Supporting Information for

Phase separation of +TIP-networks regulates microtubule dynamics

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Movies S1 to S16

Supplementary Figures S1-S6

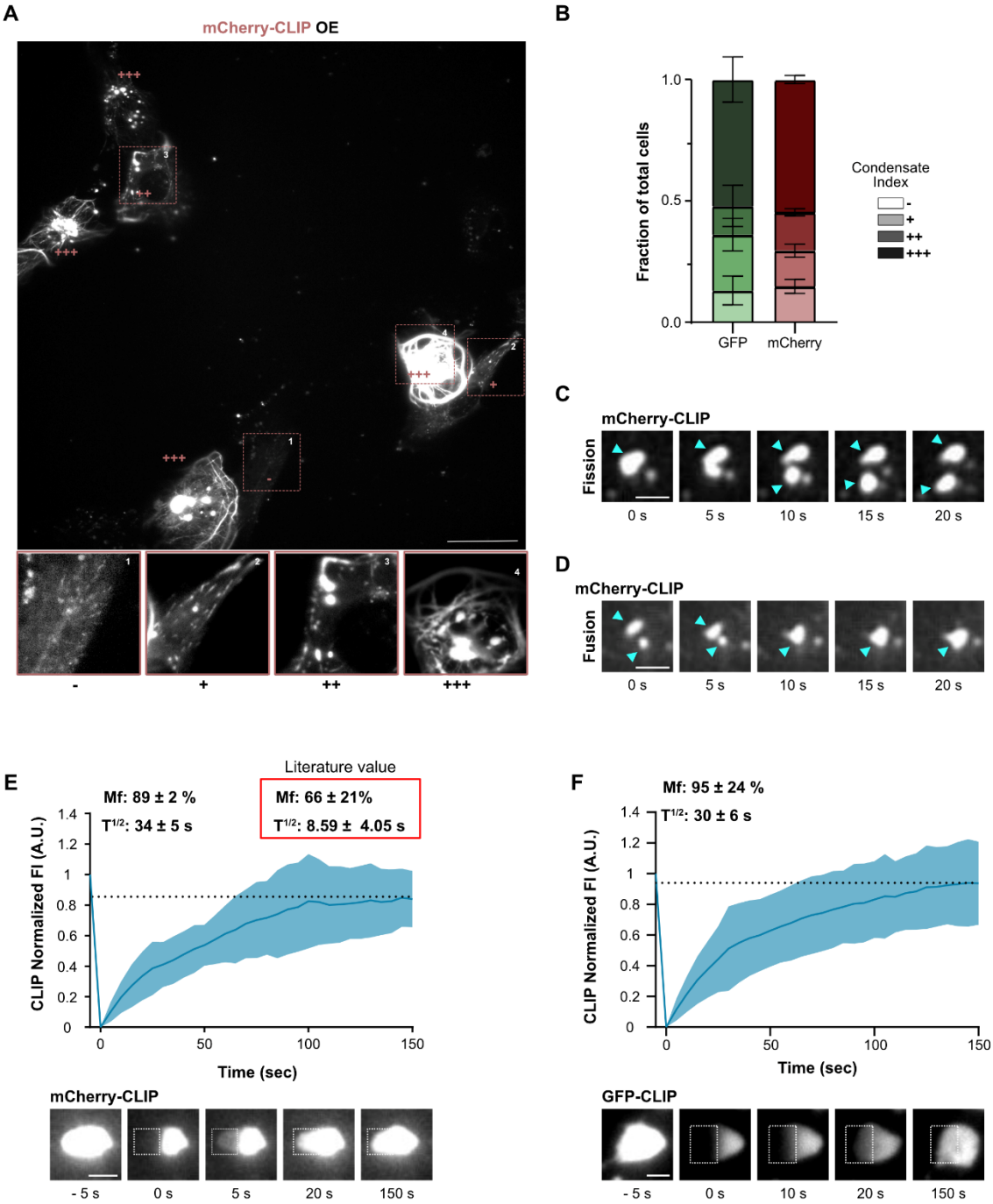
A
mCherry-CLIP OE
-
+
++
+++
B
Fraction of total cells
1.0
0.5
0.0
GFP
mCherry
Condensate Index
-
+
++
+++
C
mCherry-CLIP
Fission
0 s
5 s
10 s
15 s
20 s
D
mCherry-CLIP
Fusion
0 s
5 s
10 s
15 s
20 s
E
Literature value
Mf: 89 ± 2 %
T1/2: 34 ± 5 s
Mf: 66 ± 21%
T1/2: 8.59 ± 4.05 s
CLIP Normalized FI (A.U.)
Time (sec)
mCherry-CLIP
- 5 s
0 s
5 s
20 s
150 s
F
Mf: 95 ± 24 %
T1/2: 30 ± 6 s
CLIP Normalized FI (A.U.)
Time (sec)
GFP-CLIP
- 5 s
0 s
10 s
20 s
150 s

Figure S1: The fluorescent tags mCherry and GFP revealed the same LLPS properties of CLIP-170 in cells.

(A) Representative confocal images (top) with zoom-in (bottom) of RPE-1 cells expressing mCherry-CLIP with indicated index for different condensation phenotypes. -: no observed cytoplasmic condensates; +: few small cytoplasmic condensates; ++: several large or many small condensates; +++: many large condensates and/or coating/bundling of microtubules. Note that zoom-in are contrast-adjusted. Scale bar: 30 µm. **(B)** Quantification of condensation index from experiments shown in A. Mean with SD from 2 independent experiments with 100 cells per experiment. (**C**) Representative TIRF time-lapse images of mCherry-CLIP-170 droplets undergoing fission. Scale bar: 2 µm. (**D**) Representative TIRF time-lapse images of mCherry-CLIP-170 droplets undergoing fusion. Scale bar: 2 µm. (**E**) 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. Literature values from (23) (**F**) Representative TIRF images (bottom) and recovery curve (top) of GFP-CLIP-170 droplets in RPE-1 cells after photobleaching (dashed box). Mean with SD of 3 individual experiments with a total of 36 droplets from 24 cells. Scale bar: 2 µm. Note that mCherry-CLIP-170 and GFP-CLIP-170 showed similar FRAP-recovery and patch formation behavior.

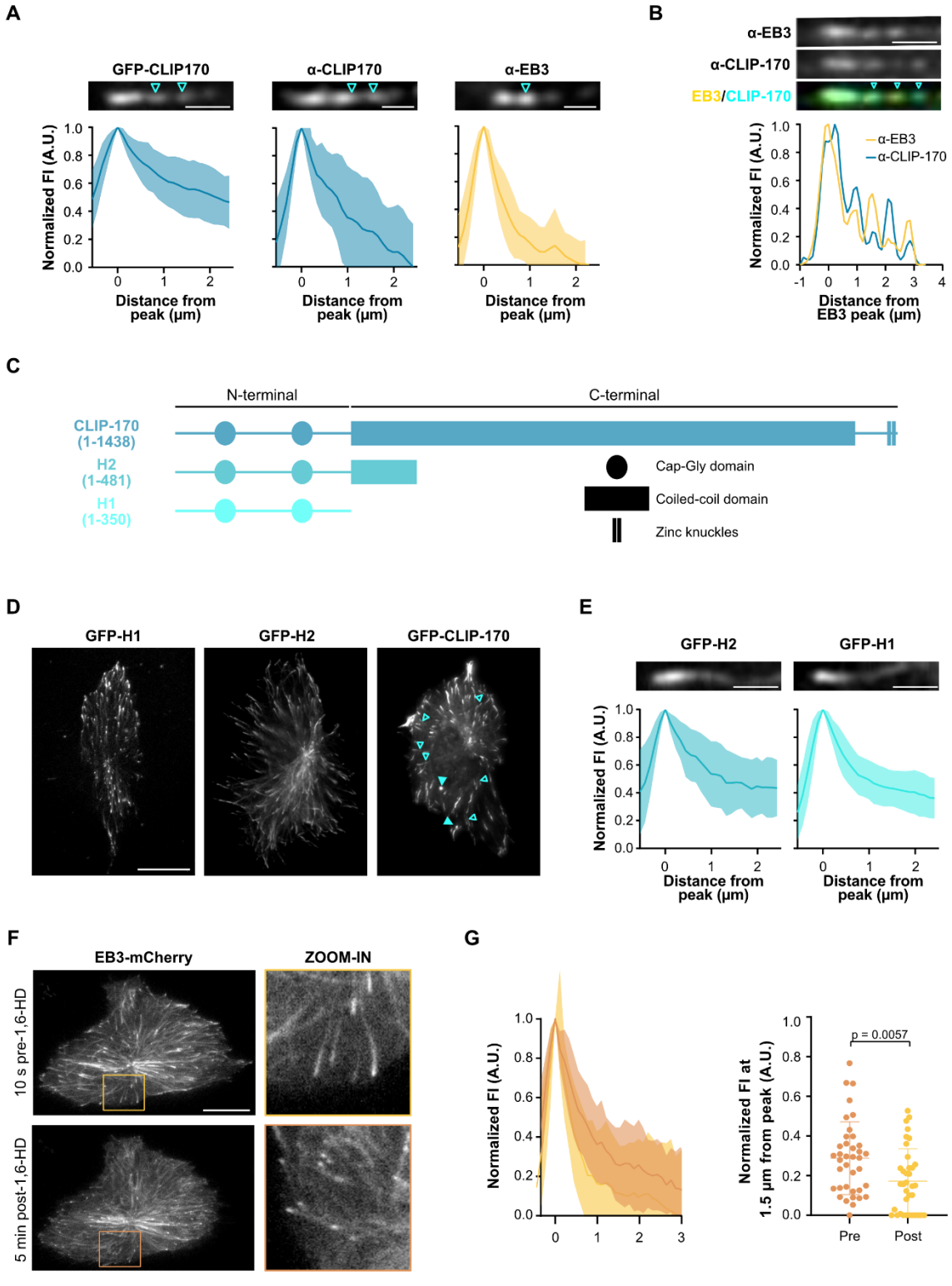
A
GFP-CLIP170
α-CLIP170
α-EB3
Normalized FI (A.U.)
Distance from peak (μm)
B
α-EB3
α-CLIP-170
EB3/CLIP-170
Normalized FI (A.U.)
Distance from EB3 peak (μm)
C
N-terminal
C-terminal
CLIP-170 (1-1438)
H2 (1-481)
H1 (1-350)
Cap-Gly domain
Coiled-coil domain
Zinc knuckles
D
GFP-H1
GFP-H2
GFP-CLIP-170
E
GFP-H2
GFP-H1
Normalized FI (A.U.)
Distance from peak (μm)
F
EB3-mCherry
ZOOM-IN
10 s pre-1,6-HD
5 min post-1,6-HD
G
Normalized FI (A.U.)
Normalized FI at 1.5 μm from peak (A.U.)
p = 0.0057
Pre
Post

Figure S2: Profile of +TIP networks at the microtubule ends.
(**A**) Representative images of fixed RPE-1 cells transfected with full length GFP-CLIP-170 or WT RPE-1 cells stained with antibodies to endogenous CLIP-170 and EB3. Cyan arrowheads indicate trailing foci. Scale bars: 2 μm. Below are quantified mean line scan 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. (**B**) Representative +TIP-network from cells stained for endogenous EB3 and CLIP-170 showing partial co-localization of EB3 and CLIP-170 trailing foci (cyan arrowheads) with corresponding fluorescence line scan below. Scale bar: 1 μm. (**C**) Secondary structure of CLIP-170 (1-1438), H2 (1-481) and H1 (1-350) based on (14, 20, 21). (**D**) Representative images of fixed RPE-1 cells transfected GFP-H1, GFP-H2 or GFP-CLIP-170. Full cyan arrowheads denote example cytoplasmic droplets and blank cyan arrowhead trailing foci in GFP-CLIP-170 expressing cell. Scale bar: 20 μm. (**E**) Representative images of fixed RPE-1 cells transfected with GFP-H2 and GFP-H1. Scale bar: 2 μm. Below are quantified mean line scan 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. **(F)** Representative images of RPE-1 cells expressing EB3-mCherry before (top panel) and after (bottom panel) 5-minute treatment with 5% 1,6-hexanediol, with insets. Scale bar: 20 μm. **(G)** Left: mean fluorescence intensity profile of +TIP-networks before and after 1,6-hexanediol treatment. Right: 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. Note that CLIP-170 patches were not dissolved by 1,6-hexanediol treatment.

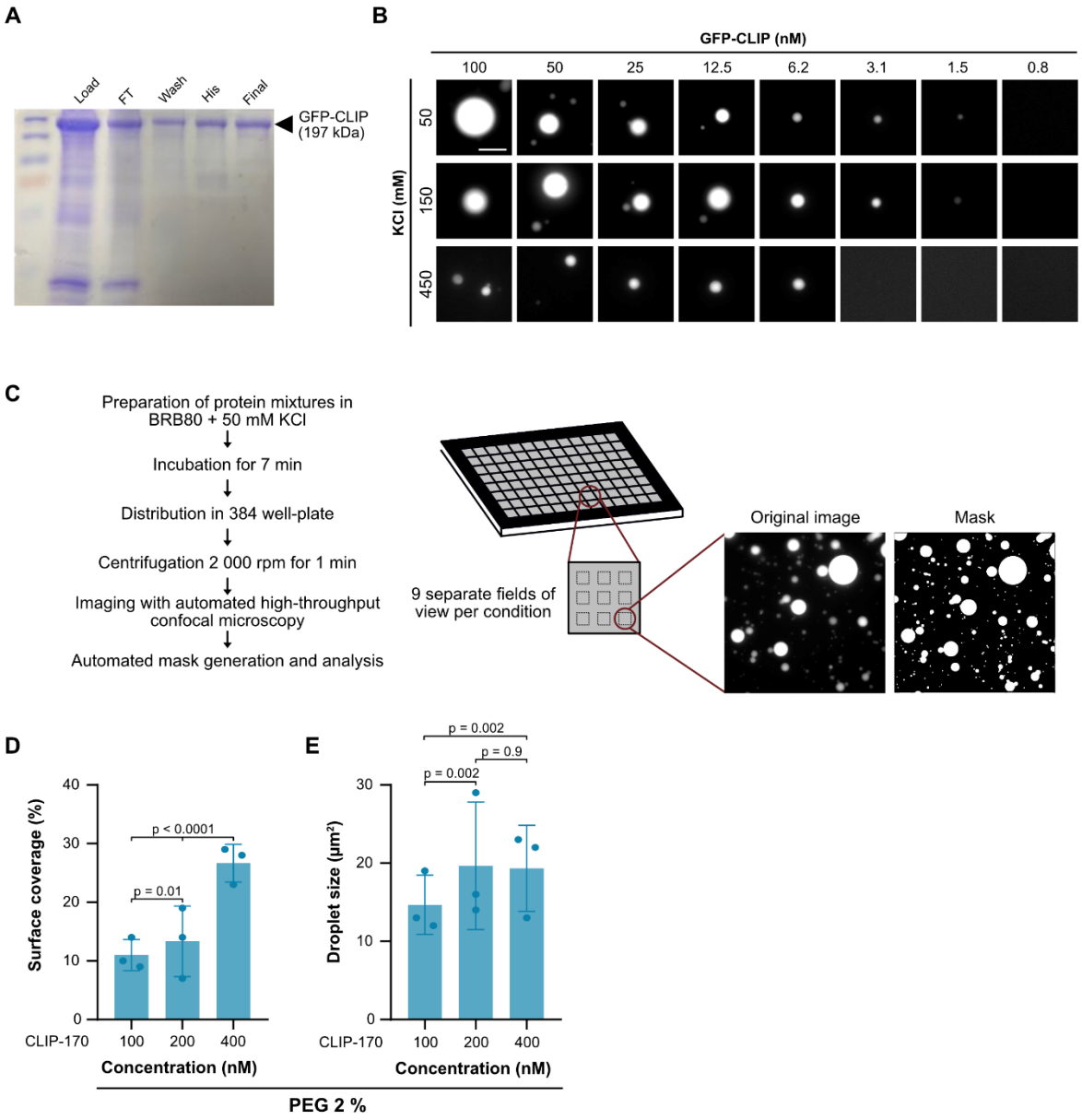

Figure S3: CLIP-170 droplet formation *in vitro* depends on molecular crowding, protein and salt concentrations.

(**A**) SDS-PAGE analysis of GFP-FL-CLIP protein purification. Load, lysate loaded onto HisTRAP column; FT, flow through; wash, fractions collected during HisTRAP-column washing; final, final concentrated protein sample post-SEC and post-concentration. (**B**) Representative confocal images of GFP-FL-CLIP phase diagram for 50, 150 and 450 mM KCl at indicated protein concentrations from 3 independent experiments. Scale bar: 10 µm. (**C**) Experimental outline for high throughput phase separation assays. For details, see materials and methods. (**D**) Coverslip surface coverage of GFP-FL-CLIP at the indicated concentrations in the presence of 2% PEG. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**E**) Droplet size (area) of GFP-FL-CLIP condensates at 100, 200 and 400 nM in the presence of

2% PEG. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test.

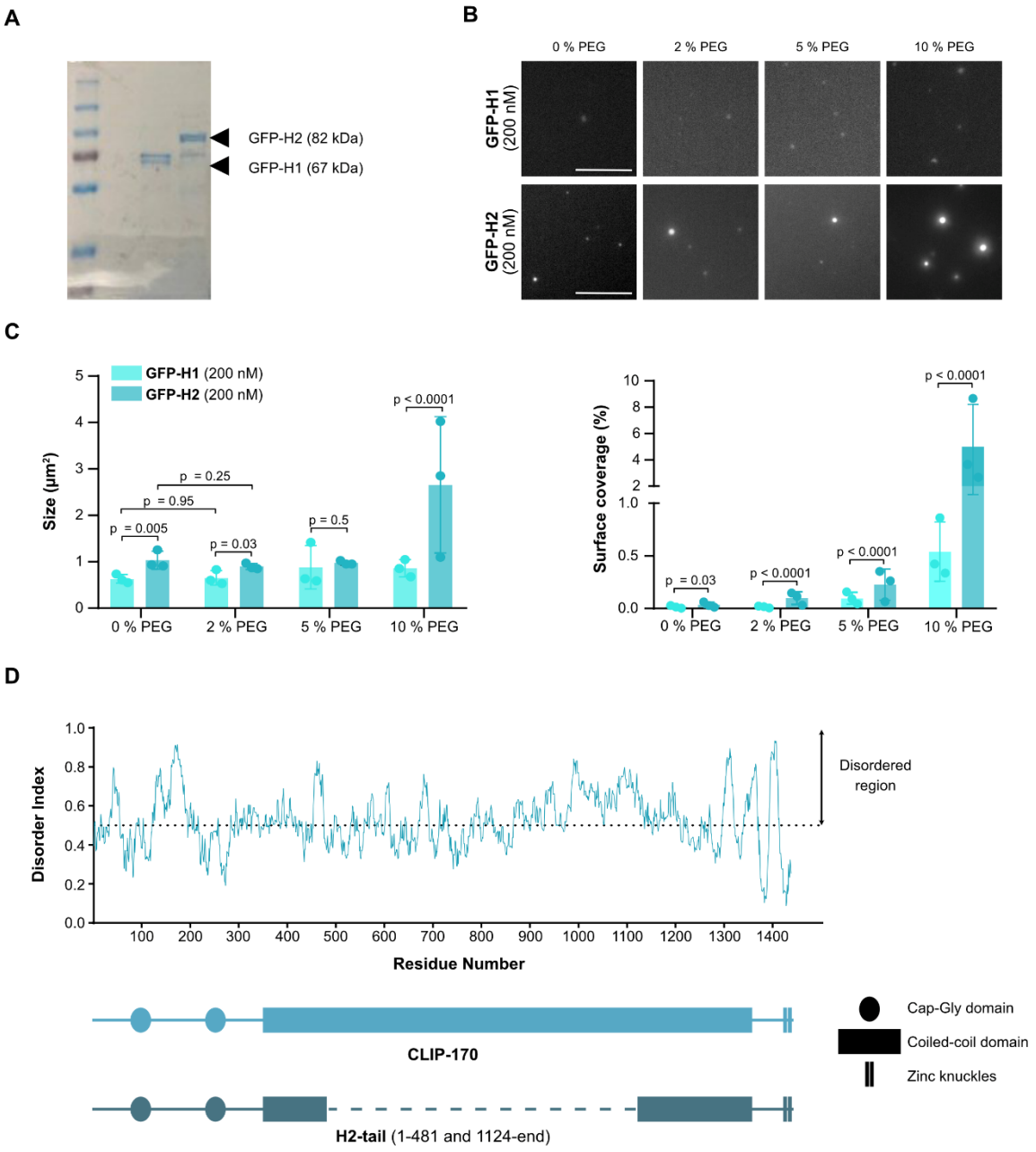
A
GFP-H2 (82 kDa)
GFP-H1 (67 kDa)
B
0 % PEG
2 % PEG
5 % PEG
10 % PEG
GFP-H1 (200 nM)
GFP-H2 (200 nM)
C
GFP-H1 (200 nM)
GFP-H2 (200 nM)
Size (µm²)
p = 0.005
p = 0.95
p = 0.25
p = 0.03
p = 0.5
p < 0.0001
0 % PEG
2 % PEG
5 % PEG
10 % PEG
Surface coverage (%)
p = 0.03
p < 0.0001
p < 0.0001
p < 0.0001
D
Disorder Index
Residue Number
Disordered region
CLIP-170
H2-tail (1-481 and 1124-end)
Cap-Gly domain
Coiled-coil domain
Zinc knuckles

Figure S4: H2 only weakly undergoes LLPS even under strong molecular crowding conditions.

(**A**) SDS-PAGE analysis of purified GFP-H1 and GFP-H2 protein. (**B**) Representative fluorescence confocal images of GFP-H1 (200nM) 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. Note that 10% PEG did increase the H2-droplet size. **(D)** Top: prediction of intrinsic disorder regions in CLIP-170, based on IUPred2A software. Values above the red dotted line (0.5) are considered as disordered (1). Bottom: Cartoon drawn to scale of CLIP-170 and mutant H2-tail secondary structures, length of H1 and H2 is indicated, black arrowhead.

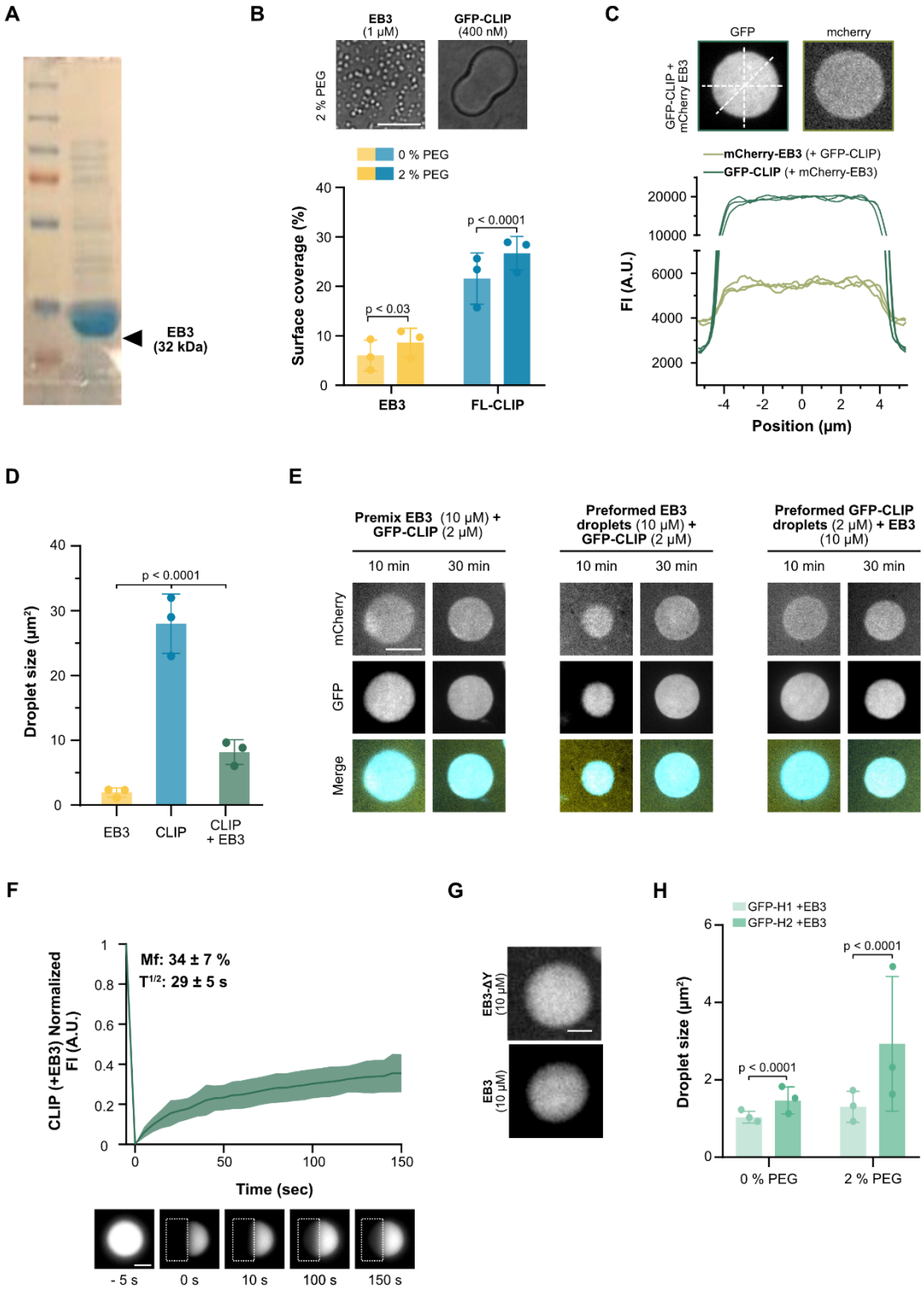
A
EB3
(32 kDa)
B
EB3
(1 µM)
GFP-CLIP
(400 nM)
2 % PEG
0 % PEG
2 % PEG
Surface coverage (%)
p < 0.03
p < 0.0001
EB3
FL-CLIP
C
GFP
mcherry
GFP-CLIP +
mCherry EB3
mCherry-EB3 (+ GFP-CLIP)
GFP-CLIP (+ mCherry-EB3)
FI (A.U.)
Position (µm)
D
Droplet size (µm²)
p < 0.0001
EB3
CLIP
CLIP
+ EB3
E
Premix EB3 (10 µM) +
GFP-CLIP (2 µM)
Preformed EB3
droplets (10 µM) +
GFP-CLIP (2 µM)
Preformed GFP-CLIP
droplets (2 µM) + EB3
(10 µM)
10 min
30 min
mCherry
GFP
Merge
F
Mf: 34 ± 7 %
T1/2: 29 ± 5 s
CLIP (+EB3) Normalized
FI (A.U.)
Time (sec)
- 5 s
0 s
10 s
100 s
150 s
G
EB3-ΔY
(10 µM)
EB3
(10 µM)
H
GFP-H1 +EB3
GFP-H2 +EB3
Droplet size (µm²)
p < 0.0001
p < 0.0001
0 % PEG
2 % PEG

Figure S5: Co-condensation of the EB3/CLIP-170.
(**A**) SDS-PAGE analysis of purified EB3. (**B**) Top: representative DIC images of EB3 (1 µM) and GFP-FL-CLIP (400 nM) in the presence of 2% PEG. Scale bar: 20 µm. Bottom: quantification of EB3 (1 µM) and GFP-FL-CLIP (400 nM) surface coverage in presence and absence of 2% PEG. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**C**) Linescan (bottom) with corresponding confocal images (top, white lines indicate the line scans) of EB3/FL-CLIP droplets showing a homogenous protein distribution. GFP-FL-CLIP (2 µM) EB3 (8 µM unlabeled EB3 + 2 µM mCherry-EB3). (**D**) Droplet size (area) of unlabeled EB3 (1 µM), GFP-FL-CLIP (200 nM) and EB3/FL-CLIP. Mean with SD of 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**E**) Representative confocal images of 2 µM GFP-FL-CLIP and 10 µM EB3 (8 µM unlabeled + 2 µM mCherry-EB3) in the denoted mixing conditions; 10 min after mixing and at coarsy steady state - 30 min after mixing. Scale bar: 10 µm. (**F**) Representative TIRF images and recovery curve of purified EB3/GFP-FL-CLIP (10 µM / 2 µM) droplets after photobleaching (dashed box). Mean with SD of 3 independent experiments with a total of 35 condensates. Scale bar: 5 µm. (**G**) Representative confocal images of EB3-ΔY condensates (8 µM unlabeled and 2 µM mCherry-EB3-ΔY) and EB3 (8 µM unlabeled and 2 µM mCherry-EB3). Scale bar: 4 µm. (**H**) 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.

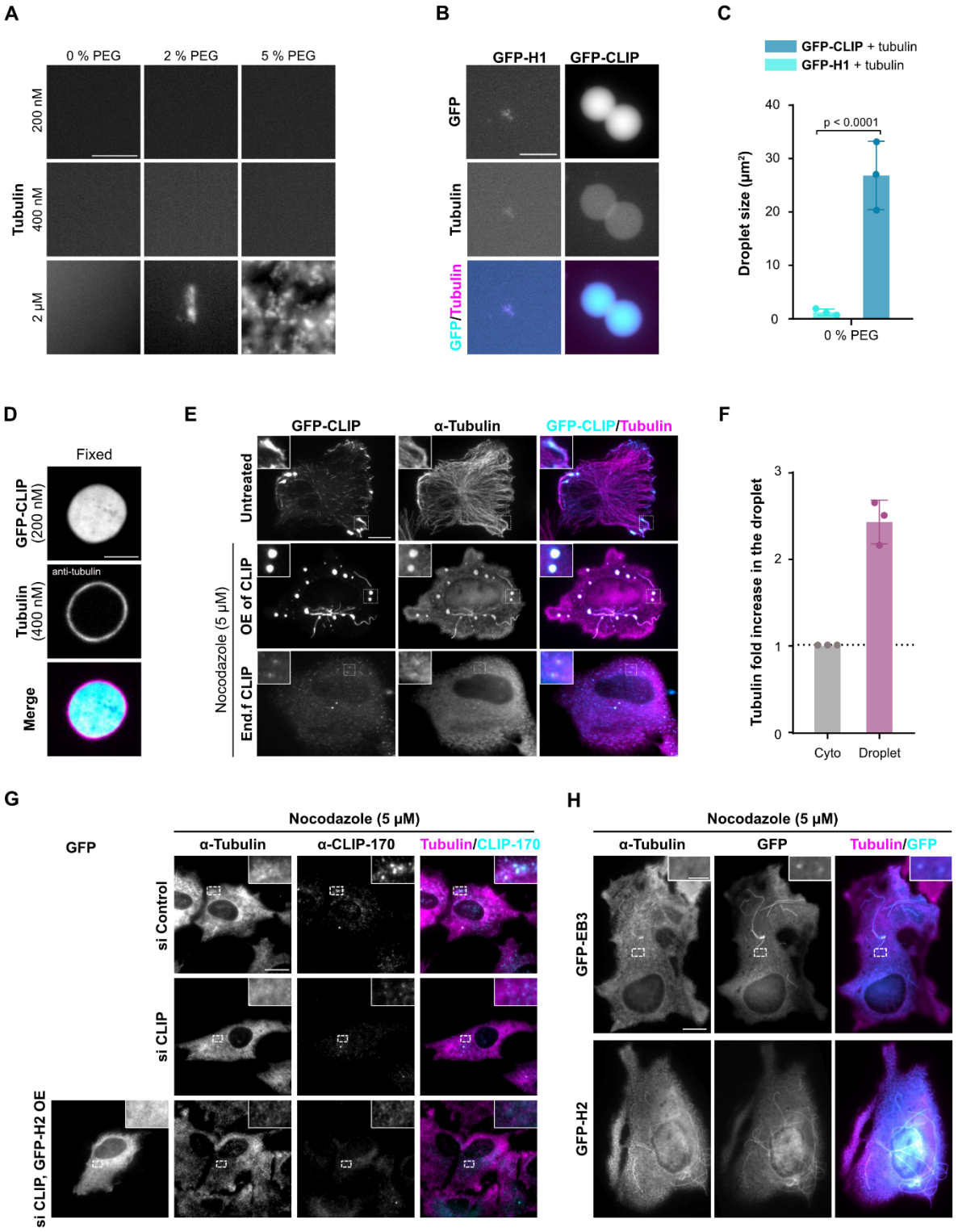
A
0 % PEG
2 % PEG
5 % PEG
200 nM
Tubulin
400 nM
2 µM
B
GFP-H1
GFP-CLIP
GFP
Tubulin
GFP/Tubulin
C
GFP-CLIP + tubulin
GFP-H1 + tubulin
p < 0.0001
Droplet size (µm²)
40
30
20
10
0
0 % PEG
D
Fixed
GFP-CLIP (200 nM)
anti-tubulin
Tubulin (400 nM)
Merge
E
GFP-CLIP
α-Tubulin
GFP-CLIP/Tubulin
Untreated
Nocodazole (5 µM)
OE of CLIP
End.f CLIP
F
Tubulin fold increase in the droplet
3
2
1
0
Cyto
Droplet
G
Nocodazole (5 µM)
GFP
α-Tubulin
α-CLIP-170
Tubulin/CLIP-170
si Control
si CLIP
si CLIP, GFP-H2 OE
H
Nocodazole (5 µM)
α-Tubulin
GFP
Tubulin/GFP
GFP-EB3
GFP-H2

Figure S6: CLIP-170 and EB3 form a tubulin-condensing network.
(A) Representative fluorescence confocal images 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**) Representative confocal images of purified GFP-FL-CLIP and GFP-H1 each at 200 nM with Atto-565-tubulin (400 nM). Scale bar: 20 µm. (**C**) Quantification of the coverslip surface coverage of tubulin (400 nM) in presence of GFP-FL-CLIP or GFP-H1 each at 200 nM. Mean with SD from 3 independent experiments with a total of 27 fields of view. Statistics: two-tailed Student's *t*-test. (**D**) Representative confocal images of tubulin/GFP-FL-CLIP droplets (unlabeled tubulin) fixed and immunostained using tubulin specific antibodies. **(E)** Representative TIRF images of fixed RPE-1 cells treated with 5 µM nocodazole for 1 hour either transfected with GFP-CLIP-170 (top panel) or stained for endogenous CLIP-170 and tubulin (bottom panel). Scale bar: 10 µm. Images are representative of 3 independent experiments. **(F)** Graph showing normalized tubulin fluorescence intensity in CLIP-170 droplets compared to cytoplasm in full-length GFP-CLIP-170 transfected RPE-1 cells treated with nocodazole. Mean with SD from 3 independent experiments with a total of 126 condensates from 26 cells. (**G**) Representative TIRF images of fixed RPE-1 cells transfected with control siRNA (top panel), CLIP-170 siRNA (middle panel), or CLIP-170 siRNA rescued with GFP-H2 (bottom panel) treated with 5 µM nocodazole for 1 hour and stained for tubulin. Zoom-in of regions indicated by dashed box. Scale bar: 10 µm. (**H**) Representative TIRF 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. Zoom-in of regions indicated by dashed box Scale bar: 10 µm.

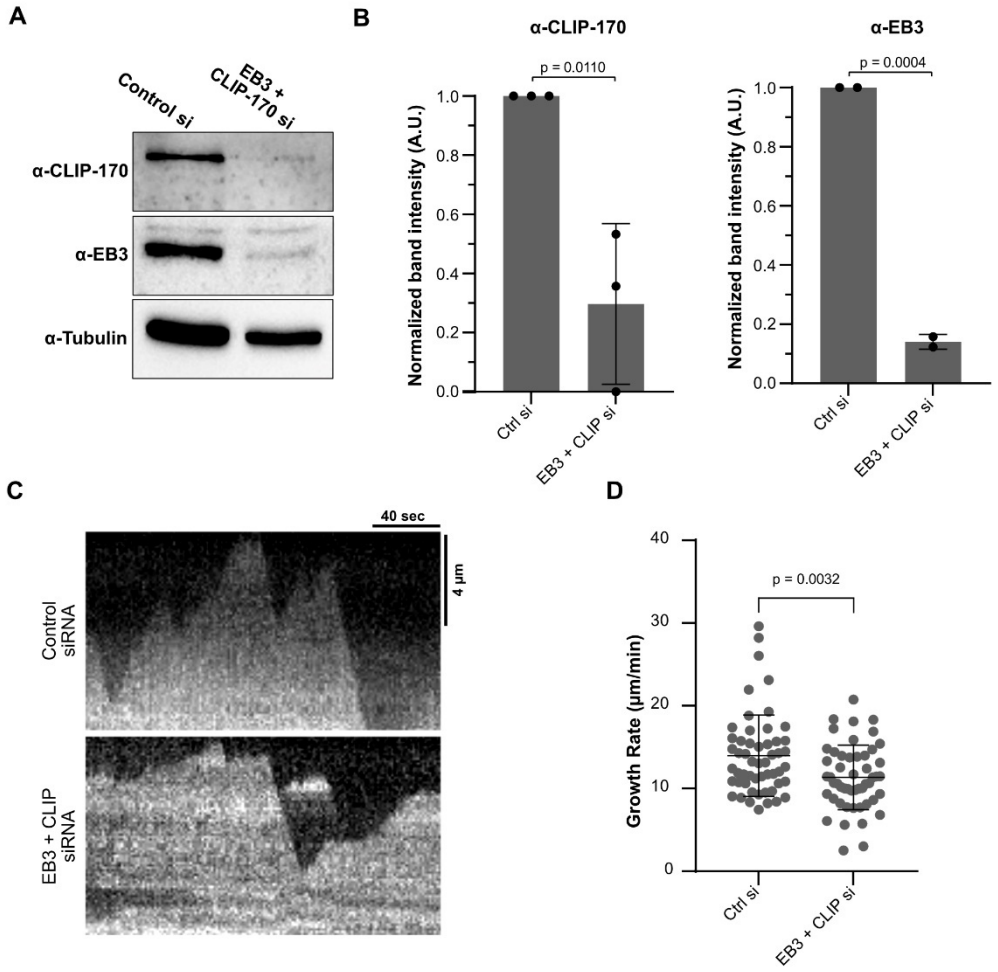

Figure S7: Depletion of EB3/CLIP-170 networks reduce microtubule growth rates in RPE-1 cells.

(**A**) Representative Western Blot of CRISPR/Cas9 knock-in GFP-Tubulin RPE-1 cells transfected with control siRNA or siRNA to CLIP-170 and EB3 simultaneously for 72 hours. (**B**) Quantification of western blot of CLIP-170 depletion (left) and EB3 depletion (right) in cells treated with either control or EB3 + CLIP-170 siRNAs. Graphs show mean with SD from 3 (α-CLIP-170) or 2 (α-EB3) individual experiments. Statistics: paired t-test. (**C**) Representative microtubule kymographs from CRISPR/Cas9 knock-in RPE-1-GFP-Tubulin cells transfected with either control (top) or EB3 + CLIP-170 (bottom) siRNAs for 72 hours. (**D**) Mean microtubule growth rate with SD from: Control – 53 microtubules from 29 cells; EB3 + CLIP siRNA – 51 microtubules from 19 cells (4 independent experiments per condition). In graph, each dot represents a single MT averaged over several different growth phases. Statistics: paired t-test.

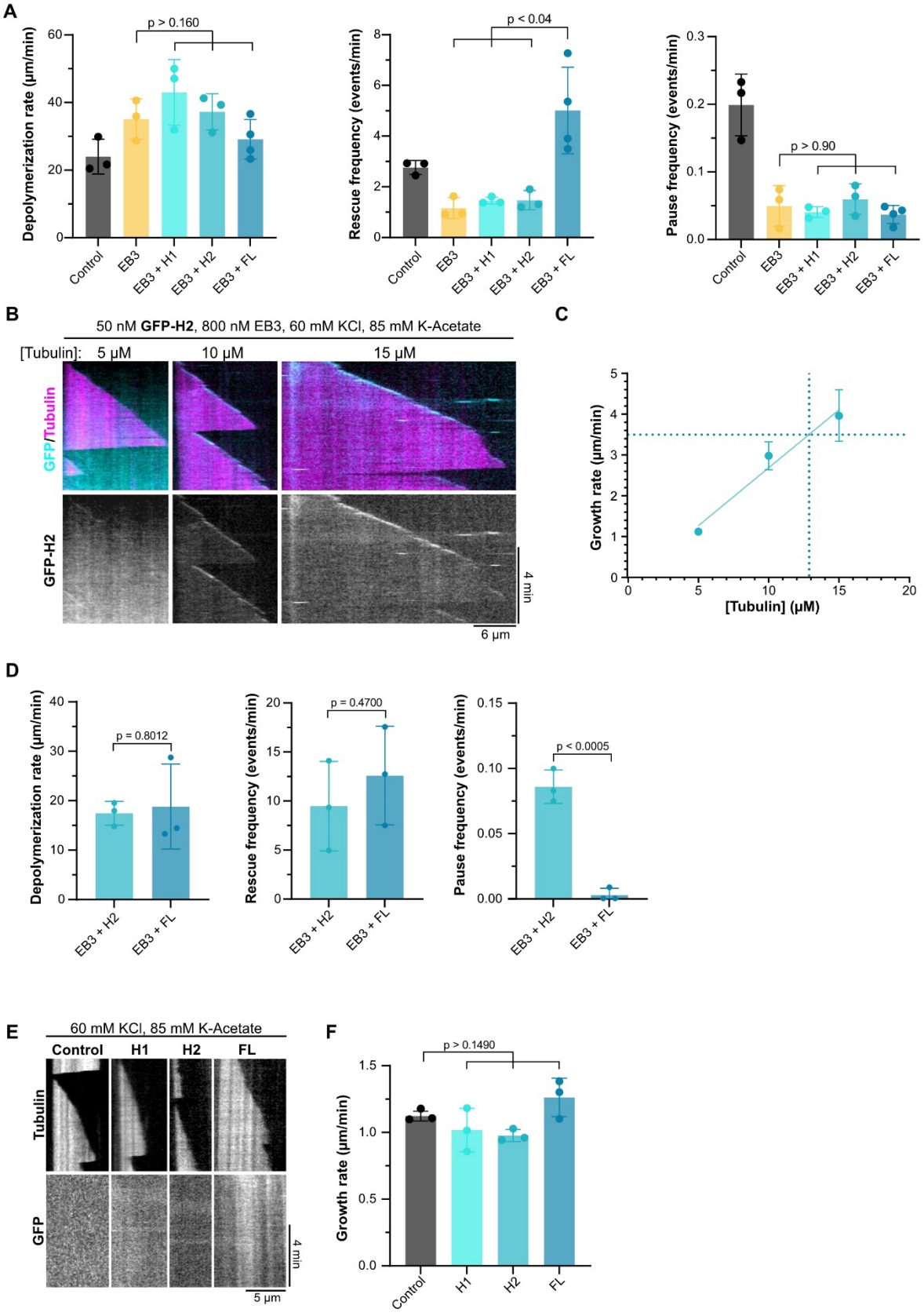
A
Depolymerization rate (µm/min)
p > 0.160
Control
EB3
EB3 + H1
EB3 + H2
EB3 + FL
Rescue frequency (events/min)
p < 0.04
Pause frequency (events/min)
p > 0.90
B
50 nM GFP-H2, 800 nM EB3, 60 mM KCl, 85 mM K-Acetate
[Tubulin]: 5 µM
10 µM
15 µM
GFP/Tubulin
GFP-H2
4 min
6 µm
C
Growth rate (µm/min)
[Tubulin] (µM)
D
p = 0.8012
p = 0.4700
p < 0.0005
E
60 mM KCl, 85 mM K-Acetate
Control
H1
H2
FL
Tubulin
GFP
4 min
5 µm
F
p > 0.1490

Figure S8: 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) in 60 mM KCl and 85 mM K-acetate (corresponding assay to Figure 6B, see here for number of analyzed microtubules). Tubulin (5 μM), EB3 (800 nM), H1 (50 nM), H2 (50 nM), FL-CLIP (50 nM). 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 (5 μM, 10 μM or 15 μM) 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 60 mM KCl (corresponding assay to Figure 6H, see here for number of analyzed microtubules). Tubulin (5 μM), EB3 (800 nM), H2 (50 nM), FL-CLIP (50 nM). Mean with SD from minimum three independent experimental replicates. Statistics: one-way ANOVA Fisher's LSD test (**E**) Representative microtubule kymographs of control (only tubulin), 5 μM 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 from three independent experiments. Total number of MTs analyzed per condition: Control – 48; H1 – 42; H2 – 41; FL – 26. Statistics: one-way ANOVA Fisher's LSD test.

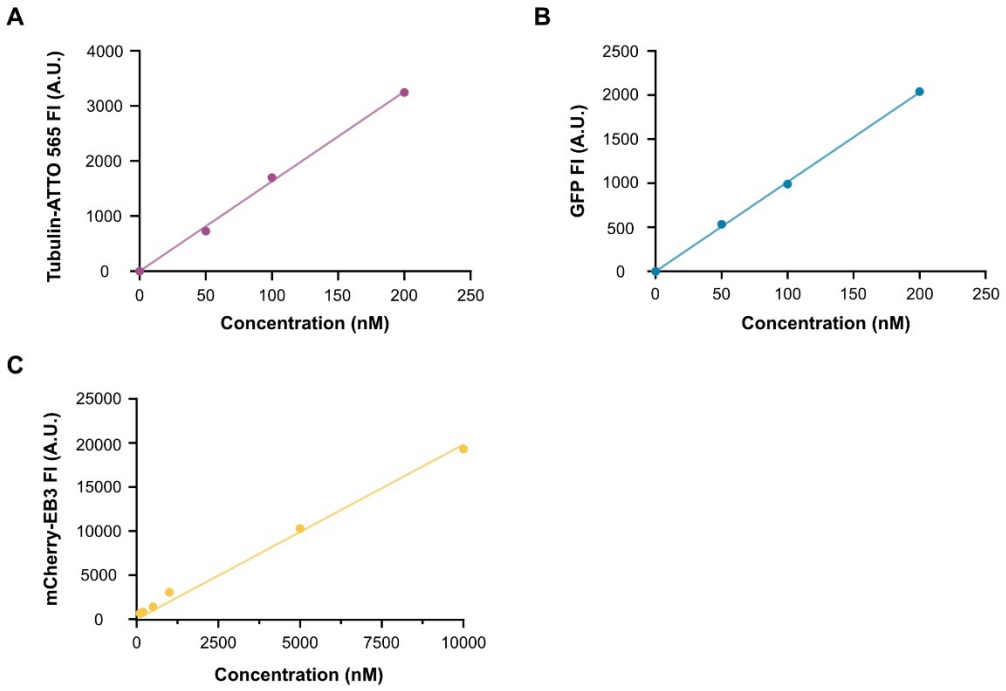

Figure S9: Calibration curve for Tubulin-ATTO 565, mCherry-EB3 and GFP.
Calibration curve between fluorescent intensity and concentration of: (**A**) Tubulin-Atto-565 at 50, 100 and 200 nM. (**B**) GFP at 50, 100 and 200 nM and (**C**) mCherry-EB3 at100, 200, 500, 5 000 and 10 000 nM.

Material and methods

Cell culture and treatments

Parental RPE-1 and CRISPR/Cas9 knock-in RPE-1-GFP-tubulin cells 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% CO_2. The cell lines were monthly checked for mycoplasma contamination. CRISPR/Cas9 knock-in GFP-tubulin RPE-1 cells were generated using the same guide RNA and protocol as in 60.

For transient expression studies of exogenous FL-CLIP, H1, H2 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 experiments in which endogenous CLIP-170 was depleted, parental RPE-1 cells were transfected with 10 nM siRNA targeting CLIP-170 (Santa Cruz Biotechnology) using lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Media was replaced the following morning, and cells were cultured for 72 hours post-transfection before

fixation, or transfected with GFP-H2 24 hours pre-fixation for "knockdown-rescue" experiments with H2. 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 GFP-FL-CLIP170 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 GFP-H1- and H2-CLIP were generated by PCR from the GFP-FL-CLIP-170 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.

For the GFP-H2-tail construct Gibson assembly was used from the GFP-FL-CLIP construct using the primer 5'- CAAGCTTCGAATTCTATGCTGAAACCCAGCGGGCTGAAGG with BstBl (Start of H2), CAGCTCTGCGTCCTTCTCTTTGTCAGCTTTGGTCTTTTCAAAGAGCAG-3' (end of H2), 5'- GCTGACAAAGAGAAGGACGCAGAGCTGGAGAAGCTGAGGAATGAG (start of tail) and CCGCGG TACCGTCGACTCAGAAGGTCTCATCGTCGTTGCAGTTGG (end of tail) using the PKPN enzyme.

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. Mutagenesis of the mCherry-6His-EB3

was used to create the EB3-ΔY281, the primer used were 5'-CAAGAAGACCAGGAC GAGTAACAGTAAAGGTGGATAC and GTATCCACCTTTACTGTTACTCGTCCTGGTCTT CTTG-3'.
For in-cell expression of EB3, EB3-mCherry vectors were obtained from Addgene (Addgene plasmid 55037).

Tubulin purification from bovine brain and labelling

Tubulin was purified from fresh bovine brain by two subsequent polymerization/depolymerization cycles as described previously (3). Tubulin labelling with biotin or ATTO-488, -565, -647 fluorophores was performed as described (3), and final labelling ratios to polymerize microtubules were 11% for ATTO-488 and 13% for ATTO-565 tubulin.

Protein purification

For purification of EB3, mCherry-EB3, EB3-ΔY and mCherry-EB3-ΔY, *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.

GFP-H1, GFP-H2 and GFP-H2-tail 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-CLIP-170-GFP was purified from insect cells as described previously (2). A plasmid encoding FL-CLIP-170-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 (61). For all purified proteins, protein concentration was measured by Bradford assay.

Imaging

Images in the same figure panel have the same brightness and contrast setting, unless otherwise stated in the figure legend.

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).

For stack acquisition and intensity measurement a 3i Marianas spinning disk confocal setup based on a Zeiss Z1 stand, a 100× PLAN APO NA 1.45 TIRF objective and a Yokogawa X1 spinning disk head followed by a 1.2× magnification lens and an Evolve EMCCD camera (Photometrics). Fast z-stack acquisition (0.5-µm steps) was obtained using a piezo stage (Mad City Labs). Single-emitter emission filters were always used to avoid bleed-through and each channel was acquired sequentially.

For the phase separation assay images were acquired using a confocal automated microscope (Molecular Device) with a 60X dry objective.

Microtubule dynamics

For *in vitro and in cell* microtubule dynamics measurements images were taken every second for 3 minutes using the TIRF microscope. Microtubules were tracked individually using the Freehand-

Line tool in ImageJ (15-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 dynamic parameters the slopes using a custom-written code. For all microtubule dynamic parameters, the average parameters were calculated across all the different growth and depolymerization phases of individual microtubules, e.g. to determine the average growth speed of a single microtubule, all the growth phases from a given kympgraph were taken into account..

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 using the TIRF microscope. 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) (1). For the fitting the solver plugin from Excel was used, using a single exponential fit $I(T) = A \times (1 - e^{(-t/\tau)})$ where A is the mobile fraction and τ a fitted parameter. The half time was calculated with the following the formula $T^{1/2} = \tau \times \ln(2)$.

+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 using the TIRF microscope. 1,6-Hexanediol (5%) was added after one minute, and cells were only analyzed whether they did not undergo any large-scale changes in morphology, as 1,6-hexanediol treatment has been noted to affect mammalian cell shape (2). 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.

Comparison between mCherry- and GFP-CLIP expression vs condensation phenotypes

Cells were plated on 96 well plates and transfected using the TransIT-X2 (Mirus) transfection reagent with 0.075 µg DNA per well according to the manufacturer's instructions**.** Images were acquired using a confocal automated microscope (Molecular Device) with a 60X water objective. The first 100 cells observed for each experiment were binned according to their condensation phenotype, as detailed in Figures S1A and B.

Correlation between whole cell fluorescence intensity and +TIP-network fluorescence intensity

For experiments in figure 1A and B, RPE-1 cells expressing GFP-CLIP-170 were imaged using a confocal automated microscope (Molecular Device) with a 60X water objective. Whole cell fluorescence intensity and +TIP-network fluorescence intensity was measured in ImageJ as

follows: whole cell fluorescence intensity was measured by measuring GFP intensity of a hand-drawn, cell shape-specific mask for each cell, and measuring the background intensity cell-adjacent using the same mask. Intensities shown on graph are the cell GFP intensity subtracted by the background intensity. For each cell measured, +TIP-network fluorescence intensity was measured by: i) randomly placing a 5 µm x 5 µm square ROI near the cell periphery, ii) performing linescans of five CLIP-170 comets (randomly selected) within that region using a line ROI (pixel width = 3), and iii) background intensity (cytoplasmic GFP) was measured in this ROI. Background was subtracted from comets by subtracting the average intensity of a 3-pixel wide line in a comet-adjacent region. The highest intensity point of the comet (the comet "peak") was averaged for all five comets measured, and this average comet peak value was plotted against the whole cell fluorescence intensity. The points were fitted to an exponential plateau with the following equation: $y= 762- (762-177, 1)e^{0.0016x}$.Cytoplasmic droplets and CLIP-170 coating / bundling microtubules were excluded from the analyses, only CLIP-170 at the growing microtubule tips was analyzed.

Phase separation assay

For in vitro phase separation assays, proteins were diluted to the appropriate concentration in BRB80 pH 6.9 supplemented with 50 mM potassium chloride and PEG 4000 (0, 2, 5 or 10% by weight) in Eppendorf tubes. For the phase diagram denoted salt concentration were used. After thorough mixing, reactions were transferred to 384-well plates (plastic well-bottom treated for cell culture, 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 microscope (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 S3C). 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. For the phase diagram, the threshold for LLPS/ no LLPS used was at least 5 droplets of more than 1 µm for one field of view.

Note that the tubulin shell (formed at the buffer-droplet interphase of tubulin/FL-CLIP droplets) disappears when the droplets sediment onto the treated plastic well-bottom. The tubulin shell remains when interacting with glass or Si-PEG, Si-PEG Biotin treated glass cover slides.

Calibration curve

To measure the concentration of proteins in the dilute and in the droplet phase, calibration curves were established for GFP, tubulin-565 and mCherry-EB3. To avoid mCherry-EB3 phase separation a buffer with 1 M NaCl was used. To establish the curve the following concentrations were used: GFP (50, 100 and 200 nM); mCherry-EB3 (100, 200, 500, 5 000 and 10 000 nM); and tubulin (50, 100 and 200 nM). The relation of the fluorescent intensity and the nanomolar

concentration was found using the linear fit. Since mcherry-EB3 (20%) was mixed with unlabeled EB3 (80%), we multiplied the concentration with 5.

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, 568, or 488 fluorophores) at room temperature for one hour. Cells were subjected to three additional PBS 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.

Fixation of FL-CLIP droplets

For experiments in which FL-CLIP droplets were fixed to observe whether unlabeled tubulin partitions into droplets (Figure S6B), GFP-CLIP (200 nM) was incubated with unlabeled purified bovine brain tubulin (400 nM) on coverslips for 15 minutes. Reactions were fixed directly on coverslips with 3% PFA for 15 minutes, washed 3 times in PBS, blocked and stained with antibodies to tubulin as described in the above immunofluorescence procedure.

Droplet-Pelleting assay

For the droplet-pelleting assays to determine amount of protein in the dense phase (Figure 5A) proteins were diluted to the appropriate concentrations (1 µM CLIP, 10 µM EB3, or 1 µM CLIP + 10 µM EB3) in BRB80 pH 6.9 supplemented with 125 mM potassium chloride and incubated at room temperature for 30 minutes. After 30 min incubation, a state is reached where droplet size equilibrated between the different conditions and droplets did not grow within the observation time. Following this incubation, reaction mixtures were centrifuged at 16,900 g at room temperature for 15 minutes using a Fresco 21 Haraeus tabletop centrifuge (Thermo Scientific).
The supernatant was collected, and pellets were resuspended in an equal volume of resuspension buffer (BRB80 pH 6.9 + 125 mM KCl). Pellets and supernatants from each reaction were run on SDS-PAGE gels, which were subsequently stained with QuickBlue Protein Stain Coomassie dye

(Lubio Science) for at least 2 hours before destaining in water. The fraction of protein in dense vs dilute phase was calculated by dividing the integrated Coomassie band intensity from the pellet or supernatant, respectively, by the sum of the integrated band intensity of the pellet and supernatant. Fold-change in pellet fraction was taken by dividing the protein fraction in the pellet for experiments with CLIP + EB3 by the protein fraction in the pellet for each protein alone.

SDS-PAGE and Western blot

Purified proteins or cell lysates were boiled, diluted into sample buffer (0.06 M Tris, 2% SDS, 10% glycerol, 5% mercaptoethanol, 50 mg bromophenol blue), and run on gels containing 10% Agarose. After protein separation, gels were stained with QuickBlue Protein Stain Coomassie dye (Lubio Science) for at least 2 hours before destaining in water.

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).

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 pH 6.9 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 pH 6.9, and microtubule seeds, then subsequently washing out unattached seeds with BRB80. Reaction buffer containing Atto-565 labelled-tubulin (1:5 ratio labelled to unlabeled; 5 µM for all assays except for Figure S6B, C) in BRB80 pH 6.9 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 10 minutes at 3 second intervals with TIRF microscopy.
We analyzed the growth rate, depolymerization rate, rescue frequency, catastrophe frequency and pause frequency for a minimum of 10 microtubules per experiment. For "low salt" assays (Figure 6E-F), the reaction buffer was supplemented with 60 mM potassium chloride. For "high salt" assays (Figure 6A-B), the reaction buffer was supplemented with 60 mM potassium chloride and 85 mM potassium acetate to facilitate tip tracking as described previously (61).

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 pH 6.9 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 (61).

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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Movies

Movie 1: Representative RPE-1 expressing mCherry-CLIP. Inset highlights fission of mCherry-CLIP droplets.

Movie 2: Representative RPE-1 expressing mCherry-CLIP. Inset highlights fusion of mCherry-CLIP droplets.

Movie 3: Fluorescence recovery after photobleaching of a mCherry-CLIP droplet in a representative RPE-1 cell.

Movie 4: Representative foci formation resembling fission in GFP-CLIP overexpressing RPE-1 cells. Scale bar: 2 μm.

Movie 5: Representative RPE-1 (magenta) expressing mCherry-H1 (cyan).

Movie 6: Representative RPE-1 (magenta) expressing mCherry-H2 (cyan).

Movie 7: Representative RPE-1 cell expressing mCherry-EB3 and treated with 5% 1,6-hexanediol (added at 00:30).

Movie 8: Fusion of two GFP-CLIP droplets (1μM) *in vitro*.

Movie 9: Fluorescence recovery after photobleaching of a purified GFP-CLIP droplet (2 μM) *in vitro*.

Movie 10: Fluorescence recovery after photobleaching of a purified EB3 (10 μM) labeled with GFP-EB3 (100 nM) droplet *in vitro*.

Movie 11: Fluorescence recovery after photobleaching of a purified EB3 (10 μM) labeled with GFP-EB3 (100 nM) mixed with GFP-CLIP (2 μM) droplet *in vitro*.

Movie 12: 3D representation of EB3 (8 μM) labelled with mCherry- EB3 (2 μM) droplets *in vitro*. Note that floating droplets moved during image acquisition.

Movie 13: 3D representation of GFP-CLIP (2 μM) droplets *in vitro*.

Movie 14: 3D representation of GFP-CLIP (2 μM) mixed with EB3 (8 μM) labelled with mCherry- EB3 (2 μM) droplets *in vitro*. Note that floating droplets moved during image acquisition.

Movie 15: Representative *in vitro* microtubule dynamics assay of Atto-565 tubulin (magenta) polymerized in the presence of 50 nM GFP-FL-CLIP (cyan) and 800 nM EB3 in high salt buffer.

Movie 16: Representative *in vitro* microtubule dynamics assay of Atto-565 tubulin (magenta) polymerized in the presence of 50 nM GFP-FL-CLIP (cyan) and 800 nM EB3 in low salt buffer.