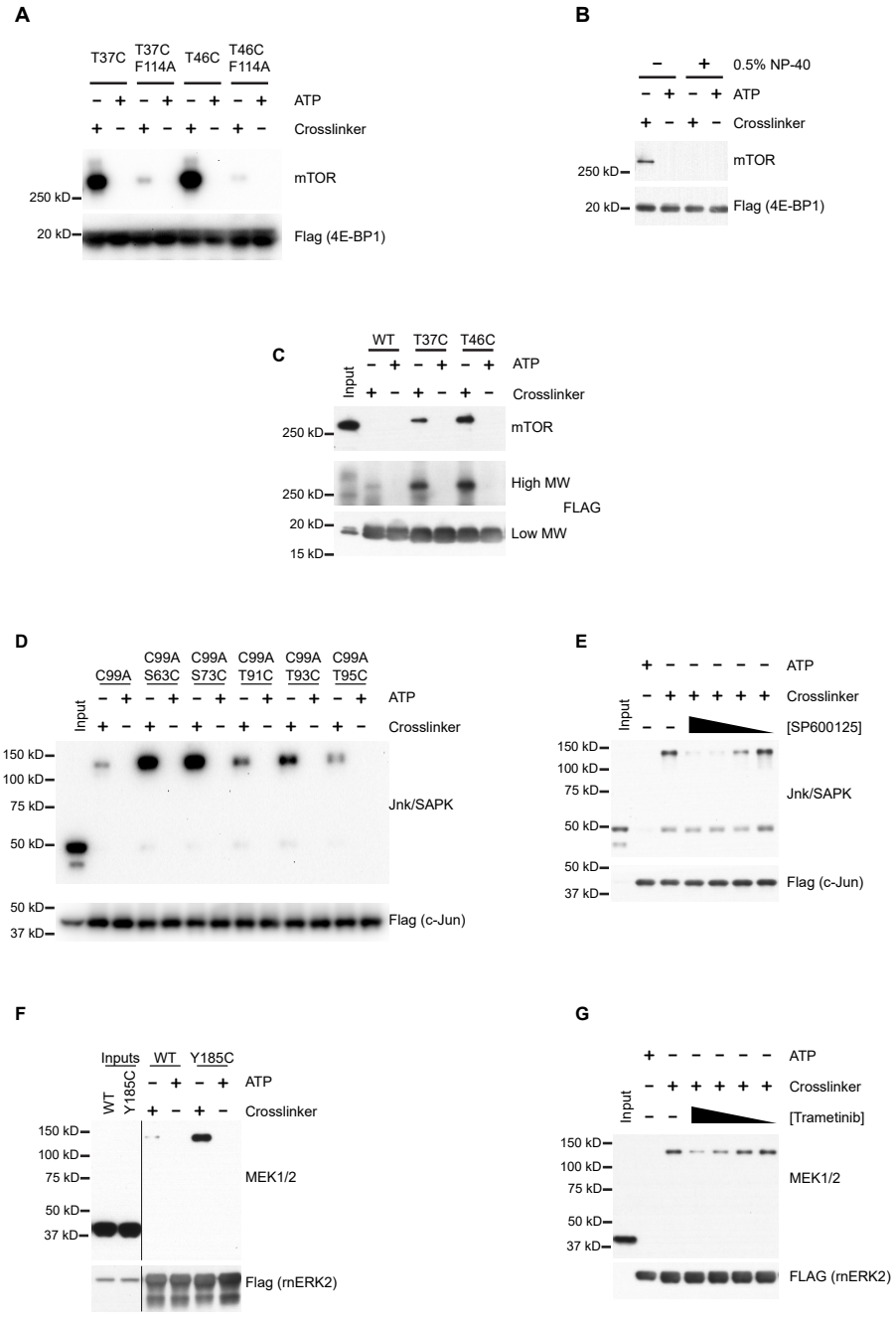



f

**Figure S1 (Related to Figure 1) | PhAXA is a broadly applicable activity-based assay for several kinase-substrate interactions**. **A)** F114 is critical for raptor-mediated recruitment of 4E-BP1 to mTOR. Mutation of this residue to alanine reduces pulldown of mTOR using the T37C and T46C probes. **B)** Non-ionic detergents reduce mTORC1 *in vitro* activity. mTOR pulldown by the T46C mutant probe is inhibited when cells are lysed in buffer containing NP-40. **C)** FLAG-4E-BP1 is detected at high molecular weights after pulldown with the T37C and T46C probes. **D)** C99A/S63C and C99A/S73C c-Jun mutants pulldown Jnk kinase. C99A was mutated to limit background pulldown from other Jnk sensitive sites near C99. **E)** SP600125 inhibition of Jnk pulldown from 5 μM – 10 nM. **F)** Y185C rnERK2 mutant pulls down MEK kinase. *Rattus norvegicus* ERK2 was used as a bait; Y185 is homologous to human Y187. **G)** Trametinib inhibition of MEK pulldown from 100 nM – 100 pM in 10-fold dilutions. Representative input samples are included in each figure to show the shift in mass upon crosslinking.

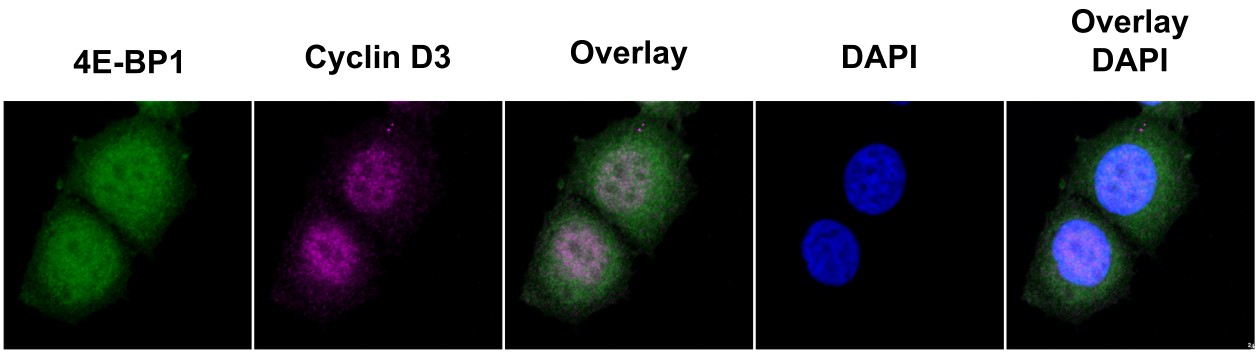



**Figure S2 (Related to Figure 2) | 4E-BP1 and Cyclin D3 are present in common cellular compartments**. Immunofluorescence imaging shows 4E-BP1 is present in the nucleus and cytoplasm, while cyclin D3 is mostly localized to the nucleus.

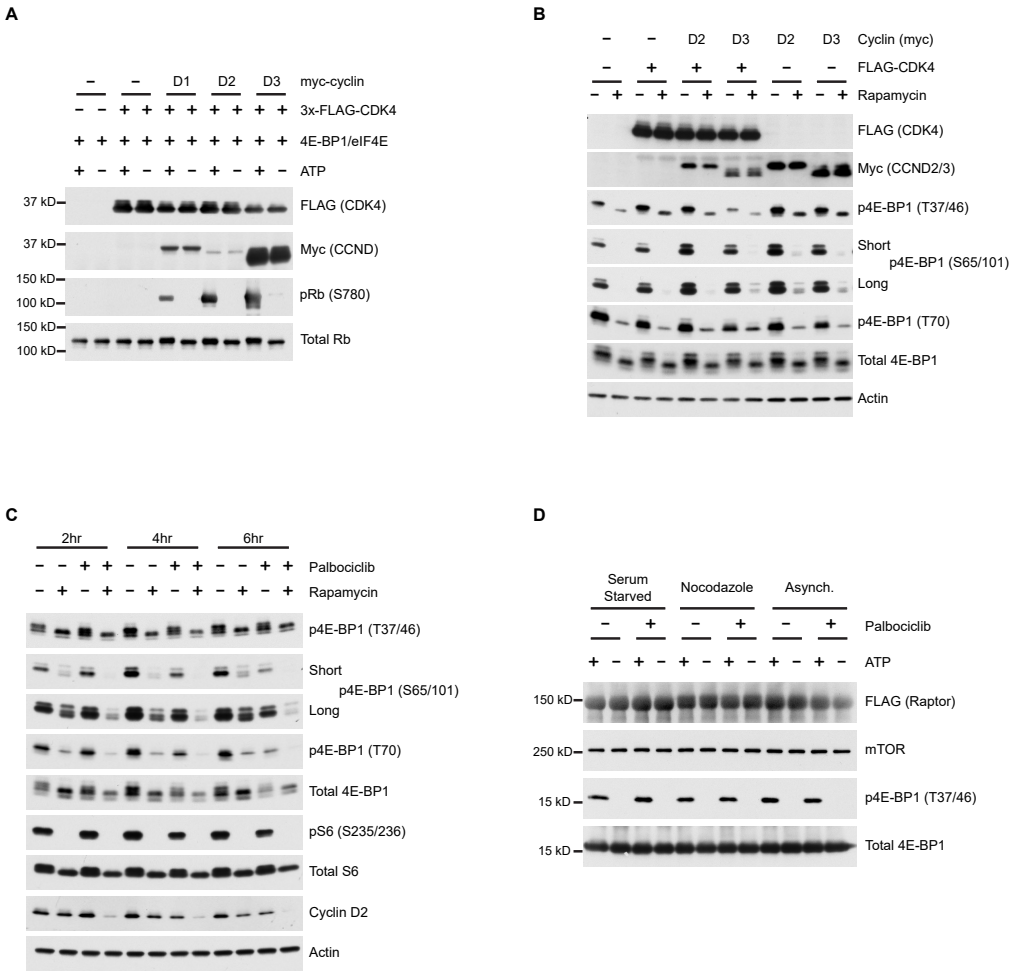



**Figure S3 (Related to Figures 3 and 4) | Cyclin D–CDK4 complexes preferentially phosphorylate non-canonical 4E-BP1 phosphorylation sites, while palbociclib does not affect mTORC1 activity *in vitro*. A)** Immunopurified FLAG-CDK4-Cyclin D complexes efficiently phosphorylate recombinant Rb *in vitro.* **B)** and **C)** Blots are repeated from **Figures 3A** and **3B**, but include T37/46 and T70 phosphorylation sites. **D)** *In vitro* activity of immunopurified mTORC1 from serum starved, mitotic, or asynchronously 293Ts using recombinant, full length 4E-BP1 (pre-incubated with HaloTag-eIF4E) as a substrate. Palbociclib (5 μM) was included in all buffers where appropriate after treating cells for 2 h.

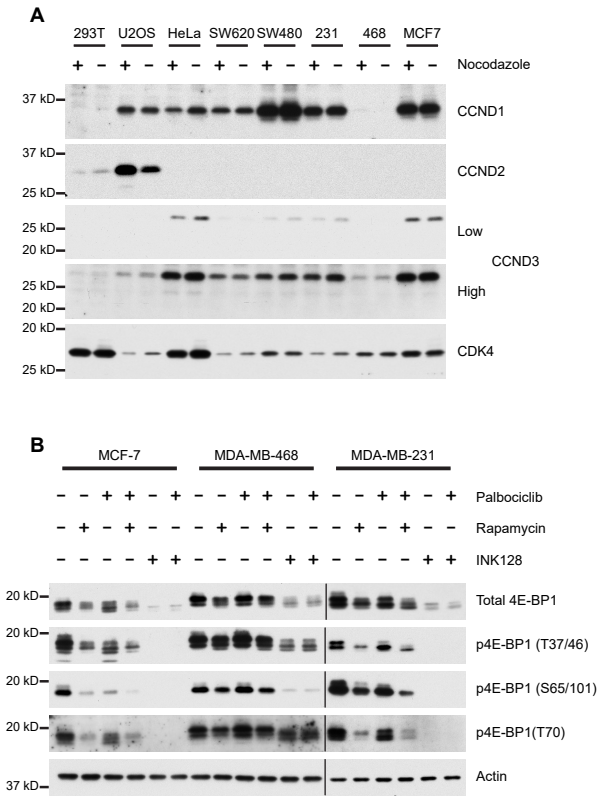



**Figure S4 (Related to Figure 4) | CDK4 and D-cyclin expression across cell lines, and active site mTOR inhibitors reduce total levels of 4E-BP1. A)** Western blot of CDK4 and D-cyclins in a variety of cell lines in asynchronous culture or arrested in prometaphase with nocodazole. **B)** Western blot of 4E-BP1 after treatment of MCF-7, MDA-MB-468, and MDA-MB-231 cells with rapamycin (100 nM) and/or palbociclib (1 μM) and/or INK128 (100 nM) for 72 h. Vertical bars denote samples run on separate blots.

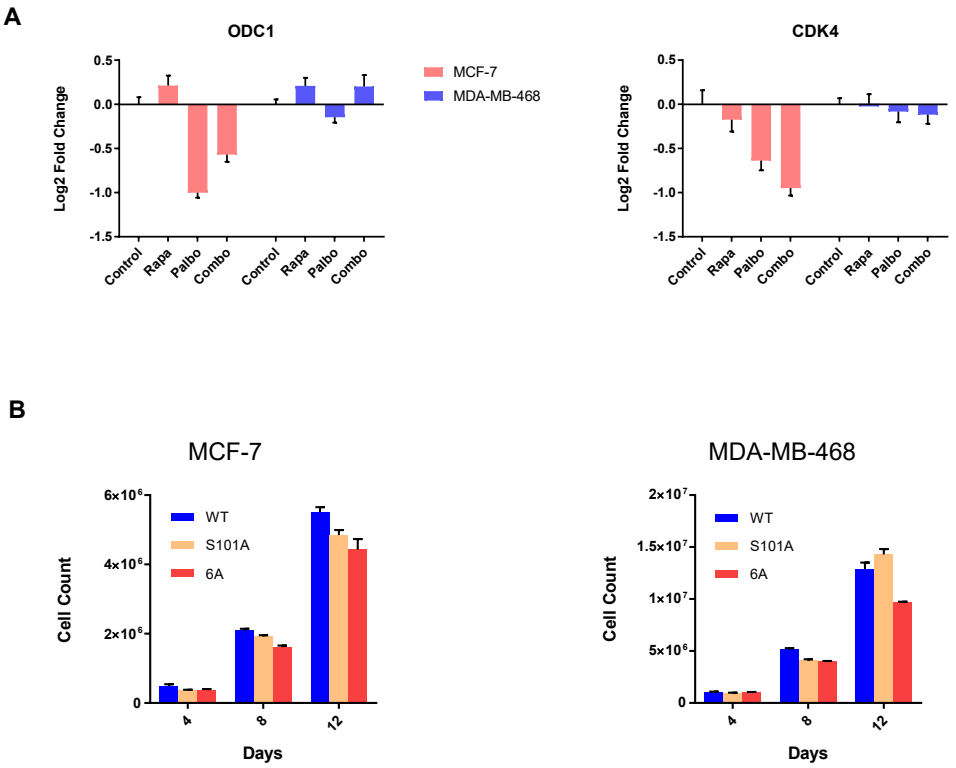



**Figure S5 (Related to Figure 5) | 4E-BP1 phosphorylation affects c-Myc activity and cell proliferation. A)** Relative RNA expression of transcripts from cells treated as in main text Figure 5B. Expression is normalized to the no treatment control sample for each cell line. UBB was used as an internal control for each gene. Error bars standard deviation (n=3). Data is representative of two independent replicates. **B)** *In vitro* proliferation of MDA-MB-468 and MCF-7 cell lines from **Figure 5C**. Cells were maintained in subconfluent culture for 12 days after plating 200,000 cells per dish. Experiment was performed in biological triplicate; data is presented as mean +/- standard deviation. 4E-BP1 expression was induced with 1.0 μg/mL and 0.1 μg/mL doxycycline for MDA-MB-468 and MCF-7, respectively. 6A mutant refers to T37A, T46A, S65A, T70A, S83A, and S101A.

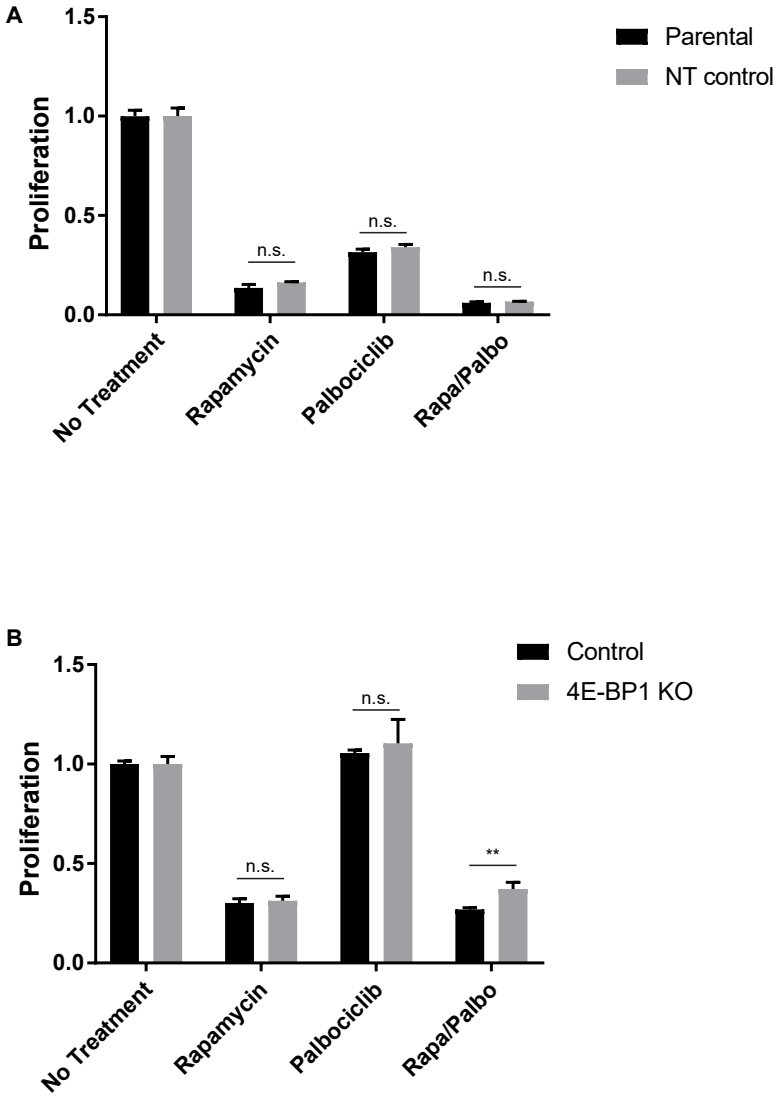



**Figure S6 (Related to Figure 6) | 4E-BP1 knockout on cell proliferation. A)** Control cell line for 4E-BP1 knockout in MCF-7 cells matches drug sensitivity of the parental MCF-7 cell line. **B)** 4E-BP1 knockout does not affect palbociclib or rapamycin sensitivity in the MDA-MB-468 cell line. For (**A**) and (**B**), Cells were treated as in **Figure 6A**. The effect on cell proliferation was assessed using Cell Titer Glo. Treated samples are shown relative to no treatment control for that cell line. Bars represent mean +/- standard deviation (n=3) ***P<0.001, ****P<0.0001 Statistical significance for all graphs was determined using an unpaired, two-tailed student's t-test. Data are representative of three biological replicates.

| | Average PSM Count | | | | Fold Change (PSMs) | | Fold Change (MS1 Integration) | |
|---|---|---|---|---|---|---|---|---|
| **Protein** | C99A + | C99A - | C99A/S63C + | C99A/S63C - | Mut+/WT+ | Mut+/Mut- | Mut+/WT+ | Mut+/Mut- |
| **MAPK8** | 5.0 | 0.0 | 28.0 | 1.5 | 5.60 | 18.67 | 5.10 +/-2.07 | 13.47 +/-6.84 |
| **MAPK9** | 22.5 | 1.0 | 30.5 | 14.5 | 1.36 | 2.10 | 2.68 +/-0.85 | 3.85 +/-0.98 |
| **CDK11B** | 1.5 | 3.5 | 1.5 | 1.0 | 1.00 | 1.50 | 1.42 (n=1) | 0.84 (n=1) |
| **NEK9** | 5.5 | 2.5 | 5.5 | 2.0 | 1.00 | 2.75 | 1.17 +/-0.12 | 2.52 +/-0.62 |
| **CDK2** | 11.5 | 0.0 | 7.5 | 0.0 | 0.65 | N/A | 1.05 +/-2.06 | - |
| **SRPK1** | 6.5 | 1.0 | 4.0 | 0.5 | 0.62 | 8.00 | 1.00 +/-0.08 | 3.61 n=1 |
| **RIO1** | 23.5 | 18.0 | 23.0 | 16.0 | 0.98 | 1.44 | 0.97 +/-0.24 | 1.35 +/-0.37 |
| **NEK1** | 0.5 | 5.0 | 1.0 | 2.5 | 2.00 | 0.40 | - | - |
| **PASK** | 0.0 | 4.5 | 0.5 | 6.0 | N/A | 0.08 | - | - |
| **CSNK2A1** | 21.0 | 24.5 | 17.5 | 20.0 | 0.83 | 0.88 | | |

**Table S1 (Related to Figures 2 and S1) | PhAXA analysis of c-Jun kinases by MS-based proteomics.** Table of all kinases identified by at least two peptides in each biological replicate following filtering of common contaminants. (See Methods) Average peptide-spectrum matches (PSMs) are from two independent biological replicates. '+' refers to samples treated with **1**, while '-' refers to ATP only controls. Dashes were added for ratios that could not be calculated due to absence of any high scoring PSMs. Ratio of MS1 intensities was determined using Skyline. Fold change is represented as +/- standard deviation (n= 2 to 18). See the Materials and Methods for more information.

| | Average PSM Count | | | | Fold Change (PSMs) | | | Fold Change (MS1 Integration) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Protein** | WT + | WT - | Y185C + | Y185C - | Y185C+ /WT+ | Y185C+ /Y185C- | Y185C- /WT- | Y185C+/WT+ | Y185C+/Y185C- | Y185C-/WT- |
| **MEK2** | 1.50 | 0.00 | 4.50 | 0.00 | 3.00 | - | - | 2.21 +/-0.65 | - | - |
| **S6KA5** | 16.5 | 0.50 | 28.50 | 0.50 | 1.73 | 57.00 | 1.00 | 1.99 +/-0.54 | - | - |
| **S6KA6** | 16.5 | 7.00 | 29.00 | 6.00 | 1.76 | 4.83 | 0.86 | 1.70 +/-0.26 | 3.82 +/-0.37 | 0.95 +/-0.21 |
| **MAPKAP** | 4.00 | 0.50 | 12.50 | 0.00 | 3.13 | - | - | 1.55 +/-0.36 | - | - |
| **Mnk1** | 4.00 | 0.00 | 7.50 | 0.00 | 1.88 | - | - | 1.38 +/-0.46 | - | - |
| **CDK4** | 4.50 | 0.00 | 3.50 | 1.00 | 0.78 | 3.50 | - | 1.36 +/-0.34 | 3.58 +/-0.16 | - |
| **S6KA4** | 8.00 | 0.50 | 15.50 | 0.50 | 1.94 | 31.00 | 1.00 | 1.34 +/-0.38 | 19.16 +/-1.14 | - |
| **Mnk2** | 2.00 | 0.00 | 5.00 | 2.50 | 2.50 | 2.00 | - | 1.29 +/-0.49 | 1.78 +/-0.20 | - |
| **Rio1** | 2.00 | 0.00 | 5.00 | 0.00 | 2.50 | - | - | 1.25 +/-0.18 | - | - |
| **S6KA1** | 49.0 | 44.5 | 39.00 | 5.00 | 0.80 | 7.80 | 0.11 | 0.76 +/-0.09 | 17.74 +/-7.46 | 0.05 +/-0.03 |
| **S6KA3** | 89.5 | 113. | 88.50 | 24.00 | 0.99 | 3.69 | 0.21 | 0.75 +/-0.13 | 10.45 +/-5.15 | 0.07 +/-0.02 |
| **MAP2K4** | 5.00 | 0.00 | 2.50 | 0.00 | 0.50 | - | - | 0.75 +/-0.22 | - | - |
| **MAP2K6** | 20.5 | 0.00 | 15.00 | 0.00 | 0.73 | - | - | 0.69 +/-0.12 | - | - |
| **MAP2K3** | 5.00 | 0.00 | 2.00 | 0.00 | 0.40 | - | - | 0.62 +/-0.004 | - | - |
| **S6KA2** | 4.50 | 8.50 | 4.00 | 2.00 | 0.89 | 2.00 | 0.24 | 0.47 +/-0.08 | 1.28 +/-0.33 | 0.27 +/-0.02 |

**Table S2 (Related to Figures 2 and S1) | PhAXA analysis of ERK2 kinases by MS-based proteomics.** Table of all kinases identified by at least two peptides in each biological replicate following filtering of common contaminants. (See Methods) Average peptide-spectrum matches (PSMs) are from two independent biological replicates. '+' refers to samples treated with **1**, while '-' refers to ATP only controls. Dashes were added for ratios that could not be calculated due to absence of any high scoring PSMs. Ratio of MS1 intensities was determined using Skyline. Fold change is represented as +/- standard deviation (n= 2 to 15). See the Materials and Methods for more information.

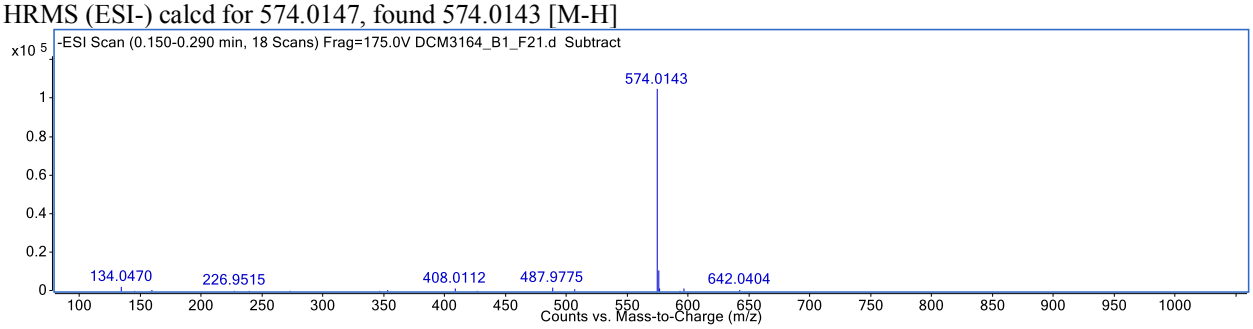
| | AVG PSM | | | | Fold Change (PSMs) | | Fold Change (MS1 Integration) | |
|---|---|---|---|---|---|---|---|---|
| Protein | WT + | WT - | S101C + | S101C - | S101C+/WT+ | S101C+/S101C- | S101C+/WT+ | S101C+/S101C- |
| CDK4 | 4 | 0 | 5 | 2 | 1.25 | 2.5 | 1.29 | 3.29 |
| Erk2 | 0 | 0 | 3 | 2 | ---- | 1.5 | ---- | 3.99 |
| mTOR | 3 | 0 | 2 | 0 | 0.667 | ---- | 1.34 | ---- |
| MAK | 0 | 2 | 0 | 0 | ---- | ---- | ---- | ---- |

**Table S3 (Related to Figure 2) | PhAXA analysis of 4E-BP1 (S101) kinases by MS-based proteomics.** Table of all kinases identified by at least two peptides in at least one sample after filtering of common contaminants. This is a single biological replicate. Ratio of MS1 intensities were determined using Skyline. (n=1 or 2)

# Data S1 | Characterization of Crosslinker 1 (Related to STAR Methods)

## High-Resolution Mass Spec. of Crosslinker 1

HRMS (ESI-) calcd for 574.0147, found 574.0143 [M-H]




## $^{1}$H NMR Spectrum of Crosslinker 1