Supporting Information

Varma et al. 10.1073/pnas.0908959107

SI Methods

Construction of Expression Plasmids. The FKBP-LC8Trap was generated using PCR to isolate a cDNA fragment containing the LC8 binding region of the rat dynein intermediate chain (IC) (isofrom 2C) residues 125–138 (REIVTYTKETQTP), and subcloning this segment into pC2Fv1E mammalian expression vector (generous gift from Ariad Pharmaceuticals Inc.) using the SpeI and BamHI restriction sites (New England BioLabs). An equivalent construct for bacterial expression was generated by isolating the cDNA for the FKBP-LC8Trap and subcloning this fragment into the Neo1 and HindIII sites of the pet21D vector (Novagen).

The FKBP-TcTex1Trap (wild type and mutants) constructs for both mammalian and bacterial expression vectors were generated using PCR by isolating the cDNA fragment containing the TcTex1 binding region of the rat DIC (residues 107–125, GRGPIKGLGMAKITOVDFPPR) and following the same protocol for the FKBP-LC8Trap.

The mutants were generated using a two-step, site-directed mutagenesis protocol (QuickChange; Stratagene). Briefly, PCR amplification was performed using a vector-specific T7 primer and the appropriate reverse mutant primer to generate extended mutant oligonucleotides. The extended oligonucleotides were purified and used for standard site-directed mutagenesis. This two-step protocol enabled the production of several mutants not obtainable using the standard protocol.

Enhanced green fluorescence protein fusions or GFP-tagged FKBP-LC8Trap and FKBP-TcTex1Trap were generated by inserting specific cDNA fragments into the SacI and EcoR1 restriction sites of the pEGFP-C1 vector (Clontech). The sequencing of each construct was confirmed by automated DNA sequencing (Kimmel Cancer Center DNA Core Facility, Philadelphia).

Protein Expression and Purification. Each positive clone was expressed in Escherichia coli BL21 (DE3)+ cells (Novagen). For the FKBP-LC8Trap and FKBP-TcTex1Trap (wild type and mutants), overexpression of an individual construct was carried out at 22 °C overnight to increase solubility, after the culture density reached A600 ~ 0.5 OD and induction with 250 μM 2-β-isopropylthio-galactosidase. Protein production for all other reagents was carried out at 37 °C. The cells were harvested by centrifugation, resuspended in 1× PBS, lysed by sonication (15% duty cycle for 0.5 min), and clarified by ultracentrifugation (40,000 × g at 4 °C). Each construct was purified to homogeneity by either ammonium sulfate fractionation or Ni affinity chromatography followed by loading on a sizing column (HiLoad 26/60 Superdex 75; GE Healthcare Life Sciences). The column was pre-equilibrated with 20 mM Tris (pH 7.5), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT. Before loading on the column, the protein samples were briefly centrifuged at 4 °C (13,200 × g). SMT-IC wild-type/mutants, SMT, and TcTex1 have elution volumes of 10.6 mL, 12.3 mL, and 12.25 mL, respectively. The 1:1 binary complexes (SMT-IC-TcTex1) elute earlier at 9.6 mL.

Analytical Size-Exclusion Chromatography. Analytical size-exclusion chromatography experiments were performed to determine the elution profiles of free proteins (e.g., SMT-IC and the mutants LC8 and TcTex1) as well as their complexes. The column used was a Superdex 75-10/300GL (GE Healthcare Life Sciences). The column was pre-equilibrated with 50 mM Tris (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT. Before loading on the column, the protein samples were briefly centrifuged at 4 °C (13,200 × g). SMT-IC wild-type/mutants, SMT, and TcTex1 have elution volumes of 10.6 mL, 12.3 mL, and 12.25 mL, respectively. The 1:1 binary complexes (SMT-IC-TcTex1) elute earlier at 9.6 mL.

Cell Culture. COS7 and COS1 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine/calf serum (Atlanta Biosciences), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). Transfection was performed in 80–90% confluent 24-h cultures of COS1 cells using Lipofectamine 2000 (Invitrogen) and Opti-MEM media (Invitrogen) according to the manufacturer's recommendations. Plasmid concentration was 0.25–0.5 μg/well. Various concentrations (10 nM to 2 μM) of AP20187 (Ariad Pharmaceuticals Inc.) were added to each experimental wells 24 h posttransfection and incubated for different periods of time before analyzing the cells.

Antibody and Reagents. Anti-EEA1 and anti-GM130 monoclonal antibodies were purchased from BD Biosciences, monoclonal anti-fibulin from Sigma, monoclonal anti GFP from Invitrogen, and monoclonal anti-LAMP2 from Santa Cruz Biotechnology Inc. A commercial rabbit polyclonal anti-LC8 antibody and monoclonal anti-dynein intermediate chain (IC 74.1) was from Chemicon. Control and LC8 siRNA were designed as previously published (2). SlowFade antifade reagent (Molecular Probes) was purchased from Invitrogen, 37% formaldehyde from Sigma, and 10× PBS from Roche Applied Science. All other chemicals used for this study were purchased from Sigma unless otherwise noted.

Immunofluorescence. For immunostaining, transiently transfected COS1 cells were fixed with 3.7% formaldehyde at room temperature for 10 min, and subsequent immunostaining was performed as described previously. COS 1 cells grown on 25-mm coverslips were washed 3× with PBS, treated with 3.7% formaldehyde in PBS for fixation, and permeabilized in 0.5% Triton X-100 in PBS at room temperature for 10 min. Cells were then incubated with blocking buffer containing 4% normal goat serum and 0.5% Triton X-100 in PBS. Anti-EEA1 or anti-GM130 monoclonal antibody was added to label either early endosome network or cis Golgi marker protein at a dilution of 1:100 in the same buffer for 30 min at 37 °C, and the coverslips were washed and incubated with rhodamine-conjugated donkey anti-mouse secondary antibody (1:100). After washing, the coverslips were mounted on slides to visualize the trapping effects. Samples were imaged using either Zeiss Axiowert S100 TV wide-field microscope system (x63 1.4 NA objective) or Zeiss LSM 510 (UV) Meta confocal microscope (x40 1.3 NA objective).

Immunoprecipitations. All IPs were performed in RIPA buffer [100 mM NaCl, 50 mM Tris, 1 mM EGTA, 1% Nonidet P-40 (pH 7.4)] at 4 °C for 2 h. IP and blotting antibodies include monoclonal anti-GFP (ab1218; Abcam), monoclonal anti-dynein intermediate chain 74.1 (MAB1618; Millipore), rabbit anti-LC8 (PIN-FL89; Santa Cruz), and rabbit anti-FKBP (ab2918; Abcam). Samples were run on 4–20% Tris-Glycine gels (Invitrogen) and transferred, and images were acquired using a LI-COR scanner and Odyssey software (LI-COR biosciences).

FACS Sorting. Cell sorting was performed on a BD FACSAria (BD Biosciences), and 30% of the cells were determined to give a strong GFP signal and collected for dynein immunoprecipitations. AP20187 was kept at a concentration of 100 nM during sorting and added to the IP buffer at a concentration of 1 μM.

Live Cell Imaging of LLC-PK1 Cells. Images were collected using transmitted light for a period of 12 h on an Olympus IX81 microscope using a Hamamatsu EM-CCD camera. The temperature was kept at 37 °C, and cells were grown in CO2-independent medium with 10% FBS (Invitrogen).


Fig. S1. Size-exclusion traces of LC8 and the LC8 trap. Chromatograms of LC8 and the LC8 trap are shown individually and as mixtures with and without AP20187. Note in the absence of AP20187, the mixture of LC8 and LC8 trap produces broad elution profile centered about the individual elution volumes, indicative of a superposition of the individual components. In other words, LC8 and the LC8 trap do not form a high-affinity complex. As a point of comparison, the same mixture shifts to an earlier elution band in the presence of AP20187 (see Fig. 2C).

Fig. S2. Dimerization dependence of the L–>A TcTex1 trap. The point mutation, L112A, effectively blocked the TcTex1 monomeric trap interaction but required significantly more AP20187 to sequester TcTex1. The bottom trace shows the analytical SEC chromatogram of TcTex1 and the L112A-FKBP-TcTex1 Trap at 1× (10 nM). At 4-fold higher concentration (40 nM), the complex begins to form (middle trace). At 16-fold higher concentration (160 nM), nearly all of the TcTex1 is sequestered (top trace). The LC8 trap effectively sequestered LC8 at 10 nM.
**Fig. S3.** Traps are specific for target. Native gel PAGE shows that the LC8 trap does not interact with TcTex1 and that the TcTex1 trap does not interaction with LC8.

**Fig. S4.** Dimerizer concentration dependence on lysosome dispersion. The number of cells with disperse lysosomes using the LC8 trap indicates the effect is saturated above 100 nM of AP20187.
**Fig. S5.** The LC8 trap binds LC8 in vivo and sequesters LC8 from the dynein complex only in the presence of AP20187. (A) Anti-GFP pulls down LC8 from LC8 trap-transfected Cos7 cells in the presence of AP20187 (cf. lanes 5 and 11). Cells were transfected for 24 h and treated with AP20187 (lanes 7–11) or ethanol (lanes 1–5) for 8 h before the IP was performed. (B) The LC8 trap and treatment with AP20187 partially depletes LC8 from the dynein complex. Cos7 cells were transfected with the LC8 trap for 24 h and treated with AP20187 (lanes 7–11) or ethanol (lanes 1–5) for 16 h followed by FACS sorting. Dynein intermediate chain was pulled down from the lysates of the GFP-expressing cells. In the presence of AP20187, there is a 55 ± 10% (n = 2) reduction of the amount of LC8 associated with the dynein complex (lanes 5 and 11). (C) Recombinant LC8 trap prevents dynein IC from associating with LC8 in the presence of AP20187. Bacterially expressed LC8 trap was added to rat brain lysates with AP20187 (lanes 6 and 7) or ethanol (lanes 4 and 5) and incubated at 37 °C for 5 min before LC8 was pulled down. LC8 fails to pull down dynein IC in the presence of AP20187 (lane 7). L, lysate; S, supernatant; P, pellet. Anti-FKBP was used to blot for the trap and also recognizes endogenous FKBP. Ethanol was used as a control because the AP20187 compound is dissolved in ethanol. AP20187 was used at a concentration of 1 μM in all experiments.

**Fig. S6.** RNAi of LC8. Western blot shows a significant knock down of LC8 after 4 days’ treatment of RNAi. The dynein intermediate chain remains constant. A comparable percentage of cells display dispersed Golgi using RNAi as observed for either LC8 or TcTex1 trap.
Fig. S7. The TcTex1 trap does not alter time spent in mitosis of LLC-PK1 cells. LLC-PK1 cells were transfected with the TcTex trap for 24 h and treated with AP20187 or ethanol for 2 h before live images were taken. The nuclear envelope breakdown to anaphase onset was determined for both transfected and untransfected cells in the absence and presence of AP20187. Error bars are SD of 10–40 cells per condition.

Fig. S8. Induction of LC traps does not affect MT organization. (A) Cos7 cells expressing a GFP-FKBP control (Top), the GFP-LC8 trap (Middle), or the GFP-TcTex1 trap (Bottom) for 24 h were treated with AP20187 for 8 h and stained with DAPI for nuclei and an anti-tubulin antibody to label microtubules. (B) Quantification of radial microtubule arrays in interphase cells expressing the control or LC traps indicates that the microtubule cytoskeleton was not affected by induction of the traps relative to controls. (Scale bars: 5 μm.)
Fig. S9. Design of a potential trap. P27-Kip1 (P27) regulates the kinase activity of Cdk2-CyclinA complex by sterically blocking the active site of Cdk2 kinase. The crystal structure of the ternary complex suggests a potential design to generate an inducible trap to inhibit Cdk2 (3). First, we note that the rapamycin analog AP21967 (Ariad Pharmaceuticals Inc.) readily dimerizes modified FKBP and FRAP (FKBP-rapamycin associated protein) (4). Thus, we propose to divide P27 into two fragments (green and blue) and tether one to FRAP and the other to FKBP. In this example, we propose to tether residues 72–93 to the C terminus of the FRAP and residues 25–71 to the N terminus of FKBP. A short gly-ser linker will be placed between the P27 peptide fragments and FKBP or FRAP to allow flexibility that may be required for binding interaction of the two P27 domains. Dimerization of the FKBP-P27 N terminus and FRAP-P27 C terminus by AP21967 would reconstitute the full-length P27 and should bind the active Cdk2-CyclinA complex with similar affinity. Specific mutations in the P27 fragments can be introduced to block posttranslational modifications that release P27 under normal physiological conditions (5). Furthermore, additional modifications may be necessary to reduce the monomeric binding affinity, as shown for the TcTex1 trap in the main text.