SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

Cell viability assay

Cells were plated in 96-well plates (3,000 to 10,000 per well) and allowed to adhere overnight. CCI-779 was added at 0.02 or 20 μM concentration. 24 and 48 h later, both adherent cells and floating cells from culture medium were collected. Live and dead cells were counted by ViaCount Assay on a Guava PCA-96 system with the Viacount Acquisition Module (Guava Technologies). This assay differentially stains viable and dead cells based on their permeability to the DNA binding dyes in the ViaCount reagent.

Assay of apoptosis

PARP cleavage was detected by immunoblotting with anti-PARP antibody (Cell Signaling). Both adherent cells and floating cells were collected for protein extraction. Equal loading was verified by Ponceau S staining. For measuring cellular caspase activation, cells (3,000 to 10,000 per well in 96-well plates) were seeded for overnight, and treated with CCI-779 for 24 h. Caspase activity was determined using Apo-ONE homogenous caspase-3 /7 assay kit (Promega) according to manufacturer's instructions. Fluorescence was measured with a Victor plate reader (Wallac-PerkinElmer).

Binding assays of FKBP12 and FRB

FKBP12 binding assay involves immobilization of FKBP12-[^{3}H]-FK506 complexes through His6-tag and direct measurement of radioactive complexes without additional

washing steps. Briefly, each binding reaction contained 50 mM Hepes (pH7.5), 0.01% Tween-20, 5 nM His6-FKBP12 and 2 nM [^{3}H]-FK506 in a final volume of 100 µl. Binding assay was incubated at room temperature for 1.5 h with gentle shaking. Bound radioactivity was quantitated in a Microbeta scintillation counter (Wallac-PerkinElmer) with a top-count program. The background values obtained from the assay wells without FKBP12 were subtracted from the total counts to calculate specific binding. To measure FKBP12-binding by rapamycin and analogs, various drug doses (in 1 µl) were added to the above binding mixture at the beginning of incubation. The FKBP12-binding EC_{50} value of the rapamycin analogs was determined as the concentration required for 50% inhibition of the [^{3}H]-FK506-FKBP12 complex.

FKBP12-rapamycin-FRB ternary complex formation assay was performed in 96-well MaxiSorp Fluoro plates (Nunc). The wells were pre-coated with His6-FKBP12 in PBS at 25 ng in100 µl volume (10 nM), and subsequently blocked with 3% BSA. The ternary-complex formation assay was initiated by adding 100 µl binding mix containing 50 mM Hepes (pH7.5), 0.01% Tween-20, 3% BSA, 2.5 nM GST-FRB and 2.5 nM rapamycin. The assay was incubated at room temperature for 1.5 h and detected using a europium-labeled anti-GST antibody labeled with europium (Eu). The assay results were read in the Victor plate reader (Wallac-PerkinElmer) using the DELFIA program.

Metabolic labeling

To measure protein synthesis, cells were grown in 96 well plates, and were serum-starved for 24 h. Cells were then incubated for 1 h in 100 µl/well of serum-free RPMI medium without methionine (Invitrogen), for 1 h followed by treatment with inhibitors for 1 h.

Cells were further stimulated for 1h with 10% dialyzed fetal bovine serum (FBS) (Invitrogen) and labeled for 1 h with 1 μCi/well of L-[Methyl-^{3}H]-Methionine (PerkinElmer). The cells were first lyzed in 50 μl of 1% Nonidet-P40 in water. Then, 10 μl of extract was transferred to new 96 well plates and proteins were precipitated on ice with equal volume of 20% trichloroacetic acid (TCA). Precipitated proteins were harvested on glass fiber mats with a Skatron 96 well harvester, and incorporated radioactivity was measured on a Wallac 1205 beta plate scintillation counter.

Polysome profiles

For polysome analysis, cells were grown on 10-cm plates until reaching ~80% confluence and treated with drugs for 3 hours. Cells were incubated with 0.1 mg/ml of cycloheximide (CHX) for 5 min at 37^{o}C, washed twice with ice cold PBS containing 0.1 mg/ml CHX and lysed in 400 μl of PEB lysis buffer [15 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 300 mM NaCl, 1% Triton X-100, 0.1 mg/ml CHX, 0.5 mg/ml heparin, and protease inhibitor cocktail (Sigma)]. Nuclei and debris were removed by centrifugation at 16,000g for 10 min at 4^{o}C. Extracts were layered onto sucrose density gradients (5 to 50% sucrose in PBE buffer lacking Triton-X100) and were centrifuged for 170 min at 3^{o}C in a Beckman SW41 rotor at 187,000g. Gradients were fractionated using Auto Densi-Flow gradient fractionator (Labconco) with continuous monitoring of the absorbance at 254 nm with a UV-6 detector (ISCO). P/M, polysomes to monosomes ratio was calculated by determining the pixel counts in corresponding areas using Adobe PhotoShop software.

SUPPLEMENTARY FIGURE AND TABLE LEGENDS

Supplementary Table 1. Effect of CCI-779 on cell viability. Indicated cell lines were plated in 96-well plates and treated with CCI-779 at 0.2, 20 μM for 24 and 48 h. Cell viability was determined by Guava ViaCount Assay, as described in Supplementary Methods. Values represent mean+/-SD (n=8) percentage of viable cells within the total cell population for each cell line.

Supplementary Figure 1. Analysis of apoptosis in CCI-779 treated cells. (**A**) Indicated cell lines were treated with DMSO, 0.02 μM CCI-779, 20 μM CCI-779 for 24 h. The cleavage of PARP and phosphorylation of S6K1 were examined by immunoblotting. Ponceau S staining is shown as a loading control. (**B)** Activation of effector caspases-3/7 in LNCap and MDA468. Cells exposed to the indicated doses of CCI-779 for 24 h were subjected to a fluorogenic assay, as described in Supplementory Methods. Data are expressed as mean+/-SD (n=8) percentage of control (DMSO treated) values.

Supplementary Figure 2. Micromolar CCI-779 elicits global repression of cellular translation and modulates phosphorylation states of translation factors. (**A**) [^{3}H]-methionine incorporation. Serum-starved HEK293 cells were treated with DMSO (Veh), 20 μM cycloheximide (CHX), 20 nM CCI-779 (CCI-L), 20 μM CCI-779 (CCI-H), 100 nM taxol in methionine-free medium for 1 h. [^{3}H]-methionine labeling and protein synthesis were measured as described in Materials and Methods. Mean values for

micromolar CCI-779 were equal to 22+/-5.9% of control in HEK293 cells. The presented graphs are based on mean values of three experiments. (**B**) Polysome profiles of HCT116 cells treated with DMSO, 20 nM and 20 μM CCI-779. The absorbance profiles at 254 nm (A_{254}) recorded during gradient fractionation of the samples are shown. The direction of sedimentation is from left to right. P/M, polysomes over monosomes ratio was calculated as described in Supplementary Methods. The profiles shown are representative of the results from three independent experiments. (**C**) Left panel, HCT116 cells were treated with the indicated doses of CCI-779 without or with 10 μM FK506 and subjected to immunoblot analysis similarly as in Fig. 6C. Right panel, HCT116 cells were transfected with eEF2K siRNA or with control siRNA as described in Materials and Methods, treated with the indicated doses of CCI-779 for 4 h and subjected to immunoblot analysis as in Fig. 6C.