Supporting Information

Identification of a Fragmented Small GTPase Capable of Conditional Effector Binding

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1. Stock Solutions

TBS buffer: 30 mM Tris-HCl (pH = 7.5), 250 mM NaCl, 5 mM MgCl₂, and 10% glycerol. Prior to use, 2 mM 2-mercaptoethanol was added.

Lysis buffer: 20 mM HEPES (pH = 7.5), 120 mM NaCl, 10% glycerol, and 2 mM EDTA.

Wash buffer: 50 mM Tris-HCl (pH = 7.6), 150 mM NaCl, 1% Triton X-100, and 20 mM MgCl₂.

2. Expression of Cdc42 Constructs in Bacterial Cells

BL21-CodonPlus (DE3)-RIPL E. coli cells were transformed with a vector expressing wild-type Cdc42 or the L61Cdc42 mutant. Protein expression was induced according to a previously described protocol.¹ ²

3. Cdc42 Nucleotide Exchange in Bacterial Cell Lysates

Cells expressing wild-type Cdc42 were normalized to an OD₆₀₀ = 2.0 in 1mL and were lysed using the B-PER™ Bacterial Protein Extraction Reagent (Life Technologies, 32 µL) following vendor provided protocols. Lysates containing 150 µg total protein were incubated with 25 mM EDTA and either 2 mM GDP or 1 mM GTP-γS for 45 min at 30 °C in order to facilitate nucleotide exchange. The reactions were then placed on ice, and quenched with 35 mM MgCl₂.

4. Preparation of PBD Beads

The Cdc42 binding domain (PBD) protein was amplified from the pET23-PBD (65-150)-N-Cys vector (Addgene, #13722),³ and cloned into a pGex-2T vector. The resulting GST-PBD protein construct was expressed in BL21 (DE3) cells by inducing with 0.3 mM IPTG for 3 hours at 37 °C.⁴ Cells were harvested by centrifugation at 3,220g for 30 min at 4 °C, washed with DI water, and frozen at -80 °C. For cell lysis, pellets were thawed and resuspended in 40 mL of 1x lysis buffer containing 1 mg/ml lysozyme and incubated for 20 min at 4 °C. The cells were then sonicated at 40% amplitude using 1 sec on and 1 sec off cycles for a total of 45 seconds. Lysates were clarified by centrifugation at 3,220g for 30 min at 4 °C, washed with DI water, and frozen at -80 °C. For cell lysis, pellets were thawed and resuspended in 40 mL of 1x lysis buffer containing 1 mg/ml lysozyme and incubated for 20 min at 4 °C. The cells were then sonicated at 40% amplitude using 1 sec on and 1 sec off cycles for a total of 45 seconds. Lysates were clarified by centrifugation at 18,000g for 30 min and glutathione resin (Glutathione Sepharose 4 Fast Flow, GE Healthcare, 17-5132-01, 600 µL per 200 mL of cell culture) was added along with NP-40 (0.5%). The resulting mixture was incubated for 1 hr with rotation at 4 °C. Beads were then collected and washed in lysis buffer with 0.5% NP-40 (5 times), and another 3 times in lysis buffer with no NP-40.⁴ The resulting GST-PBD beads can be stored at 4 °C for up to one week.

5. Pull-Down of Cdc42 from Bacterial Cell Lysates

Total protein concentrations were adjusted to 3 µg/µL with wash buffer (excluding 1% Triton X-100). GST-PBD beads (30 µg immobilized protein) were added to 300 µL of cell lysate and the mixture was incubated for 30 min at 4 °C with rotation. GST-PBD beads were then collected via centrifugation at 800g for 5 min and washed four times with wash buffer. Beads were resuspended in 60 µL of SDS-PAGE loading dye, followed by denaturing at 95 °C for 10 minutes. The samples were centrifuged at 17,000g for 5 min and cooled to room temperature before loading on a 15% SDS-PAGE gel. Samples were transferred from the SDS-PAGE gel to
a PVDF membrane using standard protocols. The membrane was blocked with 5% milk in 1x TBST for 1 hr at room temperature. The membrane was washed three times with 1x TBST (15 minutes per wash), before incubating with a 1:10,000 dilution of anti-6X His-tag antibody conjugated to HRP (Abcam, ab1187) in 1x TBST containing 5% milk overnight at 4 °C with rotation. The next morning, the membrane was washed three times with 1x TBST (1 hr for the first wash, and 15 min for the second and third wash). Bands were visualized using SuperSignal West Dura Extended Duration Substrate (Life Technologies, 34075) on a Gel Doc XR+ system.

6. Cloning and Expression of the Cdc42 Fragment Library in Rabbit Reticulocyte Lysates

FRB and FKBP were amplified and cloned into pET-45b (Novagen, 71327-3) and pRSF-1b (Novagen, 71330-3) plasmids, respectively. L61Cdc42 N-terminal fragments were fused to the C-terminus of FRB in pET-45b, yielding a FRB-N-terminal Cdc42 fragment construct. L61Cdc42 C-terminal fragments were fused to the N-terminus of FKBP in pRSF-1b, yielding a Cdc42 C-terminal fragment-FKBP construct. These resulting constructs were amplified by PCR and a T7 RNA polymerase promoter and Kozak sequence were added according to previously published procedures. mRNA was transcribed from the resulting template DNA using the T7 Ribonuclease inhibitor (Promega, P1320) and analyzed by agarose gel electrophoresis. The resulting mRNAs for potentially complementary fragments were translated in rabbit reticulocyte lysates (Promega, L4960) at a final reaction volume of 50 µL. These reactions contained 33 µL of rabbit reticulocyte lysate, 1 µL amino acid mixture minus methionine, 40 µM 35S-methionine (PerkinElmer, NEG709A001MC, 1200Ci/mmol), 10 mM Mg(CH3COO)2, 70 mM KCl, 1 µL RNasin Ribonuclease inhibitor (Promega, N2111), 2.5 µL N-terminal Cdc42 fragment mRNA (2 µg), 2.5 µL C-terminal Cdc42 fragment mRNA (2 µg), and 1 µM rapamycin where indicated. The reactions were adjusted to 50 µL with nuclease-free water. Constitutively active L61Cdc42 and dominant negative N17Cdc42 were used as positive and negative controls. Reaction mixtures were incubated at 30 °C for 90 min.

7. Pull-Down of Reassembled Cdc42 from In Vitro Translation Reactions

The entire translation reaction mixture was incubated with 10 µL of glutathione sepharose beads containing immobilized GST-PBD (2.95 µg/µL) for 30 min at 4 °C with rotation. Reassembled Cdc42 fragments that bound to immobilized GST-PBD were purified by centrifugation at 800g for 5 min at 4 °C. Beads were then washed 4 times with wash buffer. Recovered proteins were analyzed by SDS-PAGE and visualized by autoradiography.
8. Supplementary Figures

Figure S1

Wild-type Cdc42 cell lysate was exchanged with GDP or GTP-γS respectively and incubated with immobilized PBD. The L61Cdc42 mutant was incubated with immobilized PBD under the same conditions. Purified Cdc42 was detected using an anti-His tag antibody (Abcam, ab1187). Lane 1: GTP-γS bound Cdc42; Lane 2: L61Cdc42 mutant; Lane 3: GDP bound Cdc42. The expected molecular weight of Cdc42 is 22 kDa.
Figure S2

Cotranslation of FRB-N-terminal Cdc42 fragment and Cdc42 C-terminal fragment-FKBP constructs in rabbit reticulocyte lysates in the presence (+) or in the absence (-) of rapamycin. Reassembled Cdc42 was purified using immobilized PBD and visualized by autoradiography. Constitutively active L61Cdc42 is used as positive control (P) and the dominant negative N17Cdc42 mutant (N) is used as negative control. Refer to Tables S1 and S2 for protein sequences. Pair numbering is indicated above lanes, C-terminal fragments have larger molecular weights than N-terminal fragments for pairs 1-7. Due to the large number of samples, multiple gels are shown.
Figure S3

Independent replicates of cotranslations of FRB-N12 and 13C-FKBP constructs in rabbit reticulocyte lysates in the presence (+) or in the absence (-) of rapamycin where exposed to immobilized PBD and analyzed by SDS-PAGE. The N12 and 13C fragments consistently show increased effector binding in the presence of rapamycin. Translations were performed with different batches of rabbit reticulocyte lysate.
9. Supplementary Tables

Table S1. Full-Length Cdc42 Sequences

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<th>Protein</th>
<th>Amino Acid Sequence</th>
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<td>Wild-Type Cdc42</td>
<td>QTIKCVVVGDGAVGKTCLLLISYTTNKFPSEYVPTVFVNAVTVMIIGGEYPYLGLFDTAGQQEYDRFLRPLSYPQTDVFVFLVCFSVVSPSSFENVKEWVPEITHHCPKTPFLVTGQIDLRDPSTIEKLNKQKQTIPETAEKLRDLKAVKYVECSALTQRLKNFDEAILAALEPPETQPKRKCICFHHHHHH</td>
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<td>L61Cdc42</td>
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Mutated amino acids are highlighted in red.
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<th>Pair</th>
<th>Fragment</th>
<th>Amino Acid Sequence</th>
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Pair 9
FRB-N115
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VKGMEFVLEPLHAMMERGPKQTLKETSNQAYGRLDMEAQUEWCR
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116C-FKBP
MGIDLRDDPSTIEKLNKQKPIPETAEKLRDLAVENTQRLKNVFEAIAALEEPPETQPKRCCIFLQGGGSGGGGGYAS
RGVQVQETISPQDGRTFKRQGTCVHYTGMLDGGKFDSRDRNKPFKFMLGKQEVIRGVEGVAQMSVQRAKLTISPDYAYGATG
HPGIIIPPHATLVDFVELLEKHHHHH

Pair 10
FRB-N120
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VKGMEFVLEPLHAMMERGPKQTLKETSNQAYGRLDMEAQUEWCR
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FRB-N131
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132C-FKBP
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Pair 12
FRB-N137
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**FRB is highlighted in blue, FKBP is shown in red, and the Cdc42 fragment is indicated in tan.**

Multiple cloning sites and linkers are left black.
10. References


