SUPPLEMENTAL INFORMATION

Supplemental Methods

**Live cell imaging and quantification of CLIP fluorescence recovery at MT ends**

COS cells were transfected with the different YFP-CLIP-170 constructs and analyzed at 37 °C using total internal reflection fluorescence (TIRF) microscopy on an inverted Nikon Eclipse TE2000E microscope, equipped with a CFI Apo TIRF 100x 1.49 N.A. oil objective (Nikon) and a QuantEM EMCCD camera (Roper Scientific), and controlled by MetaMorph 7.1 software (Molecular Devices). For excitation, we used a 488 nm argon laser (Spectra-Physics Lasers) and a Chroma ET-GFP filter cube. YFP-CLIP170 constructs were imaged at 30 fps. Comet-like dashes, representing CLIP-170 on growing MT ends, were located in real time and bleached for ~1 ms at 100% laser power (i.e. "FRAP-on-the-fly"). 16-bit images were projected onto the CCD chip at a magnification of 0.067 μm/pixel. Images were exported to ImageJ (Rasband, W. S., National Institute of Health, Bethesda, MD, online available at http://rsb.info.nih.gov/ij), converted to 8-bit and analyzed with a home-written plug-in for ImageJ, which is partly based on the ImageJ kymograph plug-in (J. Rietdorf, European Molecular Biology Laboratory, Heidelberg, Germany). Further analysis was performed with the Aabel statistical software package (Gigawiz). For each construct, at least five transfected cells expressing low levels of the YFP fusion were imaged. To determine YFP-CLIP-170 fluorescence decay resulting from the bleaching of YFP-CLIP-170 fluorescence during imaging, we first subtracted the cytoplasmic background values, normalized curves to 1, and obtained an average curve from many individual YFP-CLIP-170-positive ends. To calculate the recovery of YFP-CLIP-170 fluorescence on MT ends after laser photobleaching, we subtracted background values and normalized curves to 1. The resulting curves represent a combination of fluorescence decay and recovery after photobleaching. We subsequently subtracted the fit of the averaged fluorescence decay to obtain a curve with only the mean fluorescence recovery. This curve could be fitted with an exponential equation, yielding \( k_{\text{recovery}} \). The half-life of recovery is calculated as \( \ln(2)/k_{\text{recovery}} \).

**Purification of rat CLIP-170**

CLIP-170 was purified by using Avidin-Biotin affinity-based separation. Primers encoding Avitag: forward 5’-CTAGCGATGGGTTGCGGTCTGAACGACATCTTCGAGGC-3’ and reverse 5’-CCGGTGCGCCCTGGAAGTATAAGTTCTCGCCACCGCCACCGGAAATGCCAGGTTCGTGCATTCCAGCTTTTCTGACGCTC-3’ were synthesized, annealed and inserted into Nhel/Agel sites in YFP-rat CLIP-170 plasmid (Avi-YFP-CLIP170). Authenticity of construct was confirmed by DNA sequencing. COS-7 cells were transiently cotransfected with
plasmids encoding Avi-YFP-CLIP170 full-length and BirA (biotin ligase) for 24 hrs. Cells were then treated with 100 nM Calyculin A for 30 min and lysed in a lysis buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.5 % NP-40, phosphatase inhibitor cocktail I & II (1:100, Sigmaaldrich), 10 mM glycerol 2-phosphate and protease inhibitor (Roche Applied Science)). Lysates were incubated with NeutrAvidin agarose beads (Thermo Fisher Scientific Inc.) for 2 hrs at 4°C. Beads were washed with lysis buffer, and bound proteins were separated by SDS-PAGE.

Sample analysis and mass spectrometry

The Coomassie-stained gel band corresponding to the Clip170 was excised, diced and divided into two tubes. One half was subjected to in-gel digestion with sequencing grade trypsin (Promega, 12.5 ng/μl) in 50mM ammonium bicarbonate pH 8.2 overnight at 37 ºC. The other half was digested with chymotrypsin (Roche Applied Science, 25 ng/μl) in 50mM ammonium bicarbonate pH 8.2 overnight at room temperature. Two different enzymes were chosen to increase the peptide coverage. Peptides were extracted with 50% acetonitrile (MeCN), 5% formic acid (FA) and then dried. Dried peptides were resuspended in 10 μl of 5%MeCN, 4% formic acid (FA), and 2 μl was loaded onto a pulled fused silica microcapillary column packed with C18 reverse-phase resin (Magic C18AQ; 5-μm particles; 200-Å pore size; Michrom Bioresources, Auburn, CA) using a Famos autosampler (LC Packings, San Francisco, CA). Once loaded, the peptides were separated using an Agilent 1100 series binary pump across a 45 min linear gradient of 6-28% CH3CN in 0.125% HCOOH at a flow rate of 600 nl/min.

Mass spectra were acquired in an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher, San Jose, CA) over the entire run using ten MS/MS scans following each survey scan. Raw data were searched against the Clip170 amino acid sequence using Sequest software with no enzyme specificity and a 1.1 Da mass tolerance. The search parameters used for posttranslational modification included a static modification of 57.02146 Da on cysteine (carboxyamidomethylation) and dynamic modifications of 15.99491 Da on methionine (oxidation) and 79.96633 Da on serine, threonine, and tyrosine (phosphorylation). The probability of correct phosphorylation site localization was determined for every site in each peptide using the Ascore algorithm (Beausoleil et al, 2006). Sites with Ascore values of >19 were considered localized with high certainty.
**Supplemental Figure 1.** Intracellular distribution of YFP-CLIP-170 and its mutants. (A) Live cell imaging of YFP-CLIP-170 in 3T3 NIH fibroblasts before and after taxol treatment. Pretreatment of cells with staurosporine induces redistribution of YFP-CLIP-170 to taxol-stabilized MTs. (B) Binding of YFP-CLIP-170 mutants to MT tips. Live imaging (upper panels) and scatter plot analysis of the intensity and length of YFP-CLIP-170-positive structures at the MT tips.

**Supplemental Figure 2.** Golgi reassembly after nocodazole-induced scattering. CHO-K1 cells expressing CFP-GalT (1,4-galactocyltransferase) and either WT, A-5 or E-5 mutants showed perinuclear localization of the Golgi apparatus at steady-state. Treatment of cells with nocodazole induced Golgi dispersion. Removal of the drug resulted in accumulation of Golgi elements in the perinuclear zone (at 0.5 hr) followed by Golgi reassembly (shown at 1 and 4 hrs). Bar, 10 μm.

**Supplemental Figure 3.** Fast FRAP analysis of YFP-CLIP-170. (A) Time-lapse images of wild type YFP–CLIP-170 (wt) and the phosphorylation-defective mutant (A-5) before (-00:02ms) and after photobleaching (00:02 and 00:18 ms for wt; 00:02 and 01:00ms for A-5). Comet-like dashes representing CLIP-170 on growing MT ends were located in real time and bleached for ~1 ms at 0 ms. Time in seconds and milliseconds are in the top right corner. Bar, 1 μm. Notice that recovery is observed behind the most proximal part of the MT end. (B) Time-versus-distance plots (kymograph analysis) of selected MT plus ends. The time of bleaching appears as a white stripe and is indicated by arrow. Note that recovery is observed behind the most proximal part of the MT end. (C) Corrected fluorescence recovery (mean ± SEM) for wt (black, 18 MT tips) and A-5 mutant (red; 31 MT tips) of YFP-CLIP-170. The mean fluorescence recovery was corrected to mean fluorescence decay in non-bleached areas and normalized to zero. (D) The curve fitting to data shown in C was generated to determine the recovery constant (shown in Fig. 4).

**Supplemental Figure 4.** Phosphorylation sites of CLIP-170 in cells. COS-7 cells transiently co-transfected with BirA and (A) Avi-tagged full-length CLIP-170 or (B) Avi-tagged CLIP-170 head were treated with 100 nM calyculin A for 30 min. Avi-tagged proteins were purified by NeutrAvidin agarose beads. Enzymatic digests of purified proteins were analyzed by tandem mass spectrometry. Detected phosphorylation sites are shown in red. Recovered peptides are in
bold and underlined. Residues that were not found in this experiment are shaded in gray. Serine residues that regulate CLIP-170 conformational changes are highlighted in green. Total number of peptides obtained from CLIP-170 full-length and head after enzymatic digestion were 99 and 111, respectively. The number of unique peptides after digestion and analysis were 75 and 80, respectively. (C) Ascores summary on peptides that contain serine residues identified as critical for CLIP-170 conformational changes. M* represents oxidized methionine residue. Residues with Ascores higher than 13 are predicted to be phosphorylated with high confidence (Beausoleil et al, 2006).

**Supplemental Figure 5.** Role of $S^{146}$ phosphorylation in regulating CLIP-170 intramolecular association. Co-IP of HA-ΔH with GFP-H2 mutants. Lysates were prepared from COS-1 cells transiently co-expressing the indicated GFP-H2 mutants together with ΔH; immunoprecipitation was performed with anti-GFP mAb. Western blots were probed with anti-HA to detect bound ΔH and with anti-GFP antibodies to detect the H2 mutants. Note that mutation of Ser 146 for Ala reduces the amount of co-immunoprecipitated ΔH.

**Supplemental Figure 6.** PKA phosphorylates $S^{311}$ in vitro. Collision-induced dissociation spectrum of peptide SPSASSLSSMSSVASSVSSKPSR amino acids 309-331 from CLIP-170 phosphorylated with PKA. +3 charged parent ion at m/z 770.37 (*). This species represents the peptide with an oxidized methionine plus one phosphate. The $y_{21}$ and $y_{22}$ ions confirm the presence of a dehydroalanine at position 311 resulting from loss of phosphate upon fragmentation.

**Supplemental Figure 7.** Mutation of $S^{311}$ inhibits effect of PKA on CLIP-170. (A) Effect of PKA activation (forskolin) and inhibition (H-89) on interaction between GFP-H2-S311A or GFP-H2-S311E and HA-ΔH domains of CLIP-170. COS-1 cells co-expressing GFP-H2-S311A or GFP-H2-S311E and HA-ΔH were treated with DMSO, 40 μM forskolin or 0.2 μM H-89. Cell lysates were used for IPs with anti-GFP antibodies and resulting precipitates were probed with antibodies against HA and GFP. PKA shows no effect on site-specific mutant. (B) Representative images of CHO-K1 cells expressing either YFP-CLIP-170A$^{309,311}$ or YFP-CLIP-170E$^{309,311}$ mutants as indicated. Cells were stained for YFP (green in overlay) and tubulin (red). Activation of PKA did not abolish the binding of YFP-CLIP-170A$^{309,311}$ to MTs in taxol-treated cells. Inhibition of phosphatases with OA had no effect on affinity of the same mutant to MT tips. Inhibition of PKA did not induce the binding of YFP-CLIP-170E$^{309,311}$ mutant to taxol-MTs.
Supplemental Figure 1.
Supplemental Figure 2.
Supplemental Figure 3.
### Supplemental Figure 4.

#### A. FL CLIP-170.

| 1  | 11 |
|----|----|
| MSMLKPGS | APTKILKPGS |
| 61 | 71 |
| GERWVNGNKG | PGFIQPLGET |
| 121 | 131 |
| PKLTKRVQA | EDEANLQTA |
| 181 | 191 |
| TPQISNLTKT | ASEISNLSE |
| 241 | 251 |
| CGVELDPELG | KNDGAVAGTR |
| 301 | 311 |
| TPASLKRSP | ASLSLSSMS |
| 361 | 371 |
| QHIEQOLLAER | DLRAEVAKA |
| 421 | 431 |
| REKVELNNQI | EEERKVEDL |
| 481 | 491 |
| KLPRELDR | VATEVSEIR |
| 541 | 551 |
| SALQKELEV | HTDHQNEVTS |
| 601 | 611 |
| NKENSVDIAL | WSKKLETAIA |
| 661 | 671 |
| EIESLQSKQ | SERSAHAKEM |
| 721 | 731 |
| SKLOEAEIKK | EKFASASEEA |
| 781 | 791 |
| KFPKEKDRED | QLVMKAKELE |
| 841 | 851 |
| ANENASLLQK | SIGEVTLKAE |
| 901 | 911 |
| KBEKASETK | IKHEILQNF |
| 961 | 971 |
| AEDAMQIMEQ | MTKETETLA |
| 1021 | 1031 |
| NQKMEEFKKE | IETIKQAAAO |
| 1081 | 1091 |
| NOLLEMKSS | PSNTRLRESEY |
| 1141 | 1151 |
| GENASAKS | SVVQTELSDK |
| 1201 | 1211 |
| SQQIMDFLNS | VIVDLQRRKQ |
| 1261 | 1271 |
| CDICDCFDLH | DTEDCPTAQ |
| 1321 | 1331 |

| 21 | 31 |
|----|----|
| TALKTPAAAA | APLEKTVSE |
| 81 | 91 |
| QFAPGQWAGI | VLDEPIKND |
| 141 | 151 |
| HARRASPLST | AAATMVSSSP |
| 201 | 211 |
| AGSVKKGERE | LKIGDRVVLG |
| 261 | 271 |
| VYQCPQKYG | FAPVHKVTKI |
| 321 | 331 |
| VASSVSSKPS | RTGLLTETSS |
| 381 | 391 |
| TSHVGEIEQE | LALARDGDQ |
| 441 | 451 |
| QFRVVEESIT | KGDLETQTKL |
| 501 | 511 |
| MELEKDLAR | VQVVAELRRR |
| 561 | 571 |
| LKVHGFTREE | MFKEIKALH |
| 621 | 631 |
| SHQQAMEELK | VSPSKGIGTD |
| 681 | 691 |
| ESMKAKLMK | IPEKEDSLEA |
| 731 | 741 |
| VSTQTSQMDT | VNLHQQKEEQ |
| 801 | 811 |
| NDAEIMKMS | GDNSSLKTM |
| 861 | 871 |
| QSOQEAHHK | EEEKELENK |
| 891 | 901 |
| SQMLVTDK | LKAAQEANRD |
| 921 | 931 |
| SLEDQTQNA | KQSELDTLK |
| 981 | 991 |
| KSQQLSAQ | ENVKLAEELG |
| 1041 | 1111 |
| KSQQLSAQ | ENVKLAEELG |
| 1101 | 1111 |
| RIKDADEEKAS | LQKSMLTS |
| 1161 | 1171 |
| VKEKLKVN | ELQKENKRO |
| 1211 | 1231 |
| DLKMKVEMMS | EGALEANGG |
| 1281 | 1291 |
| MESDPHPSTH | HGRSSEERPY |
| 21  | 31  |
| 91  | 101 |
| 141 | 151 |
| 201 | 211 |
| 261 | 271 |
| 321 | 331 |
| 381 | 391 |
| 441 | 451 |
| 501 | 511 |
| 561 | 571 |
| 621 | 631 |
| 681 | 691 |
| 731 | 741 |
| 801 | 811 |
| 861 | 871 |
| 891 | 901 |
| 921 | 931 |
| 981 | 991 |
| 1041 | 1111 |
| 1101 | 1111 |
| 1161 | 1171 |
| 1211 | 1231 |
| 1281 | 1291 |

| 41 | 51 |
|----|----|
| KASPPSSET | QEEFVDDFR |
| 101 | 111 |
| GSVAGVRYQ | CEPLKGIPTR |
| 161 | 171 |
| ATPSNIPQKP | SQPVAKETSA |
| 221 | 231 |
| GTKAGVVRFL | GETDFAKGEW |
| 281 | 291 |
| 341 | 351 |
| RYARIGT | ALQEALEK |
| 401 | 411 |
| HVLEEAWM | OLRMVEAAD |
| 461 | 471 |
| EHARIKELEQ | SSLFEKT |
| 521 | 531 |
| LESSKPFGDV | ELSRSLDA |
| 581 | 591 |
| AATELSKE | IELRRLYQOH |
| 641 | 711 |
| SAFAELKTE | QHLVEMEEM |
| 701 | 771 |
| VAKRLTAED | RLENLDMEA |
| 761 | 831 |
| FNLSSELEK | VEELQLKLT |
| 821 | 891 |
| HDQOLDK | SHYQCQLD |
| 941 | 951 |
| IMQDMEEK | QADKAKAQT |
| 991 | 1011 |
| ENNLKTEEL | NKSSKLNE |
| 1051 | 1071 |
| RTRDEVTSQH | KLEEEESLN |
| 1121 | 1131 |
| LLTEKDAELE | KLRNEVTLR |
| 1181 | 1191 |
| LSSSSGNTDV | QTEEDERA |
| 1241 | 1251 |
| PNSYDDQE | QSKKPRLE |
| 1301 | 1311 |
| CEICEMFGHW | ATCNCNTETF |
### B. CLIP-170 H2 domain.

| Peptide | Residue | Ascore |
|---------|---------|--------|
| RSPSpASSLSSMSSVASSVSSKPSR | S311 | 20.91 |
| SpPSASSLSSMSSVASSVSSKPSR | S309 | 17.36 |
| SPSpASSLSSM*SSVASSVSSKPSR | S311 | 14.49 |
| SPSpASSpLSSM*SSVASSVSSKPSR | S311 & S313 | 14.49 & 7.53 |
| RSPSpASSpLSSM*SSVASSVSSKPSR | S311 & S313 | 38.75 & 2.22 |
| KRSPSpASSL | S311 | 18.87 |
| MATTPASLKRSPASSpL | S314 | 16.2 |
| KRSPSpASSL | S311 | 24.75 |
| KRSPSpASSpL | S311 & S314 | 21.3 & 26.38 |

### C.

| Peptide | Residue | Ascore |
|---------|---------|--------|
| RSPSpASSLSSMSSVASSVSSKPSR | S311 | 20.91 |
| SpPSASSLSSMSSVASSVSSKPSR | S309 | 17.36 |
| SPSpASSLSSM*SSVASSVSSKPSR | S311 | 14.49 |
| SPSpASSpLSSM*SSVASSVSSKPSR | S311 & S313 | 14.49 & 7.53 |
| RSPSpASSpLSSM*SSVASSVSSKPSR | S311 & S313 | 38.75 & 2.22 |
| KRSPSpASSL | S311 | 18.87 |
| MATTPASLKRSPASSpL | S314 | 16.2 |
| KRSPSpASSL | S311 | 24.75 |
| KRSPSpASSpL | S311 & S314 | 21.3 & 26.38 |
Supplemental Figure 6.
Supplemental Figure 7.

A

|        | IP: GFP |
|--------|---------|
| DMSO   | +       |
| forskolin | +       |
| H-89   | -       |

WT | S311A | WT | S311E |

Western Blot

HA

GFP

B

YFP-CLIP-170A<sup>309, 311</sup>

| forskolin 20μM | + OA 100nM + taxol | OA 1μM | H-89 200nM + taxol |

YFP

Tubulin

Merge