrometry.

For StrepTactin pull-down assays with purified proteins, Strep-tagged CENP-E was purified from HEK293T cells. To minimize co-purifying host proteins, a stringent purification buffer (300 mM KCl and 1% Triton X-100) was used. Strep-tagged CENP-E immobilized on StrepTactin beads was then incubated with purified His-MBP-tagged proteins in binding buffer (20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) at 4 °C for 2 h. Beads were subsequently washed three times with 1 mL of binding buffer each time and bound proteins were boiled in SDS-PAGE loading buffer for SDS-PAGE and western blot analysis.

NMR experiments

The NMR experiments for MATCAP were performed as previously described⁸⁰. Briefly, ¹⁵N- and ¹³C-labelled NMR samples were obtained with LR medium prepared with ¹⁵NH₄Cl and ¹³C-glucose. The IDR construct was cloned into the pET28 his-SUMO tag vector and expressed in BL21(DE3) *E.coil* at 16 °C for 24 hours. The purification protocol was the same as above, and His-SUMO was cleaved by ULP1 enzyme with a 50:1 (protein:enzyme) molar ratio at 4 °C overnight. The final product was further purified on an HPLC C18 column. The sample was freeze-dried in a freeze dryer and stored at -80 °C. All purified proteins were analysed and confirmed with SDS-PAGE.

NMR experiments were performed with a Bruker AVANCE III 600 spectrometer equipped with a TXI cryogenic probe at 288 K. All NMR samples contained BRB80 buffer (pH 6.8, 100

mM KCl, 2 mM dithiothreitol in 95% H₂O and 5% D₂O). All NMR spectra were processed with the software package NMR Pipe and analysed with Sparky. The backbone assignments of IDR were mainly obtained from standard multidimensional NMR methods as HNCACB, CBCA(CO)NH, HN(CA)CO, HNCO and (H)N(COCA)NNH. The chemical shifts difference in ¹H and ¹⁵N dimensions were combined as $\sqrt{(\Delta N * 0.15)^2 + \Delta H^2}$. The intensity ratios were defined as I_b/I_0 , where I_b is the peak intensity of a particular residue in the presence of the ligand and I_0 is the intensity of the same residue when the ligand is absent. The error of I_b/I_0 was calculated according to Gauss' error propagation, in the same way as in the previous publication. SNR_b and SNR₀ are signal-to-noise (SNR) of the peaks in the presence and absence of ligands, respectively.

Immunofluorescence and time-lapse imaging

For detyrosinated-tubulin staining, cells were fixed in cold methanol (−20 °C) for 5 min. For Astrin staining, cells were pre-extracted for 5 min in 0.1% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO₄, pH 6.8), followed by fixation in 4% formaldehyde in PHEM for 10 min. Fixed cells were blocked in PBST (PBS + 0.05% Tween-20) containing 1% bovine serum albumin (Sigma) for 45 min at room temperature, incubated with primary antibodies in a humidified chamber for 1 h at room temperature or overnight at 4 °C, and then with secondary antibodies for 1 h at 37 °C. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Images were captured using DeltaVision softWoRx software (Applied Precision) and processed by deconvolution and z-stack projection. All images in figures are presented as maximum intensity projections.

TIRF microscopy

Tyrosinated porcine brain tubulin (Cytoskeleton) was prepared by incubation with recombinant human TTL as described previously⁸¹. The reactions were set up on the ice by mixing chimeric tubulin (final 10 μM) with human TTL (final 0.1 μM) in tyrosination buffer (1X BRB80, 5% glycerol, 2 mM β-mercaptoethanol, 2.5 mM MgCl₂, 0.1 mM Tyrosine, 1 mM GTP, 2 mM ATP).

GMPCPP-stabilized Double - cycled microtubule seeds were prepared as described previously⁸². Briefly, 8 μL tubulin reaction mixture in MRB80 buffer (80 mM PIPES, 4 mM MgCl₂, and 1 mM EGTA, pH 6.8), containing 14 μM unlabeled tyrosinated tubulin, 2.4 μM rhodamine-tyrosinated tubulin or HiLyte647-tyrosinated tubulin (Cytoskeleton), 3.6 μM biotin-tyrosinated

tubulin (Cytoskeleton), and 1 mM GMPCPP (Jena Biosciences), was incubated at 37 °C for 30 min to allow polymerization. Then, seeds were pelleted by centrifugation in an Airfuge (Beckman) at ~ 28 psi (90,000 rpm, 160,000 g) for 5 min. After carefully removing the supernatant, the pellet was resuspended with 6 µL MRB80 buffer and depolymerized for 20 min on ice. Subsequently, 1 mM fresh GMPCPP was added, and a second-round of polymerization was performed at 37 °C for 30 min. MT seeds were then pelleted as described above, resuspended in 60 µL MRB80 buffer supplemented with 10% glycerol, snap-frozen in liquid nitrogen, and stored at -80 °C.

Assays were performed following the procedure previously described⁸³. Flow chambers were assembled from plasma-cleaned coverslips and slides using double-sided tape. The coverslip surface was functionalized with 0.2 mg/mL PLL-PEG-biotin (Susos AG) and 1 mg/ml neutravidin (Invitrogen) in MRB80 buffer. Subsequently, GMPCPP seeds were attached to the coverslip via biotin-neutravidin linkage and chambers were blocked with 1 mg/mL κ -casein. Reaction mixture, containing purified protein in MRB80 buffer supplemented with 100 mM KCl, 1 mM GTP, 0.2 mg/mL κ -casein, 0.1% methylcellulose, and oxygen scavenger mix (50 mM glucose, 400 µg/mL glucose oxidase, 200 µg/mL catalase and 4 mM DTT), was cleared by centrifugation in an Airfuge at ~ 28 psi for 5 min and introduced into the chamber, which were sealed with vacuum grease and imaged immediately at 30 °C on a Nikon Eclipse Ti2-E TIRF microscope with perfect focus, a CFI Apo TIRF 100× 1.49 NA oil objective, Prime 95B camera (Photometrics), SOLE laser engine (four lasers: 405, 488, 561, and 638 nm; Omicron), controlled by NIS-elements software (Nikon).

Single-molecule motility assays in fixed cells

Single-molecule motility assays in fixed cells were performed as previously described³⁸. U2OS cells were plated on 35-mm glass-bottom dishes (MatTEK) for 24 h and transfected with FLAG-MATCAP, FLAG-MATCAP^{ΔIDR}, VASH1/2-mCherry+FLAG-SVBP plasmids. 24 h later, cells were permeabilized for 1 min at 37 °C in pre-warmed extraction buffer (BRB80: 80 mM K-PIPES, 1 mM MgCl₂, 1 mM EGTA; pH 6.8) supplemented with 1M sucrose and 0.15% [v/v] TritonX-100. Pre-warmed fixation buffer (BRB80 supplemented with 2% [w/v] formaldehyde (FA) and 0.1% of glutaraldehyde) was added and mixed by gentle pipetting for 1 min. The extraction-fixation solution was exchanged for pre-warmed wash buffer (BRB80 with 1 µM Taxol) and incubated for 1 min. This wash step was repeated three additional times. The wash buffer was then replaced with

pre-warmed imaging buffer (BRB80 supplemented with 583 $\mu\text{g/mL}$ catalase, 42 $\mu\text{g/mL}$ glucose oxidase, 1.7% [w/v] glucose, 1 mM DTT, 2 mM methyl viologen, 2 mM ascorbic acid, 1 μM Taxol, and 10 μM ATP) containing ~ 1 nM GFP-CENP-E¹⁻⁴⁸³. Time-lapse images were taken using an ELYRA-7 system (Carl Zeiss) with a 100 $\times$ objective, in a humidified chamber maintained at 37 $^{\circ}\text{C}$. Frames were acquired every 100 ms with 100 ms exposure, and the typical duration of imaging was 5 min.

In vitro detyrosination assays

Reactions (50 μL) were performed by incubating purified recombinant enzyme with 2 μg tubulin or microtubules in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, and 10% glycerol at 37 $^{\circ}\text{C}$ for 2 h. Samples were then mixed with loading buffer and boiled for 5 min at 95 $^{\circ}\text{C}$ for western blot analysis.

Quantification of immunofluorescence samples

For fluorescence quantification, specifically the levels of microtubule detyrosination and CENP-E at the spindle, measurements were performed using sum projections of the raw image data using Fiji/ImageJ. Signals of interest were normalized to total α -tubulin within an identical ROI drawn around the spindle. Background correction used the mean intensity of three random regions surrounding the spindle. Normalized intensity was calculated as:

$$\text{Normalized Intensity}_x = \frac{\text{Raw Intensity}_x - (\text{Area} * \text{Noise}_x)}{\text{Raw Intensity}_{\text{tub}} - (\text{Area} * \text{Noise}_{\text{tub}})} \quad (3)$$

where x is the PTM or marker of interest, Area is the ROI area (identical for both signals), and Noise is the mean of the mean background intensity⁷¹. Colocalization was assessed by Pearson correlation coefficient using the Fiji plugin EzColocalization⁸⁴⁻⁸⁷. Colocalization analyses were restricted to cells within comparable MATCAP expression levels.

Ultra-coarse-grained computational modelling

A multiscale, coarse-grain method was used for MATCAP modelling⁵⁹. Wherein each amino acid was represented as a single spherical bead. To account for structural domain effects, MATCAP carboxypeptidase domains were constructed as rigid bodies matching their crystal structure (PDB: 7Z5H). Intrinsically disordered regions (IDRs) were modeled as flexible chains based on sequence data, connected to adjacent domains. Partial charge parameters were systematically assigned according to the intrinsic physicochemical properties of amino acid residues; each bead serves as

an independent interaction site.

All simulations were performed in a cubic box with periodic boundary conditions, using reduced units of $kBT = 1$. Box length was set as 50 nm for simulations other than the slab simulation. Interparticle bonded interactions were modeled using a harmonic potential with a force constant of $9.6 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$ and an equilibrium bond length of $3.81 \text{ \AA}$, consistent with the characteristic peptide bond length in biological systems. Non-bonded interactions comprised: Truncated Lennard-Jones potential (Cutoff radius: $0.1 \text{ \AA}$); Energy parameter (ϵ): 0.15 kcal/mol ; Size parameter (σ): $6.0 \text{ \AA}$, Coulombic potential (Cutoff radius: $0.6 \text{ \AA}$). Debye-Hückel screening (Screening parameter (κ): $18.0 \text{ \AA}^{-1}$).

Statistics and reproducibility

All data were obtained from at least three independent experiments. Statistical analyses were performed using a Student's *t*-test (unpaired two-tailed) for comparisons between two groups and one-way analysis

of variance (ANOVA) for multiple comparisons with GraphPad Prism 8. All statistics are described in the figure legends. No statistical method was used to pre-determine sample size. No data were excluded from the analyses. Data collection and analysis were not performed blind to the conditions of the experiments. Images were mounted in figures with Photoshop and Microsoft Office PowerPoint.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data Availability

Previously published structures are available from the Protein Data Bank under accession codes MATCAP carboxypeptidase domain (PDB entry 7Z5H). NMR data have been deposited to the Biological Magnetic Resonance Data Bank (BMRB: 53834) at https://bmr.io/data_library/summary/index.php?bmrId=53834. Source data are provided with this paper.

Code availability

The custom code for coarse-grained computational modelling and statistical analysis along with its output dataset are available at <http://zenodo.org> with the identifier <https://doi.org/10.5281/zenodo.20483544>.

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AUTHOR CONTRIBUTIONS

X.L. and X.Y. conceived the project; T.Y. designed and performed most of the biochemical experiments, live-cell imaging and IF analyses. P.X. and S.X. performed NMR experiments. W.H., Y.Z., T.Y. and K.J. performed in vitro TIRF assays. Z.W. made the MATCAP antibody. H.L. and Z.H. performed molecular modelling. C.X., F.X., Y.L. and K.R. contributed to reagents. T.Y., with inputs from X.L., Z.W., L.Z. and K.J. drafted the initial manuscript; X.Y., L.Z., X.L. and T.Y. reviewed and edited the manuscript. All authors had commented and approved the manuscript.

COMPETING INTEREST STATEMENT:

The authors declare that they have no competing interests.

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

- a**, Representative immunofluorescence images of U2OS cells expressing mCherry, VASH1/2-mCherry + FLAG-SVBP, or mCherry-MATCAP. Scale bar, 5 μ m.
- b**, Immunofluorescence staining of CENP-E and detyrosinated tubulin in U2OS cells following individual or combined knockdown of MATCAP and VASH1/2 by shRNA. Scale bar, 5 μ m.
- c**, Quantification of CENP-E intensity on spindles as described 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 $\pm$ s.e.m. Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. **** $P < 0.0001$; NS, not significant ($P > 0.05$).
- d**, Representative live-cell images showing chromosome movements in U2OS cells stably expressing mCherry-H2B and transfected with PLKO-Control-GFP, PLKO-shMATCAP-GFP or PLKO-shVASH1/2-GFP. Time shown in minutes. White arrowheads indicate misaligned chromosomes. Scale bar, 10 μ m.
- e**, Immunoblot analysis of tubulin detyrosination in U2OS cells following individual or combined knockdown of MATCAP and VASH1/2 using shRNA. IB, immunoblot.
- f**, Statistical analysis of time from nuclear envelope breakdown (NEBD) to anaphase onset (or end of imaging) as described in **d**. n (cells) = 32 (Control), 33 (shMATCAP), 32 (shVASH1/2), 31 (shMATCAP + shVASH1/2); pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Statistical test: Ordinary one-way ANOVA with Tukey's multiple comparisons. NS, not significant; **** $P < 0.0001$; 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 misalignment as described in **d**. The number of scored cells and the statistical test were described in **f**. NS, not significant; **** $P < 0.0001$; * $P = 0.0187$.
- h**, Immunofluorescence staining of Astrin and centromere (ACA) in mitotic U2OS cells transfected with control, MATCAP or VASH1/2 shRNA. DNA was counterstained with DAPI. Scale bar, 5 μ m.
- i**, Quantification of the percentage of cells with Astrin-negative kinetochores (KTs) per cell in **h**. n (cells) = 60 (Control), 72 (shMATCAP), 63 (shVASH1/2), 60 (shMATCAP + shVASH1/2);

pooled from three independent experiments.

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Fig. 2 N-terminal IDR of MATCAP mediates CENP-E recruitment and motility on microtubules

- a,** Immunofluorescence staining of MATCAP and CENP-E in HeLa cells from different mitotic stages. Scale bar, 5 μ m.
- b,** Endogenous immunoprecipitation from mitotic cell lysates using control IgG or rabbit anti-CENP-E (HpX) antibody. Immunoprecipitates were resolved by SDS-PAGE, followed by Western blot analyses using mouse anti-CENP-E and rabbit anti-MATCAP antibodies to confirm the interaction between MATCAP and CENP-E. IB, immunoblot.
- c,** Co-immunoprecipitation of Strep-CENP-E with Cherry, mCherry-MATCAP, or VASH1/2-mCherry+FLAG-SVBP, from HEK293T cells. Their interactions were tested by Western blot with indicated antibodies.
- d,** Schematic of human MATCAP domain architecture (upper) and disorder prediction (lower). Disorder score cutoff: 0.5 (≥ 0.5 , disordered). VLXT, disorder predictor; IDR, intrinsically disordered region.
- e,** Schematic representation of full-length (FL) or truncated constructs of CENP-E.
- f,** Affinity pull-down using purified recombinant Strep-CENP-E to capture bacterially expressed His-MBP, His-MBP-MATCAP or His-MBP-MATCAP ^{Δ IDR}. Bound fractions were analyzed by western blot; input levels shown by Coomassie brilliant blue (CBB).
- g,** FLAG-CENP-E truncations expressed in HEK293T cells were incubated with His-MBP-MATCAP; interactions were assessed by pull-down and anti-FLAG Western blot.
- h,** TIRF microscopy images showing recruitment of GFP-CENP-E¹⁻⁴⁸³ (green) to GMPCPP-stabilized, Cy5-labeled microtubules (blue) in the presence of Alexa Fluor 594-labeled MATCAP, VASH1, or VASH2 (red). Scale bars, 5 μ m.
- i,** Quantification of CENP-E fluorescence intensity on microtubules, normalized to microtubule signal as shown in **h**. $n = 15$ microtubules per group, pooled from three independent experiments. Data represent mean $\pm$ s.e.m., Statistical significance was assessed by Ordinary one-way ANOVA followed by Tukey's multiple comparisons. NS, not significant; **** $P < 0.0001$.
- j,** Representative fluorescence kymographs showing CENP-E recruitment and motility under

the indicated conditions. Horizontal scale bar, 2 μm ; vertical scale bar, 10 s.

- k,** Characteristic run length and mean velocity of CENP-E motor on microtubules as shown in **j**. For Control, $n = 44$; MATCAP, $n = 50$; MATCAP ^{ΔIDR} , $n = 46$; VASH1, $n = 42$; VASH2, $n = 41$, n represents CENP-E molecules pooled from three independent experiments. Data are mean $\pm$ s.e.m. Ordinary one-way ANOVA with Tukey's multiple comparisons. NS, not significant; **** $P < 0.0001$.

Fig. 3 MATCAP undergoes phase separation via its N-terminal IDR

- a,** Time-lapse images of U2OS cells depleted of endogenous MATCAP and expressing Cry2olig-MATCAP or Cry2olig-MATCAP ^{ΔIDR} , before and after blue-light activation. Time shown in seconds. Scale bar, 10 μm .
- b,** Immunofluorescence staining of MATCAP in metaphase U2OS cells treated with 1.5% 1,6-Hexanediol (1,6-HD) or PBS (Control) for 2 min. Scale bar, 5 μm .
- c,** Quantification of MATCAP signal intensity normalized to the total tubulin signal in **b**. Control, $n = 23$; 1,6-HD, $n = 26$; n represents the number of cells pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Statistical significance was assessed by two-sided unpaired Student's t -test. **** $P < 0.0001$.
- d,** Top, Schematic of protein fusions used for the in vitro phase separation assay. Bottom, phase separation of 5 μM Alexa Fluor 488-labeled full-length and truncated MATCAP proteins induced in HEPES buffer (pH 7.4) containing 150 mM KCl following cleavage of the N-terminal His-MBP tag by PreScission protease. Scale bar, 10 μm .
- e,** Representative images of 5 μM Alexa Fluor 488-labeled MATCAP droplet fusion in HEPES buffer as described in **d**. Time shown in minutes. Scale bar, 2 μm .
- f,** FRAP analysis of 5 μM Alexa Fluor 488-labeled MATCAP droplets in HEPES buffer as described in **d**. Upper, time-lapse images from the FRAP experiment. Scale bar, 2 μm . Lower, fluorescence recovery curve after photobleaching inside droplets over time. Data are presented as mean $\pm$ s.e.m. at each timepoint from three independent repeats.
- g,** In vitro phase separation of 5 μM Alexa Fluor 488-labeled MATCAP as described in **d**. Subsequent addition of 1,6-hexanediol disrupted the condensates. Scale bar, 10 μm .

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- h,** Statistical analysis of MATCAP droplet areas in **g**. For Control, $n = 79$; 1,6-Hexanediol, $n = 98$; n represents the number of MATCAP droplets pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Two-sided unpaired Student's t -test. **** $P < 0.0001$.
- i,** Summary of in vitro phase separation behavior of Alexa Fluor 488-labeled MATCAP in HEPES buffer (pH 7.4). Red circles indicate phase separation (above saturation concentration); blue circles indicate no phase separation (below saturation concentration).
- j,** LLPS diagram of the Alexa Fluor 488-labeled MATCAP ^{Δ IDR} mutant as in **i**.

Fig. 4 MATCAP condensation facilitates MT binding and detyrosinase activity

- a,** Schematic annotation of chimera design. The N-terminal 1–137 aa (IDR) of MATCAP were replaced with exogenous domains containing distinct features.
- b,** In vitro phase separation of 5 μ M Alexa Fluor 488-labeled His-MBP-MATCAP variants (MATCAP, MATCAP ^{Δ IDR}, MATCAP ^{Δ IDR}-LEM2^{IDR}, MATCAP ^{Δ IDR}-Tau^{PRD}, MATCAP ^{Δ IDR}-EB1^{IDR} or MATCAP ^{Δ IDR}-EB1^{IDR}-KR6Q) following cleavage of the N-terminal His-MBP tag by PreScission protease in HEPES buffer (pH 7.4) containing 150 mM KCl. Scale bar, 10 μ m.
- c,** Immunofluorescence images of U2OS cells expressing GFP vector or GFP-MATCAP constructs (wild type, IDR-deleted truncation, or engineered chimeras). Cells were stained for detyrosinated and total α -tubulin. DNA was stained with DAPI. Scale bar, 10 μ m.
- d,** Quantification of the detyrosination signal normalized to total tubulin in spindles expressing GFP-MATCAP constructs as described in **c**. n (cells) = 19 (MATCAP), 15 (MATCAP ^{Δ IDR}), 20 (MATCAP ^{Δ IDR}-LEM2^{IDR}), 18 (MATCAP ^{Δ IDR}-Tau^{PRD}), 16 (MATCAP ^{Δ IDR}-EB1^{IDR}), 18 (MATCAP ^{Δ IDR}-EB1^{IDR}-KR6Q); pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons. NS, not significant; * P = 0.0165; **** P < 0.0001.
- e,** Representative real-time imaging chromosome movements in U2OS cells stably expressing mCherry-H2B and transfected with MATCAP shRNA plus GFP-MATCAP constructs (MATCAP, MATCAP ^{Δ IDR}-EB1^{IDR}, MATCAP ^{Δ IDR}-EB1^{IDR}-KR6Q). Time in minutes. White arrowheads indicate misaligned chromosomes. Scale bar, 10 μ m.
- f,** Examination of tubulin detyrosination in cells expressing MATCAP chimeric variants (MATCAP, MATCAP ^{Δ IDR}-EB1^{IDR}, MATCAP ^{Δ IDR}-EB1^{IDR}-KR6Q) after depletion of endogenous MATCAP. IB, immunoblot.
- g,** Statistical analysis of the percentage of cells with chromosome misalignment defects in **e**. n (cells) = 30 (MATCAP), 36 (MATCAP ^{Δ IDR}-EB1^{IDR}), 38 (MATCAP ^{Δ IDR}-EB1^{IDR}-KR6Q); pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. **** P < 0.0001; *** P = 0.0005; * P = 0.0287.

Fig. 5 The N-terminal aromatic and charged residue clusters govern MATCAP phase separation

- a,** NMR titration experiments showing weak multivalent interactions among MATCAP. The horizontal axis represents the ^{15}N -labeled IDR domain. HSQC peak intensity changes of IDR in the presence of IDR (IDR versus IDR), indicating the intermolecular weak multivalent interactions between IDR-IDR domain. Asterisk denotes prolines. The error bars were calculated as in Methods.
- b,** NMR titration experiments showing weak multivalent interactions among MATCAP. The horizontal axis represents the ^{15}N -labeled IDR domain. HSQC peak intensity changes of IDR in the presence of the carboxypeptidase domain (IDR versus MATCAP $^{\Delta\text{IDR}}$), indicating the interaction between IDR and the carboxypeptidase domain. Asterisk denotes prolines. The error bars were calculated as in Methods.
- c,** Schematic summary of interactions among different MATCAP regions. Residues within the IDR that potentially mediate multivalent interactions were indicated.
- d,** In vitro phase separation of MATCAP $^{\text{WT}}$ or MATCAP $^{8\text{A}}$ at the indicated protein concentrations. Scale bar, 10 μm .
- e,** Summary of in vitro phase separation behavior of Alexa Fluor 488-labeled MATCAP $^{8\text{A}}$. Red circles indicate phase separation (above saturation concentration); blue circles indicate no phase separation (below saturation concentration).
- f,** Ultra-coarse-grained modelling analyses indicate cluster formation of MATCAP $^{\text{WT}}$ and MATCAP $^{8\text{A}}$.
- g,** The phase separation diagram of MATCAP $^{\text{WT}}$ and MATCAP $^{8\text{A}}$ judged by ultra-coarse-grained modelling.
- h,** Quantitative analyses of the intra- and intermolecular interactions of MATCAP $^{\text{WT}}$ and MATCAP $^{8\text{A}}$ based on ultra-coarse-grained computational modelling. For each group, $n = 30$ MATCAP molecules pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary two-way ANOVA followed by Bonferroni's multiple comparisons test was used to determine statistical significance. **** $P < 0.0001$, ** $P = 0.002$.

Fig. 6 MATCAP condensation ensures accurate chromosome alignment

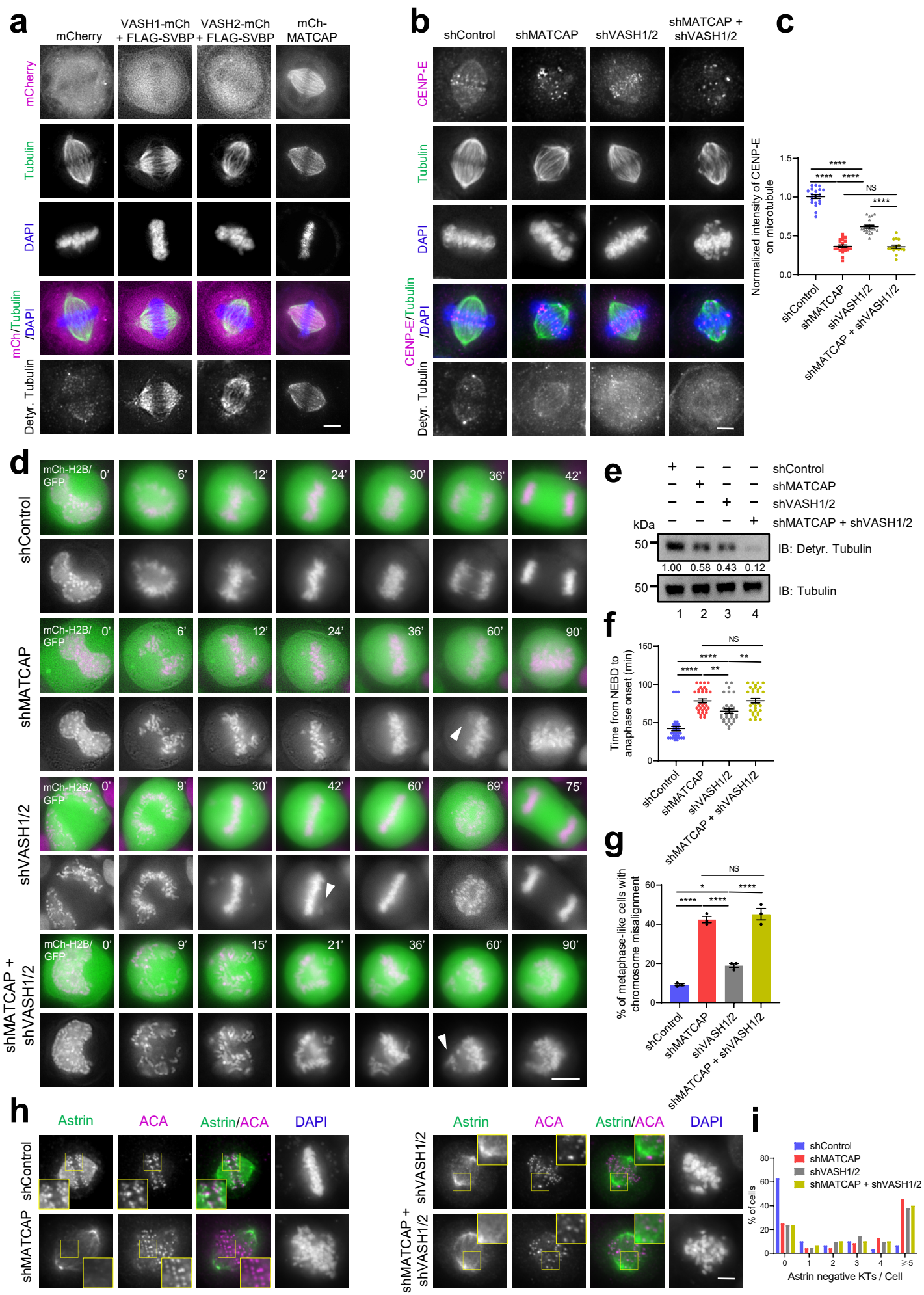
- a**, Representative real-time images showing chromosome movements in U2OS cells stably expressing mCherry-H2B and transiently transfected with MATCAP shRNA and GFP-MATCAP constructs. Time in minutes. White arrowheads indicate misaligned chromosomes. Scale bar, 10 μ m.
- b**, Statistical analysis of the percentage of cells with chromosome misalignment defects from **a**. n (cells) = 51 (MATCAP), 58 (MATCAP ^{Δ IDR}), 53 (MATCAP^{8A}); pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. *** P = 0.0004; ** P = 0.007; * P = 0.0303.
- c**, Immunofluorescence staining of Astrin and centromeres (ACA) in U2OS cells expressing FLAG-MATCAP or mutants after depletion of endogenous MATCAP. DNA was counterstained with DAPI. Scale bar, 5 μ m.
- d**, Quantification of the percentage of cells with Astrin-negative kinetochores (KTs) per cell as described in **c**. n (cells) = 27 (MATCAP), 23 (MATCAP ^{Δ IDR}), 18 (MATCAP^{8A}); pooled from three independent experiments.
- e**, Immunofluorescence staining of CENP-E, detyrosinated tubulin and total α -tubulin in U2OS cells expressing FLAG-MATCAP or mutants in the absence of endogenous MATCAP. DNA was stained with DAPI. Scale bar, 5 μ m.
- f**, Quantification of CENP-E intensity on spindles from **e**. n = 15 (MATCAP), 18 (MATCAP ^{Δ IDR}), 16 (MATCAP^{8A}); n represents the number of cells pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. **** P < 0.0001.
- g**, TIRF images showing the localization of Alexa Fluor 594-MATCAP (red) and GFP-CENP-E¹⁻⁴⁸³ (green) on GMPCPP-stabilized Cy5-labeled microtubules (blue). Scale bars, 5 μ m.
- h**, Quantification of CENP-E fluorescence intensity along microtubules as shown in **g**. Data were normalized to the corresponding microtubule signal. For each group, n = 15 microtubules, pooled from three independent experiments. Data represent mean $\pm$ s.e.m. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. NS, not significant; **** P < 0.0001; ** P = 0.0012.

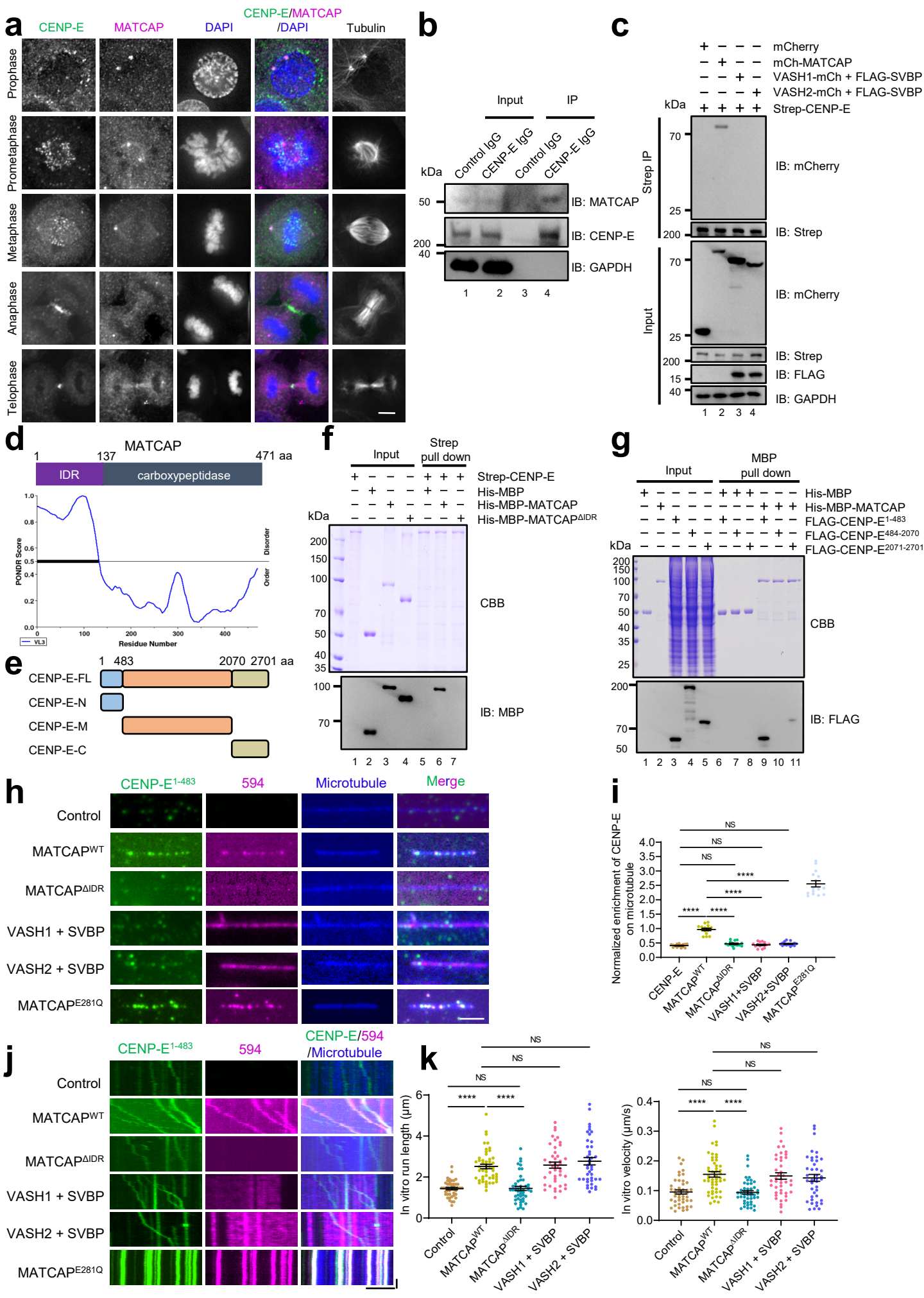
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- i,** Representative fluorescence kymographs showing the movement of CENP-E motor along microtubules in the presence of the indicated MATCAP mutants.

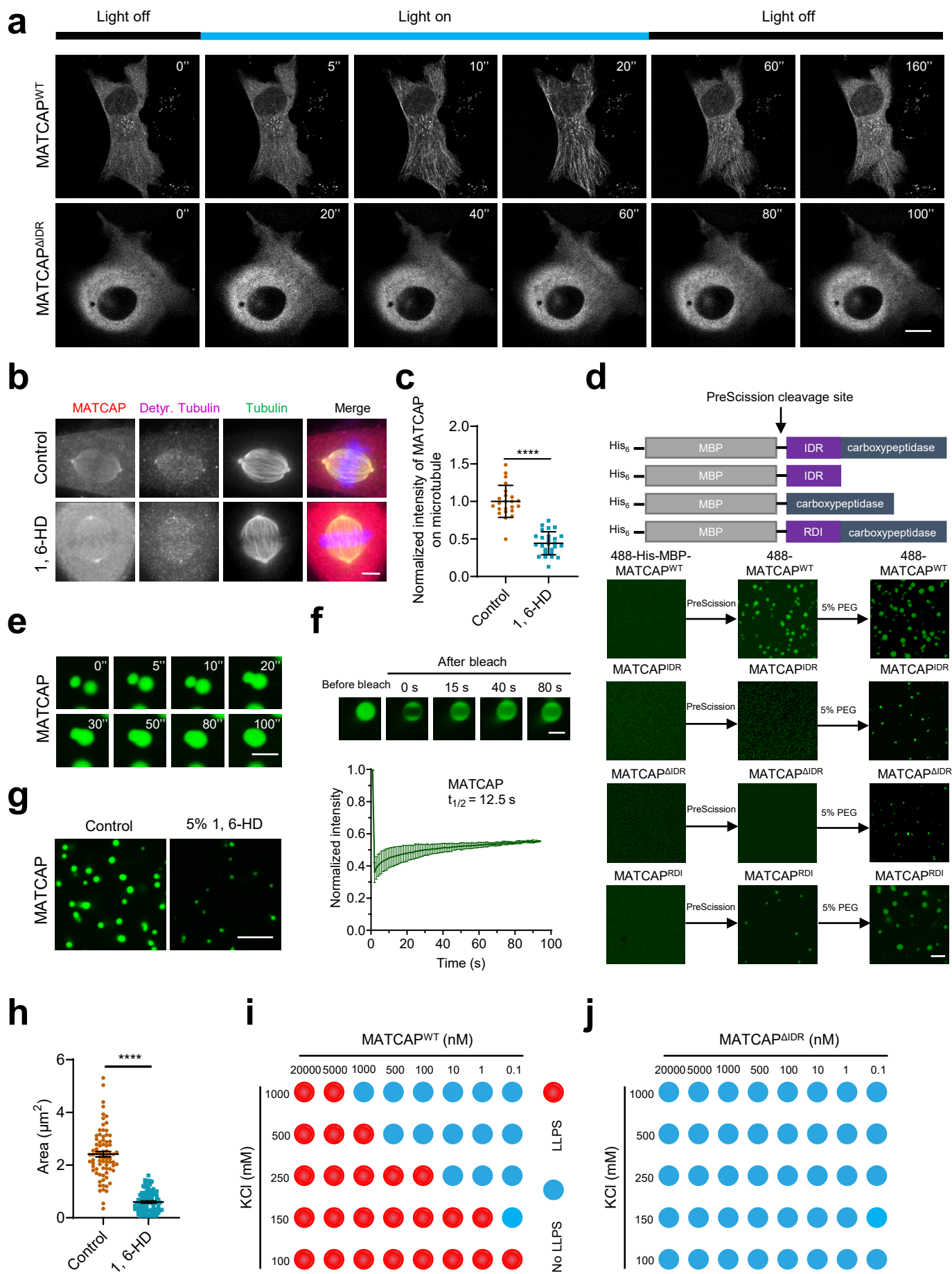
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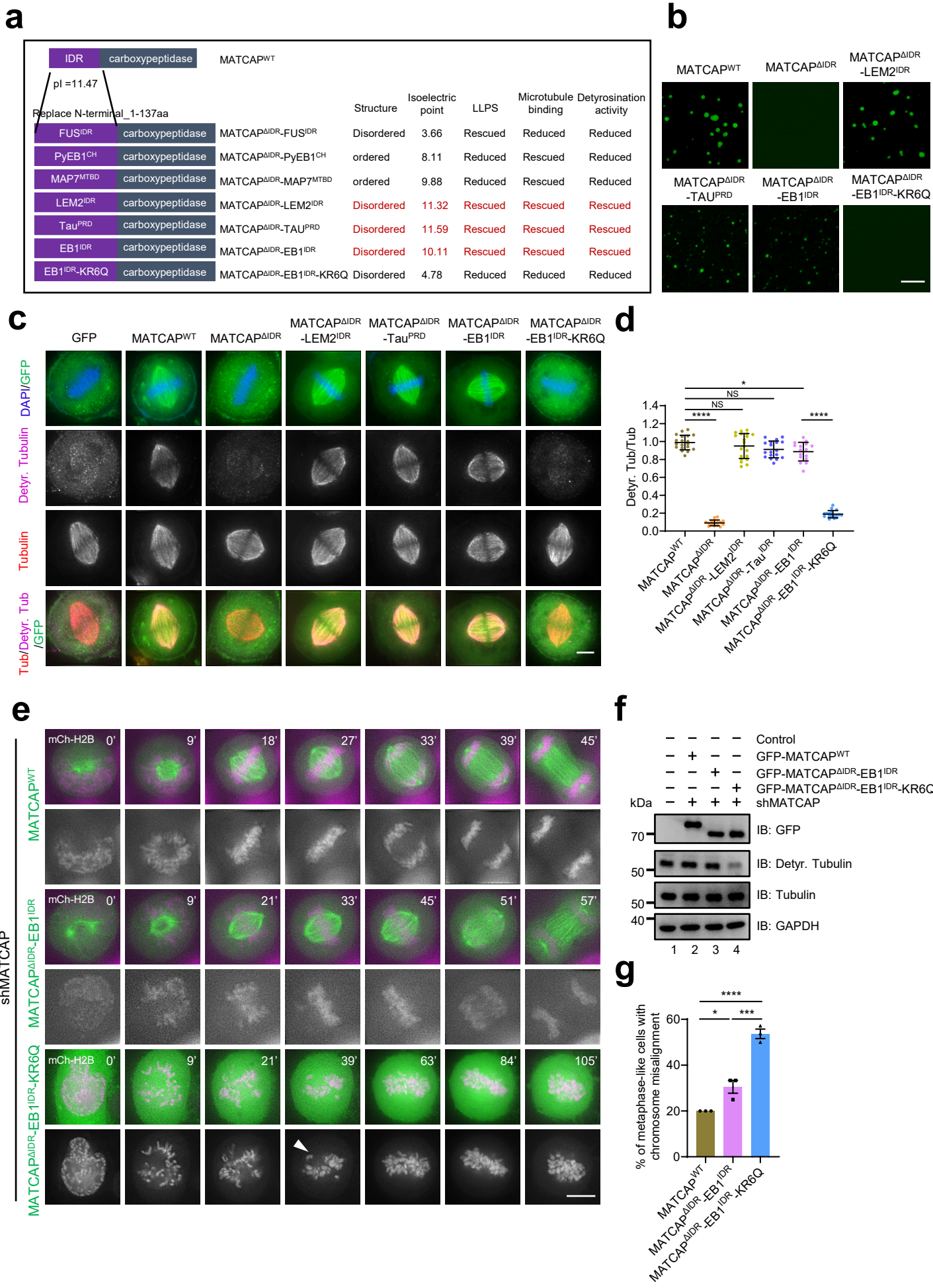
Fig. 7 A working model elucidating that MATCAP condensation regulates chromosome congression

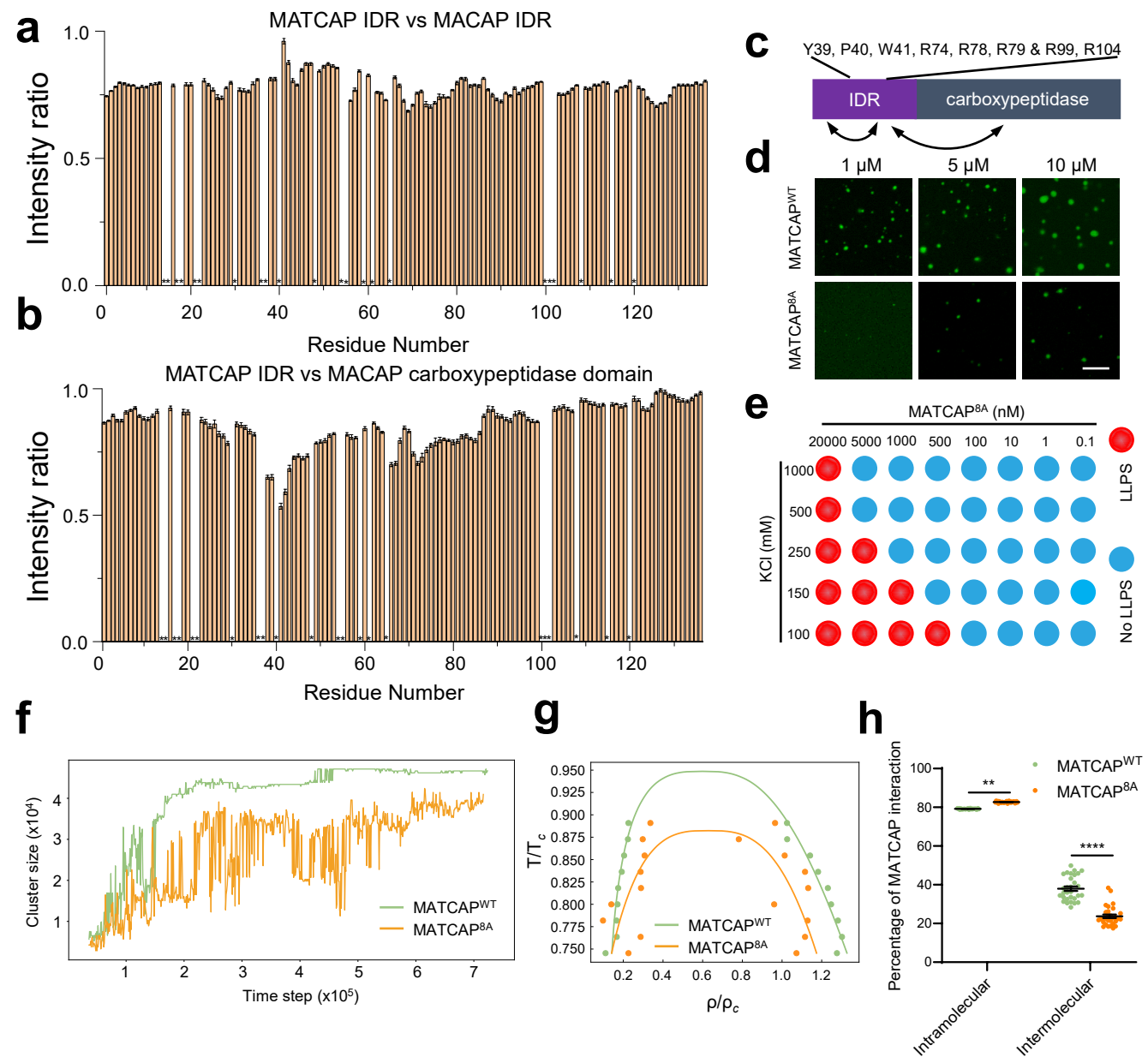
- a,** During mitotic entry, MATCAP forms condensates on microtubules via N-terminal IDR, serving as a hub that concentrates tubulin and CENP-E. MATCAP condensation promotes microtubule detyrosination. As the cell cycle progresses, CENP-E-driven chromosome congression leads to chromosome alignment at the metaphase spindle. Illustrations created in BioRender. TT, Y. (2026) <https://BioRender.com/csfruui>.
- b,** Disruption of MATCAP condensate formation reduces its microtubule association, decreases CENP-E recruitment and microtubule detyrosination, and results in defective chromosome congression. Illustrations created in BioRender. TT, Y. (2026) <https://BioRender.com/csfruui>.

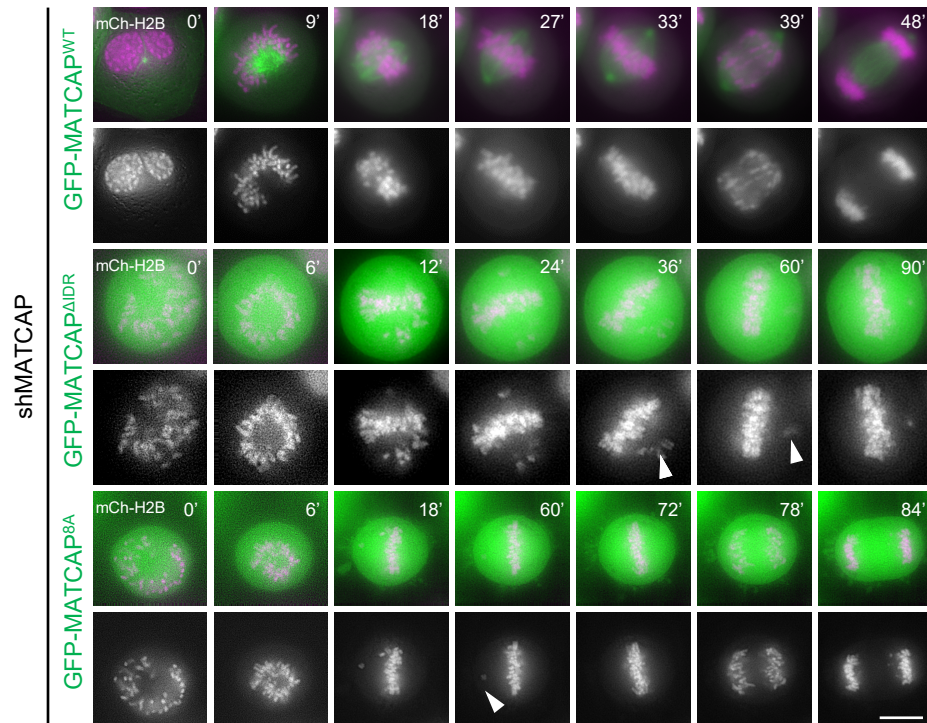
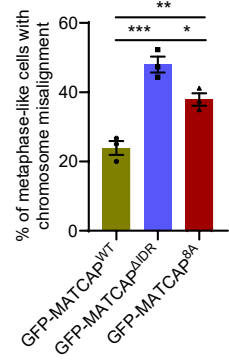
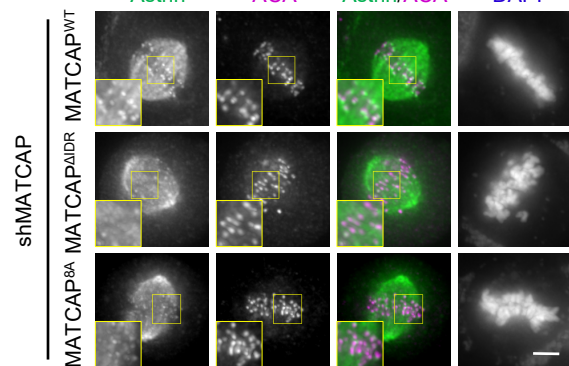
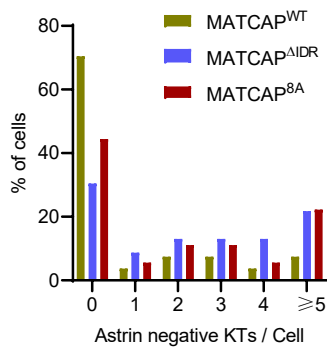
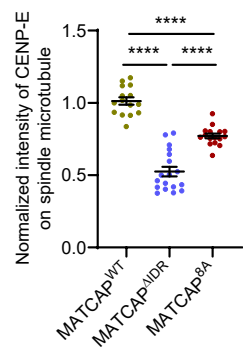
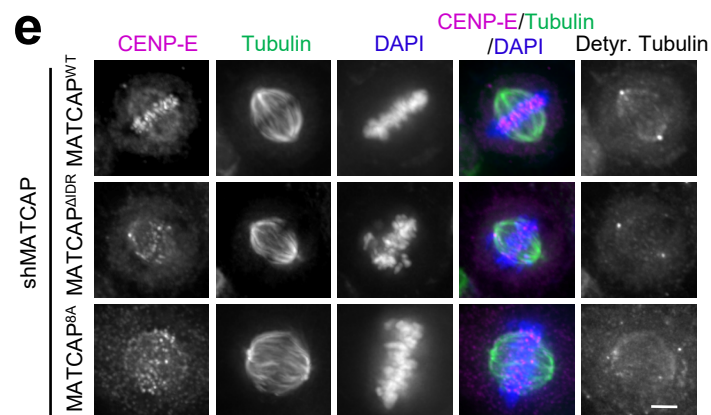
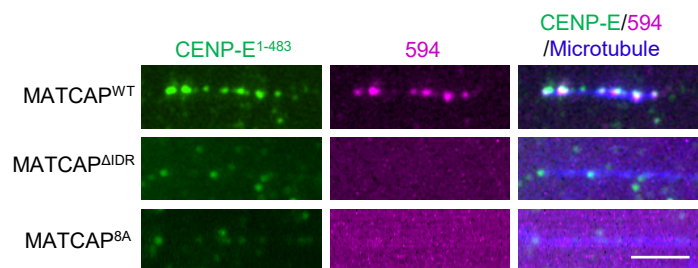
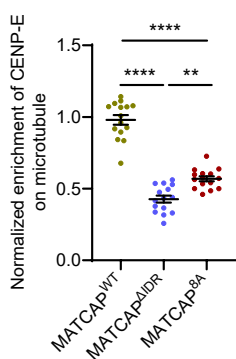
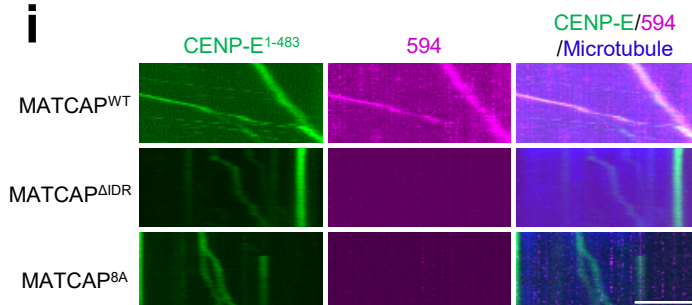


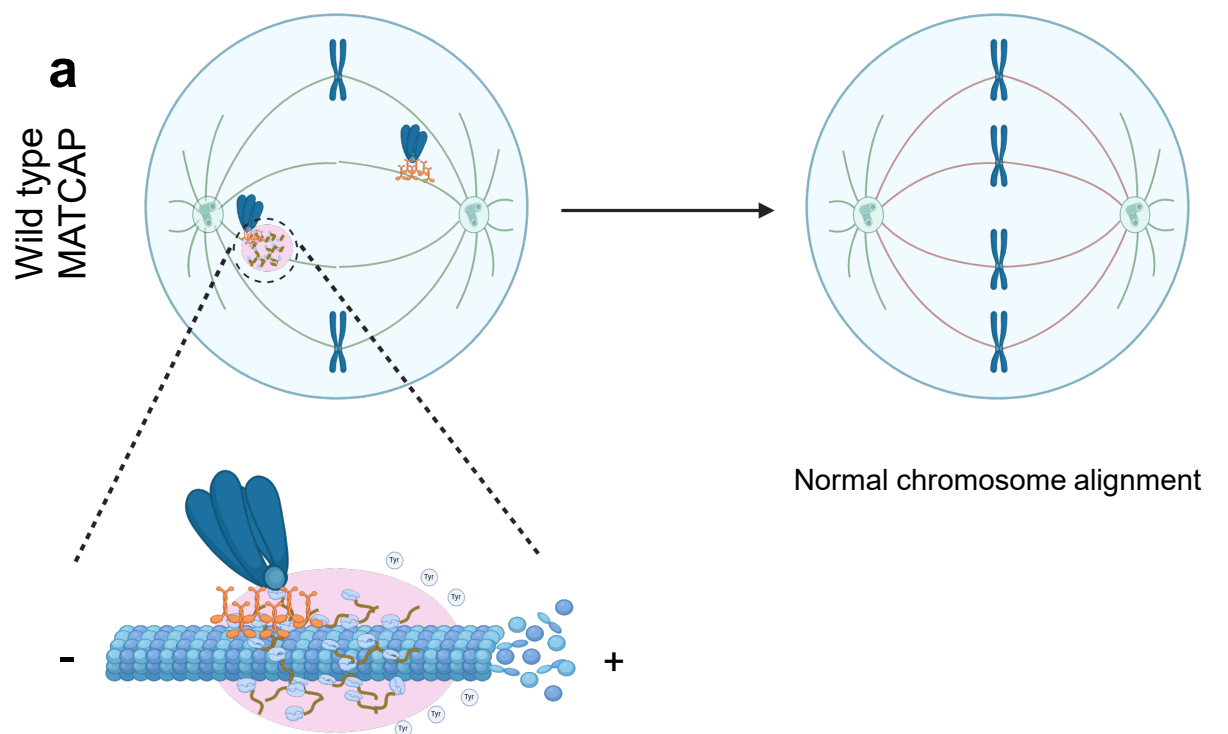




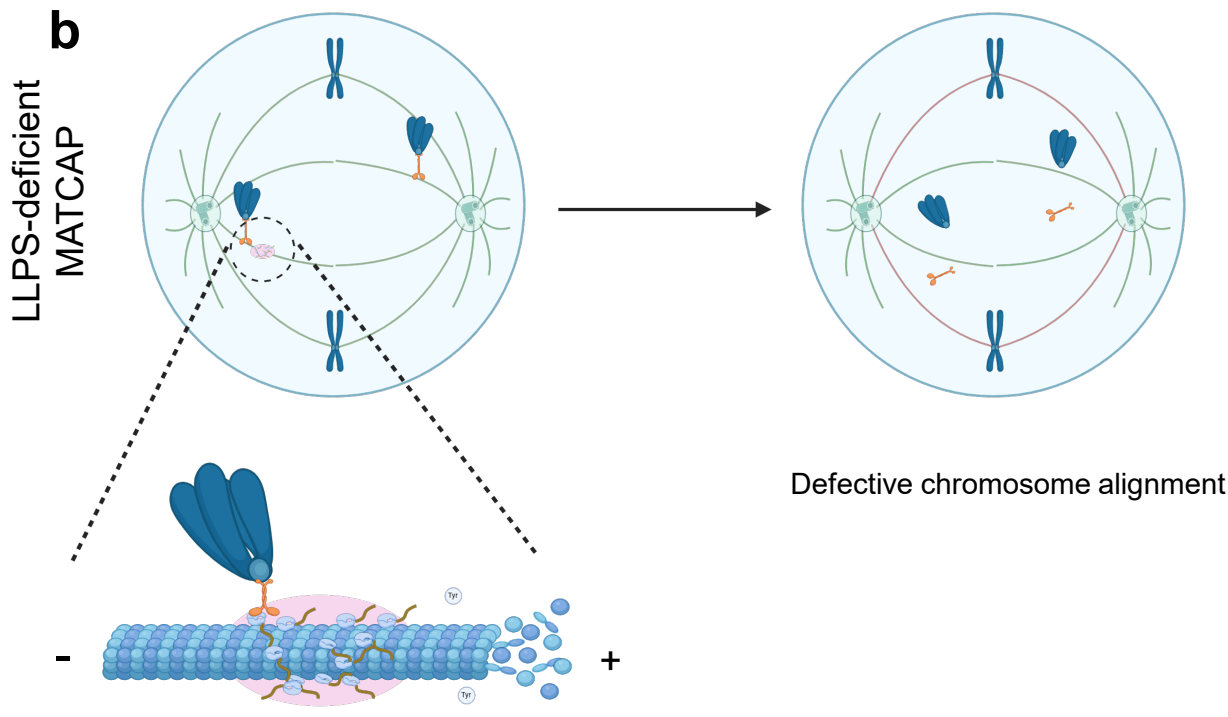




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Coordinated MATCAP condensate formation
CENP-E binds to MTs and walks processively



Attenuated MATCAP condensate formation
CENP-E detaches from MTs and walks less processively

