SUPPLEMENTAL DATA

Plasmids

The bacterial expression vector for NC-biotinylated CasSD was derived from pET-28a(+) (Novagen) and encoded a protein; AviTag, CasSD (rat Cas 115-420) and AviTag from the amino- to carboxy-terminal. The vector for C-biotinylated CasSD lacked the amino-terminal AviTag sequence. The bacterial expression vector for NY/CY-NC-biotinylated CasSD was also derived from pET-28a(+) and encoded a chimeric protein; YFP-N (EYFP-V68L/Q69K 1-154), AviTag, CasSD, AviTag, and YFP-C (EYFP 155-238) from the amino- to carboxy-terminal. The vector for NY/CY-C-biotinylated CasSD lacked the amino-terminal AviTag sequence. The vectors for His_6-YFP and His_6-YFP-N were constructed by subcloning the sequences encoding full-length YFP (EYFP-V68L/Q69K 1-238) (Miyawaki et al., 1999) and YFP-N to pET-28a(+), respectively. GST-YFP-C was constructed by subcloning the sequence encoding YFP-C into pET-41a(+) (Novagen). The bacterial expression vectors for cdb3 and GST-RalGDS-RBD were provided from P. S. Low (Purdue University) and A. Sakakibara (University of Virginia), respectively. The eukaryotic expression vectors for human c-Src and Fyn were provided from T. Yamamoto (University of Tokyo). The expression vector for SrcY527F, a mutant form of Src that has tyrosine 527 mutated to phenylalanine, was provided from H. Hirai (University of Tokyo). The expression vectors for human c-Yes and GFP-Rap1 were provided from M. Sudol (Mount Sinai Medical Center, New York) and M. Matsuda

(Kyoto University), respectively. The expression vectors for monomeric RFP (Clontech) and RFP-Cas were constructed by PCR using pCAGGS (Niwa et al., 1991) as a parent vector. The expression vector for RFP-Cas15YF in pCAGGS was constructed by PCR using an expression vector for a mutant form of Cas that has all fifteen YxxPs in the substrate domain mutated to FxxPs (Shin et al., 2004) as a template. The expression vector for GFP-CasΔSD encoding GFP fused to Cas lacking the substrate domain (Nakamoto et al., 1996) was constructed using pSSRα (Toyoshima et al., 1993) as a parent vector.

In Vitro Kinase Assay of Immunoprecipitated Src

4×10^5 SYF cells or SYF cells stably expressing c-Src were either stretched or left unstretched. 1 min after stretching or without stretching, cells were washed with cold PBS, solubilized with a lysis buffer (1% NP-40, 0.25% sodium deoxycholate, 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 20 μg/ml aprotinin) and cleared by centrifugation. Equivalent small portions of each lysate were mixed with SDS sample buffer and subjected directly to SDS-PAGE to analyze for Cas phosphorylation. Remaining portions of each lysate were incubated with an anti-Src antibody (GD11) for 2 h at 4 °C, then protein A/G immobilized on gel (UltraLink® Immobilized Protein A/G, PIERCE) was added and incubated another 1 h at 4°C. The complex bound to the gel was washed 3 times with the lysis buffer and 3 times with the kinase buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 20 μg/ml aprotinin, 0.5 mM EGTA). Immunoprecipitates thus obtained were incubated with acid-treated enolase in the kinase buffer containing 0.5 mM ATP, 3 mM $MnCl_2$, 1 mM

DTT, 1 mM Na_3VO_4 for 10 min at 30 °C. The kinase reaction was terminated by the addition of SDS sample buffer and equivalent portions of each sample were subjected to SDS-PAGE. Src kinase activity was analyzed by measuring the phosphorylation of enolase with anti-phospho-tyrosine (4G10, Upstate Biotechnology) immunoblotting. Immunoprecipitated Src, i.e. Src protein in the kinase reaction, was quantified by anti-Src immunoblotting.

Preparation of Biotinylated Proteins

Various forms of biotinylated CasSD were prepared using Biotin AviTag™ technology (Avidity). The Biotin AviTag sequence consists of 15 residues (GLNDIFEAQKIEWHE), which is specifically and efficiently biotinylated by the protein biotin ligase, Bir A (Schatz, 1993). Biotinylated AviTag-fused proteins were obtained by co-expression with BirA in bacteria (BL21 Star™, Invitrogen) cultured in NZCYM medium containing d-biotin (50 μM, Research Organics) at the time of IPTG induction. We measured the biotinylation of each AviTag-fused protein with SDS-PAGE followed by Coomassie brilliant blue staining and affinity blotting using avidin (horseradish peroxidase-conjugated streptavidin, GE Healthcare). The molar ratio of biotin to AviTag-fused protein was determined using biotinylated bovine serum albumin (BSA) (Sigma; the molar ratio of biotin to protein was 8: 1) as a standard and confirmed to be 2: 1 in NC-biotinylated CasSD and NY/CY-NC-biotinylated CasSD, and 1: 1 in C-biotinylated CasSD and NY/CY-C-biotinylated CasSD.

Covalent Avidin-coating of Latex Membrane and Preparation of Biotinylated

Proteins Specifically Bound to Avidin-coated Latex Membrane

The amine-reactive groups were introduced onto the surface of latex membrane using Friedal-Crafts chemistry by treatment with an aqueous solution containing 40% (w/w) glutaraldehyde (Sigma-Aldrich), 10% (w/w) Dioxane (Sigma-Aldrich) and 0.04% (w/w) cerium trichloride (Sigma-Aldrich) at 90 °C for 3 h. After 4 washes with 99.5% ethanol, the membrane was set in an adjustable tension ring (Coats & Clark, Greer, SC). The central area of the upper surface of the membrane (defined with a glass cylinder: inner diameter = 13 mm, see Figure 3A bottom) was incubated with 0.05% (w/w) avidin (Neutravidin, PIERCE) in 20 mM phosphate buffer (pH 7.6) containing 4 mM TCEP HCl (PIERCE) at room temperature overnight. Unbound avidin was cleared and reactive groups remaining on the latex surface were quenched by washing 4 times with 3 x concentrated TBS (1 x TBS: 20 mM Tris pH 7.4, 137 mM NaCl) containing 1% Triton X-100 and 3 mM TCEP HCl. The avidin-coated latex surface was incubated with 1% Triton X-100, 5% BSA in PBS containing 3.5 mM TCEP HCl for 1 h at room temperature to block non-specific protein binding. To bind biotinylated proteins, avidin-coated latex surface was incubated with biotinylated proteins (0.8 μM for YFP amino-terminal swapping assay and 0.2 μM for in vitro phosphorylation or antibody binding experiments) in 0.25% Triton X-100, 2% BSA in PBS containing 20 μg/ml aprotinin, 2 mM TCEP HCl and 0.5 mM EGTA at 4 °C overnight. Unbound proteins were cleared by washing 5 times with 0.25% Triton X-100, 5% BSA in PBS containing 20 μg/ml aprotinin, 2 mM TCEP HCl and 0.5 mM EGTA.

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Supplemental Figure Legend

Figure S1. Anti-Cas Antibodies

(A) Schematic description of epitope regions of three different anti-Cas antibodies.

(B) αCas1 binding to CasSD was not altered by phosphorylation of CasSD.

Equivalent portions of the samples analyzed in the first four lanes in Figure 4B were re-analyzed by αCas1 immunoblotting.

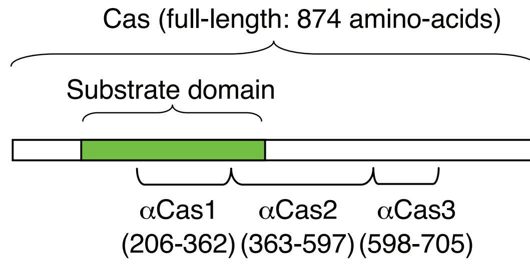
(C) Specificity of αCas1 and αCas3 immuno-reactivity.

Cells in a mono-layer culture (6-well plate) were solubilized with SDS sample buffer and equivalent portions of each sample were subjected to SDS-PAGE followed by αCas1, αCas3 and anti-actin immunoblotting. Whole blots are shown for αCas1 and αCas3. Lane 1: Cas-deficient fibroblasts (Cas-/-). Lane 2: Cas-deficient fibroblasts transiently expressing RFP-Cas (Cas-/- + RFP-Cas). Lane 3: Cas-deficient fibroblasts transiently expressing RFP-Cas15YF (Cas-/- + RFP-Cas15YF). Lane 4: Cas-deficient fibroblasts transiently expressing GFP-CasΔSD (Cas-/- + GFP-CasΔSD). Lane 5: SYF cells.

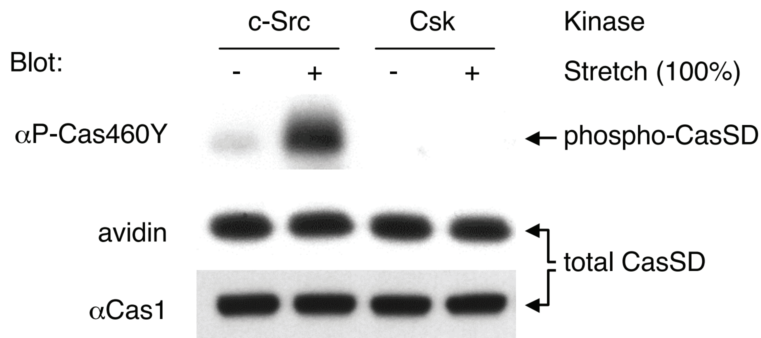
Fig. S1

Sawada et al.

A



B



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