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SOCS36E specifically interferes with Sevenless signaling during *Drosophila* eye development

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ABSTRACT

During the development of multicellular organisms the fate of individual cells is specified with great precision and reproducibility. Although classical genetic approaches led to the identification of many of the signaling pathways contributing to cell fate specification, they have provided little insight into the mechanisms that ensure robustness and reproducibility. We have used the specification of the R7 photoreceptor cells controlled by the Sevenless receptor tyrosine kinase (Sev) pathway to screen for modulators of pathway activity and to uncover the mechanisms underlying the robustness of cell fate decisions. Here we provide genetic evidence that the *Drosophila* SOCS36E adaptor protein containing an SH2 domain and a SOCS box acts as an attenuator of Sev signaling. Overexpression of *Socs36E* strongly suppresses the specification of extra R7 photoreceptor cells in response to constitutive activation of Sev, and loss of *Socs36E* function suppresses the loss of R7 cells when Sev activity is impaired. In a wild-type background, however, loss and gain of *Socs36E* function exhibits little effect on R7 specification. We also show that SH2 domain of SOCS36E is essential for this function in inhibiting Sev action and that *Socs36E* expression is suppressed by high Sev pathway activity. In our model, only the cell able to activate high levels of receptor tyrosine kinase signaling will repress SOCS36E expression, reduce the negative effect on Sev signaling and allow this cell to differentiate into R7. In contrast, the remaining cells fail to receive high signaling, and thus maintain high levels of SOCS36E. This represses residual Sev activity and blocks R7 development. Therefore, *Socs36E* constitutes a novel partially redundant feedback mechanism that contributes to the robustness of R7 specification. The SOCS family of adaptor proteins may have evolved as modulators of specific signaling pathways that contribute to the robustness and precision of cell fate specification.

Introduction

Cell-to-cell signaling is commonly regarded as the most important mechanism to drive cell specification. Many signaling pathways and their associated signal transduction molecules have been genetically and biochemically characterized. However, little is known about how these pathways are regulated to ensure the robustness and reproducibility of cell fate decisions. Signaling events must be regulated in space and time to activate specific genetic programs at the right place and moment in order to avoid wrong developmental decisions. Very likely a network of regulatory molecules will limit the range and duration of signaling activity that drives cell specification. Feedback mechanisms and specific signaling inhibitors of receptor activation are examples that could account for such a network of controlling molecules (Freeman and Gurdon, 2002).

The development of the fly retina has been a key model to isolate and identify molecules involved in signaling between cells. The specification of photoreceptor cells occurs in the third instar eye imaginal disc with great precision to ensure proper function of the visual system. The eight photoreceptors of each ommatidium are recruited in a stepwise fashion by local cell interactions. High activity of the Ras/MAPK pathway controlled by the EGF receptor is important for the specification of R1–R7 photoreceptor cells (Freeman, 1996). But in the case of R7 specification an additional burst of Ras/MAPK controlled by the Sevenless (Sev) receptor tyrosine kinase is required (Freeman, 1996; Simon et al., 1991). The Sev receptor is expressed in nine cells in each ommatidial cluster, the precursors of the R1/R6, R3/R4, R7 photoreceptors and four cone cells, known as the Sev equivalence group (Tomlinson et al., 1987). However, only the R7 precursor will activate the Sev receptor by the Bride of sevenless (Boss) ligand, which is expressed in the adjacent R8 cells (Reinke and Zipursky, 1988). Sev null alleles lack R7 cells in all ommatidia (Tomlinson and Ready, 1986) and the precursors that normally should differentiate into R7 will now trigger the non neural cone cell fate. Conversely, the constitutive activation of Sev induces additional cells of the Sev equivalence group to adopt an R7 photoreceptor cell fate (Basler et al., 1991). The tight spatial and temporal regulation of Ras/MAPK activity is therefore essential to ensure the specification of the precise number and arrangement of photoreceptor cells.

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In search for novel negative regulators of the Sev pathway we performed an EP based genetic screen for suppressors of constitutively activated *sev* transgene and isolated *Suppressor of cytokine signaling 36E* (*Socs36E*) as a gene involved in Sev signaling modulation. SOCS proteins are conserved from flies to mammals and were initially identified as inhibitors of cytokine signaling pathways by acting through a negative feedback loop involving the inhibition of Janus kinase activity (JAK/STAT signaling 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 *SOCS-5* (Callus and Mathey-Prevot, 2002; Karsten et al., 2002) and, like other members of the SOCS protein family, contains a SH2 domain flanked by a variable N-terminal domain and a conserved C-terminal domain, termed the SOCS box. The SH2 domain binds phosphorylated tyrosine residues, whereas the SOCS box participates in an ubiquitin ligase complex to promote the degradation of target proteins (Zhang et al., 1999). In addition to the JAK/STAT pathway, SOCS proteins may also regulate signaling pathways, including receptor tyrosine kinases (Baetz et al., 2004; Callus and Mathey-Prevot, 2002; Kario et al., 2005; Krebs and Hilton, 2003; Rawlings et al., 2004). However, little is known about how SOCS36E can modulate those receptors in physiological conditions.

Because cells can respond to Sev only for a restricted period of ommatidial recruitment (Basler and Hafen, 1989a,b; Bowtell et al., 1989) and because only the R7 precursor activates Sev, we decided to explore whether SOCS36E is involved in the regulation of Sev activity in the equivalence group to single out the R7. We provide genetic evidence that SOCS36E specifically acts as attenuator of Sev signaling in cells that express the Sev receptor but do not differentiate into R7. Moreover, SOCS36E targets the Sev receptor through its SH2 domain to block signaling transduction. Additionally, high levels of Ras/MAPK repress *Socs36E* in precursors of R7. Our results show that SOCS36E constitutes a novel partially redundant feedback mechanism that contributes to the robustness of R7 specification.

Materials and methods

Fly constructs and mutants

The insertion site of the *EP(34-120y^{+})* line was determined by plasmid rescue after EcoRI or XbaI digestion of genomic DNA. *EP(34-120y^{+})* is inserted 2 kbp upstream of the *Socs36E* open reading frame. The activated *sev* construct *sev^{S11}* (Basler et al., 1991) and recombinant flies *sev-Gal4 sev^{S11}/TM3* were used for the screening and genetic interactions. The following fly strains were used: *UAS-Socs36E*, *UAS-Socs36E-SH2**, *UAS-Socs36E-ΔSB* (Callus and Mathey-Prevot, 2002), *UAS-Socs44A* (Rawlings et al., 2004), and the hypomorphic allele *sev$^{d2(\Delta 22)}$* which consists of a null *sev^{d2}* allele partially rescued by a constitutive sev$^{\Delta 22}$ construct (Bohmann et al., 1994). Activation of *Egfr* was achieved using the gain-of-function alleles *Elp^{B1}*, *Elp^{1}*, or ectopically activation of the following constructs: *UAS-tor^{D}EGFR* (Dominguez et al., 1998) or *UAS-λtop* (Queenan et al., 1997); *sev-Ras^{V12}* (Fortini et al., 1992) and *sev-Raf^{torY9}* (Dickson et al., 1996) for activation of the pathway; *UAS-IR* against *Socs36E* was used to induce RNAi (VDRC stock center); *Df(2L)Exel7070* (Bloomington Stock Center) is a deficiency that uncovers a region including *Socs36E*; the EP line *Drk$^{EP(2)2477}$* (Bloomington stock center) to ectopically activate Drk; the JAK/STAT alleles *os^{upd1}*, *hop^{c111}*, *Stat92E^{06346}* (all from Bloomington) and *UAS-upd* (from Hou, S); *sev-N^{nucl}* and *sev-N^{act}* (Fortini et al., 1993) and *sev-lz* (Flores et al., 2000) transgenes were used to ectopically express *Notch* and *lozenge* respectively under the *sev* enhancer; *GMR-Gal4* (Hay et al., 1994) and *sev-Gal4* were used to drive expression of transgenes. CantonS was used as wild type. All crosses and fly culture were done in standard fly medium. When single UAS transgenes had to be compared to double transgenes, a *UAS-GFP* construct was added to avoid effects of titration of the Gal4.

P-element excision

The *P{EPgy2}Socs36E^{EY06665}* flies carry an insertion in the second exon of *Socs36E*. These flies were crossed to Δ2-3 transposase to excise the inserted element and to obtain a loss-of-function mutation of *Socs36E*. Genomic DNA extraction and PCR were performed with a pair of primers flanking the insertion point (upper primer: gccggcg-gaagtgcgtcag; lower primer: cagcgtgggcggtgtgga) in order to check whether those excisions resulted in complete or partial removal of the inserted element. The partial or imprecise excisions were sequenced using the same primers to molecularly define the deletions obtained.

Whole-mount in situ hybridization

In situ hybridization using digoxigenin-labeled antisense RNA probes was carried out using standard protocols. DIG-labeled riboprobes for *Socs36E* were synthesized using a complete cDNA clone from DGC (SD04308), sequenced using primers from the SP6 and T7 promoters and linearized with EcoRI for antisense probe, and SalI for the sense probe. To test the specificity of the riboprobe we used *ap-Gal4* to drive expression either *UAS-Socs36E* or *UAS-Socs44A* in the dorsal compartment of the wing disc, allowing us to compare the dorsal with the ventral domain of the same disc. Antisense probe strongly hybridized in the *ap* domain only when the *Socs36E* transgene was activated (data not shown).

Scanning electron microscopy and histology

Flies were dehydrated in 25, 50, 70, 90, 95 and 100% ethanol for 24 h each to prepare samples for scanning electron microscopy (SEM). To get rid of accumulated debris in the eyes, flies were sonicated for 30 s in an ultrasound bath followed by a final change of 100% ethanol. Flies were critical-point dried and coated with gold to be examined in a Hitachi S-2300 scanning microscope.

Adult flies were fixed, dehydrated and embedded in Spurr's medium. Semithin sections were obtained and stained with methylene blue for analysis under a Leica DMLB microscope. For each genotypic combination, ommatidia from 3 to 5 different eyes were counted in blind analysis. A Chi square test on contingency tables was performed. This allowed us to see significant differences in number of R7 per ommatidium comparing control versus experimental flies.

Antibody production and western blot analysis

5′ cDNA of *Socs36E* was inserted into a pPRO-EX-HTa expression vector (Invitrogen) to produce a fusion protein with 6×His residues. A histidine-tagged protein of ~48 kDa from bacterial extract was purified using His-Select Nickel Affinity Gel (Sigma). The purified SOCS36E protein was injected into rabbits and rats to generate polyclonal antibodies.

To test the SOCS36E antibody, total protein extracts were obtained from embryos (0–24 h) by homogenizing 50 µl of dechorionated embryos in standard loading buffer. The extracts were processed and analyzed using SDS-PAGE and Western blot transfer standard protocols. Immunodetection was performed using rabbit anti-SOCS36E antibody (1:3000) and detected with goat anti-rabbit peroxidase (1:3000) secondary antibody with EZ-ECL system (Biological industries Ltd., Kibbutz Beit Haemek, Israel). For immunohistochemistry, rat or rabbit anti-SOCS36E were used 1:500 in blocking buffer and incubated overnight at 4 °C.

Immunohistochemistry and bioimaging

The *P(GawB)NP5170-5-1* line (Drosophila Genetic Resource Center) has inserted a *Gal4* sequence in the first intron of the *Socs36E* gene, which promotes the expression of *UAS-GFP* to trace cells expressing *Socs36E*.