Phase separation of +TIP-networks regulates microtubule dynamics

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SUMMARY

Regulation of microtubule dynamics is essential for diverse cellular functions, and proteins that bind to dynamic microtubule ends can regulate network dynamics. Here we show, that two conserved microtubule end-binding proteins, CLIP-170 and EB3, undergo phase separation and form dense liquid-networks. When CLIP-170 and EB3 act together the multivalency of the network increases, which synergistically increases the amount of protein in the dense phase. *In vitro* and *in cells* these liquid networks can condense tubulin. *In vitro* in the presence of microtubules, EB3/CLIP-170 phase separation can co-condense tubulin all along the microtubule. At this condition microtubule growth speed increases up to two-fold and depolymerization events are strongly reduced, compared to conditions with phase separation deficient networks. Our data show that phase separated EB3/CLIP-170 networks impact microtubule growth dynamics beyond direct protein-microtubule interactions.

Keywords: Microtubule, CLIP-170, EB3, microtubule dynamics, tubulin condensation, in vitro, liquid-liquid phase separation

Introduction

The microtubule cytoskeleton engages in a plethora of cellular processes, from organelle transport to cell division. To do so, the network dynamically modifies its structure in response to external cues and adapts its architecture to specific cellular functions. Microtubules themselves are highly dynamic polymers that can rapidly cycle between phases of polymerization and depolymerization, a characteristic which is critical for cytoskeletal re-organization (reviewed in Brouhard and Rice, 2018). In cells, microtubules polymerize at their plus-end by addition of GTP-tubulin. After GTP-tubulin addition, GTP is gradually hydrolyzed, resulting in a GDP-tubulin shaft behind the tip. Once the stabilizing GTP-tubulin "cap" disappears from the plus-end, the microtubule switches from growing to shrinking, an event termed catastrophe (Howard and Hyman, 2009; Brouhard et al., 2015; Gudimchuk and McIntosh, 2021). Conversely, microtubules can stop shrinking and switch to regrowth, an event termed rescue. The balance between growth and shrinkage is

intimately linked to the addition of free tubulin to the growing microtubule (Walker et al., 1991; Voter et al., 1991).

In addition to these intrinsic modes of regulation, microtubule dynamics can be fine-tuned by Plus Tip-Interacting Proteins (+TIPs) (reviewed in Akhmanova and Steinmetz, 2010). +TIPs are functionally independent and structurally diverse microtubule regulators that concentrate at growing microtubule ends while exhibiting a weak affinity for the microtubule shaft. It is generally accepted that the unique localization of +TIPs at microtubule ends results from their specific binding to the GTP cap (Akhmanova and Steinmetz, 2015). A combination of *in vivo, in vitro* and *in silico* work has indicated that this cap size is 1-60 tubulin layers, indicating a length of < 500 nm (Voter et al., 1991; Walker et al., 1991; Drechsel and Kirschner, 1994; Schek et al., 2007, Seeptapun et al. 2012; Rickman et al., 2017). Puzzlingly, in cells +TIP networks bind to a region at the microtubule end up to 4-fold longer than this, with network lengths up to 2 µm (Komarova et al. 2009; Seepatun et al., 2012; Roth et al. 2018). The discrepancies between +TIP network profile and the size of the GTP-cap prompts the question of whether networks densities and organizational properties could impact network extension.

Key integrators of +TIP-networks are the End-Binding proteins (EBs), as they autonomously bind to GTP-tubulin at growing microtubule ends and recruit a battery of non-autonomously binding +TIPs (reviewed in Galjart, 2010). Within the EB family, higher eukaryotes express three proteins termed EB1, EB2, and EB3. These proteins increase microtubule plus-end dynamics by promoting catastrophes and increasing growth speed *in vitro* (Bieling et al., 2008; Vitre et al., 2008; Komorova et al., 2009; Gouveia et al., 2010). Interestingly, extensive measurements have shown that in vitro ~64 EB1 bind to the microtubule tip (Rickman et al., 2017), while in cells ~270 EB1 can be found within the +TIP network (Seetapun et al, 2012). What causes this non-stoichiometric binding of EBs in cells is not known.

A key accessory protein recruited to plus ends by the EBs is the Cytoplasmic Linker Protein of 170 kDa (CLIP-170), which increases microtubule rescue frequency and growth speeds (Perez et al., 1999; Arnal et al., 2004; Bieling et al., 2007; Bieling et al., 2008; Komorova et al., 2002, 2005). CLIP-170 consists of a microtubule binding "head" domain in its N-terminus, followed by a central coiled-coil region and a zinc-knuckle domain, hereafter referred to as the "C-terminal region" (Pierre et al., 1992; Pierre et al., 1994; Diamantopolous et al., 1999). Most studies of CLIP-170 function *in vitro* have focused on truncated versions, containing only the monomeric head domain (H1) or the head domain with a small extension that allows dimerization (H2) (Figure 1A); thus, it is unclear how full-length CLIP-170 contributes to microtubule dynamics. Early studies of CLIP-170 expression in cells revealed that in addition to its microtubule plus-end localization, it also formed cytoplasmic "patches" that co-localized with EB1 and the dynein-activating protein dynactin (Pierre et al., 1994; Goodson et al., 2003). Based on the physical properties of these CLIP-170 patches, it has recently been suggested that they form by liquid-liquid phase separation (LLPS) (Wu et al., 2021; Jijumon et al., 2022).

LLPS is the process by which molecules spontaneously condense into droplets and de-mix from their surrounding solution, resulting in the co-existence of two unique liquid phases (reviewed in Boeynaems et al., 2018; Hyman et al., 2014; Shin and Brangwynne, 2017). Recently, LLPS has been implicated in driving microtubule-related processes, including spindle assembly (Zhang et al., 2015), nucleation of acentrosomal and branched microtubules (Hernández-Vega et al., 2017; King and Petry, 2020), and centrosome maturation (Woodruff et al., 2017; Jiang et al., 2021). A key shared feature of these processes is the co-condensation of tubulin with LLPS-potent microtubule-associated proteins to catalyze biochemical reactions. Along these lines, condensation of a microtubule +TIP-network has been proposed based on transient multivalent interactions between the +TIPs (Akhmanova and Steinmetz, 2015; Wu et al., 2021). Despite this, LLPS of +TIPs and its function is still unclear.

In this study, we use tandem in-cell and *in vitro* approaches to investigate +TIP condensation and their function. We provide evidence that +TIP networks in cells may have liquid properties. We demonstrate that the +TIPs EB3 and CLIP-170 undergo LLPS at nanomolar concentrations in the absence of crowding agents. The EB3 and CLIP-170-containing droplets co-condense tubulin, and depend on protein regions promoting multivalent interactions. In the presence of microtubules, EB3/CLIP-170 promote rapid growth speeds while reducing catastrophe and pausing frequencies at the microtubule plus-end. Taken together, our results suggest a mechanism whereby +TIP LLPS plays a role in regulating microtubule dynamics.

Results

+TIP-networks exhibit properties reminiscent of phase separation

Overexpressed EB3 and CLIP exhibit atypical network formation at the growing microtubule tip, leaving trailing protein "remnants" behind the leading network edge (Mustyatsa et al., 2019; Henrie et al., 2020). One explanation for these remnants is that they form by binding to tubulin "islands" remaining in the GTP-state behind the GTP-cap (Perez et al., 1999; de Forges et al., 2016; Henrie et al., 2020). As overexpressed CLIP-170 has the potential to undergo LLPS (Wu et al., 2021; Jijumon et al., 2022), another possibility would be that CLIP-remnants split off from the leading network in a process resembling fission of condensates. Having these two possibilities in mind, we revisited these experiments by overexpressing GFP-CLIP-170 in RPE-1 cells. Analysis of plus-end fluorescence intensity profiles in cells revealed that 80 % of the GFP-CLIP-170 networks left behind remnants (Figures 1B, C). Dynamic analysis of CLIP networks revealed that the protein remnants remain bound to the microtubule shaft dissolved over time (Figure 1D and Movie 1). We next probed the material properties of +TIP-networks by treating cells with the aliphatic alcohol 1,6-hexanediol to reduce hydrophobic interactions (Kroschwald et al., 2017). While untreated cells exhibited 3 µm long +TIP-networks, upon hexanediol treatment, the profile of the +TIP-network reduced to ~1.5 µm foci (Figures S1A, B and Movie 2), indicating that hydrophobic interactions influence +TIP network organization.

To rule out that the observed remnant formation is not an artifact of overexpression, we measured plus-end fluorescence intensity profiles of endogenous CLIP-170. In line with overexpression studies, we observed that for endogenous CLIP-170, ~70% of the +TIP-networks had remnants (Figures 1B, C). EB3, another prominent +TIP, followed a comparable fluorescence profile, with ~75% of the endogenous EB3 networks having remnants (Figures 1B, C), consistent with a recent overexpression-based study (Mustyatsa et al., 2019). Co-staining of EB3 and CLIP-170 revealed that most networks displayed fluorescence profiles with distinct EB3 and CLIP-170 remnant co-localization (Figure 1F).

When we overexpressed the CLIP-170 truncated H2- and H1-mutants (Figure 1A), both tracked the growing GTP-microtubule tip, as previously reported (Figure 1E) (Komarova et al., 2002; Folker et al., 2005). However, the presence of remnants behind the leading edge of the H2- and H1- networks was dramatically reduced compared to FL-CLIP (Figure 1C). This suggests that the C-terminal region of CLIP influences network organization at microtubule ends. Since H1- and H2-CLIP retain binding to the growing GTP-tip, this result implies that remnant formation cannot be solely explained by the presence of GTP-islands in the shaft and might also result from material properties of the multivalent +TIP-network.

CLIP-170 forms biomolecular condensates in cells

In addition to the characteristic comet profiles, previous studies have reported that CLIP-170 overexpression leads to cytosolic “patches” that may resemble biomolecular condensates (Pierre et al., 1994; Goodson et al., 2003; Wu et al., 2021; Jijumon et al., 2022). Indeed, overexpression of mCherry-CLIP-170 in CRISPR/Cas9 knock-in GFP-tubulin RPE-1 cells resulted in cytosolic CLIP-170 patch formation in addition to microtubule plus-end tracking (Figure 2A). These patches displayed liquid properties, undergoing fusion and fission within 5 sec (Figure 2B and Movies 3 , 4). Fluorescence recovery after photobleaching (FRAP) revealed that CLIP-170 diffuses highly within these patches and showed a high protein exchange rate with the pool outside the patch, with a recovery half-life and mobile fraction of 27.5 seconds and 93 ± 32 %, respectively (Figure 2C and Movie 5). These properties lead us to conclude that CLIP-170 patches are biomolecular condensates in cells, hereafter called droplets.

CLIP-170 forms biomolecular condensates in vitro

To address whether CLIP-170 alone undergoes phase separation, we purified recombinant full-length GFP-CLIP-170 (FL-CLIP) from insect cells and reconstituted its phase separation properties *in vitro*. In the absence of other proteins, FL-CLIP condensed into spheres at concentrations as low as 100 nM (Figure 2D, upper panel). To estimate the amount of FL-CLIP in the dense phase we used a high throughput confocal microscopy-based approach. Briefly, i) protein mixtures were incubated in 384-well plates, ii) proteins in the dense phase were centrifuged onto the bottom of the wells, iii) wells were imaged using an automated confocal microscope, and iv) surface coverage of protein in the dense phase on well-bottoms was measured (Figure S2A; see

Methods for details). A hallmark property of LLPS-potent proteins is that they condense in a concentration-dependent manner and in response to the presence of crowding agents (Alberti et al., 2018). In line with this, the sphere size and amount of FL-CLIP in the dense phase increased in a concentration-dependent manner even in the absence of crowding agents (Figures 2D and S2B). Addition of a crowding agent, 2 % polyethylene glycol (PEG), increased the amount of FL-CLIP in the dense phase by 1.5-fold but reduced sphere size (Figures S2C, D).

In line with the case in cells, FL-CLIP spheres displayed liquid properties such as fusion, although 5-times slower than what we observed in cells (Figure 2E and Movie 6). We further interrogated the liquid properties of these spheres by FRAP, which confirmed that FL-CLIP diffuses within the spheres with a half-life of 27.5 s, identical to the recovery speed in cells. However, FL-CLIP exchange dynamics were reduced three-fold with a mobile fraction of 28 ± 12% (Figure 2F and Movie 7). Collectively, these results show that FL-CLIP undergoes LLPS and forms droplets *in vitro* at nanomolar concentration and in the absence of further proteins or crowding agents. We hypothesize that the discrepancies between the material properties of the droplets in cells and *in vitro* are due to the presence of additional proteins and/or CLIP-170 post-translational modifications in the cell droplets.

The CLIP-170 C-terminal region drives CLIP-170 into the dense phase

We next investigated which domains of CLIP-170 drive droplet formation. We expressed GFP-tagged H2- and H1-CLIP mutants in RPE-1 cells where the coiled-coil domain is truncated or fully removed (Figure 1A) (Pierre et al., 1992, 1994; Diamantopoulos et al., 1999; Goodson et al., 2003). In line with previous observations, we saw that H1 and H2 displayed microtubule plus-end tracking activity in cells, but did not form any cytosolic droplets even at high expression levels (Figure 3A and Movies 8, 9) (Pierre et al., 1994; Goodson et al., 2003). To understand to which extent the C-terminal region is necessary for CLIP-170 to undergo LLPS, we purified recombinant human H1 and H2 in bacteria and measured their ability to condens *in vitro* at nanomolar concentrations. While FL-CLIP phase separated in the absence of other factors, H1 showed faint irregular-shaped aggregation but no droplet formation, even in presence of 10 % PEG (Figures 3B, C and S3B, C). In contrast to the case in cells, at nanomolar concentration H2 underwent condensation *in vitro*, although with a 300-fold reduction of protein in the dense phase and a 25-fold reduction in droplet size compared to FL-CLIP (Figures 3B, D and S3A). Addition of 2 % PEG increased H2 condensate formation by 3-fold but did not affect condensate size (Figures 3B, C and S3B, C). These results show that while the monomeric H1 is sufficient to track the growing microtubule tip, the dimeric form of CLIP-170 is necessary to undergo LLPS and that the C-terminal region robustly drives CLIP-170 condensation.

EB3 undergoes LLPS and co-condenses with CLIP-170

CLIP-170 requires the presence of EBs to localize to growing microtubule ends (Dixit et al., 2009, Bieling et al., 2008). This prompted us to ask whether purified FL-CLIP and EB3 could co-

condense *in vitro*. We first studied EB3 alone and observed that it has the capacity to undergo LLPS at micromolar concentrations: compared to 48 ± 17 μm^2 large droplets observed for 400 nM FL-CLIP, at 1 µM EB3 phase separated into many small 1.9 ± 0.6 μm^2 droplets, resulting in a 3.5-fold reduced surface coverage (Figures 4A and S4A). Both the droplet size and EB3 fraction in the dense phase were comparable between 0 and 2 % PEG (Figure 4A and S4A).

Given their individual propensities for condensation, we hypothesized that a multivalent EB3/FL-CLIP network may robustly drive phase separation even further. Indeed, EB3/FL-CLIP networks formed droplets that covered the surface 2.5-fold higher than FL-CLIP droplets alone (Figure 4B). Importantly, EB3/FL-CLIP networks had a synergistic effect on droplet formation, increasing the surface coverage by 40% when compared to the sum of the surface coverages of EB3 alone plus FL-CLIP alone (Figure 4C). Repeating the EB3 experiment with H2 reduced surface coverage by 1.5-fold compared to the presence of FL-CLIP, and H1 further decreased surface coverage by 2.5-fold (Figures 4D and S4B). These data demonstrate that CLIP's C-terminal region is essential for a highly multivalent EB3-CLIP network formation. We further show that EB3 and CLIP-170 can undergo LLPS both independently, and that when acting as an ensemble, the amount of proteins in the dense phase synergistically increases.

CLIP-170 and EB3 condense tubulin in vitro

A CLIP-170 dimer has as many as 8 tubulin binding sites, can bind tubulin *in vitro*, and colocalizes with tubulin in cells (Figure 5A) (Pierre et al., 1994 et al., 1999; Perez et al., 1999; Gupta et al., 2010). We therefore asked if CLIP-170 droplets can co-condense tubulin *in vitro*. Tubulin alone did not form droplets at micromolar concentrations; even in the presence of 5% PEG only aggregation was observed (Figure S5A). However, when 200 nM FL-CLIP was mixed with 400 nM Atto561-tubulin, the two proteins phase separated into 26.8 ± 12 μm^2 droplets (Figures 5B and S5B). We repeated these experiments with H1 and found irregular-shaped aggregation with tubulin but no droplet formation (Figure 5B).

We next asked whether EB3 could condense tubulin, based on its intrinsic phase separating capacity (Figure 4A). Indeed, EB3 also co-condensed tubulin, although into much smaller 1 ± 0.07 μm^2 droplets (Figures 5C and S5C). Strikingly, FL-CLIP-droplets enriched tubulin 40-fold more than EB3 droplets, even at 5-fold lower FL-CLIP concentrations (Figure 5C). Based on the multivalency of a EB3/CLIP-170/tubulin network (Figure 5A), we then asked whether FL-CLIP and EB3 could cooperatively increase the amount of tubulin condensation compared to FL-CLIP alone. Interestingly, we observed that EB3/FL-CLIP networks were 4-fold less efficient at condensing tubulin than pure FL-CLIP (Figure 5C). We hypothesize that addition of tubulin to EB3/FL-CLIP droplets partially competes for EB3/FL-CLIP interactions and thereby reduces the valency of the network. Collectively, these results show that FL-CLIP can condense tubulin effectively and that EB3 reduces the tubulin condensation capacity of FL-CLIP droplets.

Tubulin co-condenses with CLIP-170 in cells

Overexpressing GFP-CLIP-170 in RPE-1 cells revealed that CLIP-170 droplets colocalized with areas of high tubulin fluorescent intensity (Figure 5D, top panel) (Perez et al., 1999). However, it was not possible to distinguish if these areas corresponded to microtubule bundles, or if the local increased signal resulted from tubulin condensation. To address this question, we depolymerized the microtubule network with 5 µM nocodazole (Figure 5D, middle panel). After microtubule depolymerization, tubulin showed robust co-localization with CLIP-170 droplets (Figure 5D, middle panel), and tubulin fluorescence intensity was 2.4-fold higher in the droplets compared to the cytoplasm (Figure 5D, graph).

To study if tubulin condensation with CLIP-droplets is due to overexpression, or if CLIP at endogenous concentration could also co-condensate tubulin, we used antibody staining after microtubule network depolymerization. Also in WT cells, small condensates of endogenous tubulin and CLIP-170 co-localized after microtubule network depolymerization (Figure 5D, bottom panel). Contrary to our *in vitro* experiments, H2 and EB3 did not co-condense tubulin in cells (Figure S5D). After depolymerization of the microtubule network EB3 was cytoplasmic with few small condensates, but showed no co-condensation with tubulin (Figure S5D). We conclude that CLIP-170, but not EB3, can co-condense tubulin in cells.

Phase separation-potent +TIP networks increase microtubule growth

To understand if LLPS of +TIPs could impact microtubule tip dynamics, we turned to *in vitro* reconstitution. We first reconstituted microtubule growth in presence of +TIP-networks with either reduced (50 nM H2 + 800 nM EB3) or minimal (50 nM H1 + 800 nM EB3) LLPS activity (Figure 4D), implying reduced or minimal co-condensation of tubulin at the growing tip (Figure 5C). To our knowledge these CLIP mutants preserve all binding domains to interact with EB3 and tubulin, implying that the difference between these networks lies primarily in their phase separation potency. As observed previously, EB3 alone increased microtubule growth speeds by 1.5-fold and catastrophe frequency by 8-fold (Figures 6A, B) (Komorova et al., 2009; Gouveia et al., 2010). Addition of H1 or H2 to the assay with EB3 resulted in tip-tracking behavior by EB3/H1 and EB3/H2 networks, but did not further change any of the dynamic parameters compared to EB3 alone (Figures 6A, B and S6A). These results show that CLIP/EB3 networks with reduced or minimal capacity to condense tubulin have the same impact on microtubule tip dynamics as EB3 alone.

When we repeated the reconstitution experiments with EB3/FL-CLIP, microtubules grew at a speed of 4 µm/min, a two-fold increase compared to analogous experiments with EB3/H2 and EB3/H1, and a four-fold increase compared to controls with tubulin alone (Figures 6A, B and Movie 10). Furthermore, catastrophe events were rarely observed under these conditions (Figures 6A, B), and when they occurred were rapidly followed by rescue events (Figure S6A). To reconstitute this fast microtubule growth in presence of a phase separation deficient EB3/H2 network, we increased the tubulin concentration while keeping the EB3/H2 concentration constant.

We measured microtubule growth speeds and found that a growth speed of 4 µm/min (the speed achieved by EB3/FL-CLIP in the presence of 5 µM tubulin) corresponds to 14 µM tubulin in presence of EB3/H2 (Figures S6B, C). These results show that phase separation-potent EB3/FL-CLIP networks increase the growth rate and reduce the catastrophe events compared to EB3/H2 networks. A phase separated +TIP network might impact the growing microtubule tip due to changes in material properties, or due to condensation of tubulin at the tip, based on our observation that EB3/FL-CLIP can condense tubulin (Figure 5C).

+TIPs undergo LLPS and condense tubulin on microtubules, promoting microtubule growth

To understand if LLPS of EB3/FL-CLIP networks could enrich tubulin on microtubules, we used microtubules as a platform to induce LLPS. A recent study showed that addition of cell lysate containing overexpressed CLIP-170 to microtubules formed CLIP-containing droplets along microtubules (Jijumon et al., 2022). We asked if this droplet formation is due to the crowding environment of the cell lysate, or a general property of CLIP. At 60 mM KCl, we repeated our assay from above to study if purified EB3 together with FL-CLIP could phase separate along microtubules. When we incubated purified CLIP-170 and EB3 with dynamic microtubules in vitro, we found that purified EB3/FL-CLIP formed droplets along the shaft (Figure 6C and Movie 11).

In line with our observations that EB3/FL-CLIP droplets condensed tubulin (Figure 5C), these networks enriched tubulin all along the microtubule and co-condensed tubulin over time into droplets on the shaft (Figures 6C-G). At this condition where tubulin condensed along the microtubule, we observed rapid microtubule growth speeds of 3.6 µm/min and very few catastrophe events or pauses in the growth phase (Figures 6H and S6D). This increase in growth speed and reduction in catastrophe events is in line with our results from above where EB3/CLIP was located at the microtubule tip. When we repeated these experiments with less potent phase separating EB3/H2-networks, we only occasionally observed condensate formation along the microtubule (Figure 6D, left panel). In presence of EB3/H2-networks growth speeds were 2-fold slower than for EB3/FL-CLIP, catastrophe frequencies were increased 7-fold and pauses increased 30-fold (Figure 6H). In the absence of EB3, we did not observe microtubule binding or increased growth speeds for any CLIP constructs (Figures S6E, F). Collectively, these experiments demonstrate that EB3/FL-CLIP networks undergo LLPS on microtubules, can condense tubulin, and drive microtubule growth.

Discussion

Rearrangements of the microtubule network architecture require spatiotemporal regulation of microtubule growth. A large body of work has highlighted the role of +TIPs, that act as microtubule polymerases (such as XMAP215) or increase microtubule tip dynamics (such as the EBs) in promoting these highly regulated changes (Gard and Kirschner, 1987; Srayko et al., 2005; Brouhard et al., 2008; Bieling et al., 2008; Vitre et al., 2008; Zanic et al., 2013; Yang et al., 2016). While these studies showed the impact of +TIPs on microtubule dynamics, it has remained unclear

why there are 4-fold more EBs in cellular +TIP networks than in vitro (Seeptapun et al. 2012; Rickman et al., 2017). LLPS allows for non-stochiometric network formation of proteins based on highly multivalent interactions. We now provide evidence that LLPS of +TIPs could be a mechanism to explain the non-stochiometric formation of the +TIP-network at the growing microtubule tip. This mechanism could be a base to locally recruit and enrich diverse +TIPs.

Here, we establish a new mechanism by which LLPS of +TIPs regulate microtubule growth. Our model of LLPS-driven microtubule growth is based on the following observations: i) the +TIPs EB3 and CLIP-170 have the capacity to undergo LLPS *in vitro* and in cells (Figures 2-4), ii) phase separated EB3/FL-CLIP droplets can condense tubulin (Figure 5), iii) microtubule growth speed is increased and catastrophe frequency reduced when EB3/FL-CLIP undergo phase separation on microtubules (Figure 6E-G), and iv) Tubulin condensed along the microtubule in presence of phase separated EB3/FL-CLIP (Figure 6F). We hypothesize that this tubulin enrichment is a mechanism to increase local tubulin availability at microtubule ends uncoupled from cytoplasmic tubulin concentrations.

Microtubules could serve as a general platform to concentrate proteins locally to such an extent that they the process to undergo LLPS is induced. We hypothesize that the mechanism of LLPS initiation at the growing tip depends on conformational properties of the growing microtubule tip, which leads to differential binding of EB3 to the tip over the shaft (Zhang et al., 2015) and subsequent recruitment of CLIP-170 to form a multivalent network. As LLPS is a concentration dependent process, the local recruitment of EB3 and CLIP could increase their concentration compared to the solution to such an extent that they undergo LLPS. We hypothesize that, upon reaching a critical CLIP-170 concentration, the +TIP-network undergoes LLPS. This +TIP-droplet has then the potential to, e.g. co-condenses tubulin, and drive microtubule growth. Once the GTP-cap is hydrolyzed, the +TIP-droplet dissolves. Whether the stoichiometry between EB3 and CLIP-170 has an impact on the LLPS process, as well as the distinct fluid properties and dynamics of an EB3/CLIP-170 droplet, merit investigation in future work.

Is phase separation a common feature of +TIPs? Studies performed in parallel to our work show that this phenomenon is conserved across evolution: +TIPs in budding yeast, fission yeast, and higher eukaryotes have recently been demonstrated to undergo phase separation (Maan et al., 2021; Meier et al., 2021; Song et al., 2021, Jijumi et al., 2022). Intriguingly, in line with our results, the yeast studies confirmed that the CLIP-170 homolog played a key role in the phase separation process, whereas LLPS potency of EB homologs varied between organisms. The role of different mammalian EB family members in regulating LLPS will be an interesting direction for future studies. The ability of +TIP-networks to phase separate depends on intrinsically disordered regions (Maan et al., 2021; Song et al., 2021) and multivalent interaction modules (Meier et al., 2021), consistent with the observation that these features are highly evolutionarily conserved across

+TIPs (Wu et al., 2021). Further studies will be necessary to investigate whether additional +TIPs contribute to the formation and regulation of +TIP-droplets.

Our work here and recent studies demonstrate that +TIP networks can behave like liquid condensates (Wu et al., 2021; Maan et al., 2021; Meier et al., 2021; Song et al., 2021). This work adds to the growing list of microtubule-related processes that are driven by LLPS and provides an exciting new paradigm for how cells can spatiotemporally control microtubule dynamics through local tubulin concentration (Zhang et al., 2015; Woodruff et al., 2017; Hernández-Vega et al., 2017; King and Petry, 2020; Jiang et al., 2021; Maan et al., 2021; Meier et al., 2021; Song et al., 2021). Interrogating the mechanical properties and composition of +TIP-droplets, as well as studying their regulation throughout the cell cycle, will be exciting avenues for future research.

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Declaration of Interests: The authors declare no competing interests.

Figures 1-6

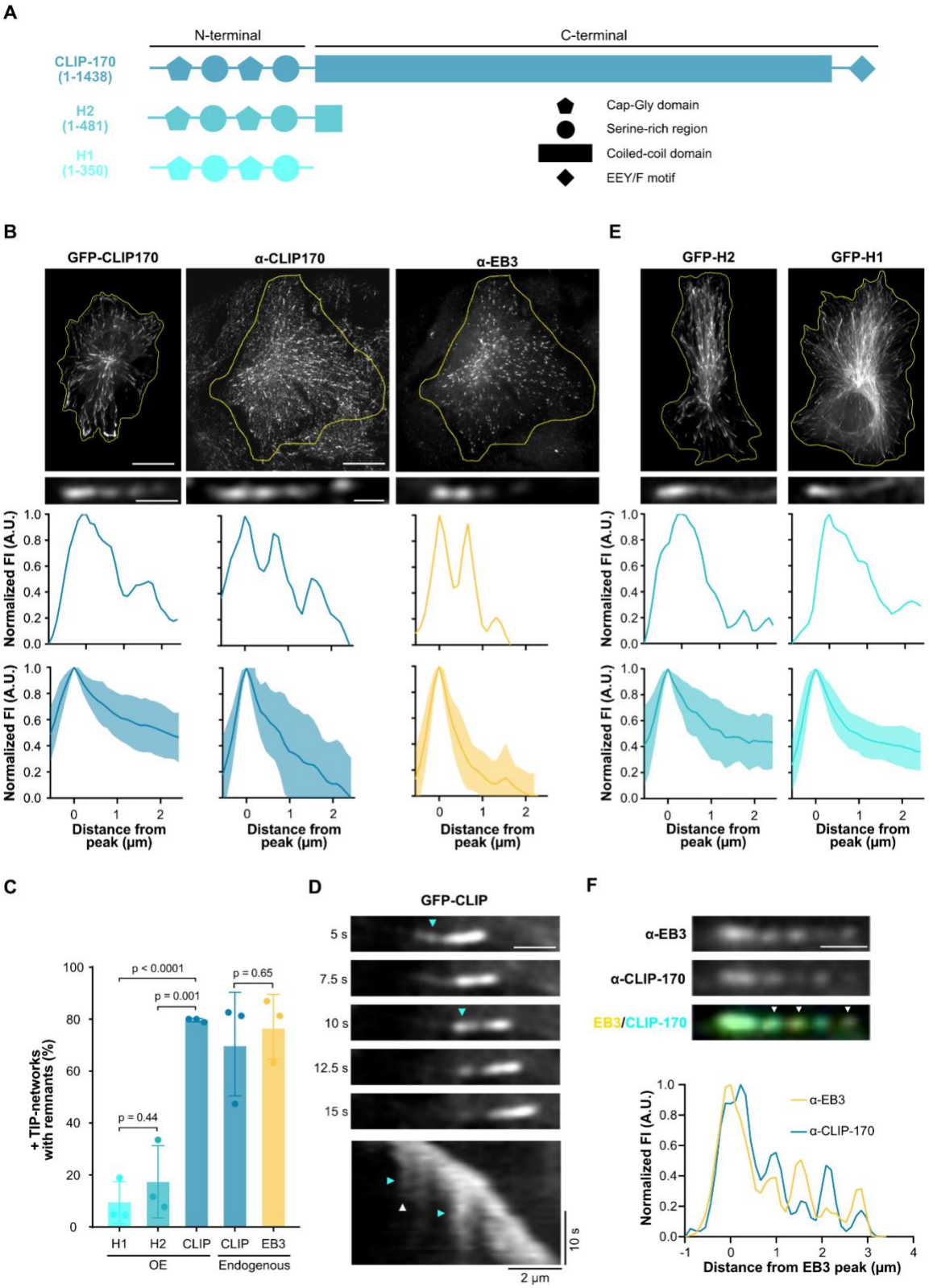

A
N-terminal
C-terminal
CLIP-170 (1-1438)
H2 (1-481)
H1 (1-350)
Cap-Gly domain
Serine-rich region
Coiled-coil domain
EEY/F motif
B
GFP-CLIP170
α-CLIP170
α-EB3
E
GFP-H2
GFP-H1
Normalized FI (A.U.)
Distance from peak (µm)
C
+ TIP-networks with remnants (%)
p < 0.0001
p = 0.001
p = 0.65
p = 0.44
H1
H2
CLIP
CLIP
EB3
OE
Endogenous
D
GFP-CLIP
5 s
7.5 s
10 s
12.5 s
15 s
10 s
2 µm
F
α-EB3
α-CLIP-170
EB3/CLIP-170
Normalized FI (A.U.)
Distance from EB3 peak (µm)

Figure 1: In cells the EB3/CLIP-170 +TIP-network displays liquid properties at microtubule tips. (**A**) Secondary structure of CLIP-170 (1-1438), H2 (1-481) and H1 (1-350) based on (Pierre et al., 1994; Diamantopolous et al., 1999; Goodson et al., 2003). (**B**) Representative images (top) of fixed RPE-1 cells transfected with full length GFP-CLIP-170 and stained with antibodies to endogenous CLIP-170 and EB3. Representative profiles of +TIP-networks with matching fluorescence linescans (bottom). Scale bars: 20 µm whole-cell, 2 µm insets. Below are quantified mean linescan profiles (dark line) with standard deviation (shaded area) from 3 independent experiments with a total of: GFP-CLIP-170 - 57 +TIP-networks from 22 cells; anti-CLIP-170 and anti-EB3 - 58 +TIP-networks from 12 cells for each condition. (**C**) Representative images (top) of fixed RPE-1 cells transfected with GFP-H2 and GFP-H1. Representative profiles of +TIP-networks with matching fluorescence linescans (bottom). Scale bars: 20 µm whole-cell, 2 µm insets. Below are quantified mean linescan profiles (dark line) with standard deviation (shaded area) from 3 independent experiments with a total of: H2 - 33 +TIP-networks from 18 cells; H1 - 47 +TIP-networks from 33 cells. (**D**) Percentage of +TIP-networks with remnants in fixed cells expressing the indicated CLIP constructs or stained with antibodies to endogenous CLIP-170 or EB3. Graph shows mean with SD from 3 independent experiments (cell/+TIP-network n-values denoted in legend from B and C). Statistics: one-way ANOVA test. (**E**) Representative timelapse images (top) and kymograph (below) of +TIP-network from GFP-FL-CLIP expressing RPE-1 cell. Cyan and white arrowheads denote remnants formation and dissolving respectively in both timelapse images and kymograph. Scale bar (2 µm). (**F**) Representative +TIP-network from cells stained for endogenous EB3 and CLIP-170 showing partial co-localization of EB3 and CLIP-170 remnants (white arrow heads) with corresponding fluorescence linescan below. Scale bar: 1 µm.

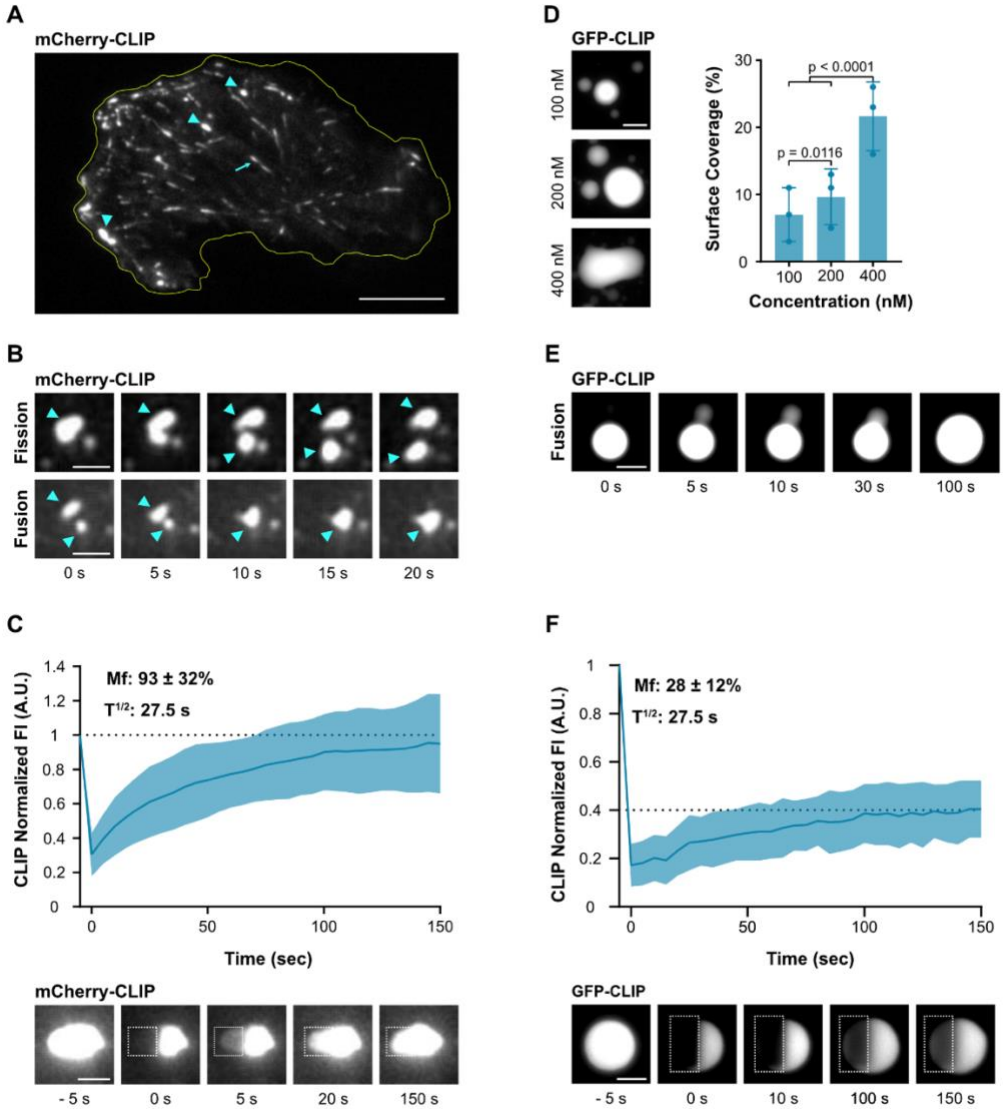
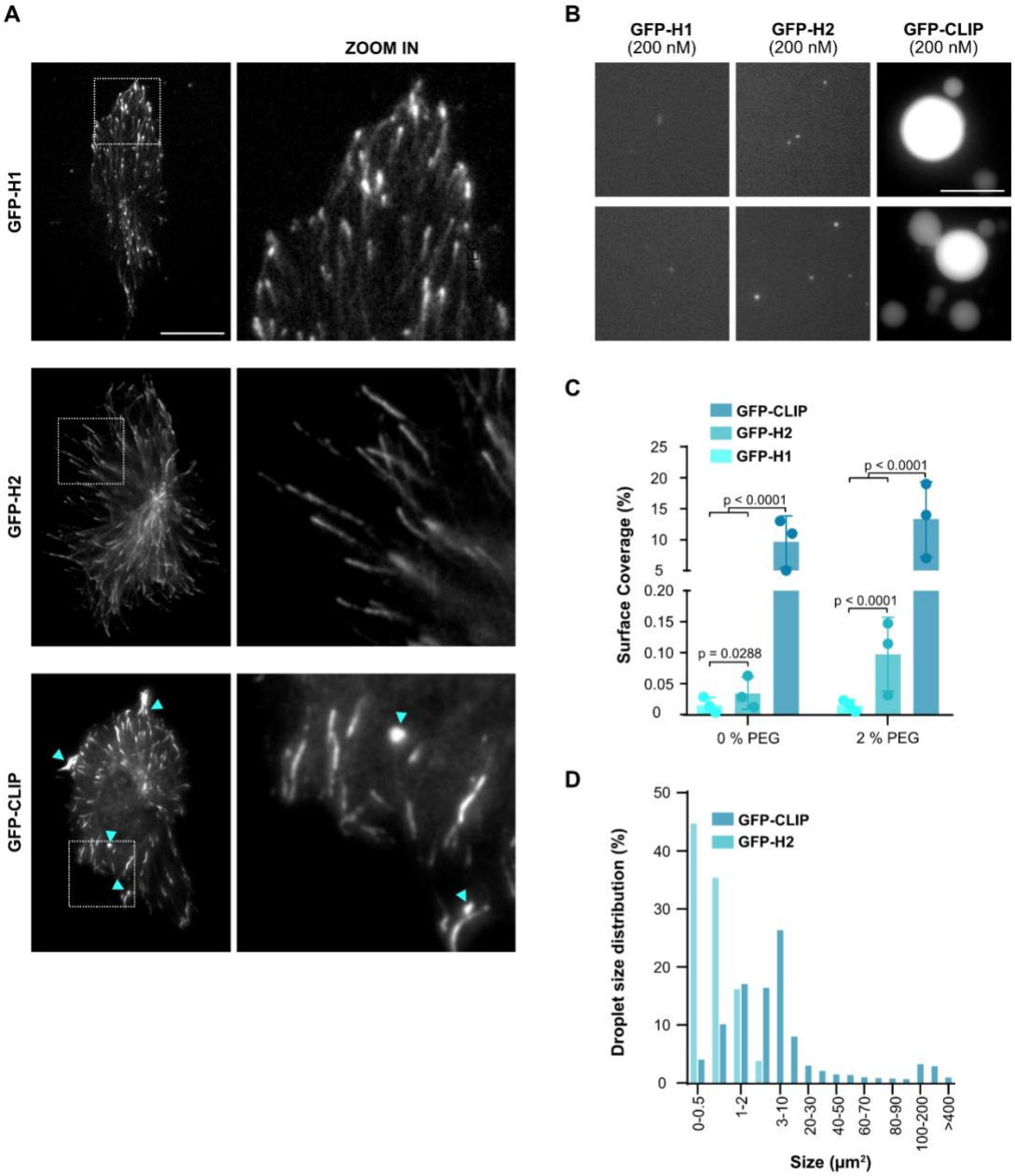
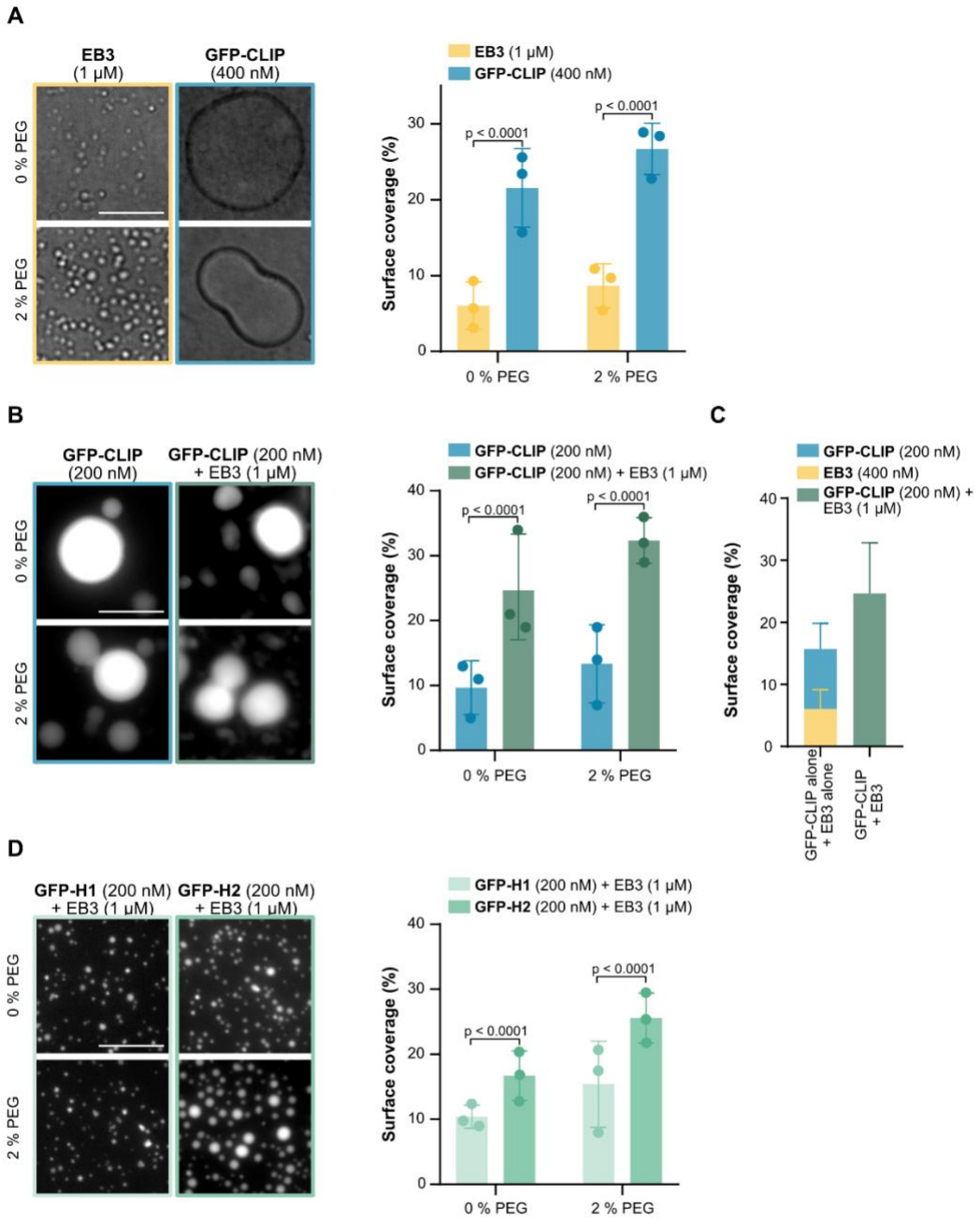


Figure 2: CLIP-170 condenses into droplets in cells and *in vitro*.
(**A**) Representative TIRF image of a RPE-1 cell transfected with mCherry-CLIP-170, showing the formation of cytoplasmic patches (cyan arrowheads) and +TIP-networks (cyan arrows). Scale bar: 10 µm. (**B**) Representative TIRF timelapse images of mCherry-CLIP-170 droplets undergoing fission (top panel, cyan arrowheads), and fusion (bottom panel, cyan arrowheads) in cells. Scale bar: 2 µm. (**C**) Representative TIRF images and recovery curve of mCherry-CLIP-170 patches after photobleaching (dashed box). Curve shows mean with SD of 5 individual experiments with a total of 38 droplets from 23 cells. Scale bar: 2 µm. **D**) Representative confocal images of purified GFP-FL-CLIP at indicated concentrations and quantification of the coverslip surface coverage by FL-CLIP. Statistics: two-tailed Student's *t*-test. Mean with SD from 3 independent experiments with a total of 27 fields of view. Scale bar: 20 µm. (**E**) Timelapse images of purified GFP-FL-CLIP (1 µM) undergoing fusion. Representative of 3 experimental replicates. Scale bar: 10 µm. (**F**) Representative images and recovery curve of purified GFP-FL-CLIP (2 µM) droplets after photobleaching (dashed box). Curve shows mean with SD of 3 individual experiments with a total of 47 condensates. Scale bar: 5 µm.


A
ZOOM IN
GFP-H1
GFP-H2
GFP-CLIP
B
GFP-H1
(200 nM)
GFP-H2
(200 nM)
GFP-CLIP
(200 nM)
C
GFP-CLIP
GFP-H2
GFP-H1
Surface Coverage (%)
p < 0.0001
p < 0.0001
p = 0.0288
p < 0.0001
0 % PEG
2 % PEG
D
GFP-CLIP
GFP-H2
Droplet size distribution (%)
0-0.5
1-2
3-10
20-30
40-50
60-70
80-90
100-200
>400
Size (µm²)

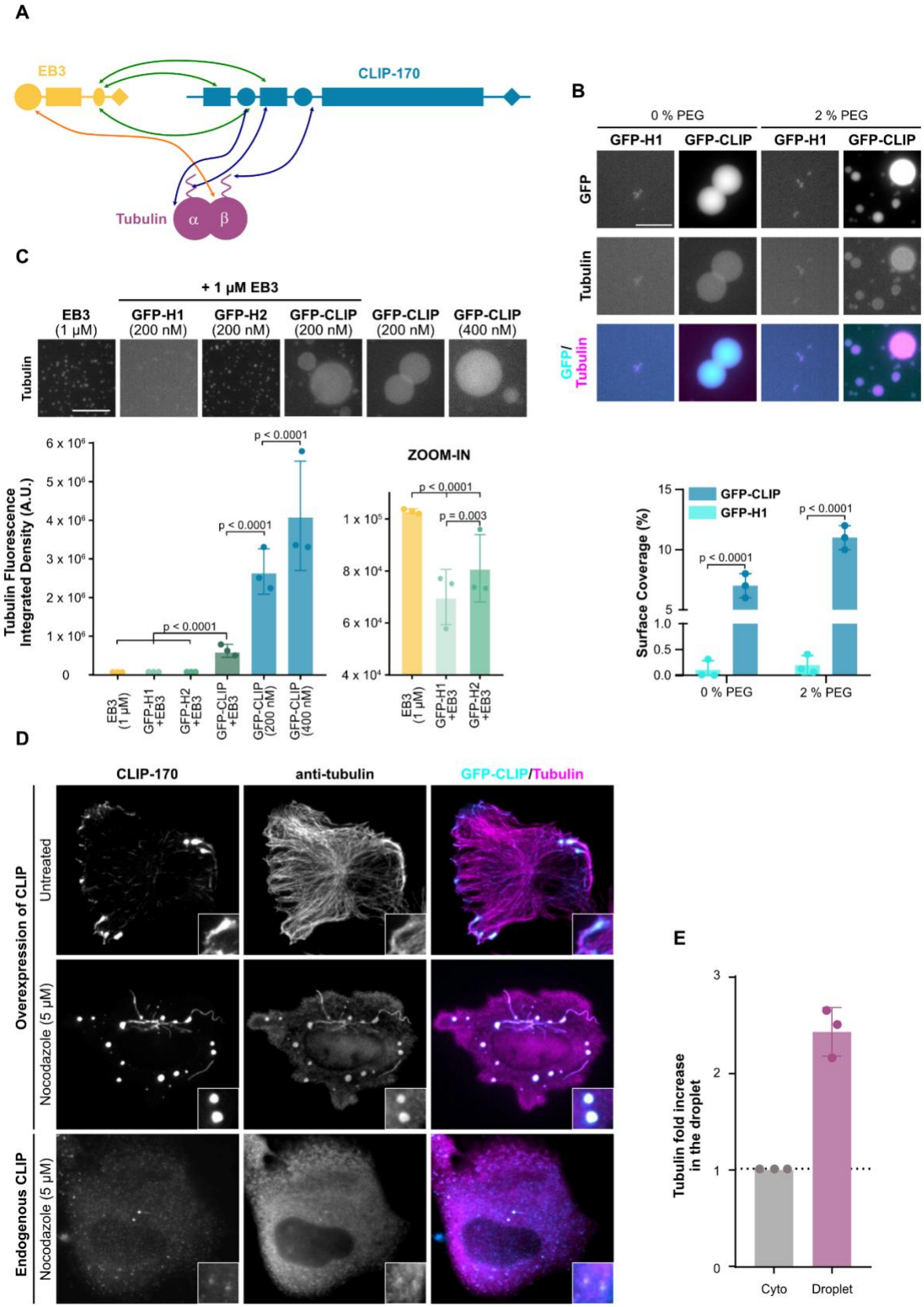
430

Figure 3: The C-terminal region drives CLIP-170 into the dense phase.
(**A**) Representative images of fixed RPE-1 cells transfected with full length GFP-CLIP-170, GFP-H2 or GFP-H1 (left panel) with insets (right panel). Cyan arrowheads denote droplet formation in GFP-CLIP-expressing cell. Scale bar: 20 µm. (**B**) Representative confocal images of purified GFP-FL-CLIP, GFP-H2 and GFP-H1 each at 200 nM in the absence (top panel) or presence (bottom panel) of 2% PEG. Scale bar: 20 µm. **(C)** Condensate surface coverage of the three constructs at indicated PEG concentrations. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**D**) Size distribution of GFP-FL-CLIP (200 nM) and GFP-H2 (200 nM) droplets in the absence of PEG. Graph shows average size distribution from 3 independent experiments with a total of 27 fields of view.


A
EB3 (1 μM)
GFP-CLIP (400 nM)
0 % PEG
2 % PEG
EB3 (1 μM)
GFP-CLIP (400 nM)
Surface coverage (%)
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG
B
GFP-CLIP (200 nM)
GFP-CLIP (200 nM) + EB3 (1 μM)
0 % PEG
2 % PEG
GFP-CLIP (200 nM)
GFP-CLIP (200 nM) + EB3 (1 μM)
Surface coverage (%)
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG
C
GFP-CLIP (200 nM)
EB3 (400 nM)
GFP-CLIP (200 nM) + EB3 (1 μM)
Surface coverage (%)
GFP-CLIP alone + EB3 alone
GFP-CLIP + EB3
D
GFP-H1 (200 nM) + EB3 (1 μM)
GFP-H2 (200 nM) + EB3 (1 μM)
0 % PEG
2 % PEG
GFP-H1 (200 nM) + EB3 (1 μM)
GFP-H2 (200 nM) + EB3 (1 μM)
Surface coverage (%)
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG

442

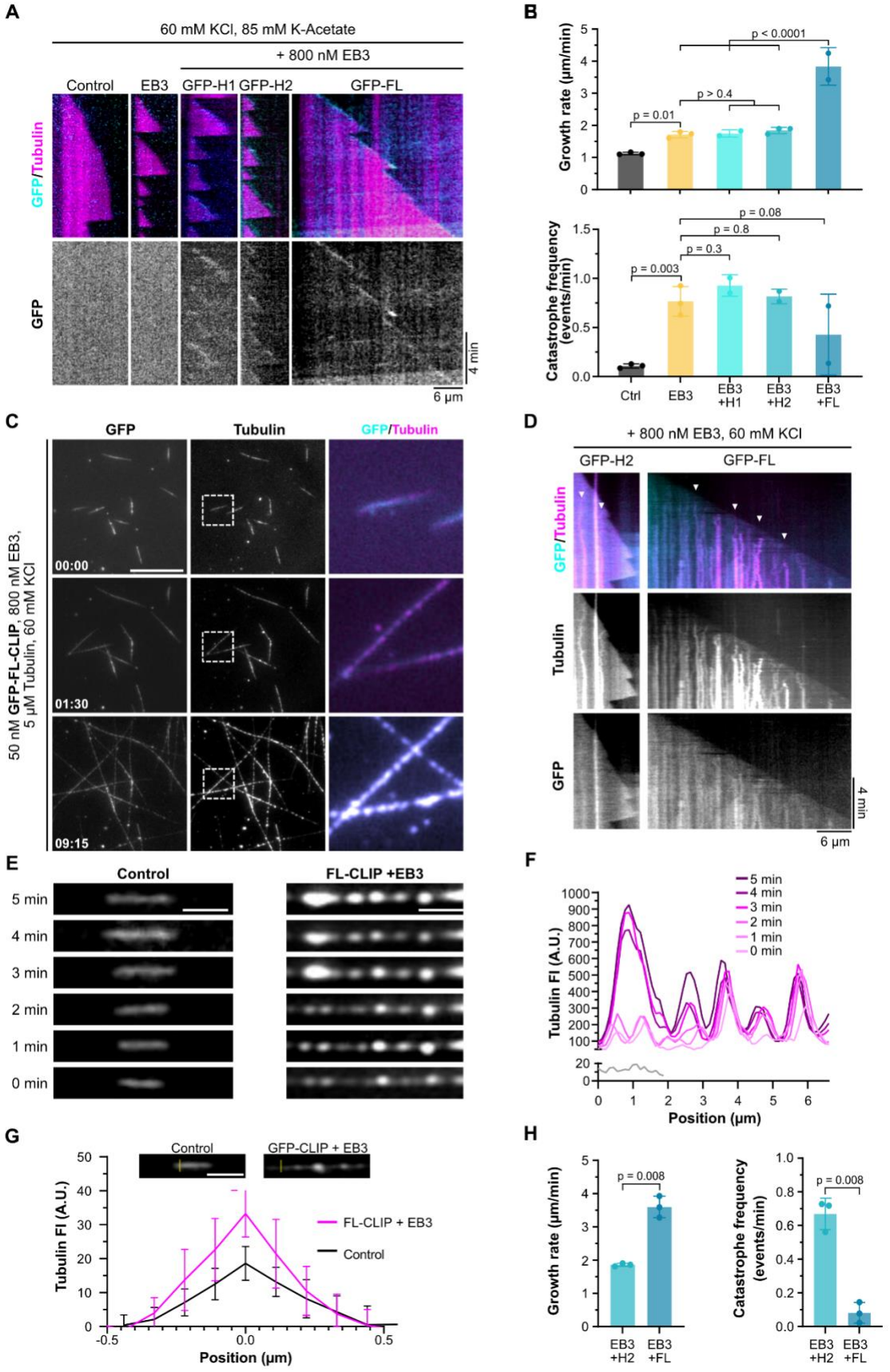
Figure 4: EB3 undergoes LLPS and co-condenses with CLIP-170 *in vitro*.
(**A**) Left: representative DIC images and quantification of purified EB3 (1 µM) and GFP-FL-CLIP (400 nM) in absence (top panel) or presence (bottom panel) of 2 % PEG. Scale bar: 20 µm. Right: coverslip surface coverage of indicated proteins at denoted PEG concentrations. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**B**) Left: representative fluorescence confocal images and quantification of purified GFP-FL-CLIP in the absence (left) or presence (right) of EB3, and in the absence (top panel) or presence (bottom panel) of 2 % PEG. Scale bar: 20 µm. Right: coverslip surface coverage of GFP-FL-CLIP (200 nM) in the dense phase with or without EB3 (1 µM) in the presence of the indicated PEG concentrations. Mean with SD of from 27 fields of view from 3 independent experiments. Statistics: two-tailed Student's *t*-test. (**C**) Quantification of droplet surface coverage of EB3 and GFP-FL-CLIP alone compared to surface coverage of EB3/GFP-FL-CLIP droplet formation when undergoing synergistic LLPS in the absence of PEG. (**D**) Left: representative fluorescence confocal images and quantification of purified GFP-H1 (left) or GFP-H2 (right) in the presence of EB3, and in the absence (top panel) or presence (bottom panel) of 2 % PEG. Scale bar: 20 µm. Right: condensate surface coverage of indicated GFP-H1 (200 nM) or GFP-H2 (200 nM) in the presence of EB3 (1 µM), and in the presence of the indicated PEG concentrations. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test.


A
EB3
CLIP-170
Tubulin
α
β
B
0 % PEG
2 % PEG
GFP-H1
GFP-CLIP
GFP-H1
GFP-CLIP
GFP
Tubulin
GFP/
Tubulin
C
+ 1 µM EB3
EB3
(1 µM)
GFP-H1
(200 nM)
GFP-H2
(200 nM)
GFP-CLIP
(200 nM)
GFP-CLIP
(200 nM)
GFP-CLIP
(400 nM)
Tubulin
Tubulin Fluorescence
Integrated Density (A.U.)
6 x 10⁶
5 x 10⁶
4 x 10⁶
3 x 10⁶
2 x 10⁶
1 x 10⁶
0
p < 0.0001
p < 0.0001
p < 0.0001
EB3
(1 µM)
GFP-H1
+EB3
GFP-H2
+EB3
GFP-CLIP
+EB3
GFP-CLIP
(200 nM)
GFP-CLIP
(400 nM)
ZOOM-IN
1 x 10⁵
8 x 10⁴
6 x 10⁴
4 x 10⁴
p < 0.0001
p = 0.003
EB3
(1 µM)
GFP-H1
+EB3
GFP-H2
+EB3
GFP-CLIP
GFP-H1
Surface Coverage (%)
15
10
5
1.0
0.5
0.0
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG
D
CLIP-170
anti-tubulin
GFP-CLIP/Tubulin
Overexpression of CLIP
Untreated
Nocodazole (5 µM)
Endogenous CLIP
Nocodazole (5 µM)
E
Tubulin fold increase
in the droplet
3
2
1
0
Cyto
Droplet

466

Figure 5: CLIP-170 and EB3 droplets condense tubulin.

(**A**) Cartoon schematic of domain interactions between EB3, CLIP-170, and tubulin based primarily on (Gupta et al., 2010; Chen et al., 2009; Bjelic et al., 2012). Interaction sites between EB3 and CLIP-170, EB3 and tubulin, and CLIP-170 and tubulin are shown with green, orange, and blue arrows, respectively. For simplification, monomers of EB3 and CLIP-170 are shown. (**B**) Top: representative confocal images of purified GFP-FL-CLIP and GFP-H1 each at 200 nM with Atto-565-tubulin (400 nM); in the absence (left panel) or presence (right panel) of 2 % PEG. Scale bar: 20 µm. Bottom: quantification of the coverslip surface coverage of tubulin in presence of GFP-FL-CLIP or GFP-H1. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**C**) Top: representative confocal images of Atto-565-tubulin (400 nM) in the presence of purified EB3 (1 µM), GFP-H1 (200 nM) and EB3 (1 µM), GFP-H2 (200 nM) and EB3 (1 µM), GFP-CLIP (200 nM) and EB3 (1 µM), and GFP-CLIP (200 nM and 400nM) alone. Scale bar: 20 µm. Bottom: quantification of the integrated density of tubulin fluorescence under denoted conditions with zoom in for the first three conditions. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**D**) Representative images of fixed RPE-1 cells transfected with full length GFP-FL-CLIP and untreated (top panel) or treated with 5 µM nocodazole for 1 hour (middle panel); endogenous CLIp-170 labelling treated with 5 µM nocodazole (bottom panel) and stained for tubulin. Scale bar: 10 µm. Images are representative of 3 independent experiments. **(E)** Graph showing normalized tubulin fluorescence intensity in CLIP-droplets compared to cytoplasm in full-length GFP-CLIP-170 transfected RPE-1 cells treated with nocodazole. Graph shows mean with SD from 3 independent experiments with a total of 126 condensates from 26 cells.


A
60 mM KCl, 85 mM K-Acetate
+ 800 nM EB3
Control
EB3
GFP-H1
GFP-H2
GFP-FL
GFP/Tubulin
GFP
4 min
6 µm
B
Growth rate (µm/min)
p < 0.0001
p > 0.4
p = 0.01
Catastrophe frequency (events/min)
p = 0.08
p = 0.8
p = 0.3
p = 0.003
Ctrl
EB3
EB3 +H1
EB3 +H2
EB3 +FL
C
GFP
Tubulin
GFP/Tubulin
50 nM GFP-FL-CLIP, 800 nM EB3, 5 µM Tubulin, 60 mM KCl
00:00
01:30
09:15
D
+ 800 nM EB3, 60 mM KCl
GFP-H2
GFP-FL
GFP/Tubulin
Tubulin
GFP
4 min
6 µm
E
Control
FL-CLIP +EB3
5 min
4 min
3 min
2 min
1 min
0 min
F
5 min
4 min
3 min
2 min
1 min
0 min
Tubulin FI (A.U.)
Position (µm)
G
Control
GFP-CLIP + EB3
FL-CLIP + EB3
Control
Tubulin FI (A.U.)
Position (µm)
H
Growth rate (µm/min)
p = 0.008
Catastrophe frequency (events/min)
p = 0.008
EB3 +H2
EB3 +FL

491

Figure 6: LLPS of +TIPs regulates microtubule dynamics through local tubulin condensation.

(A) Representative microtubule kymographs of denoted +TIP-networks in higher salt buffer (60 mM KCl and 85 mM K-acetate, see materials and methods). Note, that tip-tracking efficiency (GFP channel) is weaker at 5 µM tubulin than at higher tubulin concentrations (Figure S6G). (**B**) Microtubule growth rate (top) and catastrophe frequency (bottom) in presence of denoted proteins in high salt buffer. Note, that the catastrophe-inhibiting activity of EB3/FL-CLIP +TIP-droplets showed fluctuations between replicates. Mean with SD of three individual experiments (except for EB3/FL-CLIP, two experiments) with the following number of analyzed microtubules: Control – 48; EB3 – 31; EB3/H1 – 28; EB3/H2 – 28; EB3/FL-CLIP - 41. Statistics: One-way ANOVA Fisher's LSD test. (**C**) Representative time-lapse TIRF images of GFP-FL-CLIP (50 nM) with Atto-565-tubulin (5 µM) in the presence of unlabeled EB3 (800 nM) and 60 mM KCl. Time denoted in minutes: seconds; scale bar: 20 µm. The zoom-ins (white dashed box) show representative droplet formation along mircorubles. (**D**)Representative microtubule kymographs in the presence of GFP-H2 or GFP-FL-CLIP (50 nM) and EB3 (800 nM) grown at 5 µM tubulin and 60 mM KCl. (**E**) Representative images of only tubulin condition (left, control) and in presence of EB3/FL-CLIP (right). Right images show tubulin condensation along microtubule over time. Scale bar: 2 µm. (**F**) Corresponding linescan of E with the gray linescan representing the 5 µM tubulin control condition and the purple colored linescans of 5 µM tubulin in presence of EB3/FL-CLIP (800 nM/50 nM). (**G**) Tubulin fluorescent intensity close to the microtubule tip in 5 µM tubulin control condition and in presence of EB3/FL-CLIP. Quantifcation of perpendicular linscans close to the microtubule tip. Mean with SD from 2 independent experiments for each condition and a total of 20 microtubules; Scale bar: 2 µm. (**H**) Quantification of microtubule growth rate (left) and catastrophe frequency (right) in the presence of EB3/H2-networks and EB3/FL-CLIP droplets in 60 mM KCl. Mean with SD of three individual experiments with the following number of analyzed microtubules: EB3/H2 – 29; EB3/FL-CLIP – 59. Statistics: paired t-test.

Supplementary Figures S1-S6

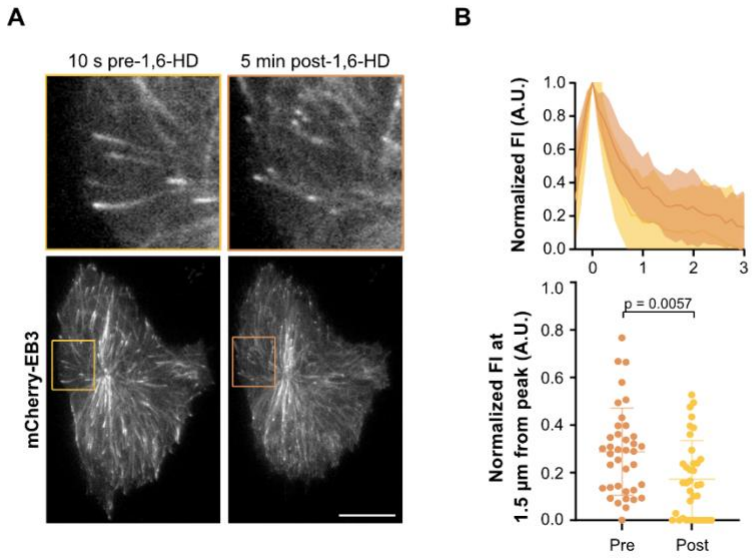


Figure S1: Decrease in EB3 comet size upon treatment with 1,6-HD: (A) Representative images of GFP-Tubulin RPE-1 cells expressing mCherry-EB3 before and after 5-minute treatment with 5 % 1,6-hexanediol, with insets. **(B)** Top: mean fluorescence intensity profile of +TIP-networks before and after 1,6-hexanediol treatment. Bottom: normalized fluorescence intensity of +TIP-networks 1.5 µm away from the peak. Mean with SD of 36-38 +TIP-networks from 6 cells from 2 independent experiments. Statistics: paired t-test. Scale bars: 20 µm.

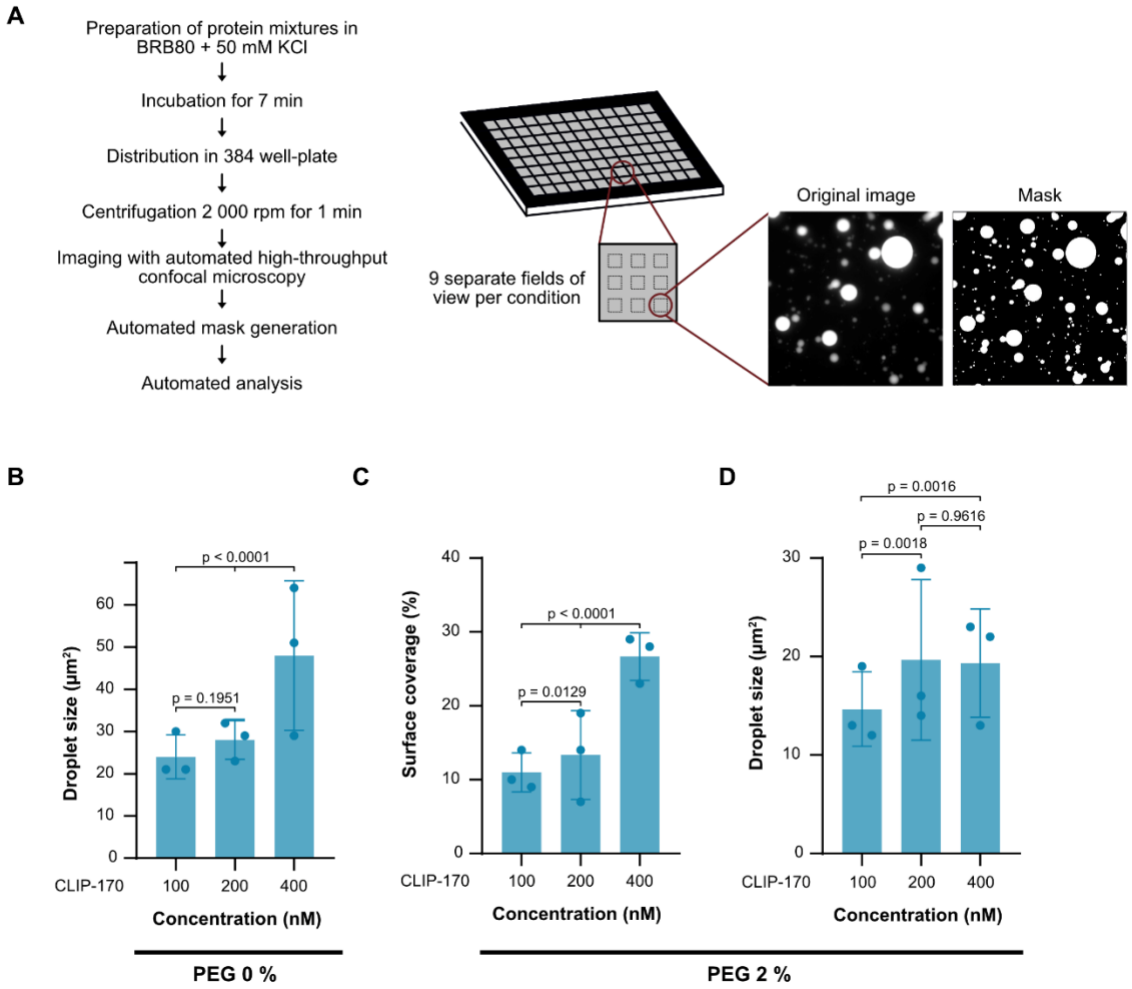


Figure S2: CLIP-170 droplet formation *in vitro* is sensitive to concentration and molecular crowding.

(**A**) Experimental outline for phase separation assays. For details, see materials and methods. (**B**) Droplet size (area) for GFP-FL-CLIP condensates at 100, 200, or 400 nM in the absence of PEG. (**C**) Coverslip surface coverage of GFP-FL-CLIP at the indicated concentrations in the presence of 2% PEG. (**D**) Droplet size (area) of GFP-FL-CLIP condensates at 100, 200 and 400 nM in the presence of 2 % PEG. All graphs show mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test.

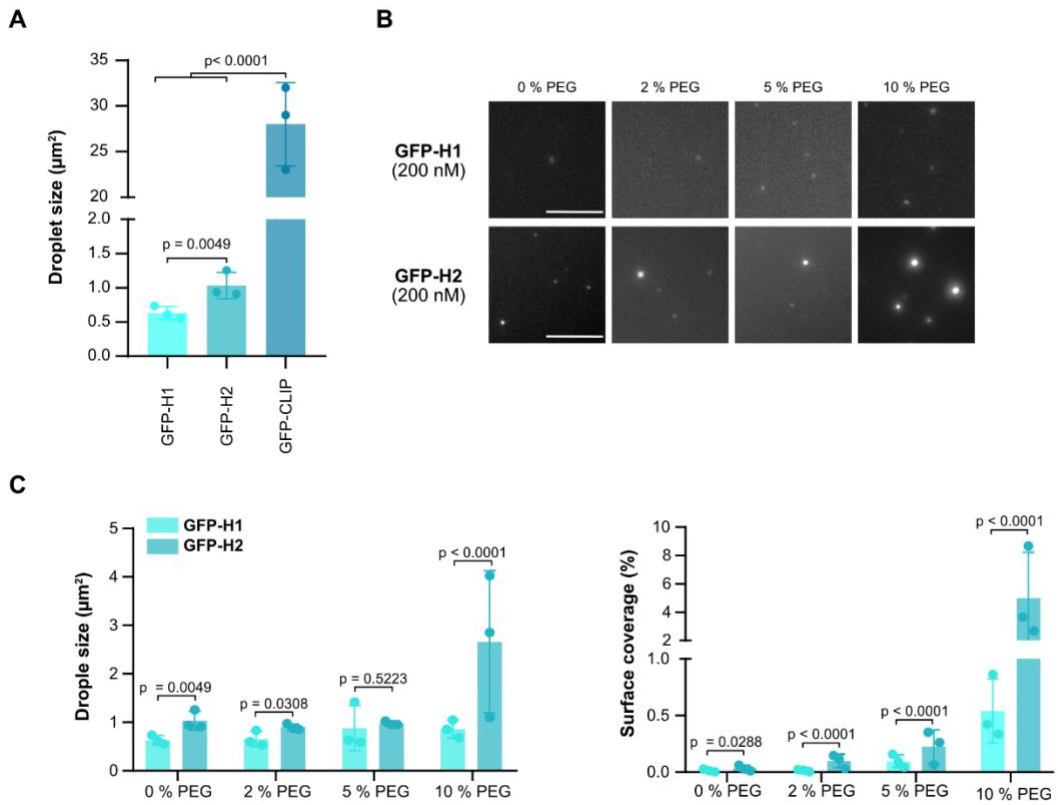


Figure S3: H2 only weakly undergoes LLPS even under strong molecular crowding conditions. (**A**) Droplet size (area) of GFP-H2 (200 nM) and GFP-FL-CLIP (200 nM), and aggregate size of GFP-H1 (200 nM) in the absence of PEG. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: one-way ANOVA test. (**B**) Representative fluorescence confocal images and quantification of purified GFP-H1 (200 nM) and GFP-H2 (200 nM) in the presence of 0, 2, 5 and 10 % PEG. Scale bar: 20 µm. (**C**) Droplet size (left graph) and surface coverage (right graph) of denoted proteins at indicated PEG concentrations. Mean with SD of from 27 fields of view from 3 independent experiments. Statistics: two-tailed Student's *t*-test.

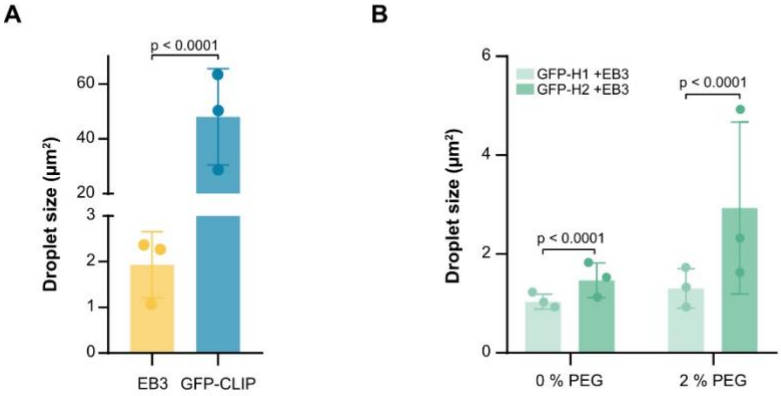
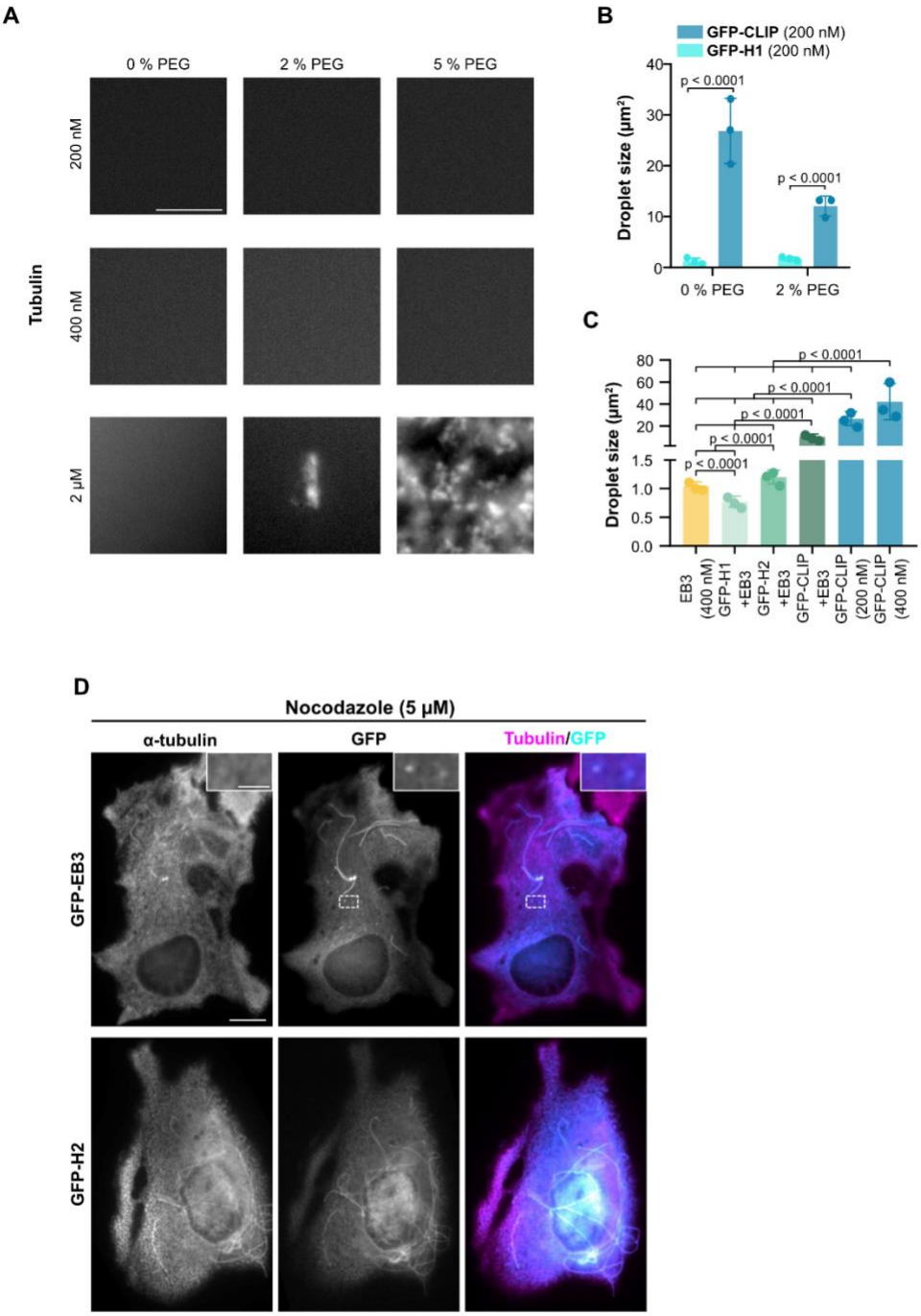
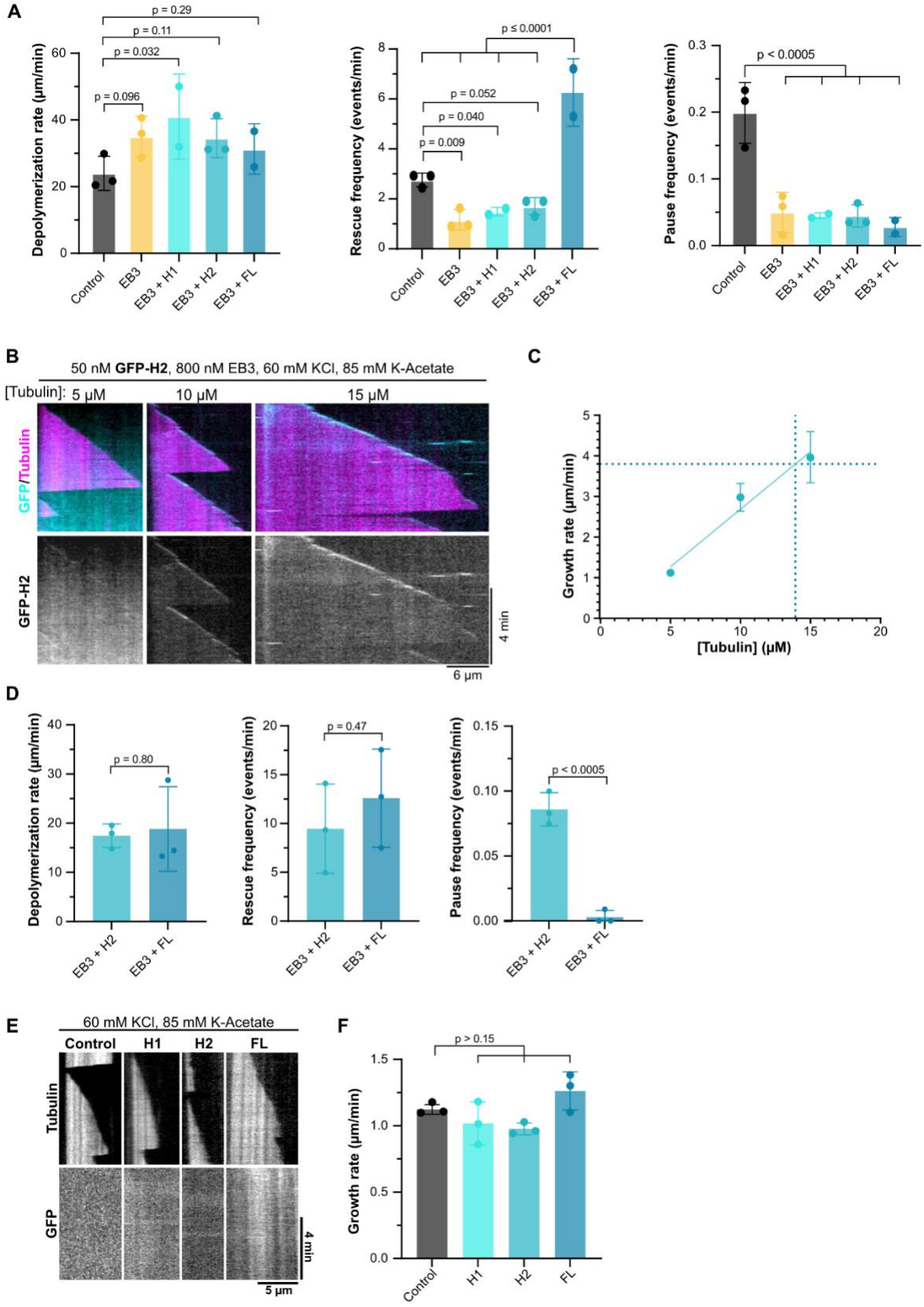


Figure S4: EB3/CLIP-170 phase separation is driven by the CLIP-170 C-terminal region *in vitro.* (**A**) Droplet size (area) of unlabeled EB3 (1 µM) and GFP-FL-CLIP (400 nM) at 0 % PEG concentrations. Graph shows mean with SD of 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**B**) Condensate size (area) of GFP-H1 (200 nM) or GFP-H2 (200 nM) in the presence of EB3 (1 µM) in the absence or presence of 2% PEG. Mean with SD of 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test


A
0 % PEG
2 % PEG
5 % PEG
200 nM
400 nM
2 µM
Tubulin
B
GFP-CLIP (200 nM)
GFP-H1 (200 nM)
Droplet size (µm²)
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG
C
Droplet size (µm²)
p < 0.0001
EB3 (400 nM)
GFP-H1
+EB3 GFP-H2
+EB3 GFP-CLIP
+EB3 GFP-CLIP (200 nM)
GFP-CLIP (400 nM)
D
Nocodazole (5 µM)
α-tubulin
GFP
Tubulin/GFP
GFP-EB3
GFP-H2

575

Figure S5: CLIP-170 and EB3 form a tubulin-condensing network.
(**A**) Representative fluorescence confocal images and quantification of purified Atto-565-tubulin at indicated concentrations in presence of 0, 2 and 5 % PEG. Note that at 2 µM tubulin, PEG caused aggregation (but not condensate formation) of tubulin. Scale bar: 20 µm. (**B**) Droplet size of Atto-565-tubulin (4 00 nM) in the presence of GFP-H1 (200 nM) or GFP-FL-CLIP (200 nM) in the absence or presence of 2% PEG. Mean with SD of from 27 fields of view from 3 independent experiments. Statistics: two-tailed Student's *t*-test (right graph). (**C**) Droplet size (area) of Atto-565-tubulin (400 nM) in the presence of purified EB3 (1 µM), GFP-H1 (200 nM) and EB3 (1 µM), GFP-H2 (200 nM) and EB3 (1 µM), GFP-FL-CLIP (200 nM) and EB3 (1 µM), GFP-FL-CLIP (200nM) alone, and GFP-FL-CLIP (400nM) alone. (**D**) Representative images of fixed RPE-1 cells transfected with GFP-H2 (top panel) or GFP-EB3 (bottom panel) treated with 5 µM nocodazole for 1 hour and stained for tubulin. Scale bar: 10 µm.


A
Depolymerization rate (µm/min)
p = 0.29
p = 0.11
p = 0.032
p = 0.096
Control
EB3
EB3 + H1
EB3 + H2
EB3 + FL
Rescue frequency (events/min)
p ≤ 0.0001
p = 0.052
p = 0.040
p = 0.009
Pause frequency (events/min)
p < 0.0005
B
50 nM GFP-H2, 800 nM EB3, 60 mM KCl, 85 mM K-Acetate
[Tubulin]: 5 µM
10 µM
15 µM
GFP/Tubulin
GFP-H2
4 min
6 µm
C
Growth rate (µm/min)
[Tubulin] (µM)
D
Depolymerization rate (µm/min)
p = 0.80
Rescue frequency (events/min)
p = 0.47
Pause frequency (events/min)
p < 0.0005
EB3 + H2
EB3 + FL
E
60 mM KCl, 85 mM K-Acetate
Control
H1
H2
FL
Tubulin
GFP
4 min
5 µm
F
Growth rate (µm/min)
p > 0.15
Control
H1
H2
FL

592

Figure S6: Phase separation potent +TIP-networks impart stronger effects on microtubule dynamics

(**A**) Microtubule dynamic parameters (depolymerization rate, left; rescue frequency, middle; and pause frequency, right) for the denoted conditions in 60 mM KCl and 85 mM K-acetate (corresponding assay to Figure 6B, see here for number of analyzed microtubules). Mean with SD of three individual experiments. Statistics: one-way ANOVA Fisher's LSD test (**B**) Representative microtubule kymographs in the presence of GFP-H2 (50 nM) and EB3 (800 nM) grown at the denoted tubulin and salt concentrations. (**C**) Microtubule growth rate from experiments in B. Mean with SD from three individual experiments. Solid cyan line shows linear regression curve fit. Dashed blue line indicates concentration of tubulin at which microtubule in presence of EB3/H2 networks grow at 3.8 µm/min (the speed achieved by EB3/FL-CLIP-droplets at 5 µM tubulin; Figure 6A). (**D**) Microtubule dynamic parameters (depolymerization rate, left; rescue frequency, middle; and pause frequency, right) for the denoted conditions in 50 mM KCl (corresponding assay to Figure 6H, see here for number of analyzed microtubules). Mean with SD. Statistics: one-way ANOVA Fisher's LSD test (**E**) Representative microtubule kymographs of control (only tubulin), 50 nM GFP-H1, 50 nM GFP-H2 or 50 nM FL-CLIP. (**F**) Microtubule growth rate from experiments in Figure E. Mean with SD of three individual experiments (dots) with the following numbers of microtubules analyzed: Control – 48; H1 – 42; H2 – 41. Statistics: one-way ANOVA Fisher's LSD test.

Movie 1: Representative remnant formation resembling fission in GFP-CLIP overexpressing RPE-1 cells. Scale Bar: 2 µm.

Movie 2: Representative mCherry-EB3 expressing RPE-1 cell treated with 5% 1,6-hexanediol (added at 00:30).

Movie 3: Representative RPE-1 cell overexpressing mCherry-CLIP imaged with TIRF microscopy. Inset highlights fusion of mCherry-CLIP droplets.

Movie 4: Representative RPE-1 cell overexpressing mCherry-CLIP imaged with TIRF microscopy. Inset highlights fission of mCherry-CLIP droplets.

Movie 5: Fluorescence recovery after photobleaching of a mCherry-CLIP droplet in a representative RPE-1 cell.

Movie 6: Fusion of two GFP-CLIP droplets (1µM) *in vitro*.

Movie 7: Fluorescence recovery after photobleaching of a purified GFP-CLIP droplet (2 µM) *in vitro*.

Movie 8: Representative plus-end tracking behavior of overexpressed mcherry-H1 (cyan) in RPE-1 GFP-tubulin cells (magenta).

Movie 9: Representative plus-end tracking behavior of overexpressed mcherry-H2 (cyan) in RPE-1 GFP-tubulin cells (magenta).

Movie 10: Representative *in vitro* microtubule dynamics assay of Atto-565 tubulin (magenta) polymerized in the presence of 50 nM GFP-FL-CLIP (cyan) and 800nM EB3 in high salt buffer.

Movie 11: Representative *in vitro* microtubule dynamics assay of Atto-565 tubulin (magenta) polymerized in the presence of 50 nM GFP-FL-CLIP (cyan) and 800nM EB3 in low salt buffer.

Methods

Cell culture and treatments

Parental RPE1 and CRISPR/Cas9 knock-in RPE1-GFP-tubulin cells (Andreu-Carbó et al., 2021) were cultured in high glucose Dulbecco's Modified Eagle's Medium F12 (DMEM, ThermoFisher, 113057) supplemented with 10 % Fetal Bovine Serum (FBS, ThermoFisher, 10270106) and 1 % penicillin-streptomycin (Gibco, 15140122) at 37°C with 5 % CO2. The cell lines were monthly checked for mycoplasma contamination.

For transient expression studies of exogenous CLIP and EB3, cells were transfected using the jetOPTIMUS transfection reagent (Polyplus) with 0.5 µg DNA according to the manufacturer's instructions. Transfection media was replaced with fresh culture media 8 hours post-transfection, and cells were imaged 15-24h after transfection.

For microtubule dynamics studies, RPE1-GFP-Tubulin cells were transfected with siRNA using Lipofectamine RNAiMAX (Thermo Fisher) for 72 hours according to the manufacturer's instructions. For control experiments, cells were transfected with Allstars Negative Control siRNA (QIAGEN). For CLIP-170 + EB3 depletion experiments, cells were transfected with dual siRNA mixes targeting EB3 (Thermo Fisher, s22683) and CLIP-170 (Santa Cruz, 43281). Transfection media was replaced with fresh culture media 8 hours post-transfection, and cells were imaged 72 hours post-transfection.

To depolymerize the microtubule network, cells were treated with 5 µM nocodazole (Sigma, M1404; diluted in culture medium) for 1 hour prior to fixation. For experiments using 1,6-hexanediol (Sigma), cells were treated with 5% 1,6-hexanediol (diluted in culture medium) for 10 minutes.

Cloning

The in-cell expression vector for mCherry-FL-CLIP170 was generated by excising GFP from a FL-CLIP170-GFP vector (a kind gift from Thomas Surrey) using AgeI/BsrG1 restriction sites and replacing it with mCherry containing AgeI/BsrG1 overhangs generated by PCR. From this vector, we generated mCherry-tagged H1- and H2-CLIP170 by PCR and reinsertion using the XhoI/KpnI restriction sites (N-terminal XhoI primer: 5'-CCGCTCGAGCTCAAGCTTCGATGAGTAT GCTGAAACCCAGCGGGCTGAA-3', C-terminal KpnI H1 primer: 5'-CGGGGTACCGTCGACTCAAGTGGTGCCCGAGATCTTGCGGGC-3', and C-terminal KpnI H2 primer: 5'-CGGGGTACCGTCGACTCATTTGTCAGCTTTGGTCTT TTCAAAGAGCAGGCTCTGTTC-3'). Protein purification vectors for H1- and H2-CLIP-GFP were generated by PCR from the FL-CLIP-170-GFP vector using a primer with an overhang for the NdeI restriction site as well as an N-terminal TEV protease site (5'-GCGGCAGCCATATGGAAAACCTGTATTTCCAGGGAAGTGCCACCATGGTGAGCAAG GGCGAGGAGCTGTTCA-3'), and C-terminal primers specific to each CLIP truncation with overhangs corresponding to the ScaI restriction site (H1: 5'-CCTTATCAAGTACTA GTGGTGCCCGAGATCTTGCGGGCGTAGCGGGAAG-3') (H2: 5'-CCTTATCAAGTACTT CATTTGTCAGCTTTGGTCTTTTCAAAGAGCAGGCTCTGTTCAAGC-3'), then cloned into

an empty pET28a-6His vector (a kind gift from Natacha Olieric, Paul Scherrer Institute) using NdeI/ScaI restriction sites.

A protein purification vector for mCherry-6His-EB3 was a kind gift from Natacha Olieric (Paul Scherrer Institute). From this vector, mCherry was excised using AgeI/BsrGI restriction sites to produce an untagged 6His-EB3 vector for purification.

For in-cell expression of EB3, mCherry-EB3 vectors were obtained from Addgene (Addgene plasmid 55037).

Imaging

Microscope

For in-cell studies and *in vitro* microtubule dynamics experiments, imaging was performed on an Axio Observer Inverted TIRF microscope (Zeiss, 3i) equipped with a Prime 95B BSI (Photometrics) using a 100X objective (Zeiss, Plan-Apochromat 100X/1.46 oil DIC (UV) VIS-IR). SlideBook 6 X 64 software (version 6.0.22) was employed to record time-lapse imaging. For *in vitro* microtubule dynamics and cell imaging, microscope stage conditions were controlled with the Chamlide Live Cell Instrument incubator (37°C for *in vitro* experiments, supplemented with 5 % CO_2 for live cell experiments).

Microtubule dynamics

For in-cell microtubule dynamic measurements images were taken every second for 3 minutes. Microtubules were tracked individually using the Freehand-Line tool in ImageJ (10-pixel width) and kymographs were built using the KymographBuilder plugin. Microtubule growth speeds were then calculated by manually tracing the slopes of kymographs using the Straight-Line tool in ImageJ and extracting the growth rate from the slopes using a custom-written code.

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) experiments in cells were performed in square regions (4x4 µm) with a 656 nm laser at 20% intensity. The normalized fluorescence intensity was calculated using the formula $F(t)_{norm} = \frac{F(t)_{ROI}-F_{bck}}{F(t)_{ctrl}-F_{bck}} \times \frac{F(i)_{ctrl}-F_{bck}}{F(i)_{ROI}-F_{bck}}$ where $F(t)_{ROI}$ and $F(t)_{ctrl}$ are respectively the ROI and the control fluorescence intensity before the FRAP, F_{bck} the background fluorescence and $F(i)_{ROI}$ and $F(i)_{ctrl}$ are respectively the ROI of the unbleached part of the condensate at one timepoint (i) (Day et al.,2012).

+TIP-network analysis in 1,6-hexanediol-treated cells

For experiments in cells treated with 1,6-hexanediol, images were taken in a single z-plane every 10 seconds for 10 minutes. 1,6-Hexanediol (5 %) was added after one minute, and cells were only analyzed if they did not undergo any large-scale changes in morphology, as 1,6-hexanediol treatment has been noted to affect mammalian cell shape (Wheeler et al., 2016). For "pre-treatment" time points, all in-focus +TIP-networks on the cell periphery were analyzed in the time frame 10 seconds prior to hexanediol addition using the Segmented-Line tool in ImageJ to obtain fluorescence intensity. For "post-treatment" time points, the same strategy was applied to the time frame 5 minutes after hexanediol addition.

Immunofluorescence

15-24 hours post-transfection (or post-seeding for non-transfected cells), cells were fixed with 100% methanol for 5 min at -20°C and then for 15 min with 3% paraformaldehyde at room temperature. Cells were then permeabilized for 10 minutes with 0.15% by volume Triton-X 100 (Sigma) in PBS followed by 10 minutes with 0.1% Tween-20 (AppliChem) in PBS, washed thoroughly in a solution of 0.05% Tween-20 in PBS (hereafter referred to as PBS-T), and subsequently blocked with 2% bovine serum albumin (in PBS) for 1h. Post-blocking, cells were incubated overnight with antibodies targeting tubulin (Sigma T6199, DM1α, 1:1000, mouse), EB1 (Millipore AB 6057, rabbit, 1:1000), EB3 (Santa Cruz Biotechnology sc-101475, KT36, rat, 1:200), or CLIP-170 (Santa Cruz Biotechnology sc-28325, F3, mouse, 1:500). Primary antibodies were diluted to the appropriate concentration in 2% bovine serum albumin in PBS. The following day, cells were subjected to three five-minute washes at room temperature in PBS-T, then subsequently incubated in secondary antibodies (Invitrogen, species-specific IgG conjugated to Alexa-647, 561, or 488 fluorophores) at room temperature for one hour. Cells were subjected to three additional PBS-T washes, and coverslips were mounted onto glass microscopy slides (Glass technology) using ProLong™ Diamont Antifade Mountant. Coverslips were sealed with nail polish and stored at 4°C until imaging.

Tubulin purification from bovine brain and labelling

Tubulin was purified from fresh bovine brain by two subsequent polymerization/depolymerization cycles as described previously (Andreu-Carbó et al., 2021). Tubulin labelling with biotin or ATTO-488, -565, -647 fluorophores was performed as described (Andreu-Carbó et al., 2021), and final labelling ratios to polymerize microtubules were 11% for ATTO-488 and 13% for ATTO-565 tubulin.

Protein purification

For purification of EB3, *E. coli* BL21 (DE3) cells were transformed with 6-His-tagged EB3-encoding plasmids and induced for expression overnight with 1 mM IPTG at 20°C under rotation at 200 rpm. All following steps were performed at 4°C. The morning after induction, cells were lysed by in lysis buffer (20 mM Tris pH 7.5, 300 mM NaCl) supplemented with 1% Triton-X 100 and protease inhibitors cocktail tablets (Roche) and sonicated. Cell debris were then cleared by ultracentrifugation. The cleared lysate was subsequently loaded onto a pre-equilibrated HisTrap column (GE Healthcare 1mL HisTrap column) using an ÄKTA Pure Protein Purification System (GE Healthcare). After washing the column in lysis buffer, elution buffer (20 mM Tris pH 7.5, 300 mM NaCl, 1 M imidazole) was applied to the column in a 1% gradient. Eluted protein fractions were pooled and concentrated using Amicon 30K Centrifugal filters (Millipore). The concentrated, cleared protein was subjected to size-exclusion chromatography using a HiLoad 16/600 Superdex column (GE Healthcare) in lysis buffer. Protein-containing fractions were harvested, pooled, and concentrated. Protein was supplemented with 20% glycerol, aliquoted, snap-frozen and stored at -80°C.

H1-CLIP-GFP and H2-CLIP-GFP were purified using the same scheme as EB3, with the following differences: (1) the lysis buffer was 50 mM potassium phosphate pH 7.5, 500 mM NaCl, 1 mM $MgCl_2$, and 1 mM β-mercaptoethanol. (2) Between applying protein to the HisTrap column and elution, the column was washed with lysis buffer supplemented with 8 mM Imidazole. (3) H1- and H2-CLIP were eluted from the HisTrap column using lysis buffer + 300 mM Imidazole, and protein-containing fractions were subjected to tobacco etch virus (TEV) protease treatment overnight to remove His tags prior to size-exclusion chromatography.

FL-CLIP170-GFP was purified from insect cells as described previously (Telley et al., 2011). A plasmid encoding FL-CLIP170-GFP in pFasBacHTa (a kind gift from Thomas Surrey) was used to generate Baculovirus, which was subsequently used to infect Sf9 cells. Cells were harvested and lysed with lysis buffer (30 mM HEPES pH 7.4, 400 mM KCl, 20 mM Arginine, 20 mM potassium-glutamate, 0.01% Birj35, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol) supplemented with 20 mM imidazole and protease inhibitor tablets (Roche) using dounce homogenization. Cell debris were cleared by ultracentrifugation, and cleared lysate was loaded onto a Histrap column (GE Healthcare). The column was washed with lysis buffer supplemented with 50 mM imidazole, then eluted with lysis buffer supplemented with 300 mM imidazole. Protein-containing fractions were pooled and further cleared by a second centrifugation, then subjected to size-exclusion chromatography using a HiLoad 16/600 Superdex column (GE Healthcare) in lysis buffer (lacking Birj35). Only proteins coming off with a clean, single peak profile from the Superdex column were used. Protein-containing fractions were pooled and concentrated and used immediately (maximal 5 hrs after purification), as FL-CLIP was prone to degradation and loss of activity after freezing as previously noted (Telley et al., 2011). For all purified proteins, protein concentration was measured by Bradford assay.

Phase separation assay

For in vitro phase separation assays, proteins were diluted to the appropriate concentration in BRB80 supplemented with 50 mM potassium chloride and PEG 4000 (0, 2, 5 or 10% by weight) in Eppendorf tubes. After thorough mixing, reactions were transferred to 384-well plates (Falcon) and incubated for 7 min. The plate was then centrifuged at 2000 rpm for 1 min to sediment proteins in the dense phase on the well bottoms. Images were acquired using a confocal automated microscopy (Molecular Device) with a 60X dry objective. For each reaction, 9 times 2048 x 2048 px fields of view were acquired with one focal plane (Figure S1A). Automated analysis was performed using MetaXpress Custom Module editor software. From fluorescence intensity, masks were generated to differentiate condensates from the background and the area sum was calculated together with the condensate versus background fluorescence intensity.

Coverslip treatment and Flow chamber preparation

For *in vitro* microtubule dynamics studies, slides and coverslips were cleaned by two successive 30-minute sonication cycles in 1 M NaOH followed by 96% ethanol with thorough rinsing in bi-distilled water between each step. After drying, slides and coverslips were plasma treated

(Electronic Diener, Plasma surface technology) and subsequently incubated for 48 hours with tri-ethoxy-silane-PEG (Creative PEGWorks) or a 1:5 mix of tri-ethoxy-silane-PEG-biotin: tri-ethoxy-silane-PEG (final concentration 1 mg/ml) in 96 % ethanol and 0.02 % HCl, with gentle agitation at room temperature. Slides and coverslips were then washed in ethanol (96 %) followed by thorough washing in bi-distilled water, then dried with an air gun and stored at 4°C. Flow chambers were prepared by affixing a silane-PEG-biotin coverslip to a silane-PEG slide using double-sided tape.

Microtubule dynamics assays in vitro

Microtubule seeds were prepared at a final concentration of 10 µM tubulin (20 % ATTO-647-labelled tubulin and 80 % biotinylated tubulin) in BRB80 supplemented with 0.5 mM GMPCPP (Jena Bioscience) for 45 minutes at 37°C. Seeds were incubated with 1 µM Paclitaxel (Sigma) for 45 minutes at 37°C, centrifuged (50,000 rpm at 37°C for 15 min), resuspended in BRB80 supplemented with 1 µM Paclitaxel and 0.5 mM GMPCPP, aliquoted and subsequently stored in liquid nitrogen.

Flow chambers were prepared by injecting subsequently 50 µg/mL neutravidin (ThermoFisher), BRB80, and microtubule seeds, then subsequently washing out unattached seeds with BRB80. Reaction buffer containing Atto-565 labelled-tubulin (1:5 ratio labelled to unlabelled; 5 µM for all assays except for Figure 6G, H) in BRB80 supplemented with an anti-bleaching buffer [10 mM DTT, 0.3 mg/mL glucose, 0.1 mg/mL glucose oxidase, 0.02 mg/mL catalase, 0.125 % methyl cellulose (1500 cP, Sigma), 1 mM GTP] was subsequently injected, and chambers were sealed with silicon grease and immediately imaged. For “low salt” assays (Figure 6B-D), the reaction buffer was supplemented with 60 mM potassium chloride. For “high salt” assays (Figure 6E-H), the reaction buffer was supplemented with 60 mM potassium chloride and 85 mM potassium acetate as described previously (Telley et al., 2011).

For assays involving recombinant EB3 and H1- or H2-CLIP-170, purified proteins were flash-thawed and spun at 50,000 rpm in a TLA-100 centrifuge at 4°C for 15 minutes to remove any large aggregates. Proteins were diluted into BRB80 immediately prior to their usage, and further diluted to the appropriate concentration in reaction buffer. Assays involving FL-CLIP-170 were carried out as noted above, but within the first 5 hours post-purification as FL-CLIP-170 activity is poorly preserved after freezing (Telley et al., 2011).

SDS-PAGE and Western blot

Cell lysates were boiled run on SDS-PAGE gels (10% acrylamide) and subsequently transferred to a nitrocellulose membrane using an iBLOT 2 Gel Transfer Device (ThermoFisher Scientific, IB21001). Nitrocellulose membranes were blocked for 1 h with 5 % dried milk resuspended in TBS-Tween 1 %, then incubated over-night with primary antibodies: anti-beta-tubulin (Sigma, T6074, 1:1000 dilution) anti-EB3 (ATLAS anti-MAPRE3, HPA-009263, 1:500 dilution), or anti-CLIP-170 (Santa Cruz Biotechnology, SC-28325, 1:1000 dilution). The following day, unbound antibodies were washed off with TBS-Tween 1%, and membranes were incubated with secondary

antibodies conjugated to horseradish peroxidase (anti-mouse or anti-rabbit; GE Healthcare 17097199 and 16951542, 1:5000 dilution) for 1 hour at room temperature. Following secondary antibody incubation, membranes were washed extensively with TBS-Tween 1% and imaged using an ECL Western blotting detection kit (Advansta) and with Fusion Solo Vilber Lourmat camera (Witec ag).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software v9 as described in figure legends. Unless otherwise noted, analyses were carried out between experimental means using one-way ANOVA Fisher's LSD test, or two-tailed Student's t-test. P-values less than 0.05 were considered statistically significant.

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