

Competition between SOCS36E and Drk modulates Sevenless receptor tyrosine kinase activity

Isabel Almudi, Montserrat Corominas and Florenci Serras*

Departament de Genètica, Facultat de Biologia and Institut de Biomedicina de la Universitat de Barcelona, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

*Author for correspondence (fserras@ub.edu)

Accepted 2 August 2010

Journal of Cell Science 123, 3857–3862

© 2010. Published by The Company of Biologists Ltd

doi:10.1242/jcs.071134

Summary

Modulation of signalling pathways can trigger different cellular responses, including differences in cell fate. This modulation can be achieved by controlling the pathway activity with great precision to ensure robustness and reproducibility of the specification of cell fate. The development of the photoreceptor R7 in the *Drosophila melanogaster* retina has become a model in which to investigate the control of cell signalling. During R7 specification, a burst of Ras small GTPase (Ras) and mitogen-activated protein kinase (MAPK) controlled by Sevenless receptor tyrosine kinase (Sev) is required. Several cells in each ommatidium express *sev*. However, the spatiotemporal expression of the *boss* ligand and the action of negative regulators of the Sev pathway will restrict the R7 fate to a single cell. The *Drosophila* suppressor of cytokine signalling 36E (SOCS36E) protein contains an SH2 domain and acts as a Sev signalling attenuator. By contrast, downstream of receptor kinase (Drk), the fly homolog of the mammalian Grb2 adaptor protein, which also contains an SH2 domain, acts as a positive activator of the pathway. Here, we apply the Förster resonance energy transfer (FRET) assay to transfected *Drosophila* S2 cells and demonstrate that Sev binds directly to either the suppressor protein SOCS36E or the adaptor protein Drk. We propose a mechanistic model in which the competition between these two proteins for binding to the same docking site results in either attenuation of the Sev transduction in cells that should not develop R7 photoreceptors or amplification of the Ras–MAPK signal only in the R7 precursor.

Key words: SOCS36E, Sevenless, Receptor regulation

Introduction

Signalling through the activation of receptor tyrosine kinases (RTKs) is one of the most critical mechanisms used by metazoans to control growth and development. Upon ligand binding, autophosphorylation of RTK cytosine residues provides the docking sites for a variety of phosphotyrosine-binding proteins. The specific recruitment of these proteins, which harbour various catalytic and/or scaffolding domains, determines the signalling output. Perturbation of this signalling, by mutations or other genetic alterations, results in deregulated kinase activity and cancer (Blume-Jensen and Hunter, 2001). A major deactivation pathway for RTKs is receptor downregulation, which involves ligand-induced internalisation by means of endocytosis, followed by degradation in lysosomes (Bache et al., 2004).

The photoreceptor specification of the *Drosophila melanogaster* retina is a model system with which to study RTK signalling. The compound eye of *Drosophila* is formed by an array of well-organised ommatidia in which clusters of cells will specify the photoreceptors and cone cells. The photoreceptor R7 is specified by high activity of the mitogen-activated protein kinase (MAPK) through the activation of the Sevenless (Sev) RTK (Banerjee et al., 1987; Hafen et al., 1987; Tomlinson and Ready, 1986), whereas cells that express Sev, but do not activate it, are specified as other photoreceptors or cone cells (Freeman, 1996; Simon et al., 1991; Tomlinson et al., 1988; Tomlinson and Ready, 1987). Sev is temporarily activated in the R7 by the action of the Boss ligand expressed in the adjacent R8 photoreceptor (Cagan et al., 1992; Hart et al., 1990; Reinke and Zipursky, 1988). It is not yet clear how the inactivation of Sev in cells that should not become R7

photoreceptors is maintained. The presence of negative regulators of Sev in those cells will provide a fail-safe mechanism in case of perturbations that could result in the wrong cell fates.

A genetic screen for negative regulators of the Sev pathway (Sese et al., 2006) identified *Suppressor of cytokine signalling 36E* (*Socs36E*) (Almudi et al., 2009). *Socs36E* is expressed in those cells that express *sev*, but do not become R7 photoreceptors, and is not expressed in the presumptive R7 (Almudi et al., 2009). SOCS proteins are conserved from flies to mammals and were initially identified as repressors of cytokine signalling through a negative-feedback loop that inhibits the cytoplasmic TK Janus (JAK–STAT signalling pathway) (Endo et al., 1997; Hilton et al., 1998; Starr et al., 1997; Yoshimura et al., 1995). *Socs36E* encodes the *Drosophila* homolog to the mammalian *SOCS5* and *SOCS4* (Callus and Mathey-Prevot, 2002; Karsten et al., 2002) and, similarly to other members of the SOCS family, contains an SH2 domain flanked by a variable N-terminal domain and a conserved C-terminal domain, termed the SOCS-box. The SH2 binds phosphorylated tyrosine residues (Tyr-P), whereas the SOCS-box participates in a ubiquitin ligase complex to promote the degradation of target proteins (Zhang et al., 1999). In addition to the JAK–STAT pathway, SOCS proteins also regulate other signalling pathways, including RTKs (Almudi et al., 2009; Baetz et al., 2004; Callus and Mathey-Prevot, 2002; Kario et al., 2005; Krebs and Hilton, 2003; Rawlings et al., 2004).

Genetic analyses demonstrated that *Socs36E* negatively regulates Sev and epidermal growth factor receptor (EGFR) pathways (Almudi et al., 2009; Callus and Mathey-Prevot, 2002; Rawlings et al., 2004). Two pieces of evidence suggest a direct interaction

between SOCS36E and Sev. First, it was demonstrated by co-immunoprecipitation assays that the mammalian orthologs of *Socs36E* bind directly to EGFR through their SH2 domains (Kario et al., 2005; Nicholson et al., 2005). Second, the *sev* gain-of-function phenotype, which results in extra R7 cells in the retina, is suppressed by overexpression of *Socs36E*, and this suppression is impaired by mutations in the SH2 domain (Almudi et al., 2009). Moreover, overexpression of the SH2-containing protein Drk enhances Sev signalling except in the presence of *Socs36E* (Almudi et al., 2009). Drk, the fly homolog to the mammalian Grb2, is the adaptor protein that links Sev to Sos and its downstream effectors Ras and MAPK (Olivier et al., 1993; Simon et al., 1993). These results suggest a mechanism for Sev modulation in which the SH2-containing proteins SOCS36E and Drk would compete for the phosphorylated Sev receptor.

To characterise the molecular mechanism of the interactions between Sev, Drk and SOCS36E, we explored whether these proteins are able to come into physical contact with one another. To this aim, we took advantage of Förster resonance energy transfer (FRET) analysis, which allows us to determine the proximity of proteins beyond the resolution of conventional optical microscopy and, therefore, monitor protein–protein interactions in living cells (Ciruela, 2008; Gordon et al., 1998). Because FRET is based on photon energy resolution, the maximum distance between proteins of interest to detect energy transfer is typically 5–10 nm. Here, we demonstrate that both Drk and SOCS36E physically interact with Sev, thus supporting a model in which SOCS36E represses the Sev pathway by competing with Drk to bind to the activated receptor.

Results and Discussion

Activated Sev stimulates tyrosine phosphorylation in transfected S2 cells

The *sev^{S11}* allele is a constitutively activated form that consists of the C-terminal Sev protein, which includes the intracellular tyrosine kinase domain, bound to the signal peptide of the *Drosophila* cuticle protein CP3 and the 10 amino acid *Myc* epitope (Basler et al., 1991). *sev^{S11}* transgenic flies exhibit a striking rough eye phenotype because of the transformation of non-neural cone cells into R7 photoreceptors (Fig. 1A,B) (Basler et al., 1991).

We generated new ligand-independent *sev* constructs containing cyan fluorescent protein (CFP) (*Sev^{S11}-CFP*; Fig. 1C). S2 cells transfected with *Sev^{S11}-CFP* showed Sev localised mainly in cytoplasmic and vesicular accumulations, probably owing to the constant turnover of the protein because of its constitutive activity (Fig. 1D,E). To confirm that this construct functions in a ligand-independent manner, similarly to *sev^{S11}* in transgenic flies, we used an antibody against Tyr-P to visualise tyrosine phosphorylation as a marker of constitutive activation of Sev and subsequent kinase activity. We found that cells transfected with *Sev^{S11}-CFP* had a higher Tyr-P signal in comparison with non-transfected cells (Fig. 1D'–D''', E'–E'''). Moreover, the *Sev^{S11}-CFP* fusion protein colocalised with Tyr-P (Fig. 1D'', E''). Furthermore, a transgene of the *Sev^{S11}-CFP* construct resulted in the formation of supernumerary R7 cells when expressed in the developing eye (Fig. 1F–H). These results indicated that *Sev^{S11}-CFP* is functional and that *Sev^{S11}* activity is not affected by the addition of a fluorochrome. Thus, transfection of *Sev^{S11}-CFP* in S2 cells provides a new and fast assay with which to study RTK interactions in vivo.

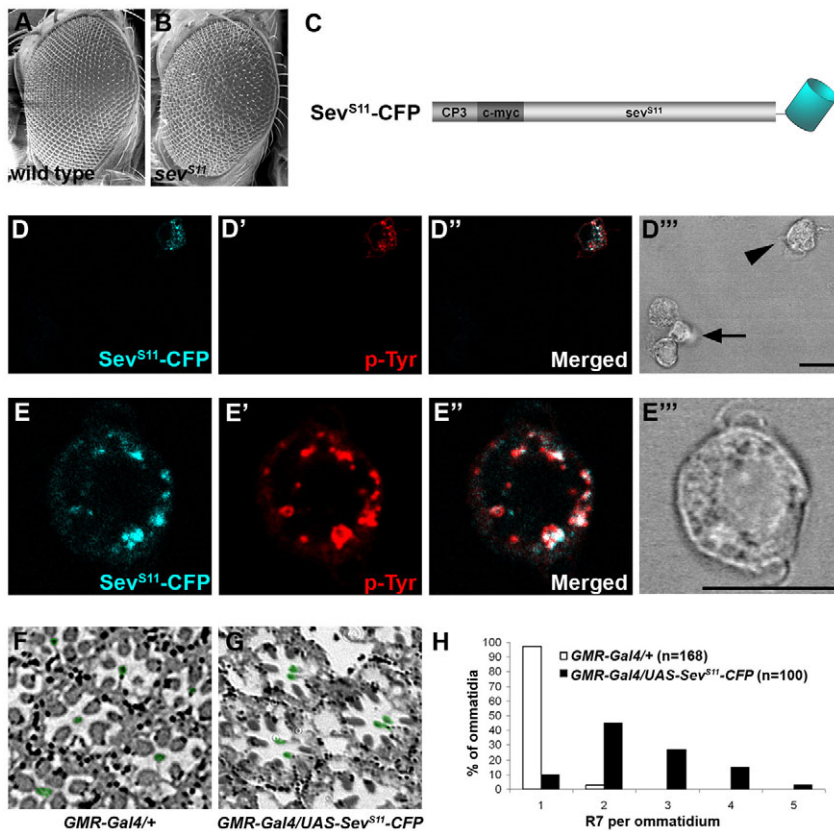


Fig. 1. *Sev^{S11}-CFP* construct promotes tyrosine phosphorylation. (A) Wild-type eye. (B) *Sev^{S11}* eye. (C) *Sev^{S11}-CFP* construct scheme. (D–D''') Transfected cell with *Sev^{S11}-CFP* (cyan) shows an increase in Tyr-P (p-Tyr; red) levels (arrowhead in bright field) compared with non-transfected cells (arrow). (E–E''') *Sev^{S11}-CFP* and Tyr-P (red) colocalisation in a zoom of a transfected cell. (F) Semi-thin section from a *GMR-Gal4* eye. (G) Semi-thin section from a *GMR-Gal4/UAS-Sev^{S11}-CFP* eye. Some ommatidia exhibit extra R7 cells (green). (H) Histogram showing distribution profiles of number of R7 cells per ommatidium in each genotype represented. Scale bars: 10 μ m.

Direct interaction between Sev and Drk

It has been previously reported that Drk interacts specifically with the phosphorylated tyrosine residue 2546 (Tyr2546) of activated-Sev RTK through its SH2 domain (Raabe et al., 1995). A direct interaction between Drk/Grb2 and another RTK, EGFR, has also been described in mammals (Sorkin et al., 2000). We used FRET analysis to monitor this interaction *in vivo* and demonstrate that Drk binds directly to Sev under our experimental conditions.

Cells were transfected with Sev^{S11}-CFP and red fluorescent protein (RFP)-Drk constructs (Fig. 2A–C). RFP-Drk was evenly distributed in the cytoplasm after a single transfection (Fig. 2C). However, in co-transfected cells, the distribution of RFP-Drk protein shifted to colocalise with Sev^{S11}-CFP (Fig. 2D–D''). In addition, colocalisation depends on Sev^{S11}-CFP concentration, because cells transfected with high Sev^{S11}-CFP concentration showed better colocalisation than cells with low Sev^{S11}-CFP (supplementary material Fig. S1). This colocalisation was due to a direct binding between Drk and Sev, because FRET analysis showed an efficiency of 14.6±2% in 95% of the examined transfected cells ($n=20$ cells; Fig. 2D'''). These results confirmed that Drk functions through a direct binding to Sev, as in vertebrates, where Grb2 interacts physically with EGFR (Sorkin et al., 2000).

Direct interaction between Sev and SOCS36E

Although the interaction between SOCS36E and Sev has been suggested to be highly specific by genetic analysis (Almudi et al., 2009), no direct evidence for physical binding has been reported. To understand the molecular mechanism of SOCS36E as attenuator of Sev, we first analysed the physical interaction between Sev^{S11}-CFP and haemagglutinin-tagged SOCS36E (HA-SOCS36E) using immunoprecipitation. S2 cells transfected with Sev^{S11}-CFP were immunoprecipitated using an antibody against Myc and the lysates analysed by western blotting. We found that HA-SOCS36E was pulled down, confirming that Sev and SOCS36E can mutually bind (Fig. 3A). To examine the domains involved in this interaction, the SH2 domain of SOCS36E and the Tyr2546 of Sev were mutated. In both cases, we detected a substantial decrease in SOCS36E immunoprecipitation. This suggests the requirement of the SH2 domain and Tyr2546 phosphorylation to achieve the interaction, similarly to the situation with Drk (Raabe et al., 1995) (Fig. 3A).

To monitor this direct binding *in vivo*, cells were transfected with Sev^{S11}-CFP and different SOCS36E constructs (Fig. 3B). We first examined the subcellular localisation of reporters after single transfections. In contrast to the accumulations found for Sev^{S11}-

CFP, single RFP-SOCS36E transfections resulted in homogeneous cytoplasmic localisation (Fig. 3C,D). Nonetheless, the subcellular localisation of RFP-SOCS36E changed dramatically when co-transfected with Sev^{S11}-CFP, because both proteins colocalise (Fig. 3H–H''). This colocalisation suggests a recruitment of RFP-SOCS36E by the Sev^{S11}-CFP receptor. When we used RFP-SOCS36E-SH2* (Fig. 3B,E), which is an SH2 mutant form that inhibits the ability to bind phosphorylated tyrosine residues, the colocalisation with Sev^{S11}-CFP was impaired (Fig. 3I–I''). This demonstrates that the SH2 domain of SOCS36E is essential for the interaction between these two proteins.

Despite the colocalisation of Sev^{S11}-CFP and RFP-SOCS36E, FRET was not detected (Fig. 3H–H''). This absence could be due to the presence of an adaptor protein responsible for the recruitment of RFP-SOCS36E or simply to the significant length of the SH2 upstream region of the SOCS36E protein (474 residues versus 58 residues in Drk).

To address this question, we designed a new SOCS36E construct, RFP-SOCS36E-ΔN, in which 415 residues of the N-terminal region of SOCS36E were eliminated (Fig. 3B,F), obtaining an SH2 upstream region of similar length (59 amino acids) to that of Drk. This modification permits us to trace energy transfer only if the protein docks through the SH2 domain at Tyr-P. We observed a decrease in the colocalisation of RFP-SOCS36E-ΔN with Sev^{S11}-CFP (Fig. 3J–J''). It is possible that there is an unknown domain in the missing N-terminal region that facilitates the proper localisation of SOCS36E. However, energy transfer was found in those subcellular areas where Sev^{S11}-CFP and RFP-SOCS36E-ΔN overlapped. Indeed, in 40% of the cells examined, an energy transfer efficiency of 15.5±5% was detected ($n=17$; Fig. 3J'''). This demonstrates the capability of the SH2 domain of SOCS36E to attach to the activated Sev.

Cells co-transfected with Sev^{S11Y2546F}-CFP mutant and RFP-SOCS36E-ΔN lacked energy transfer [FRET efficiency (FRETeff) <3%; $n=16$], confirming that both SOCS36E and Drk share the same Sev docking site (Fig. 3K).

SOCS36E competes with Drk to bind to Sev

As Drk and SOCS36E bind directly to Sev, we hypothesised that the intermediate phenotype observed in *UAS-Socs36E-UAS-drk; sev-Gal4 sev^{S11}* eyes of adult flies (Fig. 4A) (Almudi et al., 2009) could be due to competition between SOCS36E and Drk for the same docking site. Thus, direct binding of SOCS36E to Sev would impede the physical interaction between Sev and Drk to trigger Sev signalling.

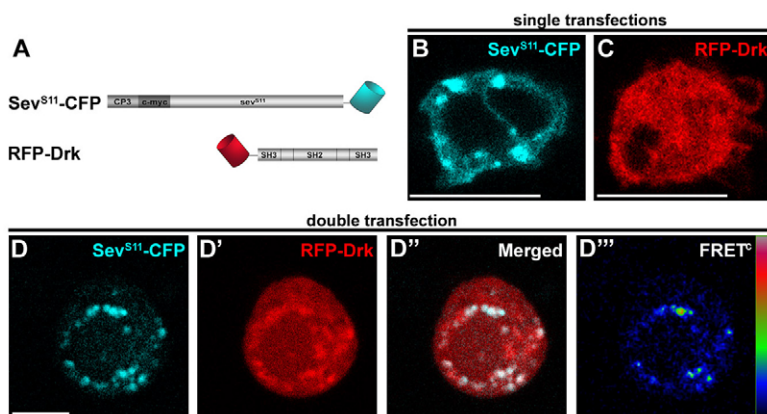


Fig. 2. Direct interaction between Sev RTK and Drk proteins.

(A) Sev^{S11}-CFP and RFP-Drk constructs. (B,C) Localisation of Sev^{S11}-CFP (cyan, B) and RFP-Drk (red, C) in cells transfected separately with each construct. (D–D'') Co-transfection of RFP-Drk and Sev^{S11}-CFP. Regions where Sev^{S11}-CFP and RFP-Drk colocalised showed the highest FRET^c intensity (color code: blue, low intensity; red, high intensity). Scale bars: 10 μm.

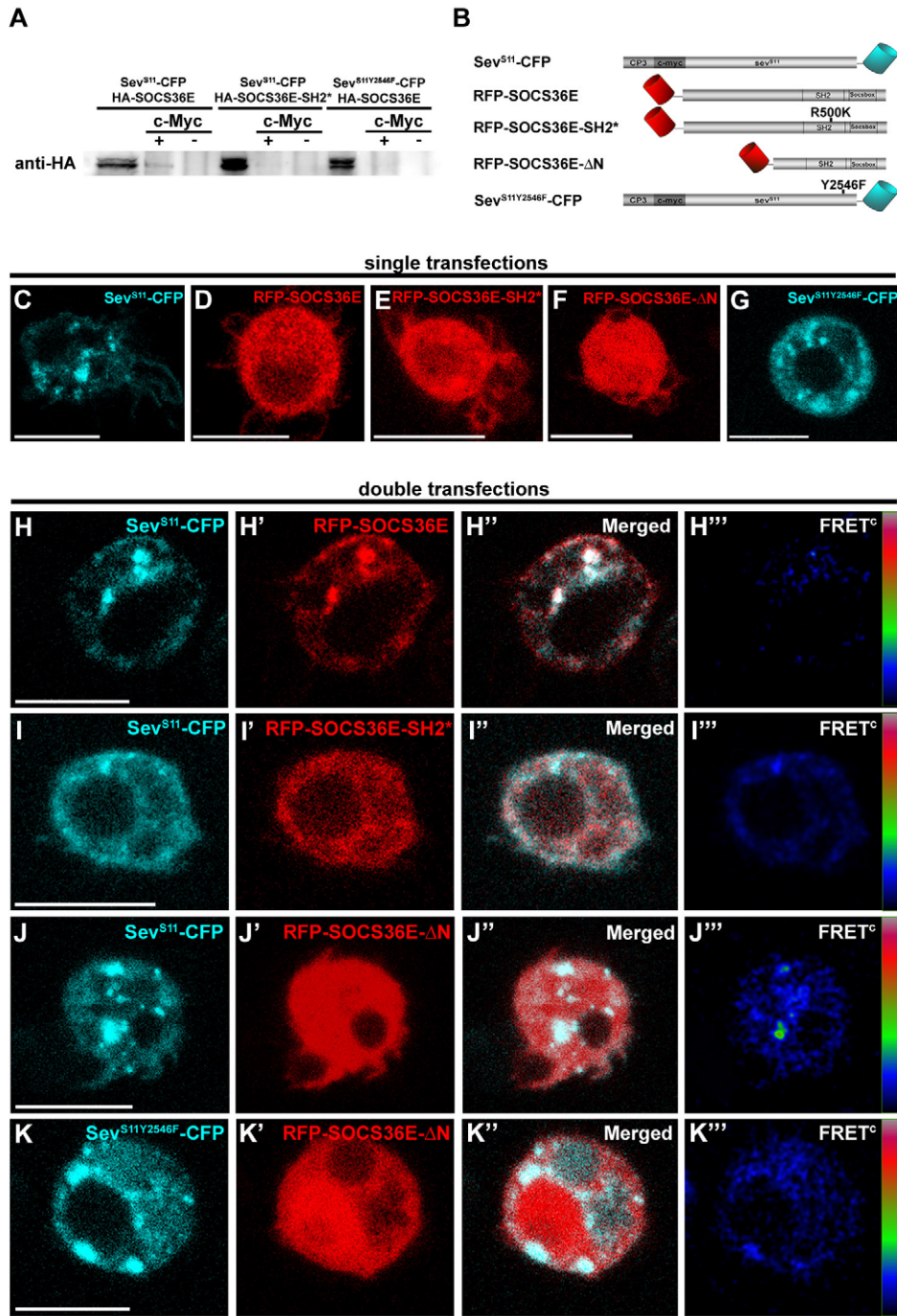


Fig. 3. Direct interaction between Sev RTK and SOCS36E proteins. (A) SOCS36E immunoprecipitates with Sev through its SH2 domain. Three immunoprecipitation conditions were performed: Sev^{S11}-CFP-HA-SOCS36E; Sev^{S11}-CFP-HA-SOCS36E-SH2*; and Sev^{S11Y2546F}-CFP-HA-SOCS36E. Note that SOCS36E immunoprecipitates only when its own SH2 domain and the Sev Tyr2546 residue are not mutated. Lanes 1, 4 and 7 are raw lysates without immunoprecipitation. (B) Sev^{S11}-CFP, RFP-SOCS36E, RFP-SOCS36E-SH2*, RFP-SOCS36E-ΔN and Sev^{S11Y2546F}-CFP constructs. (C–G) Localisation of Sev^{S11}-CFP (C), RFP-SOCS36E (D), RFP-SOCS36E-SH2* (E), RFP-SOCS36E-ΔN (F) and Sev^{S11Y2546F}-CFP (G) fusion proteins in single transfections. (H–H''') Sev^{S11}-CFP (cyan) and RFP-SOCS36E (red) colocalise, although they lack FRET. (I–I''') Colocalisation between Sev^{S11}-CFP and SOCS36E is lost when the SOCS36E SH2 domain is mutated. No FRET was detected. (J–J''') Sev^{S11}-CFP and RFP-SOCS36E-ΔN (red) showing FRET in Sev puncta. (K–K''') Energy transfer is lost in Sev^{S11Y2546F}-CFP (cyan) and RFP-SOCS36E-ΔN cells. Scale bars: 10 μm.

To test this hypothesis, we analysed the energy transfer between Sev^{S11}-CFP and RFP-Drk in the presence of SOCS36E. As a negative control, we transfected MAPK together with Sev^{S11}-CFP and RFP-Drk, because MAPK is known to occur downstream of the pathway (Biggs et al., 1994). Double transfection of RFP-MAPK and Sev^{S11}-CFP resulted in the absence of colocalisation and energy transfer (supplementary material Fig. S2). Triple transfections showed that the presence of MAPK did not interfere with the interaction between Sev^{S11}-CFP and RFP-Drk. Every triple-transfected cell observed showed energy transfer (Fig. 4B). However, triple transfection of Sev^{S11}-CFP, RFP-Drk and SOCS36E resulted in a significant reduction in the number of cells that exhibited energy transfer between Sev^{S11}-CFP and RFP-Drk

(19% of cells; Fig. 4C). Moreover, we observed a decrease in FRETeff to $\sim 11 \pm 3\%$ in Sev^{S11}-CFP, RFP-Drk and SOCS36E versus the $\sim 19 \pm 5\%$ FRETeff obtained in Sev^{S11}-CFP, RFP-Drk and MAPK cells (Fig. 4B''', C''').

We next checked for the ability of the SOCS36E-SH2* mutant to inhibit competition and found energy transfer between Sev^{S11}-CFP and RFP-Drk in all cells examined ($n=15$, FRETeff $\sim 16 \pm 4\%$; Fig. 4D). This shows that the SH2 domain is necessary for docking SOCS36E to Sev.

Conclusions

By using FRET technology in *Drosophila* S2 cells, we provide evidence for the physical interaction of two adaptor proteins, Drk

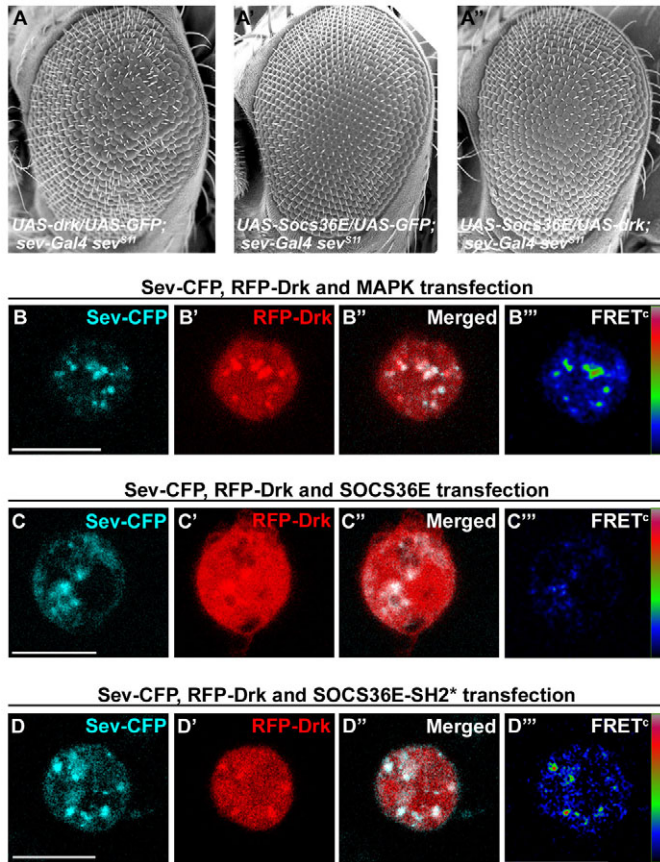


Fig. 4. SOCS36E impedes energy transfer between Sev^{S11}-CFP and RFP-Drk. (A–A'') Overexpression of *drk* (*sev-Gal4-UAS-drk*) in *sev^{S11}* genetic background results in strong rough eye phenotype due to extra R7 cells in each ommatidium (A). *Socs36E* ectopic activation (*sev-Gal4-UAS-Socs36E*) rescues *sev^{S11}* rough eye phenotype (A'). *Socs36E* and *drk* overexpression promotes an intermediate rough eye phenotype (A''). (B–B'') Sev^{S11}-CFP (cyan, B) and RFP-Drk (red, B') colocalisation (B'') in triple-transfected cells (Sev^{S11}-CFP, RFP-Drk and MAPK). Regions where Sev^{S11}-CFP and RFP-Drk colocalised showed the highest FRET^c. (C–C'') FRET^c decreased in regions where Sev^{S11}-CFP and RFP-Drk colocalised in cells triple transfected with SOCS36E, Sev^{S11}-CFP and RFP-Drk. (D–D'') The inactivity of the SH2 domain in SOCS36E-SH2* construct allows the energy transfer between Sev^{S11}-CFP and RFP-Drk in Sev^{S11}-CFP, RFP-Drk and SOCS36E-SH2* triple-transfected cells.

and SOCS36E, with activated Sev. Although both proteins share the same target, they act as positive and negative regulators, respectively. These results, together with previous genetic interaction studies (Almudi et al., 2009), demonstrate that SOCS36E directly binds to the intracellular domain of Sev and that its suppressor effect strictly depends on the presence of the SH2 domain, similarly to the interaction of SOCS-5 with EGFR in mammalian cells (Nicholson et al., 2005). In addition, recruitment of SOCS-6 by the active form of the cytoplasmic tyrosine kinase p56^{lck} has been recently reported (Choi et al., 2010). Therefore, SOCS interaction with activated tyrosine kinases could be a mechanism of signal attenuation. Our results support a model by which SOCS36E suppresses the Sev pathway in precursor cells that should not develop R7 photoreceptors. In such cells, the binding of SOCS36E to the activated receptor attenuates Ras levels, preventing the interaction between Sev and Drk that is

required to transduce the signal. This is not the case for the R7 precursor, where the absence of SOCS36E (Almudi et al., 2009) allows Drk to dock at Sev, promoting the high levels of Ras signalling required for the R7 specification. The competition between SOCS36E and Drk for Sev provides the robustness of the RTK pathway, where attenuation or amplification can result in a switch between different developmental programs.

Materials and Methods

Cell culture, transfection and immunocytochemistry

S2 cells were maintained at 25°C in *Drosophila* Schneider's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cells were transfected using Cellfectin (Invitrogen) according to manufacturer's protocol and collected 48 hours later. Transfected cells were prepared for immunocytochemistry as previously described (Beltran et al., 2007). We used mouse anti-pTyr (PY99; 1:500, Santa Cruz Biotechnology) and goat anti-mouse Rhodamine Red (1:200, Jackson ImmunoResearch Laboratories) antibodies for immunodetection.

sev^{S11}, drk, Socs36E and MAPK constructs

PCRs using primers shown in supplementary material Table S1 were carried out using genomic DNA from *sev^{S11}* flies, full-length cDNA of *drk* and *MAPK* from wild-type flies, *Socs36E* cDNA (SD 04308 from *Drosophila* Gold Collection, Berkeley *Drosophila* Genome Project) and genomic DNA from *UAS-Socs36E-SH2** flies [which carry a substitution of the conserved arginine in the SH2 domain (Arg500) for a lysine (Callus and Mathey-Prevot, 2002)]. To mutate phosphorylation in Sev, we generated a Sev^{S11}Tyr2546F-CFP construct, which is a substitution of Tyr2546 for phenylalanine. PCR products were introduced into pDONRTM221 (Invitrogen) via Gateway cloning to create pEntry-vectors. In the subsequent reactions, these vectors were combined with the pAWC, pARW, pAHW and pPWC vectors (T. D. Murphy, unpublished results; obtained from the *Drosophila* Genomics Research Center) to recombine the constructs with the CFP, RFP proteins and HA tag and express them under the *Drosophila Actin5C* and *UAS* promoters. DNA was purified using a Qiagen Midiprep Kit.

Fly transgenes and stocks

Activated *sev* construct *sev^{S11}* (Basler et al., 1991), *sev-Gal4 sev^{S11}-TM3* (Almudi et al., 2009) and *UAS-Socs36E* (Callus and Mathey-Prevot, 2002) were used for genetic interactions. *drk^{EP(2)2477}* (Bloomington Stock Center) is an insertion containing upstream activation sequence (*UAS*) sites that drive expression of *drk*; hereafter called *UAS-drk*. *UAS-GFP* transgene was added to avoid titration of Gal4 when comparing with double transgene experiments. The *Sev^{S11}-CFP* sequence used for S2 cell transfection was cloned via Gateway in a pPWC vector to drive its expression under the *Gal4/UAS* system and injected in *white* strain *Drosophila* embryos. The eye-specific Glass multimer reporter *GMR-Gal4* (Hay et al., 1994) was used to drive transgene expression.

Scanning electron microscopy and histology

Adult flies were prepared for scanning electron microscopy and semi-thin sections made as previously described (Almudi et al., 2009).

SOCS36E immunoprecipitation

Transfected cells were collected and lysated using standard lysis buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40, protease inhibitor mix) and incubated for 1 hour. Immunoprecipitation was performed with Protein-G-agarose bound to mouse anti-Myc. The extracts were processed and analysed using SDS-PAGE and western blot transfer standard protocols. Immunodetection was performed using mouse anti-HA antibody (1:1000; Abcam) and detected with goat anti-mouse peroxidase (1:3000) secondary antibody with an EZ-ECL system (Biological Industries, Kibbutz Beit Haemek, Israel).

FRET and co-localisation analysis

For FRET analysis, 0.65 µg of donor molecule and 1.35 µg of acceptor molecules were transfected in all experiments. To check for concentration-dependent localisation in the Drk-Sev interaction experiment, we used additional 0.3 µg and 1.5 µg of donor Sev^{S11}-CFP. To examine interactions between Sev, Drk and SOCS36E proteins, the FRET sensitised emission method was used. S2 cells were plated onto MatTek plates, CFP was used as donor molecule and RFP₁ as acceptor molecule (Campbell et al., 2002). Data were acquired and analysed using a Leica TCSSP2 confocal microscope. Normalised FRET was calculated as FRET^c = FRET - (a × CFP) - (b × RFP)/RFP, where FRET^c is corrected FRET (includes corrections for background fluorescence and crossover of donor and acceptor fluorescence through FRET values), and *a* and *b* are the fraction of bleed-through of CFP and RFP fluorescence, respectively, through the FRET filter channel (Jiang and Sorkin, 2002). FRET^c values are presented as FRET efficiency (FRETeff). FRETeff values were averaged from regions of interest (ROIs) observed in cells from at least three independent experiments (*n*>10) per condition and represented as a mean ± s.d. For

our sensors, we considered $\text{FRET}_{\text{eff}} \geq 10\%$ as positive FRET (Galperin et al., 2004; van der Krogt et al., 2008). However, it is generally accepted that absence of FRET delivers values $< 3\%$. FRET° values were presented in pseudocolor mode according to a temperature-based lookup table with blue (cold) indicating low values and red (hot) indicating high values. We used the Colocalisation Finder plug-in from NIH ImageJ software to analyse the grade of colocalisation between Sev^{S11} -CFP and RFP-Drk.

We are grateful to María Calvo, Antonio Felipe, Imre Gaspar and Manel Bosch for help with the FRET technique. We thank Silvia Pérez-Lluch for advice in cell culture. We also thank Cherie Byars Baker, Ada Repiso and Ignacio Maeso for critically reading the manuscript. We also acknowledge Carnegie Institution, the *Drosophila* Genomics Research Center and the Berkeley *Drosophila* Genome Project for vectors. I.A. was supported by a Universitat de Barcelona fellowship. This work was supported by BFU2006-07334/BMC and BFU2009-09781 grants from the Spanish Ministerio de Ciencia y Innovación.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/22/3857/DC1>

References

- Almudi, I., Stocker, H., Hafen, E., Corominas, M. and Serras, F. (2009). SOCS36E specifically interferes with Sevenless signaling during *Drosophila* eye development. *Dev. Biol.* **326**, 212-223.
- Bache, K. G., Slagsvold, T. and Stenmark, H. (2004). Defective downregulation of receptor tyrosine kinases in cancer. *EMBO J.* **23**, 2707-2712.
- Baetz, A., Frey, M., Heeg, K. and Dalpke, A. H. (2004). Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. *J. Biol. Chem.* **279**, 54708-54715.
- Banerjee, U., Renfranz, P. J., Pollock, J. A. and Benzer, S. (1987). Molecular characterization and expression of sevenless, a gene involved in neuronal pattern formation in the *Drosophila* eye. *Cell* **49**, 281-291.
- Basler, K., Christen, B. and Hafen, E. (1991). Ligand-independent activation of the Sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye. *Cell* **64**, 1069-1081.
- Beltran, S., Angulo, M., Pignatelli, M., Serras, F. and Corominas, M. (2007). Functional dissection of the *ash2* and *ash1* transcriptomes provides insights into the transcriptional basis of wing phenotypes and reveals conserved protein interactions. *Genome Biol.* **8**, R67.
- Biggs, W. H., 3rd, Zavitz, K. H., Dickson, B., van der Straten, A., Brunner, D., Hafen, E. and Zipursky, S. L. (1994). The *Drosophila* rolled locus encodes a MAP kinase required in the sevenless signal transduction pathway. *EMBO J.* **13**, 1628-1635.
- Blume-Jensen, P. and Hunter, T. (2001). Oncogenic kinase signalling. *Nature* **411**, 355-365.
- Cagan, R. L., Kramer, H., Hart, A. C. and Zipursky, S. L. (1992). The Bride of Sevenless and Sevenless interaction: internalization of a transmembrane ligand. *Cell* **69**, 393-399.
- Callus, B. A. and Mathey-Prevot, B. (2002). SOCS36E, a novel *Drosophila* SOCS protein, suppresses JAK/STAT and EGF-R signalling in the imaginal wing disc. *Oncogene* **21**, 4812-4821.
- Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882.
- Choi, Y. B., Son, M., Park, M., Shin, J. and Yun, Y. (2010). SOCS-6 negatively regulates T cell activation through targeting p56LCK to proteasomal degradation. *J. Biol. Chem.* **285**, 7271-7280.
- Ciruela, F. (2008). Fluorescence-based methods in the study of protein-protein interactions in living cells. *Curr. Opin. Biotechnol.* **19**, 338-343.
- Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H. et al. (1997). A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387**, 921-924.
- Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87**, 651-660.
- Galperin, E., Verkhusha, V. V. and Sorkin, A. (2004). Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nat. Methods* **1**, 209-217.
- Gordon, G. W., Berry, G., Liang, X. H., Levine, B. and Herman, B. (1998). Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* **74**, 2702-2713.
- Hafen, E., Basler, K., Edstroem, J. E. and Rubin, G. M. (1987). *Sevenless*, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236**, 55-63.
- Hart, A. C., Kramer, H., Van Vactor, D. L., Jr, Paidhungat, M. and Zipursky, S. L. (1990). Induction of cell fate in the *Drosophila* retina: the Bride of Sevenless protein is predicted to contain a large extracellular domain and seven transmembrane segments. *Genes Dev.* **4**, 1835-1847.
- Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**, 2121-2129.
- Hilton, D. J., Richardson, R. T., Alexander, W. S., Viney, E. M., Willson, T. A., Sprigg, N. S., Starr, R., Nicholson, S. E., Metcalf, D. and Nicola, N. A. (1998). Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proc. Natl. Acad. Sci. USA* **95**, 114-119.
- Jiang, X. and Sorkin, A. (2002). Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. *Mol. Biol. Cell* **13**, 1522-1535.
- Kario, E., Marmor, M. D., Adamsky, K., Citri, A., Amit, I., Amariglio, N., Rechavi, G. and Yarden, Y. (2005). Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. *J. Biol. Chem.* **280**, 7038-7048.
- Karsten, P., Häder, S. and Zeidler, M. P. (2002). Cloning and expression of *Drosophila* SOCS36E and its potential regulation by the JAK/STAT pathway. *Mech. Dev.* **117**, 343-346.
- Krebs, D. L. and Hilton, D. J. (2003). A new role for SOCS in insulin action. Suppressor of cytokine signaling. *Sci. STKE* **2003**, PE6.
- Nicholson, S. E., Metcalf, D., Sprigg, N. S., Columbus, R., Walker, F., Silva, A., Cary, D., Willson, T. A., Zhang, J. G., Hilton, D. J. et al. (2005). Suppressor of cytokine signaling (SOCS)-5 is a potential negative regulator of epidermal growth factor signaling. *Proc. Natl. Acad. Sci. USA* **102**, 2328-2333.
- Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. and Pawson, T. (1993). A *Drosophila* SH2-SH3 adaptor protein implicated in coupling the Sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73**, 179-191.
- Raabe, T., Olivier, J. P., Dickson, B., Liu, X., Gish, G. D., Pawson, T. and Hafen, E. (1995). Biochemical and genetic analysis of the Drk SH2/SH3 adaptor protein of *Drosophila*. *EMBO J.* **14**, 2509-2518.
- Rawlings, J. S., Rennebeck, G., Harrison, S. M., Xi, R. and Harrison, D. A. (2004). Two *Drosophila* suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities. *BMC Cell Biol.* **5**, 38.
- Reinke, R. and Zipursky, S. L. (1988). Cell-cell interaction in the *Drosophila* retina: the bride of sevenless gene is required in photoreceptor cell R8 for R7 cell development. *Cell* **55**, 321-330.
- Sese, M., Corominas, M., Stocker, H., Heino, T. I., Hafen, E. and Serras, F. (2006). The *Cdi/TESKI* kinase is required for Sevenless signaling and epithelial organization in the *Drosophila* eye. *J. Cell Sci.* **119**, 5047-5056.
- Simon, M. A., Bowtell, D. D., Dodson, G. S., Laverty, T. R. and Rubin, G. M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* **67**, 701-716.
- Simon, M. A., Dodson, G. S. and Rubin, G. M. (1993). An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to Sevenless and Sos proteins in vitro. *Cell* **73**, 169-177.
- Sorkin, A., McClure, M., Huang, F. and Carter, R. (2000). Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* **10**, 1395-1398.
- Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A. et al. (1997). A family of cytokine-inducible inhibitors of signalling. *Nature* **387**, 917-921.
- Tomlinson, A. and Ready, D. F. (1986). *Sevenless*: a cell-specific homeotic mutation of the *Drosophila* eye. *Science* **231**, 400-402.
- Tomlinson, A. and Ready, D. F. (1987). Cell fate in the *Drosophila* ommatidium. *Dev. Biol.* **123**, 264-275.
- Tomlinson, A., Kimmel, B. E. and Rubin, G. M. (1988). rough, a *Drosophila* homeobox gene required in photoreceptors R2 and R5 for inductive interactions in the developing eye. *Cell* **55**, 771-784.
- van der Krogt, G. N., Ogink, J., Ponsioen, B. and Jalink, K. (2008). A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. *PLoS ONE* **3**, e1916.
- Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T. and Miyajima, A. (1995). A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* **14**, 2816-2826.
- Zhang, J. G., Farley, A., Nicholson, S. E., Willson, T. A., Zugaro, L. M., Simpson, R. J., Moritz, R. L., Cary, D., Richardson, R., Hausmann, G. et al. (1999). The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc. Natl. Acad. Sci. USA* **96**, 2071-2076.