Amano et al., http://www.jcb.org/cgi/content/full/jcb.201412008/DC1

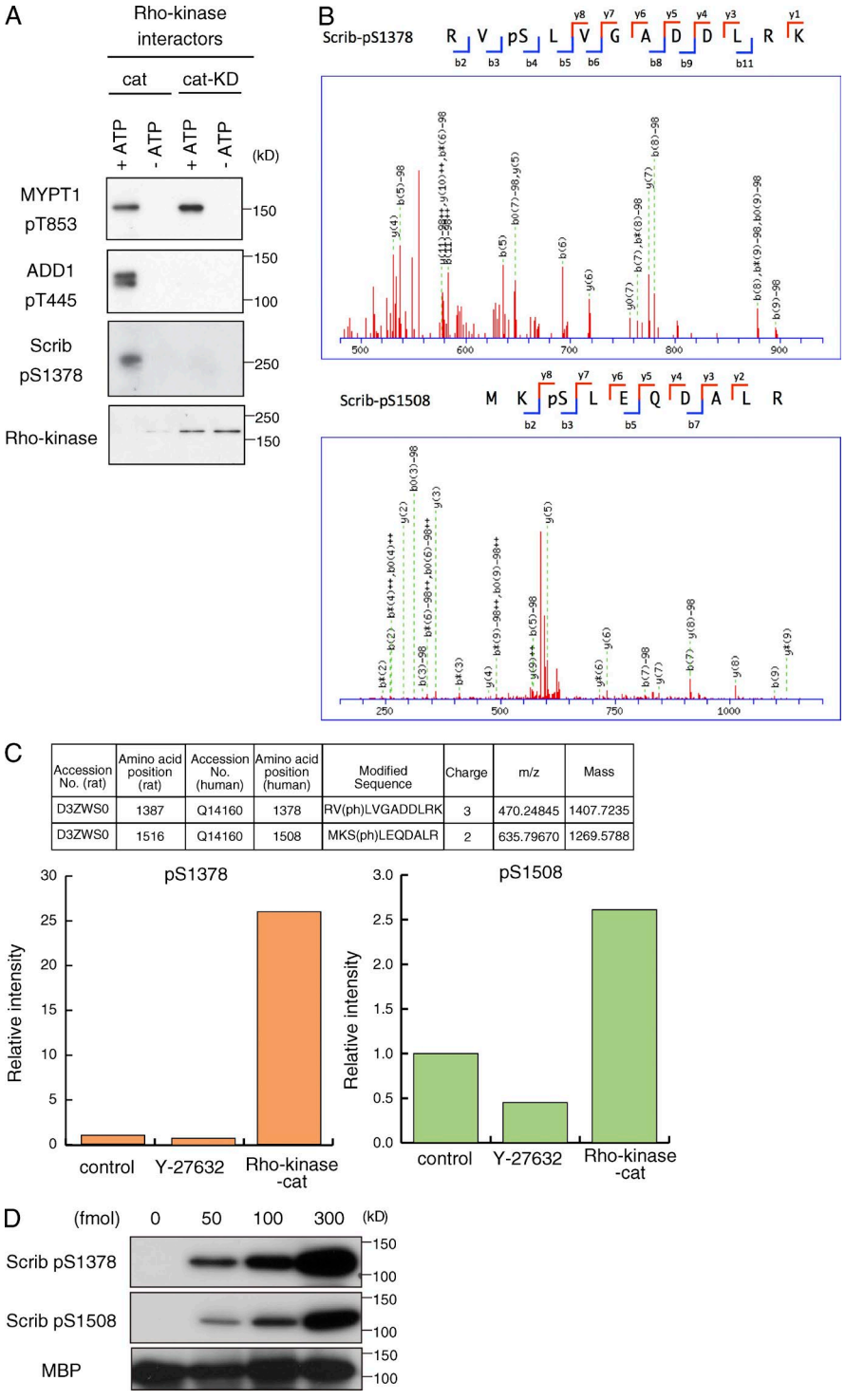

Figure S1. **Phosphorylation of Scrib at Ser 1378 and Ser 1508 by Rho-kinase.** (A) The phosphorylation levels of substrates complexed with Rho-kinase-cat or Rho-kinase-cat-KD. After the formation of the kinase–substrate complexes, they were incubated in the presence or absence of ATP. The phosphorylation at MYPT1-pT853, ADD1-pT445, and Scrib-pS1378 and endogenous Rho-kinase associated with beads were examined by immunoblot analyses. (B) The representative MS/MS spectra of Scrib peptides with pS1378 and pS1508. The data shown are from a single representative experiment out of four independent experiments. (C) Semiquantification of phosphopeptides containing pS1378 and pS1508 of Scrib in COS-7 cells. GST-Scrib was expressed in COS-7 cells, precipitated, and analyzed by LC/MS/MS after tryptic digestion. The intensities of precursor ion for phosphopeptide containing pS1378 (RVpSLVGADDLRK) and that containing pS1508 (MKpSLEQDALR) were measured from LC/MS/MS raw data by PinPoint software. The peak intensities of phosphopeptides were normalized by the averages of six peptides (LPDGFTQLR, LVCLDVSENR, GLGFSIAGGK, AGDAGIFVSR, ALAAVPSAGSVQR, and YFELEVR) derived from Scrib. The intensities of both phosphopeptides were diminished in the Y-27632–treated cells and elevated in the cells coexpressing constitutively active Rho-kinase. The data shown are from a single representative experiment out of two independent experiments. (D) Specificity of the antibodies against Scrib phosphorylated at Ser 1378 (anti-pS1378 antibody) and at Ser 1508 (anti-pS1508 antibody). 1 pmol of purified MBP-Scrib-C containing the indicated amounts of phosphorylated MBP-Scrib-C was subjected to SDS-PAGE, followed by immunoblot analysis with anti-pS1378 antibody (top), anti-pS1508 antibody (middle), or anti-MBP antibody (bottom).

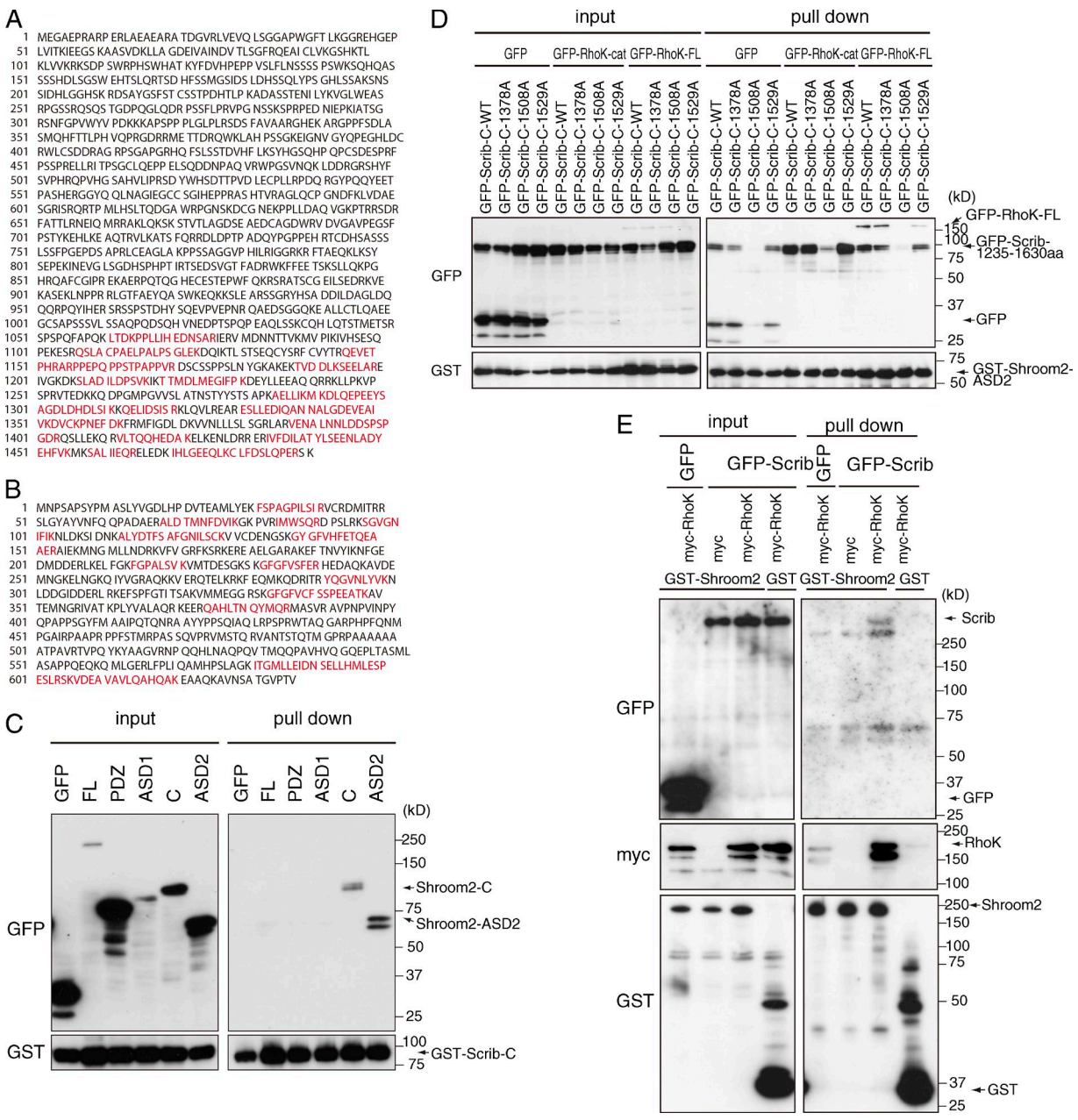

Figure S2. **Shroom2 as a novel interactor with Scrib.** (A and B) GST, phosphorylated GST-Scrib-C, or nonphosphorylated GST-Scrib-C immobilized on glutathione Sepharose beads was incubated with rat brain lysate. After washing, the bound proteins were eluted with 1 M NaCl. The eluates with 1 M NaCl and the remaining proteins on beads were separately subjected to tryptic digestion and the following LC/MS/MS analyses. Peptides derived from Shroom2 and Pabpc1 were preferentially detected in the samples of phosphorylated GST-Scrib-C. Matched peptides with Shroom2 and Pabpc1 are shown in red in A and B, respectively. (C) Various Shroom2 fragments fused with GFP were expressed with GST-Scrib-C in COS-7 cells, and GST-Scrib-C was precipitated with glutathione Sepharose beads. Precipitates were analyzed by immunoblot analysis with anti-GFP and anti-GST antibodies. (D) GFP-Scrib-C WT or mutants at phosphorylation sites were expressed with GST-Shroom2-ASD2 and/or GFP-Rho-kinase in COS-7 cells, and GST-Shroom2-ASD2 was precipitated with glutathione Sepharose beads. Precipitates were analyzed by immunoblot analysis with anti-GFP and anti-GST antibodies. (E) Ternary complex formation of Scrib–Shroom2–Rho-kinase. GFP-Scrib and myc-Rho-kinase were coexpressed with GST or GST-Shroom2 in COS-7 cells, and GST or GST-Shroom2 was precipitated with glutathione Sepharose beads. Precipitates were analyzed by immunoblot analysis with anti-GFP, anti-myc, and anti-GST antibodies.

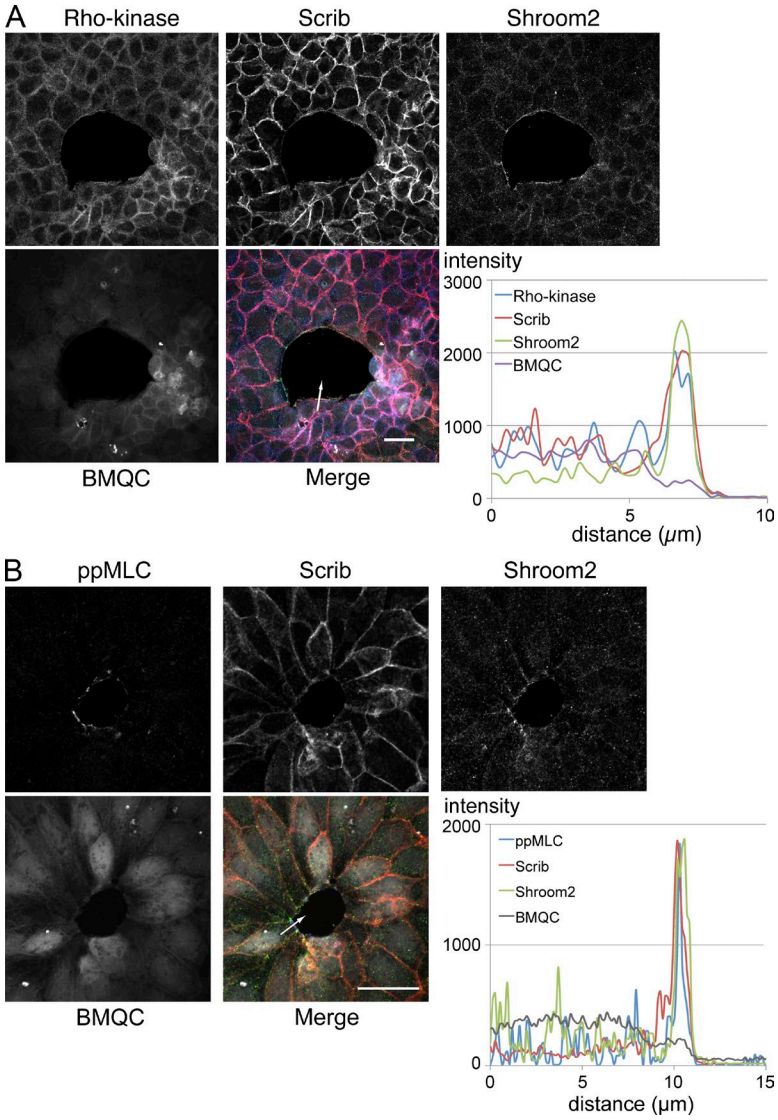

Figure S3. **Colocalization of Scrib with Shroom2, Rho-kinase, and ppMLC at the edge during wound hole closure.** Endogenous Rho-kinase (blue), Scrib (red), and Shroom2 (green; A) or ppMLC (blue), Scrib (red), and Shroom2 (green; B) were immunostained with the cytosolic marker CellTracker Violet BMQC in MDCK II cells. Intensities of all the fluorescence along the white arrows are shown. Bars, 20 μm. The data shown are from a single representative experiment out of at least three independent experiments.

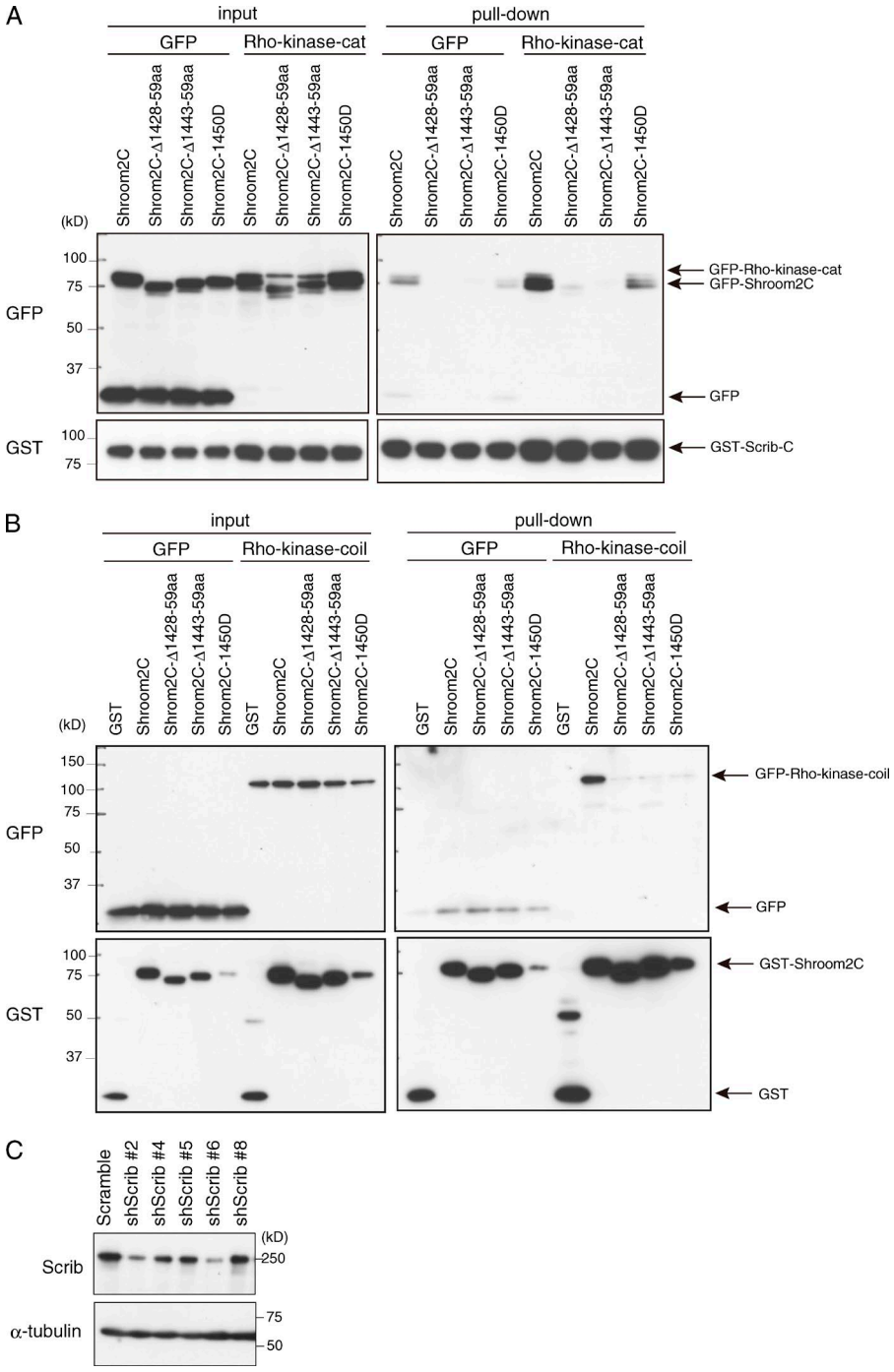

Figure S4. **Shroom2 mutants lacking the binding abilities against Scrib and Rho-kinase.** (A) Shroom2-C or Shroom2-C mutant fused with GFP was co-expressed with GST-Scrib-C in COS-7 cells and/or GFP-Rho-kinase-cat, and GST-Scrib-C was precipitated with glutathione Sepharose beads. Precipitates were analyzed with anti-GFP and anti-GST antibodies. (B) Shroom2-C or Shroom2-C mutant tagged with GST was coexpressed with GFP or GFP-Rho-kinase-coil (residues 421–1,137) in COS-7 cells, and GST-Shroom2-C was precipitated with glutathione Sepharose beads. Precipitates were analyzed with anti-GFP and anti-GST antibodies. (C) shRNA against Scrib was transfected into MDCK II cells, and cell lysates were subjected to immunoblot with anti-Scrib and anti–α-tubulin antibodies.

Table S1. **Interactors with the C terminus of Scrib phosphorylated by Rho-kinase**

	GST	GST-Scrib-C		
Rho-kinase	+	+	+	−
ATP	+	+	−	−
Accession number, gene name	**Number of unique peptides**			
D4A053, Shroom2	0	20	3	0
P29341, Pabpc1	0	13	2	4

Table S2. **Plasmids used in this study**

Plasmid name	Features	Insert (Protein)	Parental vector
pEF-BOS-GST	Mammalian expression vector, EF-1α promoter, GST tag	–	pEF-BOS
pEF-BOS-GST-CaMKK2	Mammalian expression vector, EF-1α promoter, GST tag	human CaMKK2, NP_757380, 1–541 aa	pEF-BOS
pEF-BOS-GSTCDC42EP4	Mammalian expression vector, EF-1α promoter, GST tag	human CDC42EP4, NP_036253.2, 1–356 aa	pEF-BOS
pEF-BOS-GST-EPB49 pEF-BOS-GST	Mammalian expression vector, EF-1α promoter, GST tag	human EPB49 variant, Q08495-3, 1–358 aa (Δ58–72 aa)	pEF-BOS
pEF-BOS-GST-FGFR1OP2	Mammalian expression vector, EF-1α promoter, GST tag	human FGFR1OP2, Q9NVK5-2, 1–215 aa	pEF-BOS
pEF-BOS-GST-LASP1	Mammalian expression vector, EF-1α promoter, GST tag	human LASP1, NP_006139.1, 1–261 aa	pEF-BOS
pEF-BOS-GST-HSP90a	Mammalian expression vector, EF-1α promoter, GST tag	human HSP90a, P07900, 1–732 aa	pEF-BOS
pEF-BOS-GST-MPP2-N	Mammalian expression vector, EF-1α promoter, GST tag	human MPP2, ABM82603, 1–218 aa	pEF-BOS
pEF-BOS-GST-MPP2-C	Mammalian expression vector, EF-1α promoter, GST tag	human MPP2, ABM82603, 219–552 aa	pEF-BOS
pEF-BOS-GST-MYO18A-N	Mammalian expression vector, EF-1α promoter, GST tag	human MYO18A, KIAA0216, 1–363 aa	pEF-BOS
pEF-BOS-GST-MYO18A-C	Mammalian expression vector, EF-1α promoter, GST tag	human MYO18A, KIAA0216, 358–1,185 aa	pEF-BOS
pEF-BOS-GST-Scrib	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 1–1,630 aa	pEF-BOS
pEF-BOS-GST-SPTBN2-C	Mammalian expression vector, EF-1α promoter, GST tag	human SPTBN2, KIAA0302, 1,905–2,390 aa	pEF-BOS
pEF-BOS-GST-TWF2	Mammalian expression vector, EF-1α promoter, GST tag	human TWF2 variant, NP_009215.1, 1–349 aa	pEF-BOS
pEF-BOS-GST-MLC	Mammalian expression vector, EF-1α promoter, GST tag	rat MLC, NP_080340, 1–172,aa	pEF-BOS
pEF-BOS-GST-Sctib-1-581aa	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 1–581,aa	pEF-BOS
pEF-BOS-GST-Sctib-578-1239aa	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 578–1,23 9,aa	pEF-BOS
pEF-BOS-GST-Sctib-C	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 1,235–1,630 aa	pEF-BOS
pEF-BOS-GST-Sctib-1235-1483aa	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 1,235–1,483 aa	pEF-BOS
pEF-BOS-GST-Sctib-1478-1630aa	Mammalian expression vector, EF-1α promoter, GST tag	human Scrib, NP_056171.2, 1,478–1,630 aa	pEF-BOS
pEGFP-c1	Mammalian expression vector, CMV promoter, GFP tag	–	pEGFP-c1
pEGFP-c1-Scrib	Mammalian expression vector, CMV promoter, GFP tag	human Scrib, NP_056171.2, 1–1,630 aa	pEGFP-c1
pEGFP-c1-Scrib-C	Mammalian expression vector, CMV promoter, GFP tag	human Scrib, NP_056171.2, 1,235–1,630 aa	pEGFP-c1
pEGFP-c1-Scrib-1478-1630aa	Mammalian expression vector, CMV promoter, GFP tag	human Scrib, NP_056171.2, 1,478–1,630 aa	pEGFP-c1
pMal-c2	*E. coli* expression vector, tac promoter, MBP tag	–	pMal-c2
pMal-c2-Scrib-C	*E. coli* expression vector, tac promoter, MBP tag	human Scrib, NP_056171.2, 1,235–1,630 aa	pMal-c2
pGEX-2T	*E. coli* expression vector, tac promoter, GST tag	–	pGEX-2T
pGEX-2T-Shroom2-ASD2	*E. coli* expression vector, tac promoter, GST tag	human Shroom2, Q13796, 1,304–1,616 aa	pGEX-2T
pEF-BOS-GST-Shroom2	Mammalian expression vector, EF-1α promoter, GST tag	human Shroom2, Q13796, 1–1,616 aa	pEF-BOS-GST
pEF-BOS-GST-Shroom2-ASD2	Mammalian expression vector, EF-1α promoter, GST tag	human Shroom2, Q13796, 1,304–1,616 aa	pEF-BOS-GST
pEGFP-c1-Shroom2	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 1–1,616 aa	pEGFP-c1
pEGFP-c1-Shroom2-PDZ	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 1–485 aa	pEGFP-c1
pEGFP-c1-Shroom2-ASD1	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 480–981 aa	pEGFP-c1
pEGFP-c1-Shroom2-C	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 978–1,616 aa	pEGFP-c1
pEGFP-c1-Shroom2-ASD2	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 1,304–1,616 aa	pEGFP-c1
pEGFP-c1-Shroom2-C-Δ1428-1459aa	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 978–1,616 aa (Δ1,428–1,459 aa)	pEGFP-c1
pEGFP-c1-Shroom2-C-Δ1443-1459aa	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 978–1,616 aa (Δ1,443–1,459 aa)	pEGFP-c1
pEGFP-c1-Shroom2-Δ1428-1459aa	Mammalian expression vector, CMV promoter, GFP tag	human Shroom2, Q13796, 1–1,616 aa (Δ1,428–1,459 aa)	pEGFP-c1
pEGFP-c1-RhoA-L63	Mammalian expression vector, CMV promoter, GFP tag	human RhoA, NP_001655, 1–193 aa	pEGFP-c1
pEGFP-c1-Rho-kinase	Mammalian expression vector, CMV promoter, GFP tag	bovine Rho-kinase, NP_776877, 1–1,388 aa	pEGFP-c1
pEGFP-c1-Rho-kinase-cat	Mammalian expression vector, CMV promoter, GFP tag	bovine Rho-kinase, NP_776877, 6–553 aa	pEGFP-c1
pEF-BOS-myc-Rho-kinase	Mammalian expression vector, EF-1α promoter, myc tag	bovine Rho-kinase, NP_776877, 1–1,388 aa	pEF-BOS
pEF-BOS-myc-Rho-kinase-cat	Mammalian expression vector, EF-1α promoter, myc tag	bovine Rho-kinase, NP_776877, 6–553 aa	pEF-BOS
pEF-BOS-myc-Rho-kinase-coil	Mammalian expression vector, EF-1α promoter, myc tag	bovine Rho-kinase, NP_776877, 421–1,137 aa	pEF-BOS
pEF-BOS-HA-Rho-kinase-coil	Mammalian expression vector, EF-1α promoter, HA tag	bovine Rho-kinase, NP_776877, 421–1,137 aa	pEF-BOS

Table S3 shows the lists of candidate phosphorylation sites detected in the screens for the indicated kinases.