Supplementary Data

Identification of a Crk mutant

The *Drosophila Crk* gene is mapped to cytogenetic region 102B1 on the fourth chromosome. One P-element insertion, KG00336, which was isolated by BDGP, maps to this same cyotogenetic region. We found that the SUPor-P, P element containing a Su(Hw) protein binding site is inserted in the first exon of *Crk*. The 5' end of the P-element is located approximately 40 bp downstream of the *Crk* transcription initiation site, which we identified by 5'RACE and RT-PCR (Supplementary Figure S3A, sequence data not shown). By performing RT-PCR using poly A^+ RNA derived from KG00336 heterozygotes, we detected the chimeric cDNA containing the sequence of Crk and SUPor-P (Supplementary Figures S3A,C). Su(Hw) protein blocks the interaction between enhancer/silencer elements and their promoter (Roseman et al. 1995); thus Crk gene expression could be suppressed, even though there is another transcription initiation site downstream of the one we have identified. Homozygotes for KG00336 P-element insertion are lethal. These data suggested that the P-insertion disrupts the Crk gene function, so we renamed the P-insertion line $Crk^{KG00336}$.

Supplementary Experimental Procedures

Fly stocks for genetic interaction experiments

The following amorphic or hypomorphic alleles were used for genetic interaction experiments with *pnr>Pvr-IR* flies: Pvr^{c2195}, $slpr^{921}$, hep^{r75}, bsk^{2}, phl^{HM7}, $Dsor1^{LH100}$, 3

Rac genes together (*Rac1*^{J10}, *Rac2*$^{\Delta}$ and *Mtl*$^{\Delta}$), *Cdc42*^{4}, *Crk*^{KG00336}, and *Mbc*$^{D11.2}$. The *puc*^{E69} allele is a P-*lacZ* enhancer-trap insertion line of the *puc* gene (Martin-Blanco et al., 1998).

Constructs for transgenic flies and S2 cell culture

Drosophila genes *Pvr, msn, slpr, hep, bsk, Rac1, Rac2, Mtl, Cdc42*, *Rho1*/*RhoA, Crk, Mbc,* and *ELMO*/*CG5336* were amplified by RT-PCR using a *Drosophila* embryo cDNA pool (synthesized with SMART cDNA Synthesis Kit, Clontech) as a template. The human *PDGFRα* cDNA was amplified from human brain Marathon-Ready cDNA (Clontech). For all PCR reactions, we used KOD-Plus-DNA polymerase according to the manufacturer's protocol (TOYOBO).

To make the *PDGFRα*/PVR-Myc chimera construct, we fused a human *PDGFRα* cDNA fragment encoding the N-terminal extracellular domain (corresponding to amino acid residues 1-523, GenBank accession no. P16234) to *Pvr* cDNA encoding a transmembrane region and the intracellular kinase domain (amino acid residues 786-509 of PVR, GenBank accession no. AAF52626). On its 3' end, 6X Myc-tag sequence (MGSSKSKPKDPSQR) was added; and the construct was then cloned as a SpeI-NotI fragment into pMT/V5-His A vector (Invitrogen). For making the Y1428F mutant PVR, nucleotides coding for amino acid residue 1428 were mutated to change the residue from tyrosine to phenylalanine by a modified QuickChange site-directed mutagenesis method using KOD-plus-DNA polymerase instead of *Pfu* DNA polymerase. Wild-type and Y1428F mutant of *Pvr* were 6X Myc-tagged on their 3' end and cloned

into the pUAST vector as NotI fragments. For the construction of Flag-Rac1 and Flag-Cdc42, the nucleotide sequence encoding the Flag-tag (MDYKDDDDK) was put to their 5' end. To construct dominant active Rac1 and Cdc42, we mutated the nucleotides corresponding to amino acid residue 61 of wild-type molecules to change the residue from glutamine residue to leucine. All *Rac1*- and *Cdc42*-constructs were inserted as EcoRI-NotI fragments into the pMT/V5-His A vector for use in the S2 cell culture system.

The nucleotide sequence corresponding to the Flag-tag was added to the 5' end of Crk (Crk[SH2S]; CG1587-PB, GenBank accession no. NM166744) and cloned into the EcoRI site of the pUAST vector. Nucleotide sequences encoding HA-tag (YPYDVPDYAST) and 6X Myc-tag were added to the 3' end of *mbc* (GenBank accession no. AF007805) and *ELMO* (*CG5336-PA*, GenBank accession no. NM135704), respectively. Both *mbc-HA* and *ELMO-Myc* constructs were cloned into the pUAST vector for making transgenic flies and into the pMT/V5-His A vector for S2 cell transfection. Mbc- ΔSH3 and Mbc- ΔPRM, which carry deletions of amino acids 1-70 and 1662-1970, respectively, were generated, HA-tagged, and cloned into the pMT/V5-His A vector. A ΔPRM version of ELMO (carrying a deletion of amino acids 708-724) was generated , 6X-Myc tagged, and cloned into pMT/V5-His A.

For generation of *Pvr-, hep-, bsk-, Crk-, mbc-, and ELMO* sequences with dyad symmetry, *Pvr* coding sequence (GenBank accession no. AJ250859, nucleotide positions 314 to 1,041), *hep* coding sequence (GenBank accession no. U05240, nucleotide positions from 646 to 1,364), *bsk* coding sequence (GenBank accession no.

U49180, nucleotide positions from 536 to 1,260), *Crk* coding sequence (GenBank accession no. NM143651, nucleotide positions from 387 to 976), *mbc* coding sequence (GenBank accession no. AF007805, nucleotide positions from 609 to 1,311), and *ELMO* coding sequence (GenBank accession no. NM135704, nucleotide positions from 123 to 827) were amplified by PCR with primers that introduced unique sequences at the products ends and cloned into the pUAST Cs1 to become those fragments duplicated in tandem. Details about the cloning procedures used for the pUAST Cs1 vector will be described elsewhere (R. U.). The constructs were injected into w^{118} embryos, resulting in several independent transformed lines.

PDGFRα/PVR-Myc transfectant S2 cell lines

To establish transfectant S2 cell lines, the cells were cotransfected with pCoHYGRO (Invitrogen) and selected with 300 μg/ml Hygromycin-B (Invitrogen) according to the manufacturer's protocol. The cloned PDGFRα/PVR-Myc chimera cell line was selected from bulk transfected cells after limited dilution. After serum-starvation and induction of the PDGFRα/PVR-Myc with 100 mg/ml $CuSO_4$ overnight, the receptor was stimulated by directly adding recombinant human PDGF-BB (Invitrogen, 100 ng/ml for the final concentration) to the medium.

RNAi in cell culture

For RNAi, S2 cells were cultured and treated with dsRNA as described (Clemens et al., 2000). Primer sequences used to generate specific dsRNAs were as follow: *Pvr*

(GenBank accession no. AJ25085), sense-primer 314-335, antisense-primer 1021-1042; *drk* (accession no. L12446), sense-primer 243-266, antisense-primer 841-861; *slpr* (accession no. AF416233), sense-primer 121-141, antisense-primer 1129-1149; *hep* (accession no. U05240), sense-primer 646-666, antisense-primer 1628-1648; *bsk* (accession no. U49180), sense-primer 536-557, antisense-primer 1244-1267, *Rac1* translated-region (accession no. Z35642), sense-primer 268-288, antisense-primer 819-840; *Rac1* untranslated-region (UTR, accession no. Z35642), sense-primer 788-808, antisense-primer 1474-1494; *Rac2* UTR (accession no. L38310), sense-primer 13-34, antisense-primer 654-634; *Mtl* UTR (accession no. AF238044), sense-primer 825-843, antisense-primer 1467-1488; *Cdc42* UTR (accession no. AY119570), sense-primer 718-738, antisense-primer 1254-1276; *Crk* (accession no, NM143651), sense-primer 164-187, antisense-primer 911-934; *mbc* (accession no. AF007805), sense-primer 561-581, antisense-primer 1539-1560; and *ELMO* (accession no. NM135704), sense-primer 153-175, antisense-primer 1150-1170. The 5'-end of each oligonucleotide contains a T7 RNA polymerase promoter sequence. dsRNAs were synthesized and purified by use of a MEGAscript RNAi kit (Ambion).

The cells were incubated at 25 °C for 3 days to allow for turnover of the target protein. To see the effects on JNK activation by the PDGFR α/PVR-Myc chimera molecule, we cultured the cells with dsRNA for 2 days and then serum-starved them overnight in Schneider's medium + 0.1% FCS supplemented with the same dsRNA and 100 mg/ml $CuSO_4$ for the induction of PDGFαR/PVR-Myc chimera receptor.

Western analysis and in vitro GEF assay

Cells were harvested and lysed in MAPK lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 50 mM NaF, 20 mM β- glycerophosphate, 1 mM EDTA, 1 mM EGTA and 1 mM Na_3VO_4, with 1/100 vol. of each of 100 mM PMSF, 1 mM leupeptin, and 1.6 mg protein/ml aprotinin added just before use) for Western analyses. *In vitro* PAK-PBD pull-down assays were performed according to the manufacturer's protocol (Cytoskeleton).

Immunoprecipitation assay

S2 cells were transfected with combinations of wild-type or mutant versions of PVR-Myc and Flag-Crk in pUAST and of Mbc-HA and ELMO-Myc in pMT/V5-His A (Invitrogen) and incubated overnight. Then, in the case of the pMT/V5-His A constructs, 100 mg/ml $CuSO_4$ was added to the medium and the cells incubated overnight in order to express the transgenes. Cells were harvested and lysed in MAPK lysis buffer. Cell lysates (500 μg of protein in 500 μl MAPK lysis buffer) were subjected to immunoprecipitation by using 2 μg of an antibody against a tag sequence

and a 20-μl bed volume of Protein G Sepharose (Amersham Bioscience).

Antibodies

Primary antibodies used were rat anti-PVR antibodies (1:1,000 dilution for immunoblotting, kind gift from Pernille Rørth, 1:200 dilution for immuno-histochemistry, kind gift from Ben-Zion Shilo), rabbit anti-Mbc antibody (1:1,000, kindly donated by Susan Abmayr), rabbit anti-Crk antibody (1:1,000), rabbit anti-ELMO antibody (1:1,000), mouse anti-pJNK (Phospho-SAPK/JNK [Thr183/Tyr185] antibody, clone G9, 1:1,000, Cell Signaling), rabbit anti-JNK1 (FL) antibody (1: 2,000, Santa Cruz), mouse anti-dpERK antibody (clone MAPK-YT, 1:1,000, Sigma), rabbit anti-MAPK antibody (1:10,000, Santa Cruz), rabbit anti-Rac1 antibody (1:1,000, Cytoskeleton), rabbit anti-Cdc42 (P1, 1:500, Santa Cruz), mouse anti-RhoA antibody (clone 26C4,1:1,000, Santa Cruz), mouse anti-pan Ras antibody (clone Ras 10, 1:2,000, Sigma), mouse anti-Grb2 antibody (clone 81, 1:1,000, Transduction), mouse anti-Flag antibody (clone M2, 1:2,500, Sigma), rabbit anti-Flag antibody (used for immunoprecipitation, Sigma), mouse anti-Myc (1:5,000, Invitrogen), rabbit anti-HA (Y-11, 1:1,000, Santa Cruz), rat anti-HA (clone 3F10, used for immunoprecipitation, Roche), mouse anti-phosphotyrosine antibody (clone 4G10, 1:1,000, Upstate), and anti-α-tubulin (clone DM1A, 1:10,000, Sigma).

A fragment encoding 2 SH3 domains of Crk was PCR amplified and cloned into pGEX4 (Promega). The GST-fusion protein was expressed in *E. coli*, purified on glutathione beads, and used as an immunogen for polyclonal antibody production in

rabbits. Oligopeptide RLLDTEGVDISKD (corresponding to amino acid residues 655-705 of ELMO/CG5336-PA, GenBank accession no. NP609548) was synthesized and used as an immunogen for polyclonal antibody production against ELMO in rabbits. Those antibodies were affinity purified and used for Western analysis.

Preparation of adult flies for scanning electron microscopy

The samples were dehydrated by passage through a graded series of ethanol (30%, 50%, 75%, 90%, and 2 x 100%) with a 12-24 hr incubation at room temperature in each step of the series. After the last 100% step, the samples were gradually rehydrated and rinsed with 10 mM phosphate buffer (pH 5.2). Then, the samples were fixed with 2% glutaraldehyde + 2% OsO_4. Subsequent to the fixation, they were dehydrated, freeze dried in the presence of t-butyl alcohol, and then sputter-coated with platinum-palladium from an ion sputter (E1030, HITACHI). Ion-coated samples were viewed with a scanning electron microscope (S4500, HITACHI).

Supplementary Reference

Roseman RR, Johnson EA, Rodesch CK, Bjerke M, Nagoshi RN, Geyer PK (1995) A P element containing suppressor of hairy-wing binding regions has novel properties for mutagenesis in *Drosophila* melanogaster. *Genetics* **141:** 1061-1074