Characterization of *split ends*, a new component of the *Drosophila* epidermal growth factor receptor signaling pathway

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Abstract

split ends (*spen*) was isolated as a strong enhancer of the rough eye phenotype associated with constitutive activation of Yan, implicating *spen* as a positive regulator of the receptor tyrosine kinase (RTK) signaling pathway. Molecular characterization of *spen* has revealed that *spen* encodes a protein with 5476 amino acids. It contains three tandem repeats of an RNA Recognition Motif (RRM) at its N-terminus, suggesting that Spen might function as an RNA-binding protein. Spen also contains a highly conserved SPOC (Spen Paralogue and Orthologue C-terminal) domain at its C-terminus. Spen-like proteins exist from worms to humans, and they likely define a novel subfamily of RNA-binding proteins based on the RRM sequence similarities. Characterization of *spen* mutant phenotypes in the context of RTK signaling suggests that *spen* function is required for normal eye development and wing vein formation, both contexts where RTK signaling has been proven to play important roles.

We have focused on the development of Drosophila embryonic midline glial cells (MGCs) and have demonstrated that *spen* is required for the normal migration and survival of MGCs. Loss of *spen* leads to aberrant migration and as a consequence, reduced number of midline glial cells. As a result, *spen* mutant embryos exhibit severe morphology and axon-guidance defects in the central nervous system, a phenotype strikingly reminiscent of those seen in *spitz* group mutants. The phenotypic analysis of *spen* mutants strongly suggests that *spen* is a positive regulator of the RTK pathway. Further supporting this hypothesis, we have shown that *spen* synergistically interacts with *pointed*. To further investigate the relationship between *spen* and the RTK pathway, we have generated a dominant negative mutant protein by truncating the C-terminus of Spen including the highly conserved SPOC domain (SpenΔC). Specific overexpression of SpenΔC in the midline glial cells causes lethality, and we have demonstrated that the lethality associated with SpenΔC can be rescued by overexpression of activated Ras^{V12} and activated DER ligand Spitz. Since SpenΔC also suppresses the lethality caused by Ras^{V12}, *spen* is likely to function genetically downstream of or in parallel to Ras. The implication of a putative RNA-binding protein downstream of the RTK/Ras pathway suggests that there might be post-transcriptional gene regulation mechanisms downstream of Ras to allow the cells quickly and precisely to respond to extracellular signals.

In order to elucidate the molecular mechanisms underlying Spen function in the RTK pathway, we have designed a genetic screen to isolate *spen*-interacting genes. By overexpression of a nuclear-localization-sequence (NLS)-tagged Spen C-terminus (CspenNLS) specifically in the eye, we have generated a rough eye phenotype. Reducing endogenous *spen* dosage enhances this rough eye phenotype, suggesting that CspenNLS functions as a dominant negative mutant *in vivo*, possibly by sequestering the *spen*-interacting proteins. Using this phenotype as a starting

background, we screened through the deficiency kit which uncovers ~ 80% of the Drosophila genome and have isolated 23 enhancing and 27 suppressing regions. Among the modifiers, there are regions uncovering known RTK pathway components, including *Draf*, *sevenless*, *vein*, *seven-up*, *pointed* and *Ras*, consistent with *spen* functioning as a component of the RTK pathway. Most interestingly, we have isolated multiple overlapping deficiencies as modifiers of CspenNLS, suggesting that these overlapping regions might contain candidate genes directly interacting with *spen*. Future genetic and biochemical analysis of these candidate genes will likely shed important light on the molecular mechanisms underlying Spen function.

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Chapter 1

Introduction

Introduction

One of the most fascinating questions in biology is how cell-cell communication coordinates the development of multicellular organisms. Individual cells must integrate both activating and inhibitory signals from the surrounding environment and translate this information into specific developmental responses. Extracellular signals are usually transduced across the cell membrane by transmembrane receptors (reviewed by van der Geer et al., 1994). The activated receptors then relay the signals through a cytoplasmic signaling cassette to the nucleus to directly modulate gene expression. These signaling cascades have been highly conserved during evolution and are used reiteratively in many different contexts during normal development. There are reportedly 17 categories of signal transduction pathways functioning in metazoans. However, most aspects of embryonic development are regulated by only five of these signaling cascades. Thus the Wnt, Transforming Growth Factor-beta (TGF-β), Hedgehog, Receptor Tyrosine Kinase (RTK), and Notch signaling pathways represent the predominant developmental cell-signaling mechanisms used by organisms ranging from invertebrates to humans (reviewed by Gerhart, 1999).

This review will summarize the wide range of biological events that are controlled by the RTK pathway and will detail the molecular mechanisms by which the RTK signaling cascade transduces extracellular signals to the nucleus. In particular, we will emphasize the contributions that genetic and biochemical analysis of RTK signaling during Drosophila development have made to the field. As specific and relevant examples, we will discuss RTK signaling during Drosophila embryonic midline glial cell, eye and wing development. Finally, we will discuss the molecular mechanisms whereby RTK-initiated signals lead to context specific developmental outcomes.

Receptor Tyrosine Kinases

Receptor Tyrosine Kinases (RTKs) are Type I integral membrane proteins with intrinsic protein kinase activity in their cytoplasmic domains (reviewed by van der Geer et al., 1994). The extracellular domains are highly divergent, allowing interaction with a broad spectrum of ligands. So far, more than 15 subfamilies of RTKs have been identified with members of each subfamily sharing unique ligand-binding domains. For example, Epidermal Growth Factor Receptors (EGFR) have two homologous Cysteine-rich regions in their extracellular regions. The Platelet-Derived Growth Factor Receptors (PDGFR) have either five or seven immunoglobulin-like (Ig) domains. The Fibroblast Growth Factor Receptors (FGFR) have two or three Ig-like domains. The Insulin Receptors (INSR) have both a Cys-rich domain and three fibronectin type III repeats. These differences in the extracellular domains presumably facilitate direct and specific binding to appropriate ligands. In contrast to the N-terminal extracellular region, the C-terminal cytoplasmic region of RTKs is highly conserved (reviewed by van der Geer et al., 1994). As discussed below, this structure allows RTKs to respond to a broad range of extracellular signals by activating a common intracellular signaling cascade.

The most common mechanism by which RTKs are activated is through receptor dimerization upon ligand binding (reviewed by van der Geer et al., 1994). Dimerization induces trans- and auto-phosphorylation of the C-terminal cytoplasmic region of the receptor, which then generates docking sites for Src-Homology 2 (SH2) domain-containing adaptor proteins (reviewed by van der Geer et al., 1994). The adaptor proteins subsequently recruit downstream signaling molecules to the proximity of the plasma membrane and activate the downstream signaling cascade. The best example is adaptor protein GRB2, whose binding to the activated RTK results in activation of the downstream Ras/Mitogen Activated Protein Kinase (MAPK) signaling pathway (reviewed by van der Geer et al., 1994). Other downstream targets activated by RTKs include the Janus kinase (JAK), phosphatidylinositol-3-kinase (PI-3-kinase), and

phospholipase C (PLC) (reviewed by Porter and Vaillancourt, 1998; van der Geer et al., 1994; Wells, 1999).

Although biochemical studies in mammalian tissue culture systems indicate that several pathways may be stimulated by RTK activation, the best-understood is the Ras small GTPase mediated transcytoplasmic signal transduction cascade. The small GTP-binding protein Ras functions as a molecular switch, which cycles between the active GTP-bound and inactive GDP-bound states (Bourne et al., 1990). The cycle of Ras is directly regulated by two proteins, Ras guanine nucleotide exchange factors (GEFs) that facilitate GTP binding to Ras and Ras GTPase activators (GAPs) that stimulate the Ras intrinsic GTP hydrolysis activity (Boguski and McCormick, 1993; Quilliam et al., 1995).

Upon RTK activation, the docking protein GRB2 brings the Ras GEF Son-of-sevenless (SOS), which is constitutively bound to GRB2, to the proximity of the membrane thereby allowing SOS to activate Ras (Schlessinger, 1993). Activated Ras relays the signals through a cascade of the cytoplasmic protein kinases. The direct effector of Ras is Raf, a protein serine/theronine kinase. Although the complete mechanistic details whereby Ras activates Raf remain to be elucidated, Ras binding to the N-terminus of Raf is thought to be critical (Dickson et al., 1992; Li et al., 1998; Morrison and Cutler, 1997). Biochemical studies in mammalian systems suggest that the Ras-Raf association serves two functions that facilitate Raf activation: first Raf is recruited to the membrane; second, the Ras-Raf association relieves the auto-inhibitory effect of the N-terminus of Raf (Morrison and Cutler, 1997). However, studies in Drosophila embryos reveal that Ras1, a Drosophila Ras homologue, is required for the activation of mutant Drosophila Raf (Draf) proteins which can no longer physically interact with Ras1, suggesting that Ras is required for aspects of Raf activation independent of membrane translocation (Li et al., 1998). Once activated, Raf phosphorylates and activates MAP kinase kinase (MEK), and activated MEK functions as a dual specificity protein kinase to activate its target MAP kinase (Crews et al., 1992; Crews and Erikson, 1993). Activated MAPK can translocate into the nucleus where it directly phosphorylates specific transcription factors, such

as Elk-1 and c-myc in mammalian systems, thereby modulating gene expression (Marais et al., 1993).

RTK signaling in vertebrate development and disease

Experiments in mammalian cultured cells have shown that RTK mediated signaling pathways trigger a wide variety of cellular responses, including mitogenesis, apoptosis, migration, protein secretion, differentiation and dedifferentiation (reviewed by Marshall, 1995; Wells, 1999). In recent years, *in vivo* immunohistochemical studies and targeted knock-out experiments in mouse have provided powerful tools to study RTK signaling during vertebrate development. For example, immunohistochemical studies revealed that EGFR is broadly expressed in diverse fetal tissues (reviewed by Gresik et al., 1998), indicating its critical role during fetal organ morphogenesis, maintenance and repair (reviewed by Wells, 1999; Gresik et al., 1998). These developmental roles have been further supported by EGFR knockouts which die neonatally due to severe immaturity of several epithelial organs (reviewed by Wells, 1999). Similar experiments indicate the Trk subfamily of Nerve Growth Factor (NGF) receptors are required for normal neural function and development (reviewed by van der Geer et al., 1994), the Ror RTKs are important for heart development and limb formation (Takeuchi et al., 2000), and Fibroblast Growth Factor (FGF) RTK function underlies lung morphogenesis (Warburton et al., 2000).

In the past two decades, extensive studies in both humans and model organisms have directly linked unregulated RTK signaling pathways to different types of human diseases. First of all, it is well established that inappropriately activated RTK signaling pathway leads to oncogenesis (reviewed by Porter and Vaillancourt, 1998). About two decades ago, ras was first

isolated as an oncogene based on its transforming activity when it is overexpressed in murine fibroblasts and human EJ bladder carcinoma cell lines (Chang et al., 1982; Parada et al., 1982). Point mutations in the ras proto-oncogene were later found in a variety of human cancers, typically at positions 12, 13, 59 or 61. In particular, a high frequency of human cancers carry a point mutation at codon 12, where the normal Glysine is substituted by either Valine, Aspartic Acid or Cysteine. It has been demonstrated that such mutations render Ras oncogenic by locking Ras in a GTP-bound constitutively active state (reviewed by Abrams et al., 1996). Unregulated Ras leads to cellular overproliferation and loss of contact inhibition, which eventually causes tumorigenesis. Recently, unregulated RTK signaling has been implicated in other aspects of cancer development as well. For example, transgenic mouse experiments and direct studies on human patients suggest that EGFR signaling is important for normal mammary gland differentiation (Schroeder and Lee, 1997), and that elevated EGFR signaling directly contributes to breast cancer invasion and metastasis (Fox and Harris, 1997; Hortobagyi, 2000; reviewed by Porter and Vaillancourt, 1998). Angiogenesis has recently been shown to be an essential step for tumor growth and metastasis, and is thought to involve up-regulation of the vascular endothelial growth factor (VEGF) receptor signaling pathway (McMahon, 2000).

Besides cancer, mutations in receptor tyrosine kinases have been implicated in an increasing number of human diseases (Robertson et al., 2000). For example, mutations in FGF receptors are associated with dwarfism syndromes, craniosynostosis syndromes and Pfeiffer syndrome. Mutations in VEGF receptors have been linked to hereditary lymphedema. Mutations in TIE receptors have been implicated in venous malformation syndrome (reviewed by Robertson et al., 2000). Given the broad spectrum of normal developmental events mediated by RTK signaling pathways together with the correspondingly broad spectrum of diseases and

developmental defects associated with inappropriately regulated RTK signaling, continued research about RTKs and their downstream signaling networks should improve our understanding of the molecular mechanisms underlying cancer and other human diseases and eventually lead to better therapeutic strategies.

Drosophila as a model organism to study RTK signaling

RTK signaling pathways are highly conserved in evolution. Similar receptor tyrosine kinases have been identified in invertebrates and vertebrates (van der Geer et al., 1994). In Drosophila at least seven different receptor tyrosine kinases families have been identified, including Drosophila epidermal growth factor receptor (DER), Drosophila FGF receptor, Drosophila insulin receptor, Drosophila Trk, Torso, Sevenless and Drosophila Ror receptor (reviewed by Perrimon and Perkins, 1997). Because of its powerful genetics and well-characterized developmental biology, Drosophila has proved to be a very useful model organism to study RTK signaling. In fact, the link between RTKs and the Ras/MAPK intracellular signaling cascade was first elucidated in Drosophila (Simon et al., 1991). Following this major contribution, genetic screens conducted in Drosophila have continued to identify new components and regulators of RTK signaling pathways (Casci and Freeman, 1999). Vertebrate homologues of most of these new genes were later found and shown to function in RTK signaling in mammalian systems. Analysis of phenotypes caused by mutations in genes involved in RTK signaling in Drosophila revealed an enormous amount of information about RTK signaling during development. Despite morphological differences between Drosophila and vertebrate development, the molecular and cellular processes underlying developmental events appear highly conserved. Therefore, studies in Drosophila have greatly enhanced our knowledge of receptor tyrosine kinases and their downstream effectors.

Like in vertebrates, RTK mediated signaling controls a broad range of developmental events in Drosophila. For instance, during oogenesis, Drosophila EGF receptor is expressed in the follicle cells where its function is required at three different stages (Van Buskirk and Schupbach, 1999). During very early oogenesis, DER signaling is required for the proper formation of the egg chambers. Later, DER signaling is activated in the follicle cells located posterior to the oocyte and determines the posterior follicle cell fate. During mid-oogenesis, signals from the oocyte activate DER in the follicle cells that contact the dorsal anterior membrane of the oocyte and lead to the establishment of the dorsal follicle cell fate. The differentiated dorsal and posterior follicle cells in turn help to establish the dorsal-ventral and anterior-posterior axes of the oocyte. Recently, DER signaling has also been implicated in the proper migration of the border follicle cells (Duchek and Rorth, 2001). The primary ligand for EGF receptor during oogenesis is Gurken, a TGF-α like protein localized in the oocyte (reviewed by Van Buskirk and Schupbach, 1999).

Throughout embryogenesis, RTK signaling is used reiteratively to instruct cells to assume specific fates in different developmental contexts. During early embryogenesis, the anterior and posterior terminal cell fates are determined by the Torso receptor tyrosine kinase mediated signaling pathway (Duffy and Perrimon, 1994). Activated Torso receptor pathway at both the anterior and posterior regions of the embryo ultimately localizes expression of two transcription factors *tailless* and *huckebein*. The expression of *tailless* and *huckebein* in turn determines the terminal cell fate. Later in embryogenesis, DER signaling and Heartless/FGF receptor signaling pathways are required for mesodermal cell fate determination (Carmena et al., 1998). A graded DER signal originates from the ventral midline to induce discrete ventral ectoderm cell fates (Gabay et al., 1996).

In addition to their functions in cell fate determination, RTK mediated pathways are equally important for cell migration and morphogenesis during embryogenesis. Drosophila FGF receptor signaling is first required for mesodermal cell migration (Forbes and Lehmann, 1999; Gisselbrecht et al., 1996). Later, Breathless/FGF receptor mediated signaling is used reiteratively

during tracheal system branching morphogenesis (Metzger and Krasnow, 1999; Skaer, 1997). DER signaling is also required for the proper migration of the ventral midline glial cells(Hummel et al., 1997; Jacobs, 2000).

RTK pathways also function similarly during larval imaginal disc development to regulate differentiation, migration and apoptosis. During Drosophila eye development, both DER and Sevenless receptor tyrosine kinase mediated signal transduction pathways play important roles during photoreceptor and non-neuronal cell differentiation (Freeman, 1997; Simon, 1994). During wing development, DER signaling is required for wing vein formation (Martin-Blanco et al., 1999). In the following sections, I will focus on the Drosophila EGFR (DER) signaling pathway and further elaborate its function during embryonic midline glial cell development, and imaginal eye and wing development.

Embryonic midline glial cell development

The midline of the Drosophila embryonic central nervous system (CNS) is analogous to the floorplate of the vertebrate neural tube (Jacobs, 2000), functioning as a key organizer of the nervous system. As in vertebrates, the Drosophila embryonic CNS is a bilaterally symmetric structure with longitudinal axons at each side and connecting commissural axons. The two sides are separated and connected by a distinct midline made of neurons as well as glial cells. A specialized set of midline cells, called the midline glial cells (MGCs) is required for CNS morphogenesis, axon guidance and subsequent ensheathment of axon bundles (Hummel et al., 1997; Jacobs, 2000; Klambt et al., 1996). Midline glial cell development involves cell fate determination, cell migration and apoptosis. Regulation of these events involves many of the major signaling pathways, including DER and Notch (Jacobs, 2000). Any perturbation in MGC development results in distinctive CNS defects making it an attractive system to study cell-cell signaling events.

Right before gastrulation, midline precursors are formed in the mesectoderm, a region which is located between the mesoderm and neuroectoderm (Jacobs, 2000). The midline

precursors first distinguish themselves from the neighboring cells by expression of the basic helix-loop-helix (bHLH) transcription factor Single-minded (Sim) and the EGF receptor activator Rhomboid (Rho). Sim functions as a "master regulatory gene" that specifies the midline lineage. Embryos lacking *sim* function fail to develop midline precursors, while ectopic expression of *sim* in other ectodermal tissues induces mesectoderm differentiation (Nambu et al., 1991). As the midline neurons start to differentiate, they stop expressing both *sim* and *rho*. In contrast, midline glial cells continue to express *sim* and *rho* throughout embryogenesis (reviewed by Jacobs, 2000).

At gastrulation, two rows of mesectodermal cells (4 cells per segment) move together towards the ventral midline, where they intermingle to form a single row of 8 cells per segment during germ-band elongation (Figure 1A, Jacobs, 2000). Shortly thereafter, the eight cells divide synchronously to generate 8 pairs of cells in each segment. At stage 12, the anterior most three pairs of precursor cells start to differentiate into midline glial cells. These are referred to as midline glia posterior (MGP), midline glia middle (MGM), and midline glia anterior (MGA). The remaining midline precursors give rise to midline neurons (Figure 1A, Jacobs, 2000). The newly differentiated midline glial cells express Netrins as attractive cues for the posterior and anterior commissure axons to across the midline (Harris et al., 1996; Kolodziej et al., 1996). They also secrete Slit as a repulsive signal to prevent the longitudinal axons from inappropriately acrossing the midline (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999).

By the end of stage 12, the structure of the axon scaffold has been established and subsets of midline glial cells start to migrate (Figure 1B). The MGPs migrate anteriorly toward the previous segment and the MGMs migrate posteriorly over the MGAs to insert themselves between the anterior and posterior commissure axon bundles (Jacobs, 2000). The result of the MGM migration is a physical separation of the anterior and posterior commissure axon bundles (Hummel et al., 1999; Jacobs, 2000). By the end of stage 13, migration of the midline glial cells is complete. As a result, the subsets of midline glial cells that make good contact with the commissure axons survive, while the rest of the midline glial cells are removed by apoptosis

(Jacobs, 2000). The surviving midline glial cells will later elongate to ensheath the commissural axon bundles, thereby keeping the anterior and posterior axon bundles well separated. Mutations affecting midline glial cell development lead to a characteristic phenotype that includes fusion of anterior and posterior commissures and inappropriate crossing of the midline by the longitudinal axons (Klambt and Goodman, 1991; Klambt et al., 1991).

The role of DER signaling in midline glia development was first revealed by the characterization of four mutations with similar neuronal and ectodermal phenotypes, *spitz*, *star*, *rhomboid* and *pointed*, now termed the *spitz* group (Jacobs, 2000; Jurgens et al., 1984; Nusslein-Volhard et al., 1984). Spitz is a Drosophila TGF-α ortholog, functioning as a transmembrane ligand for the EGF receptor (Rutledge et al., 1992). Spitz must be activated by cleavage, in a process involving Star and Rhomboid (Rutledge et al., 1992; Schweitzer et al., 1995). *star* and *rhomboid* function genetically either upstream or in parallel to *spitz* (Pickup and Banerjee, 1999; Ruohola-Baker et al., 1993; Sturtevant et al., 1993). Star is a type I transmembrane protein that is proposed to facilitate Spitz cleavage (Pickup and Banerjee, 1999). Rhomboid is a seven transmembrane protein that appears to restrict Spitz signaling to specific domains on the cell surface (Lanoue and Jacobs, 1999; Ruohola-Baker et al., 1993). Finally, Pointed is an Ets-domain transcription factor, required for the transcriptional response to DER signaling (O'Neill et al., 1994; Scholz et al., 1993). Mutations in any of these genes result in characteristic phenotypes with respect to CNS morphology and axon guidance, including fusion of the commissure axons, reduced separation between the longitudinal pathways and inappropriate axons crossing the midline (Klambt et al., 1991).

In the past decade, extensive studies have been conducted to understand DER signaling during midline glia development. In summary, DER signaling is required at three different levels (reviewed by Jacobs, 2000). Before stage 12, a low level of DER signaling is required for establishment of the midline glia precursor pool. Vein, a Neuregulin-like activating ligand for DER, is expressed transiently in the midline precursors (Lanoue et al., 2000; Schnepp et al., 1996). Embryos lacking *vein* function have lower than normal numbers of midline glial cell

precursors, although later midline glia development appears normal (Lanoue et al., 2000). Higher levels of DER signaling are required for midline glial cell migration. Mutations in major components of the DER signaling pathway, like *pointed*, cause aberrant migration of midline cells, while the initial midline glia differentiation is relatively normal (Klambt, 1993; Klambt et al., 1991). Finally, sustained DER signaling is necessary to suppress apoptosis. Most or all midline glial cells die in *rhomboid* mutants (Sonnenfeld and Jacobs, 1994), while sustained overexpression of *rho* results in extra MG cells (Lanoue and Jacobs, 1999). Interruptions in *rhomboid* misexpression result in apoptosis (Lanoue and Jacobs, 1999). Similarly, overexpression of Ras causes supernumerary midline glial cells. In contrast, ubiquitous expression of the DER inhibitor Argos removes most of the midline glial cells (Lanoue and Jacobs, 1999). Interestingly, *argos* expression is induced by the DER pathway itself in the midline glial cells, thereby generating a feedback loop to restrict the number of midline glial cells (Golembo et al., 1996). The other important survival signals for midline glial cells come from the axon contacts (also known as trophic signals) (Noordermeer et al., 1998). It has been noticed that axon contact signals may intersect with DER signaling. Loss of axon contact causes excessive MG cell death; however, over activation of DER signaling can partially suppress this phenotype (Stemerdink and Jacobs, 1997). It has also been reported that the early axon contact may help localize subsequent DER signaling although the mechanism underlying this interaction remains to be resolved (Noordermeer et al., 1998).

Eye development

The Drosophila compound eye provides another excellent system to study cell-cell signaling. First, the fly eye features a highly regular and reiterative structure. Mutations that only cause subtle defects within each unit eye, or ommatidium, will be amplified a few hundred fold in the entire eye, usually resulting in rough eye phenotypes. Second, the eye is dispensible for viability and fertility, which makes it easy to study and maintain the mutations affecting eye development. Third, the structure and development of the eye have been described at single cell

resolution. All these features make the Drosophila eye amenable to both genetic and cell biological approaches.

The Drosophila adult eye is composed of approximately 800 hexagonal ommatidia (Figure 2A) (Ready et al., 1976). Each ommatidium is an exact 19-cell assembly consisting of 8 photorecepors and 11 accessory cells. The overall structure of the ommatidium is concentric, reflecting its radial recruitment of photoreceptors and accessory cells (Figure 2B). The center core of each ommatidium consists of 8 photoreceptors which can be divided into three classes based on their morphology, axon projection and spectral sensitivity: the R1-R6 outer photoreceptors form a trapezoid ring surrounding the two central photoreceptors; R7 is the distal central cell; and R8 is the proximal central cell (Ready et al., 1976; Tomlinson, 1985). Each photoreceptor contains a stack of microvillar membranes called a rhabdomere (Ready et al., 1976). On top of the photoreceptors are four lens-secreting cone cells. Primary, Secondary and Tertiary pigment cells surround the photoreceptors and cone cells in an hexagonal shape to form the boundary of each ommatidium (Ready et al., 1976; Tomlinson, 1985).

This highly stereotyped structure arises from a single cell-layer epithelium, called the eye imaginal disc (Tomlinson and Ready, 1987). Patterning of the eye disc starts at the third instar larval stage, initiating at the posterior margin of the disc. A wave of differentiation, associated with a distinct indentation called the morphogenetic furrow, moves from the posterior to the anterior end of the eye imaginal disc. Posterior to the furrow, the photoreceptors and supporting cells are recruited sequentially to the developing ommatidial cluster. R8 is the first cell to be determined and is followed pairwise by R2 and R5, R3 and R4, R1 and R6, and finally R7 (Figure 2C). After photoreceptor differentiation, the cone cells and pigment cells are added to the cluster to complete the ommatidial development (Tomlinson and Ready, 1987; Wolff and Ready, 1991). Undifferentiated cells are removed by apoptosis during pupal development to tighten the precisely patterned lattice structure (Wolff and Ready, 1991).

Eye development also requires a precise coordination between cell proliferation and cell differentiation. Cells anterior to the furrow divide asynchronously, and then arrest in the furrow

to form R8, then R2, R5, R3 and R4. After this five cell precluster is established, the remaining undifferentiated cells undergo another round of synchronized division known as the second mitotic wave (Ready et al., 1976). It has been postulated that the second mitotic wave functions to generate enough cells to complete an ommatidium (de Nooij and Hariharan, 1995). The cells that differentiate after the second wave include R1, R6, R7 and all the accessory cells.

It was initially thought that the cells within an ommatidium were clonally related. However, analysis of clones generated by somatic recombination and marked with the eye pigmentation gene *white* revealed that ommatidia along the mosaic border can contain any mix of pigmented and unpigmented cells (Wolff and Ready, 1991). This result ruled out the possibility of a clonal origin for cells within each ommatidium, and led to the suggestion that differentiation of all the cell types depends exclusively on cell-cell signaling. It has been well established that the development of the Drosophila eye involves most of the major signaling pathways, including Notch, Wingless, Hedgehog, Dpp and RTK (Cagan and Ready, 1989; Dickson and Hafen, 1994; Heberlein et al., 1993; Pignoni and Zipursky, 1997; Treisman and Rubin, 1995). For RTK signaling, both the Sevenless and the EGF receptor tyrosine kinases function during eye development (reviewed by Freeman, 1997; Zipursky and Rubin, 1994). Sevenless receptor tyrosine kinase mediated signaling specifically controls R7 photoreceptor differentiation. Loss of *sevenless* function results in ommatidia specifically missing R7 photoreceptors (Tomlinson and Ready, 1986). Another mutation causing the exact same phenotype is in a gene known as *bride of sevenless* (*boss*) (Reinke and Zipursky, 1988). Mosaic analysis suggested that *sevenless* functions autonomously in the presumptive R7 cell (Tomlinson et al., 1987), while *boss* function is required in the R8 photoreceptor that is adjacent to R7 (Reinke and Zipursky, 1988). This result strongly suggested that Boss might function as a ligand for the Sevenless receptor. Further molecular characterization and biochemical analysis confirmed this hypothesis (Zipursky and Rubin, 1994). It is now clear that R7 and the four cone cells arise from the same pool of competent precursor cells. All of them express the Sevenless receptor, however, the Sevenless pathway is only activated in the one cell adjacent to R8 through

this specific Boss-Sevenless interaction. The activated Sevenless pathway determines the R7 photoreceptor cell fate, while the remaining four cells develop into cone cells.

In contrast to Sevenless, the DER mediated signaling pathway is used reiteratively throughout eye development (Freeman, 1997). The first indication of DER involvement in eye development was the identification of an activated mutation of DER called *Ellipse* (Baker and Rubin, 1989). *Ellipse* mutants have a reduced number of ommatidia, suggesting a function for DER in the initial establishment and spacing of R8 cells. Immunostaining with an antibody specific to diphospho-ERK revealed that the highest DER signaling activity occurs in the intermediate cells within the morphogenetic furrow, indicating that DER signaling functions in the furrow to suppress R8 specification (Lesokhin et al., 1999). However, clonal analysis of DER within the furrow suggested that DER function is not required for the initial steps of ommatidial assembly as R8 photoreceptors are specified normally within the DER mutant clones (Dominguez et al., 1998).

Despite the conflicting data concerning R8 specification, DER function is absolutely required for the subsequent recruitment of the other photoreceptors (Freeman, 1996). DER null mutant cells fail to differentiate as photoreceptors, and in fact do not survive(Xu and Rubin, 1993). To further clarify whether DER is required for general viability or specifically for cell differentiation, Freeman expressed a dominant negative form of DER in the eye to block DER function after cell proliferation has ceased. He found that DER function is required for the differentiation of all the cell types in the ommatidium except R8 (Freeman, 1996). Furthermore, he demonstrated that DER activation is sufficient to induce all the different ommatidial cell fates. Thus when DER is activated in the cells near the morphogenetic furrow, it induces outer photoreceptor differentiation. Further away from the furrow, it induces R7s and cone cells. If DER is activated in pupae, it induces pigment cells (Freeman, 1996). These results are consistent with two earlier observations. First, Spitz, a DER ligand, is required for the formation of all the photoreceptors except R8 (Freeman, 1994; Tio et al., 1994; Tio and Moses, 1997). Although Spitz is ubiquitously expressed in the eye, its activation requires cleavage which is

likely mediated by two membrane proteins Rhomboid and Star (Freeman, 1994; Schweitzer et al., 1995). The expression of both proteins is initially limited to the first three photoreceptors, R8, R2 and R5, which are probably the early source of the activated ligand Spitz (Freeman et al., 1992; Heberlein et al., 1993). Second, Argos, a secreted DER inhibitor, inhibits differentiation of both photoreceptors and non-neuronal cell types (Freeman, 1994; Freeman et al., 1992). Interestingly, *argos* expression is activated by DER signaling itself, generating a negative feedback loop (Golembo et al., 1996).

Taking all these results together, Freeman proposed the following model: Photoreceptor differentiation takes place by successive cycles of induction, with each round giving rise to different types of cells (Freeman, 1997). In the early ommatidia, the centrally located R8, R2 and R5 produce active ligand Spitz which can diffuse over a short range to induce the neighboring cells to differentiate as R1, R3, R4, R6 and R7. As each cell responds to DER signaling, it produces Argos, a secreted inhibitor of DER. Argos diffuses further than Spitz and therefore prevents cells far from R8 from activating the DER signaling cascade. As the ommatidia mature, the newly recruited photoreceptor cells became the source of Spitz and Argos, inducing the adjacent cells to become cone cells. Finally, the source of Spitz expands again to recruit the outermost cells as pigment cells (Figure 3A).

This model raises an interesting question about what determines cell fate specificity given the reiterative use of DER signaling during ommatidial assembly. In contrast to the Sevenless pathway where a cell-type specific signal induces a specific cell fate, this model suggests that cell fate is not specified by the type of signal but rather by the time at which the DER signal is activated. In this view, specificity is probably achieved by the developmental history and competence of each cell at the time it receives the DER signal. This state is presumably defined by the presence of different transcription factors and other signaling pathways with the interplay between them ultimately determining the specific cell fate (discussed in more detail below).

In addition to its involvement in cell fate determination, DER signaling has been implicated in promoting cell survival (Miller and Cagan, 1998). During eye development, cell

death normally occurs to remove all surplus cells after the pigment cells are recruited (Wolff and Ready, 1991). It has been proposed that a balance of DER and Notch signals originating from the cone and pigment cells precisely regulates cell death at this late stage of eye development (Miller and Cagan, 1998). Earlier during eye development, DER signaling provides not only differentiation signals but also survival signals for the developing photoreceptors. Loss of DER function posterior to the furrow causes massive cell death (Xu and Rubin, 1993). This is indicated by the small and narrow shape of the DER mutant clones and the clusters of TUNEL positive staining observed within the clone (Dominguez et al., 1998; Xu and Rubin, 1993). Mechanistically, it has been demonstrated that the DER/Ras signaling pathway antagonizes cell death by directly inactivating Hid, one of the key component of the fly cell death machinery, possibly by direct phosphorylation by MAPK (Bergmann et al., 1998; Kurada and White, 1998).

Wing development

Like the eye, Drosophila wings emerge from a proliferating epithelial sheet (Garcia-Bellido, 1975). During early larval stages, DER signaling is required for cell proliferation in the wing and haltere discs (Clifford and Schupbach, 1989). The major DER ligand functioning at this stage is Vein, a neuregulin-like protein (Sturtevant et al., 1993). Null mutations in *vein* result in tiny wing discs(Simcox et al., 1996). Later, restricted DER signaling controls wing vein formation and intervein cell differentiation (Martin-Blanco et al., 1999). The earliest marker to distinguish between future vein and intervein cells is Rhomboid (Sturtevant et al., 1993). Localized Rhomboid expression along the vein primordia triggers high levels of DER signaling to specify vein cell fate. Loss of *rho* function results in truncated wing veins, while ubiquitous expression of *rho* leads to ectopic vein formation. Similarly, truncated and missing vein phenotypes are observed in DER mutants and ectopic veins are induced by misexpression of DER (Diaz-Benjumea and Garcia-Bellido, 1990). Although it has been proposed that Rhomboid

activates DER function through facilitating Spitz cleavage in other systems (Schweitzer et al., 1995), Spitz is not required for vein differentiation (Simcox, 1997). Therefore, Rhomboid function in the wing may be to facilitate processing or presentation of Vein.

During late larval and early pupal stages, a high level of DER signaling is detected within the presumptive vein primordia (Martin-Blanco et al., 1999). During late pupal stages, DER signaling is downregulated in vein territories by a combination of transcriptional downregulation of DER and the accumulation of Argos protein and is upregulated in the intervein cells in response to Vein (Martin-Blanco et al., 1999). It has been demonstrated that downregulation of DER signaling at late pupal stages is necessary to maintain the vein cell fate. Overexpression of DER signaling pathway components within the presumptive vein territories at late pupal stages causes a truncated vein phenotype (Martin-Blanco et al., 1999). It has been proposed that the final differentiation of vein cells depends on the expression of Dpp, which is triggered in vein primordia by DER signaling during early pupal stages and maintained by an autoregulatory loop. In contrast, Dpp expression is inhibited in the intervein regions by elevated DER signaling during late pupal stage (Figure 4; Martin-Blanco et al., 1999).

Ras/MAPK cascade in Drosophila

One of the most useful applications of genetics is to isolate genes that are functionally related. Although studies in mammalian cell culture systems have suggested the connection between RTKs and the different downstream signaling pathways, the first solid evidence to support the idea that Ras mediated pathways transduce signals from RTKs *in vivo* came from the studies of R7 photoreceptor development in Drosophila. Simon et al. designed a genetic screen

to isolate mutations that would further enhance the loss of R7 phenotype associated with a weak *sevenless* mutation (Simon et al., 1991). The logic behind this screen was that the effects of reducing by half the dose of a gene functioning elsewhere in the *sevenless* pathway would be sufficient, in the already sensitized background, to enhance the phenotype. This screen isolated Ras, Drosophila GRB2 and the Ras guanine nucleotide exchange factor Son-of-sevenless (Simon et al., 1991; Simon et al., 1993). It was subsequently confirmed that Ras/Raf/MAPK pathway transduces signals from Torso, EGF and other receptor tyrosine kinases in Drosophila (Perrimon, 1994; Perrimon and Perkins, 1997). The Drosophila counterpart of Raf, MEK and MAPK are also known as Draf, Dsor and Rolled, respectively, and they function similarly to their mammalian homologues (Figure 5; Biggs et al., 1994; Brunner et al., 1994; Dickson et al., 1992; Tsuda et al., 1993)

Nuclear Effectors

There are several nuclear proteins implicated downstream of Rolled/MAPK in Drosophila (Figure 5; Dickson, 1995). Two of them, Pointed and Yan have been well studied (Brunner et al., 1994; O'Neill et al., 1994; Rebay and Rubin, 1995). Both Pointed and Yan are Ets-domain transcription factors (Klambt, 1993; Lai and Rubin, 1992). Genetically, *pointed* and *yan* act antagonistically (Gabay et al., 1996). *pointed* appears to be a positive regulator of the RTK/Ras pathway, while *yan* functions as a negative regulator. Loss of *pointed* function leads to phenotypes very similar to those of DER and *spitz* mutants. For example, *pointed* is required for eye development, for midline glial cell migration and survival, and ventral ectoderm patterning (Gabay et al., 1996; Klaes et al., 1994; Lee et al., 1999; O'Neill et al., 1994). In contrast, loss of *yan* causes similar phenotypes associated with activated RTK signaling, consistent with it functioning antagonistically to the pathway. Hypomorphic *yan* alleles cause extra R7

photoreceptors in the eye (Lai and Rubin, 1992). Loss of *yan* function expands the ventral most cell fate during ectoderm patterning (Gabay et al., 1996). A more detailed phenotypic analysis was performed using a null allele of *yan* that was isolated as an enhancer of activated DER (Rogge et al., 1995). By mosaic analysis, it has been found that cells within the *yan* null clones undergo extra rounds of cell divisions and fail to differentiate as neurons. The overproliferated cells die before adulthood leaving a scar in the adult fly retina. Similar phenotypes were observed in the embryos. Loss of *yan* leads to overproliferation and loss of normal differentiation in the dorsal head ectoderm. Consequently, head involution fails to happen normally, resulting in an anterior open phenotype. These results suggest that *yan* functions in certain contexts to allow cells to choose between proliferation and differentiation decisions (Rogge et al., 1995).

Both genetic and biochemical data strongly suggest that Pointed and Yan are direct targets of MAPK (Brunner et al., 1994; O'Neill et al., 1994; Rebay and Rubin, 1995). *pointed* encodes two alternatively spliced transcripts resulting in two protein isoforms, PointedP1 and PointedP2 (Klambt, 1993). The two proteins share a common C-terminus which contains an Ets DNA binding domain, but have distinct N-termini which may explain the differences in their activity. PointedP1 is constitutively active, while PointedP2 is activated via phosphorylation by MAP kinase (Brunner et al., 1994; O'Neill et al., 1994). PointedP2 contains one conserved MAPK phosphorylation site within the N-terminal Pointed box and is phosphorylated by MAP Kinase at this site *in vitro*. Mutation of this site abolishes responsiveness to MAPK signaling *in vivo* (Brunner et al., 1994; O'Neill et al., 1994). Yan contains 8 consensus MAPK phosphorylation sites. Like pointedP2, Yan is phosphorylated by MAP kinase *in vitro*. Mutations of all 8 putative sites generate a constitutively active form of Yan, Yan^{ACT} (Rebay and Rubin, 1995). Overexpression of Yan^{ACT} blocks both neuronal and non-neuronal cell differentiation throughout development. For instance, during eye development, overexpression of Yan^{ACT} blocks photoreceptor differentiation resulting in a rough eye phenotype. This

observation is consistent with the hypothesis that Yan is a negative regulator of RTK/Ras signaling pathway and that its function is downregulated by the activated MAP kinase. One possible mechanism of MAPK-mediated inactivation of Yan is through regulation of Yan subcellular localization. It has been observed that in S2 cells, Yan subcellular distribution shifts from nuclear to cytoplasmic upon Ras/MAP kinase activation. In contrast, Yan^{ACT} remains nuclear upon Ras/MAPK activation (Rebay and Rubin, 1995).

As downstream effectors of the RTK pathway, it has been proposed that Pointed and Yan compete for the same promoter region of the target genes. Pointed functions as a transcription activator while Yan is a repressor. The first supporting evidence came from an assay done in S2 cells (O'Neill et al., 1994). Using a reporter construct containing multiple Ets-1 binding sites, it was shown that PointedP2 can activate transcription. In the same assay, Yan acts as a competing repressor. The Yan repressor activity is greatly down regulated upon co-transfection of activated Ras or MAPK. *In vivo* evidence supporting this model has recently been published (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). These three groups mapped the promoter regions of three target genes downstream of the DER pathway, *prospero*, D-*Pax2* and *even-skipped*, and found ETS consensus sites in all three. *In vitro* gel shift assays demonstrated that both Yan and Pointed bind to these sites. Mutations affecting the Ets consensus sites abolish the *in vitro* binding by both Yan and Pointed. When the same mutations were introduced *in vivo*, they abrogate the DER-activated expression of each of the three genes (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). Both *in vitro* and *in vivo* data suggest the following model. Before RTK/Ras activation, Yan physically binds to the promoters of the target genes and represses their transcription. RTK signals activate MAPK to downregulate Yan function and simultaneously activate Pointed to compete away Yan and to initiate target gene transcription.

In addition to Pointed and Yan, several other nuclear proteins have been implicated genetically downstream of MAP kinase. However, it is less clear how their functions are regulated by MAPK (Dickson, 1995). One such protein is Tramtrack, a zinc-finger transcription

factor (Xiong and Montell, 1993). Like Pointed, it has two isoforms, TTK69 and TTK88 (Harrison and Travers, 1990). Mutations specifically abolishing the TTK88 product cause extra R7 photoreceptors in the eye, similar to the phenotype associated with hypomorphic *yan* alleles. Epistasis analyses suggest Ttk functions genetically downstream of *yan* (Lai et al., 1996). It has been shown that down-regulation of Ttk is required for normal R7 photoreceptor differentiation and is mediated by two additional nuclear proteins, Phyllopod and Sina (Li et al., 1997; Tang et al., 1997). Phyllopod is a novel nuclear protein required for R1, R6 and R7 fate determination during eye development (Chang et al., 1995; Dickson et al., 1995). Sina is a novel nuclear protein containing a RING finger domain, whose function is specifically required for R7 differentiation (Carthew and Rubin, 1990). Both *phyl* and *sina* function genetically downstream of MAPK. It has been proposed that activated Ras/MAPK induces expression of Phyl, which then forms a complex with Sina and Ttk88 to target Ttk88 for ubiquitin-mediated degradation (Li et al., 1997; Tang et al., 1997).

Other regulators

The initial modifier screen for mutations affecting signaling downstream of *sevenless* identified major components of the RTK/Ras signaling pathway (Simon et al., 1991; Simon et al., 1993). Similar screens that were subsequently conducted focusing on different parts of the pathway successfully isolated many more regulators of the pathway (reviewed by Casci and Freeman, 1999). Phenotype based genetic screens also identified additional components of the RTK/Ras signaling pathway (reviewed by Freeman, 1998). The list of regulators includes tyrosine phosphatases like Corkscrew (Perkins et al., 1992), protein kinases like Kinase suppressor of Ras (Ksr) (Therrien et al., 1995), different kinds of adaptor proteins such as Daughter-of-sevenless (Dos) and Connector enhancer of Ksr (Cnk) (Herbst et al., 1996; Raabe et al., 1996; Therrien et al., 1998), and negative regulator like Kekkon-1 and Sprouty (Casci et al.,

1999; Hacohen et al., 1998; Queenan et al., 1997). Some of these proteins are receptor specific regulators, whereas others are general factors. The emerging picture is that the RTK/Ras pathway is no longer a simple linear pathway, but instead involves feedback loops and networks linking to the other pathways (Figure 5). Discussion of these important regulators is beyond the scope of this review.

Control of Specificity

One of the most fascinating questions about RTK signaling is what controls the specificity. Not only does the universal Ras/Raf/MAPK cascade transduce signals from many different receptors, but the same receptor mediates a variety of signaling events in different contexts. For instance, DER signaling is used reiteratively throughout development controlling a variety of developmental events (see above). How can DER mediated cell-cell communication elicit such diverse outcomes?

Although the complete answer to this question is not yet known, specificity is probably controlled by a combination of many different factors. The first major factor contributing to specificity is the existence of tissue-specific ligands for DER (Perrimon and Perkins, 1997). So far, there are three different activating ligands identified in *Drosophila*. For example, Gurken is the major DER ligand during oogenesis (Nilson and Schupbach, 1999). Spitz is the major ligand during embryogenesis, although in certain contexts, Vein functions as a minor ligand (Golembo et al., 1999; Perrimon and Perkins, 1997; Yarnitzky et al., 1998). During wing development, Vein serves as the primary ligand (Schnepp et al., 1996). Different ligands might activate the receptor at different levels, resulting in different signaling strengths. The existence of tissue-specific ligands may thus generate tissue-specific signals. In the case of Spitz, another level of regulation is added, because membrane associated Spitz needs to be cleaved to become active

(Rutledge et al., 1992; Schweitzer et al., 1995). The Spitz cleavage appears to be facilitated by two other membrane proteins, Star and Rhomboid (Pickup and Banerjee, 1999; Ruohola-Baker et al., 1993; Sturtevant et al., 1993; Heberlein et al., 1993). The cleaved Spitz can form a gradient, which will in turn generate a strength gradient of DER signals. As a consequence, the cells along the gradient will adopt different cell fates. It has been proposed that the distinct ventral ectoderm cell fates are specified mainly by this mechanism (Gabay et al., 1996).

The second factor contributing to the specificity is regulation by feedback loops. There exist both positive and negative feedback loops which regulate DER activity (Figure 5) (reviewed by Casci and Freeman, 1999). Evidence supporting this model came primarily from studies on Argos and Rhomboid. Argos is a secreted protein with an atypical EGF motif, which functions as a DER inhibitor based on both genetic and biochemical studies (Schweitzer et al., 1995). Argos transcription is directly regulated by the DER signaling pathway, thus generating a negative feedback loop (Gabay et al., 1996; Golembo et al., 1996). While Rhomboid is important for processing DER ligand Spitz (Ruohola-Baker et al., 1993), its expression is also controlled directly by the DER pathway, thus generating a positive feedback loop (Ruohola-Baker et al., 1993).

The function and interplay of both positive and negative feed back loops is best illustrated by the model proposed for dorsal appendage positioning in the follicle cells (Figure 4B) (Wasserman and Freeman, 1998). During oogenesis, the dorsal follicle cells initially response to the Gurken signal from the oocyte to activate the DER signaling pathway. DER signaling activates *rhomboid* expression in those cells, which in turn activates Spitz. As a result of positive feedback, the DER signal is amplified in the dorsal midline and adjacent cells. This elevated DER signaling then induces the expression of the inhibitor Argos in the dorsal midline cells, resulting in local suppression of DER signaling. The integration of both positive and negative feed back regulation generates two peaks of DER signaling, which eventually specifies the position of dorsal appendages (Figure 3B).

The third and probably the most important contributing factor ensues from interactions between the RTK signaling pathway and other signaling pathways (Simon, 2000). As discussed above, DER signaling is used reiteratively during eye development. The same signal induces different cell types at different times (Freeman, 1997). It has been proposed that the specificity is controlled not by the cell-type specific signals, but instead by the developmental histories of the cells. Specifically, different cells containing distinct sets of transcription factors that have been activated by other signaling pathways, upon RTK pathway activation will generate distinct responses. The best supporting evidence for this model was provided most recently by studies of specific gene responses in specific cell types during eye and mesoderm development (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000).

During eye development, as discussed above, the cells in each ommatidium are recruited sequentially, starteding with R8 and followed by R2, R5, R1, R6, R7, cone cells and pigment cells. DER signal is responsible for the induction of all cell types except R8 (Freeman, 1997). Characterization of different cell types reveals that cell fates are marked by specific sets of gene expression. For example, Prospero, a transcription factor, is specifically expressed in the R7 equivalence group that includes R7 and the cone cells (Xu et al., 2000), while D-Pax2, a homeobox transcription factor, is specifically expressed in cone cells and pigment cells (Flores et al., 2000). Xu et al. found that DER signaling in conjunction with the transcription factor Lozenge is necessary for the cell-type specific expression of Prospero, and that Sevenless RTK signaling is required for Prospero upregulation in R7 as compared to the cone cells (Xu et al., 2000). They defined an enhancer region within the *prospero* promoter which is responsible for the DER and Lozenge regulation. They further identified and biochemically proved that there are functional ETS-binding sites and Lozenge binding site within that region. Moreover, they demonstrated that mutations in any of those sites abolish the cell specific expression of *prospero*. They also provided evidence that the presence of the Sevenless signaling pathway in the R7 photoreceptor induces R7 specific upregulation of *prospero*. Together these results suggest that

the integration between DER signaling and another transcription factor Lozenge, together with R7-specific signaling by the Sevenless RTK, contributes to the specificity of cellular response.

Similarly, Flores et al. examined the regulation of D-Pax2 expression in the developing eye (Flores et al., 2000). They found that Notch signaling, DER signaling and Lozenge were all required for the specific expression of D-Pax2 in cone cells. They then defined the enhancer region in the D-Pax2 promoter that binds Lozenge and the transcription factors downstream of DER, Yan and Pointed, and Notch signaling, Suppressor of hairy (Su(H)). The authors further demonstrated that the "on" and "off" combinations of these three pathways determines different cell fate specification outcomes within the ommatidium. For example, the Notch pathway is normally off in R7 precursors, while the DER and Lozenge mediated pathways are on. Ectopic activation of Notch signaling in R7 is sufficient to induce D-Pax2 expression, which promotes the cone cell fate.

A similar mechanism is also suggested for the specification of embryonic muscle and cardiac progenitors (Halfon et al., 2000). During early mesoderm development, the precluster cells form in the dorsal mesoderm region where *wingless* and *dpp* signals overlap. Later, groups of cells within the precluster that receive localized Heartless/FGFR signals differentiate into cardiac progenitors, while cells that receive both Heartless and DER signals become muscle progenitors. As a result, the heart and muscle precursors express *even-skipped* as a cell fate marker. Halfon et al. showed that there are enhancer regions within the promoter of *even-skipped* that can directly bind to the transcription factors downstream of each of these signaling pathways, including one site for dTCF of the Wingless pathway, multiple sites for MAD of the Dpp pathway and multiple ETS binding sites for Pointed and Yan. They also identified binding sites for the mesoderm-specific transcription factors Twist and Tinman. Importantly, they found that mutations affecting any of these binding sites abolishes *even-skipped* expression. Therefore, the combinatorial effects of transcription factors from different signaling pathways ultimately determines the specificity of cellular response.

Summary

The evolutionarily conserved RTK signaling pathway has been extensively studied in different systems. Research in Drosophila and other model organisms has contributed enormously to our knowledge. Despite great progress, there are still important questions remaining to be answered. First of all, the mechanisms controlling how specific outcomes from general RTK signals are achieved are far from understood. Recent work in Drosophila eye and mesodermal development has shed important light on this question, however, the studies are limited to the transcriptional regulation of a small number of target genes. Whether this model applies to the general transcriptional regulation of RTK downstream targets remains to be tested. Furthermore, RTK mediated signaling also elicits many cellular responses other than differentiation, including migration, metabolic changes and survival. The mechanisms underlying this great variety of responses is unclear.

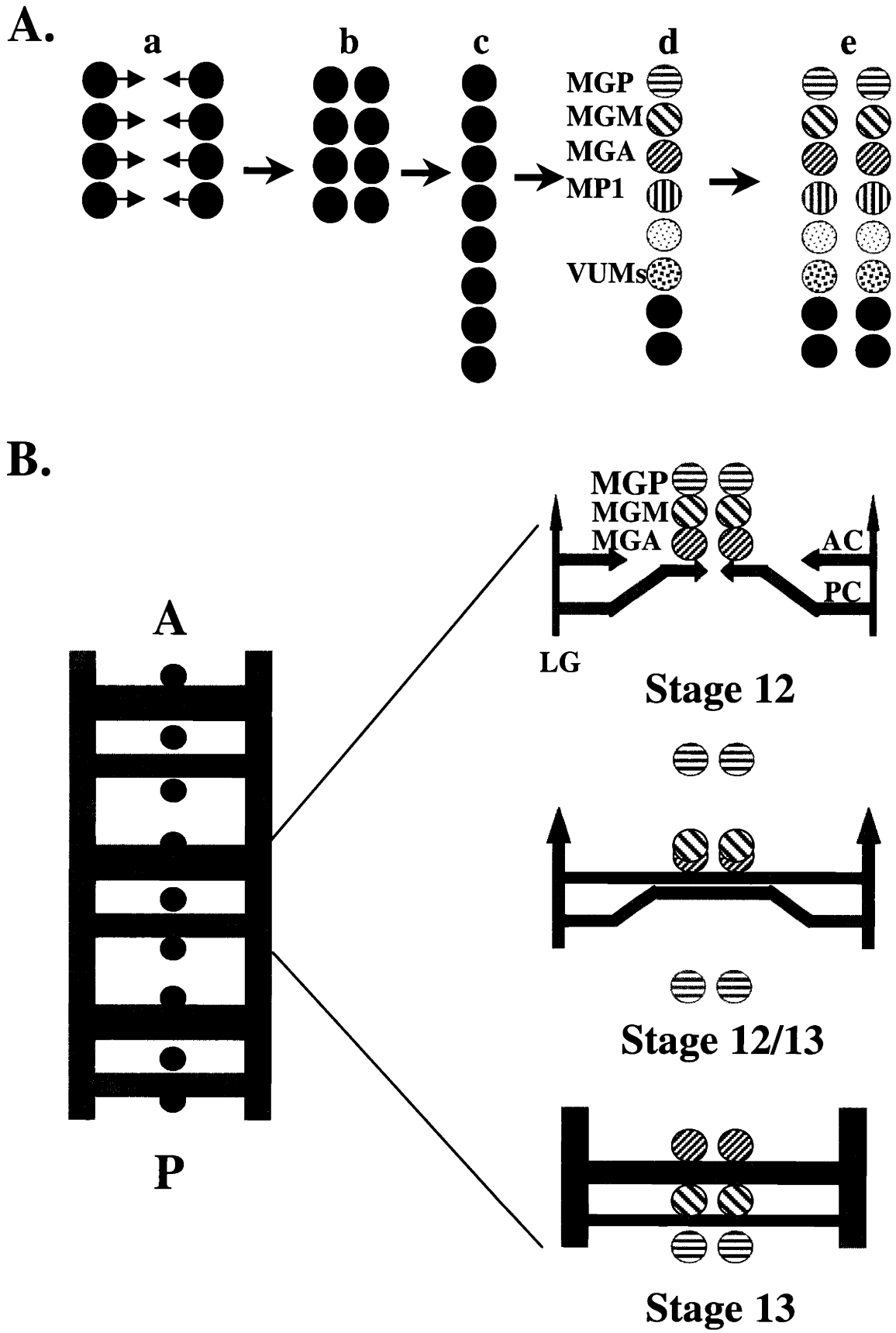
Second, our understanding of the mechanisms mediating crosstalk between the RTK pathways and other signaling pathways remains limited. The model of combinatorial transcription regulation recently presented by three groups suggests one possible way to integrate signals from several pathways (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). However it is likely that this is not the only mechanism. Genetic and biochemical studies have identified many adaptor proteins such as CNK, DOS and SHC downstream of receptor tyrosine kinases, suggesting that the signals from the receptors may branch out to different cytoplasmic signaling cascades. Thus different pathways might intersect each other at different points.

In the rest of my thesis, I will report the molecular and genetic characterization of a gene known as *split ends* (*spen*), which was isolated as a putative positive regulator of the RTK pathway (Rebay et al., 2000). The initial molecular characterization revealed that Spen is a putative RNA-binding protein, leading to the speculation that Spen might be involved in post-transcriptional gene regulaton mechanisms downstream of the RTK/Ras pathway (Rebay et al.,

2000). Post-transcriptional regulation usually includes protein modification as well as RNA splicing, stabilization and targeting. The advantage of such mechanisms is that they allow cells to respond quickly to the extracellular signals and to increase the complexity of the signaling events without involving additional rounds of transcription (Siomi and Dreyfuss, 1997). Just as protein modification, especially phosphorylation, is commonly used during signal transduction, RNA-binding protein mediated post-transcriptional gene regulation is involved in many important developmental events. For example, during Drosophila sex determination, the RNA-binding protein Sex-lethal directly regulates the pre-mRNA splicing of itself and of a downstream target gene *transformer* to control female specific differentiation (Valcarcel et al., 1993). In the RTK signaling pathway, it has been demonstrated that targeted *gurken* mRNA localization by an RNA-binding protein Squid is important to initiate DER signaling during oogenesis (Norvell et al., 1999).

To date, RNA binding protein mediated mechanisms have not been implicated downstream of Ras to directly regulate gene expression. Despite lack of evidence, it is plausible that such mechanisms may exist downstream of Ras. First, RTK signaling is highly dynamic. It regulates cell fate determination in a both qualitative and quantitative manner. Different strengths of the signals induce distinct cell fates both *in vitro* and *in vivo*. It therefore seems likely that "fine-tuner" mechanisms must exist in the pathway to allow quick and precise adjustments to the outcomes in response to fast changing extracellular stimuli. Second, two transcription factors that function genetically downstream of MAPK, *pointed* and *tramtrack*, are known to be alternatively spliced. Although the regulation of the alternative splicing of the two genes is unclear, it remains an intriguing hypothesis that RTK/Ras signaling, perhaps acting via Spen, might be directly responsible.

Figure 1. Development and migration of midline glial cells. (A) During mid gastrulation, two rows of midline glial cells (4 cells per segment) move together towards the ventral midline (a-b), intermingle into single rows of 8 cells per segment during germ band elongation (c), and, at early stage 12, the anterior most three will differentiate into midline glial cells: midline glial posterior (MGP), midline glial middle (MGM) and midline glial anterior (MGA). The rest of the cells will assume midline neuronal fates (d). Shortly after, the 8 MG cells divide synchronously to generate 8 pairs of cells in each segment (f). (B) By the end of stage 12, MGCs start to migrate. MGPs migrate anteriorly to the next segment, MGM migrate posteriorly over MGA to insert themselves between the commissure axon bundles to separate them. The migration completes at the end of stage 13. Subsets of MGCs make good contacts with the commssiure axons. (Panel A is adapted from Klambt et al. 1991).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Stage 12/13
Stage 13

Figure 2. Drosophila eye development. (A) The external morphology of a wild type fly compound eye. (B) Schematic view of an ommatidial unit. A longitudinal section is shown on the left and cross sections at three different levels are shown on the right. (C) Schematic representation of the assembly of one ommatidial unit. B, Bristle; C, liquid-filled pseudocone; CC: cone-cells; L; lens; M; basal membrane; PC, posterior cone cell; PLC, polar cone cell; PP, primary pigment cells; Rh, rhabdomere; SP, secondary pigment cells; TP, tertiary pigment cells; (1-8), photoreceptor cells.

(Modified from Dickson and Hafen, 1993).

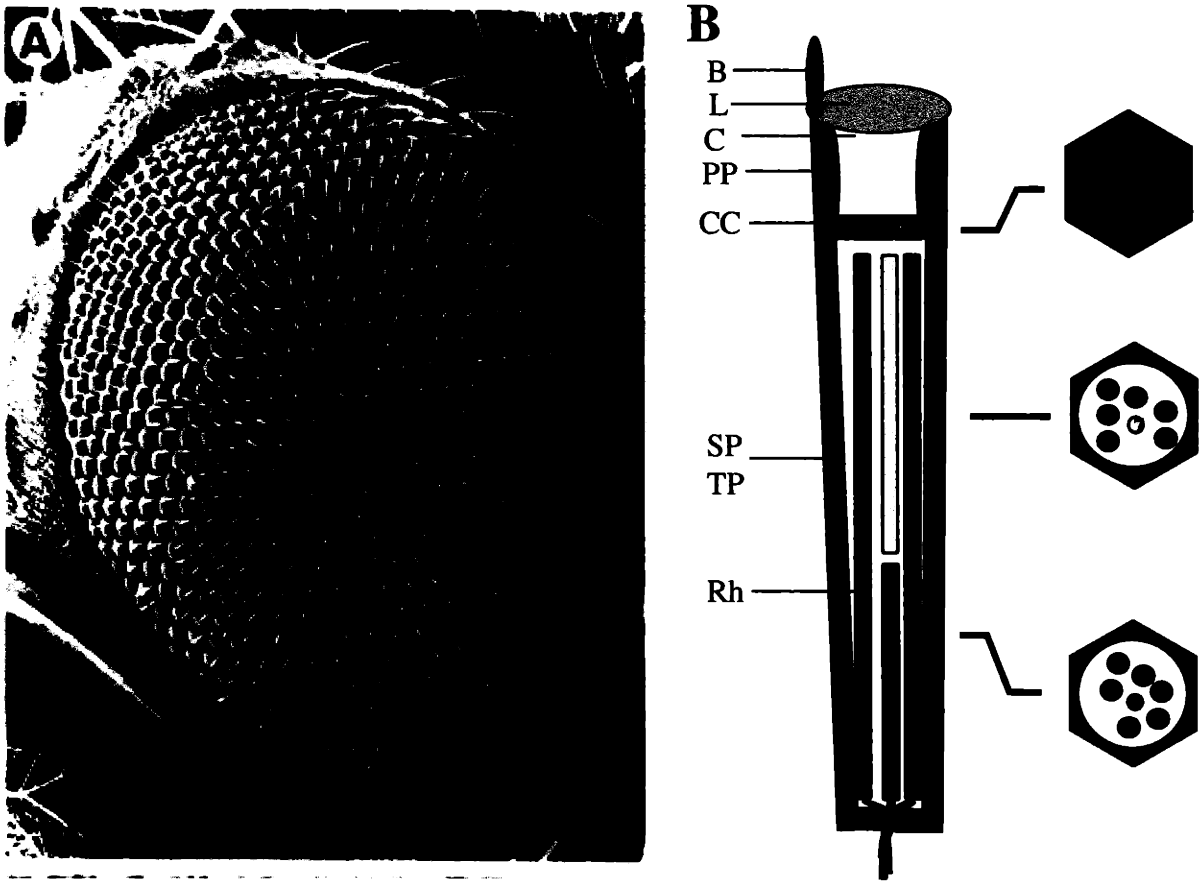
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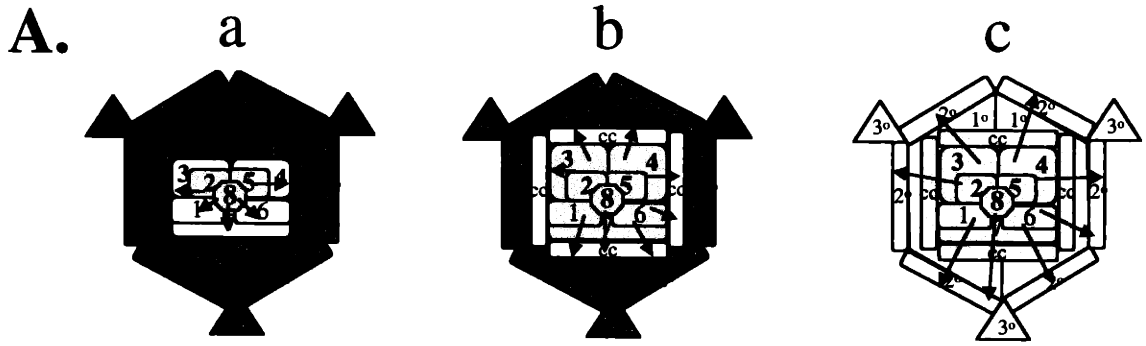
Figure 3. Two models for DER signaling pathway. (A) DER signals are used reiteratively during eye development. In the early ommatidia(a), the centrally located R8, R2 and R5 produce active ligand Spitz (arrows) to induce the neighboring cells (marked as yellow cells) to differentiate as R1, R3, R4, R6 and R7. As each cell responds to DER signaling, it produces Argos, a secreted inhibitor of DER. Argos diffused further than Spitz and therefore prevents cells far from R8 (gray cells) from activating the DER signaling cascade. As the ommatidia mature, the newly recruited photoreceptors become the source of Spitz and Argos, inducing the adjacent cells to become cone cells(b). Finally, the source of Spitz expands again to recruit the outermost cells as pigment cells(c) (this panel is adapted from Freeman, 1997). (B) The interplay between the positive and negative feedback loops specifies the dorsal appendages in the follicle cells. During oogenesis, the dorsal follicle cells initially response to the Gurken signal from the oocyte to activate the DER signaling pathway. DER signaling activates rhomboid expression in those cells, which in turn activates Spitz. As a result of positive feedback, the DER signal is amplified in the dorsal midline and adjacent cells. The elevated DER signaling then induces the expression of the inhibitor Argos in the dorsal midline cells, resulting in local suppression of DER signaling. The integration of both positive and negative feed back regulation generates two peaks of DER signaling, which eventually specifies the position of the dorsal appendages (this panel was adapted from Wasserman and Freeman, 1998).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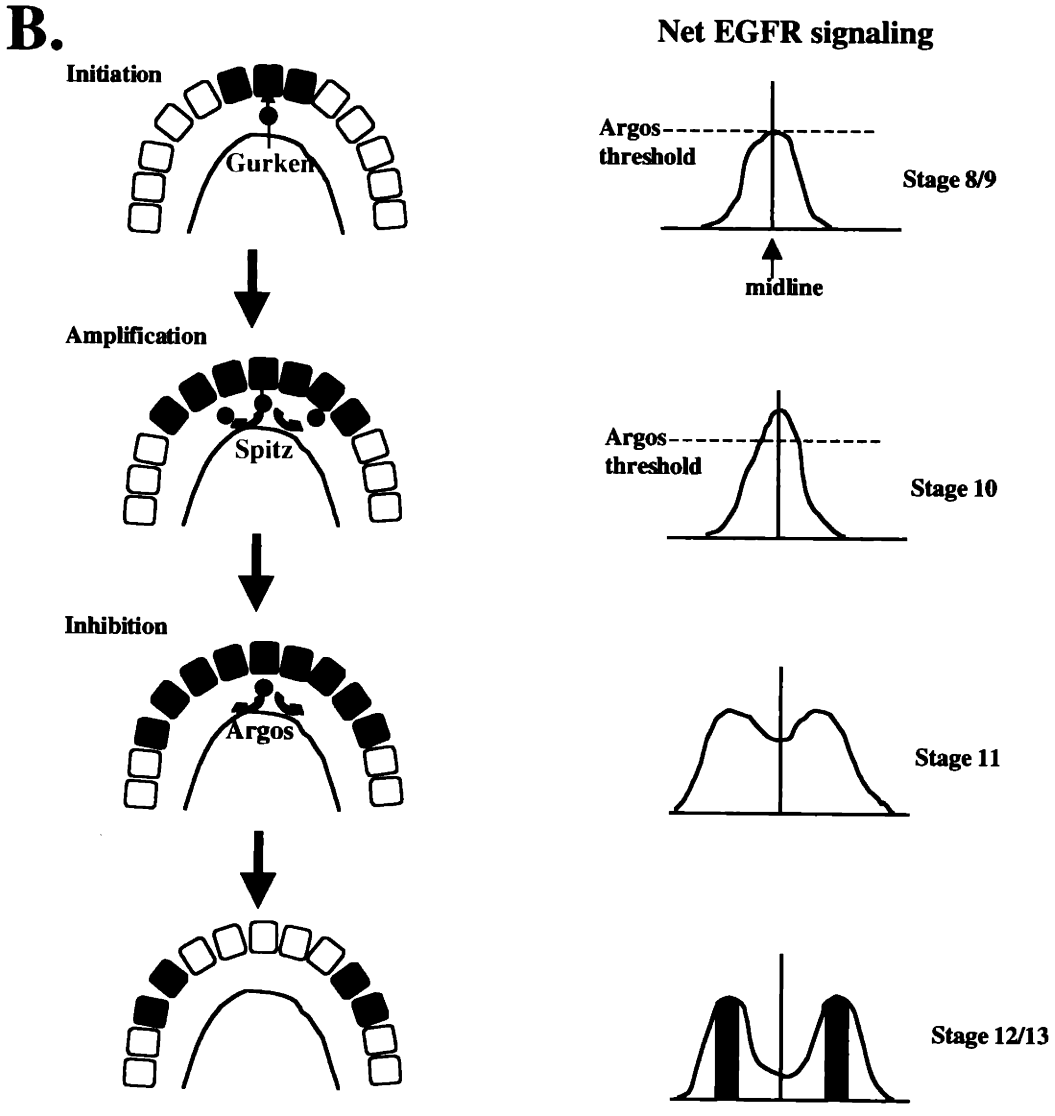
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Initiation
Gurken
Amplification
Spitz
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Stage 8/9
midline
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threshold
Stage 10
Stage 11
Stage 12/13

Figure 4. Regulated DER signaling determines the vein and intervein cell fates in the developing wing. During late larval and early pupal stages, a high level of DER sigaling is detected within the presumptive vein primordia. During late pupal stages, DER signaling is downregulated in vein territories by a combination of transcriptional downregulation of DER and the accumulation of Argos protein. In contrast, DER signaling is upregulated in the intervein cells in response to Vein. (Adapted from Martin-Blanco et al 1999).

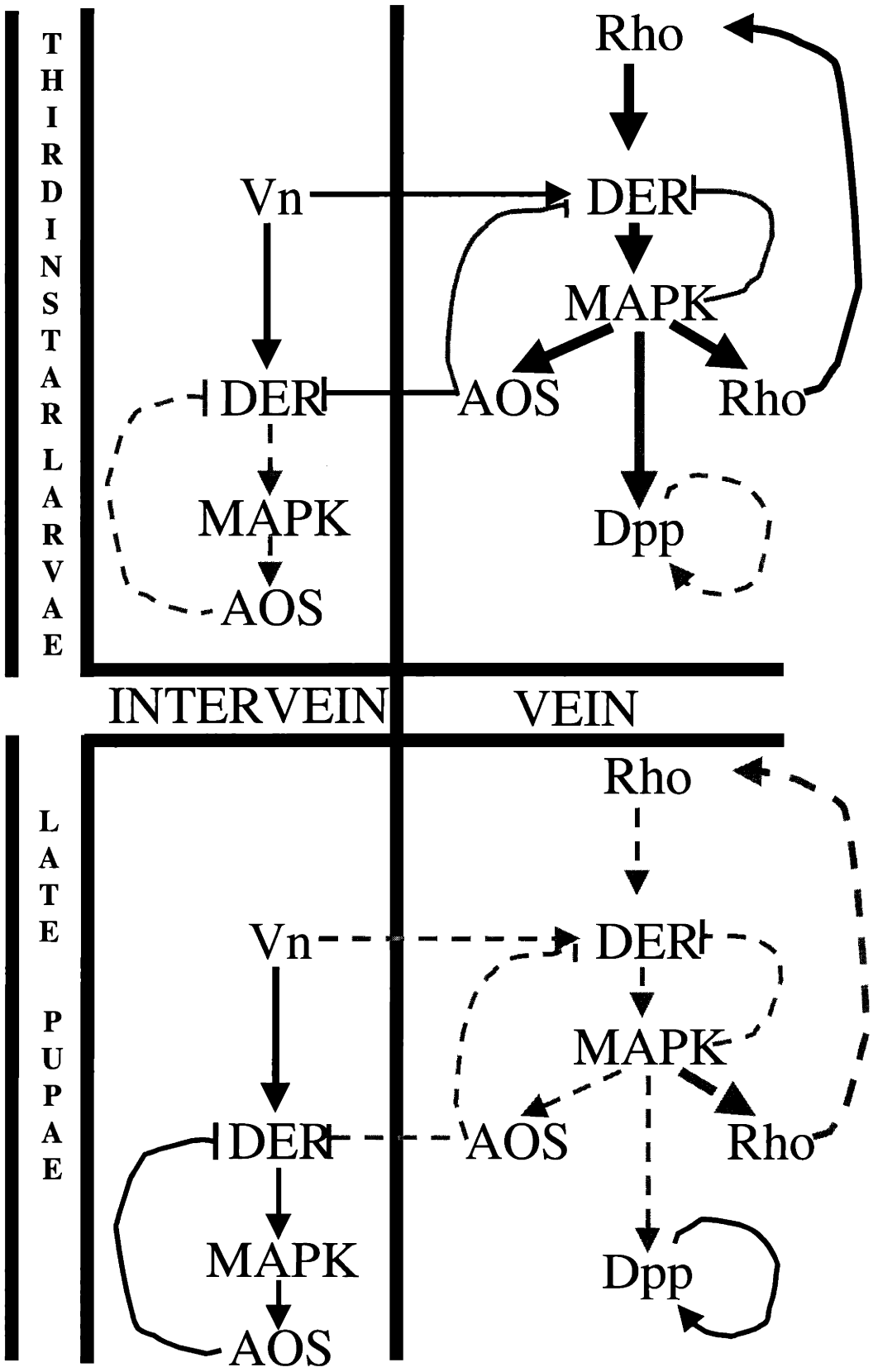
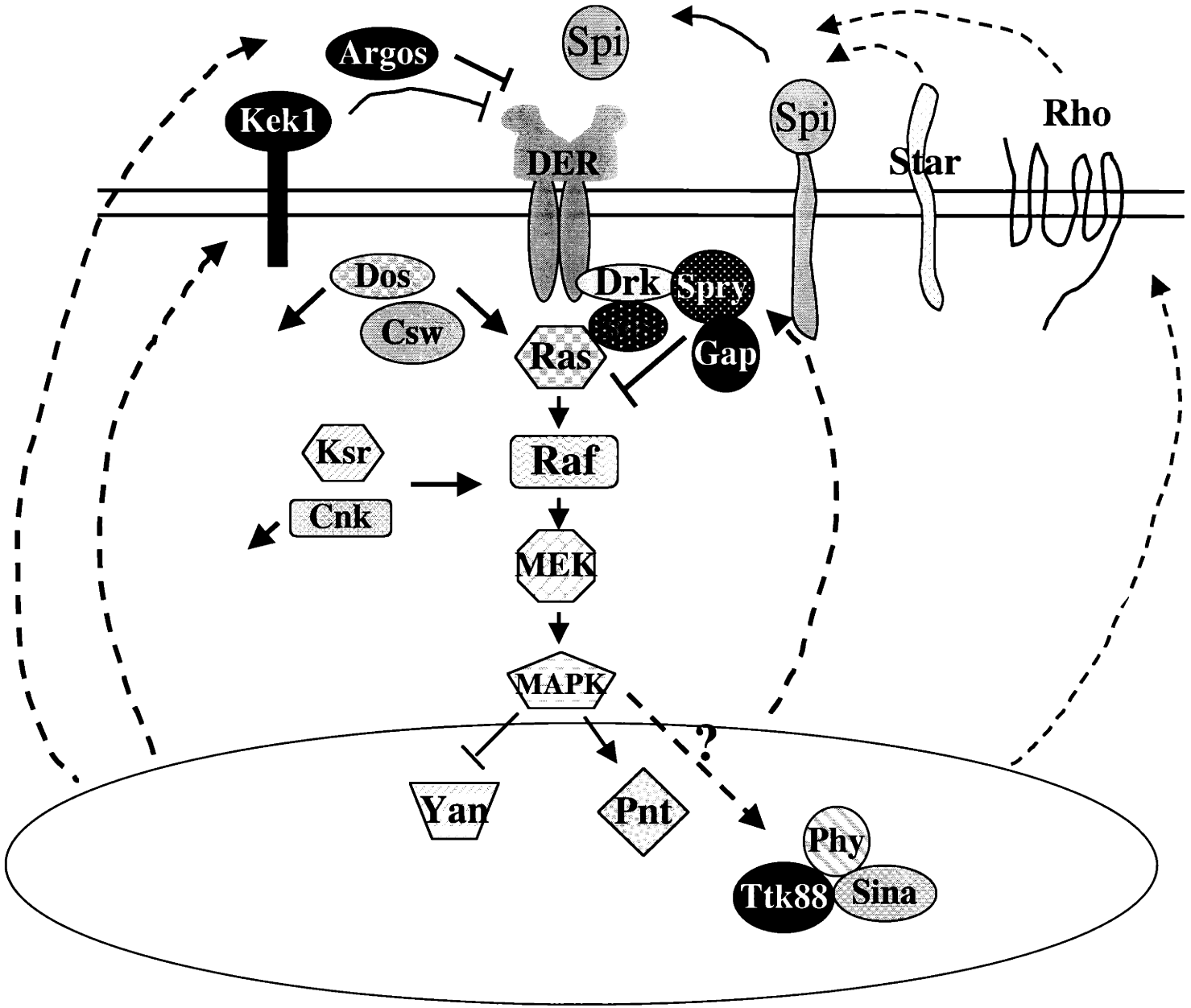

THIRD INSTAR LARVAE
Rho
Vn
DER
MAPK
DER
AOS
Rho
MAPK
Dpp
AOS
INTERVEIN
VEIN
LATE PUPAE
Rho
Vn
DER
MAPK
DER
AOS
Rho
MAPK
Dpp
AOS

Figure 5. The regulators of the Drosophila EGF receptor (DER) pathway. The receptor (DER) is activated by dimerization upon ligand Spitz binding. Star and Rhomboid (Rho) are required to activate Spitz. The activated receptor recruits the Ras guanine nucleotide exchange factor Son-of-sevenless (SOS) via the adaptor protein Drk. SOS activates Ras and Ras relays the signals by activating a cascade of protein serine/theorine kinases including Raf, MEK and MAPK. Activated MAPK translocates into the nucleus to directly modulate the activities of transcription factors such as Yan and Pointed. MAPK may also activate Phyllopod and Sina to downregulate Tramtrack. Parallel positive regulators of the pathway include Daughter-of-sevenless (DOS), Corkscrew (Csw), Kinase suppressor of Ras (Ksr), Connector enhancer of Ksr (Cnk). There are also negative regulators of the pathway: Kekkon-1 (Kek1) and Sprouty (Spry). The two parallel lines represent the plasma membrane, the oval circle represents the nucleus, and in between represents the cytoplasm. The arrows from the nucleus represent the feedback loops. The solid arrows indicate the events with known molecular mechanisms. The dashed arrows indicates the events with unclear molecular mechanisms.


Argos
Spi
Kek1
DER
Spi
Star
Rho
Dos
Drk
Spry
Csw
Ras
Gap
Ksr
Raf
Cnk
MEK
MAPK
?
Yan
Pnt
Phy
Ttk88
Sina

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Chapter 2

split ends, a novel putative RNA binding protein, functions during eye development

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Note:

*** Francis Hsiao helped to isolate the C-terminal spen cDNA**
 # Andrina Williams did the sections in figure 7.
& Ilaria Rebay performed the screen that isolated *spen,* cloned most of the *spen* cDNA sequence and analyzed the Spen protein sequence.
The isolation and molecular characterization of *spen* reported in this chapter has been published in Rebay, I., Chen, F., Hsiao, F., Kolodziej, P. A., Kuang, B. H., Laverty, T., Suh, C., Voas, M., Williams, A., and Rubin, G. M. (2000). A genetic screen for novel components of the Ras/Mitogen-Activated protein kinase signaling pathway that interact with the *yan* gene of Drosophila identifies *split ends*, a new RNA recognition motif-containing protein. Genetics *154*, 695-712.

Abstract

split ends(spen) was isolated as an enhancer of Yan^{ACT} in a genetic screen designed to identify novel components of the RTK/Ras signaling cascade, implicating *spen* as a positively acting component of the pathway. Molecular characterization of *spen* revealed that *spen* encodes a predicted protein of ~5500 amino acids. Spen contains three RNA Recognition Motifs (RRMs) at its N-terminus suggesting that Spen is a putative RNA binding protein. It also contains a highly conserved C-terminal SPOC (Spen Paralogue and Orthologue C-terminal) domain. Spen-like proteins exist from *C. elegans* to humans. Thus Spen is the founding member of a novel family of RNA binding proteins defined by both N-terminal RRMs and a C-terminal SPOC domain. To explore its potential role as a new component of the RTK/Ras signaling pathway, we studied *spen* mutant clones during both eye development and wing vein formation where RTK/Ras signaling pathways play essential roles. We found that loss of *spen* function during eye development results in a disorganized retina, malformed photoreceptors, and failure in cone cell fate determination. In addition, loss of *spen* during wing development leads to missing or thinner wing veins. Our results suggest that *spen* is required for normal Drosophila eye development and wing vein formation which is consistent with our hypothesis that *spen* is a potential positive regulator of the RTK signaling pathway.

Introduction

Receptor tyrosine kinase (RTK) triggered activation of the Ras/MAPK cascade is one of the major routes through which extracellular signals are transduced to the nucleus. This signaling pathway is evolutionarily conserved from nematodes to humans and is used reiteratively in many different developmental contexts to regulate a broad range of cellular events including

proliferation, differentiation, migration and survival. For example, in Drosophila, RTK/Ras signaling is required during oogenesis to establish anterior-posterior and dorsal-ventral polarity (Nilson and Schupbach, 1999; Queenan et al., 1997; Schupbach and Roth, 1994), during embryogenesis to regulate cell fate decisions in the ventral ectoderm (Gabay et al., 1996; Raz and Shilo, 1993), mesoderm(Buff et al., 1998) (Carmena et al., 1998), midline of the central nervous system (Scholz et al., 1997) (Jacobs, 2000; Stemerdink and Jacobs, 1997), and tracheal system (Metzger and Krasnow, 1999) (Skaer, 1997; Sutherland et al., 1996), and during larval and pupal imaginal development to control proliferation, patterning, and cell fate induction (Freeman, 1996) (Hafen et al., 1993) (Nagaraj et al., 1999). This diverse array of developmental events is regulated by a small set of RTKs including Torso, Sevenless, the fibroblast growth factor receptors Breathless and Heartless, and the Drosophila epidermal growth factor receptor (DER). Of these five RTKs, DER appears to have the most pleiotropic functions(Schweitzer and Shilo, 1997).

One particular context in which RTK-mediated signaling has been extensively studied is Drosophila eye development (See Chapter 1). Both DER and Sevenless RTK mediated signaling are essential for photoreceptor differentiation (Freeman, 1997; Zipursky and Rubin, 1994). DER signaling also controls cell fate determination of non-neuronal cells in the eye (Freeman, 1997).

While the downstream effectors of the RTK/Ras/MAPK pathway remain largely elusive in most systems, a primary consequence of MAPK activation in Drosophila is activation of Pointed and inactivation of Yan, both members of the ETS family of transcription factors (O'Neill et al., 1994). Inactivation of Yan in the developing eye, as in many tissues throughout development, is an essential prerequisite for their differentiation (Rebay and Rubin, 1995).

In the course of a genetic screen designed to identify novel components downstream in the Ras/MAPK signaling pathway, we identified 37 alleles of the gene *split ends* (*spen*) (Rebay et al., 2000). *spen* was isolated as an enhancer of the rough eye phenotype associated with a constitutively active form of Yan (referred to as Yan^{ACT}) that is no longer responsive to MAPK phosphorylation (Rebay and Rubin, 1995). Specifically, whereas wild type Yan is both a negative regulator of RTK signaling and is itself inactivated upon phosphorylation by MAPK, the Yan^{ACT} product is no longer a substrate for MAPK and thus remains constitutively active, repressing the differentiation of the cell types in which it is expressed and generating a rough eye phenotype. Thus recovery of *spen* as an enhancer of Yan^{ACT} suggests a possible function for Spen as a positively acting factor in, or in parallel to, the RTK/Ras/MAPK/Yan pathway. Here we show the molecular characterization of *spen* and the results of phenotypic clonal analyses designed to study *spen* function in tissues where RTK-mediated signaling is known to play an important role.

Results

Isolation of *spen* as an enhancer of Yan^{ACT} implicates *spen* as a positive regulator of RTK signaling

spen was isolated as a strong enhancer of Yan^{ACT} in a genetic screen designed to identify novel components of the RTK/Ras signal transduction pathway (Rebay et al., 2000). The Yan^{ACT} transgene encodes an engineered protein in which all 8 MAPK consensus sites have been mutated to a non-phosphorylatable form, rendering the Yan^{ACT} product insensitive to down-regulation by MAPK (Rebay and Rubin, 1995). Eye-specific overexpression of Yan^{ACT} under the Sevenless promoter (Sev-Yan^{ACT}) results in a moderate rough eye phenotype (Figure 1A, B). Removal of one copy of *spen* dominantly enhances the phenotype, producing a much smaller and

more disorganized eye (Figure 1C). Tangential sections through the adult eyes further confirm the direction of interaction between Yan^{ACT} and *spen*. Because *yan* is a negative regulator of the RTK/Ras signaling pathway, overexpression of Sev-Yan^{ACT} blocks the differentiation of a subset of the photoreceptor neurons and accessory cells (Figure 1D, E). Reduction in the dose of *spen* increases the numbers of missing photoreceptors and disrupts the pigment cell lattice surrounding each ommatidium, resulting in a much more disorganized retina (Figure 1F). The direction of interaction between Yan^{ACT} and *spen* suggests that *spen* antagonizes *yan*, perhaps by functioning as a positive regulator of the RTK/Ras/MAPK pathway.

spen encodes a novel putative RNA binding protein with three N-terminal RRMs and a conserved C-terminal SPOC domain

spen was cloned by P-element mediated plasmid rescue method (Rebay et al., 2000). Molecular characterization of the *spen* genomic region revealed that *spen* spans ~50 kb of genomic DNA (Figure 2). Conceptual translation of the cDNA predicts a protein with 5496 residues (Rebay et al., 2000). Blast search analysis suggests that the predicted Spen protein contains three tandem repeats of an RNA Recognition Motif (RRMs) at the N-terminus implying that Spen might function as an RNA binding protein (Figure 3). Further sequence analysis also revealed that Spen contains a highly conserved but novel motif at the C-terminus. This ~100 a.a. domain is referred to as the SPOC (Spen Paralogue and Orthologue C-terminal) domain (Figure 4 and Wiellette et al., 1999). Spen-like proteins (defined by three N-terminal RRMs and a C-terminal SPOC domain) exist from *C. elegans* to humans indicating that Spen is a founding member of a novel family of putative RNA binding proteins. It is worth mention that the sequence similarity among Spen orthologues is limited to the RNA Recognition Motifs and the

SPOC domain. There is little conservation outside these regions, and the size of Spen-like proteins in different species varies widely. Spen is so far the largest with ~5500 amino acids. The Spen mouse orthologue (MINT) contains 3576 amino acids, human Spen contains 3349 amino acids and *C. elegans* Spen contains 2738 amino acids (Figure 5; Kuang et al., 2000). Interestingly, homology searches revealed the existence of a second much shorter (~800 a.a.) Spen-like protein (Dm44A) in Drosophila (Figure 5).

spen is required for normal photoreceptor development

In order to investigate its potential role as a positive regulator of RTK/Ras signaling pathway, we decided to study *spen* biological function in several developmental contexts known to require RTK/Ras signaling. For that purpose, we generated *spen* mutant clones using the FLP-FRT system in the Drosophila adult retina (Xu and Rubin, 1993). Using *eyeless* FLPase, which expresses the FLP recombinase specifically in the eye, we generated large-sized *spen* mutant clones in the adult retina. The external morphology of the *spen* mutant clonal patches is rough as compared to the surrounding wild type tissues. Tangential sections of those retinas reveal that the ommatidia within *spen* mutant clonal patches are disorganized (Figure 6). Eleven *spen* alleles were used to examine the eye phenotypes and consistent phenotypes are observed in all of them, although there is a range in the severity of the defects. For example, in null or strong alleles like $spen^{XFM911}$ and $spen^{AH393}$, the rhabdomeres are elongated and sometimes degenerated (Figure 6A, B). There are ommatidia with missing photoreceptors as well as ommatidia with extra photoreceptors. This apparently contradictory combination of missing and extra photoreceptors led us to ask whether both phenotypes could be a consequence of the degenerated rhabdomeres. To address that possibility, we did longitudinal sections of the retina and found

that rhabdomeres within the clonal patches are degenerated, split and twisted suggesting that the missing and extra photoreceptors seen in the tangential sections could be caused by the degeneration and splitting of the rhabdomeres, respectively (Figure 7). Thus *spen* appears important for later phases of retinal development. In clones of hypomorphic alleles like $spen^{U284}$, the shape of the rhabdomere is closer to normal, with only an occasional rhabdomere appearing elongated (Figure 6C). In these weak alleles, missing photoreceptors is the more common phenotype (Figure 6C). These results suggest *spen* may also be required for the early cell-fate decision events mediated by RTK signaling.

In order to determine whether *spen* functions during early photoreceptor differentiation, we analyzed *spen* clones in the larval eye imaginal discs. We found that in both strong and weak alleles there are developing ommatidial rosettes clearly missing photoreceptors (Figure 8A, B). In strong alleles like $spen^{AH393}$, the ommatidial rosettes are irregularly spaced and often fused (Figure 8A). In weak alleles, the pattern of the developing ommatidial rosettes is normal (Figure 8B). The complexity of the *spen* phenotypes in the eye suggest that *spen* is required at multiple times during eye development. Particularly, the rabdomere elongation and degeneration defects observed in *spen* mutants suggest that *spen* might be involved in non-RTK-mediated events since there is no other known RTK pathway component that causes similar defects.

spen function is necessary for cone cell fate determination

In addition to its role in neuronal specification, DER/Ras signaling also controls non-neuronal cone cell fate determination during eye development (Freeman, 1996). We therefore examined cone cell fate determination in *spen* clones by immunostaining eye imaginal discs with an early cone cell fate marker, anti-Cut. We found that Cut staining is greatly reduced, if not

completely gone, within the *spen* mutant clonal eye tissues (Figure 9A, B). In contrast, Cut expression in the antennal disc is not affected by *spen* mutations (Figure 9C, D). Therefore, the loss of Cut expression in the cone cell precursors within *spen* clones is likely to be an indication of a cell fate alteration rather than simply loss of expression of a particular marker. One protein known to be involved in cone cell fate determination is Prospero, which is a downstream target of Pointed and Yan (Kauffmann et al., 1996; Xu et al., 2000). To find out whether *spen* directly affects Prospero expression in cone cell precursors, we stained the eye discs with anti-Prospero antibody and found that Prospero expression in *spen* clones is not affected (Figure 10). This result suggests that *spen* functions either downstream of or in parallel to *prospero* to regulate cone cell fate determination.

spen is required for wing vein formation

In order to determine whether *spen* is involved in RTK/Ras pathway signaling events in tissues other than the eye, we studied its effects on wing vein formation, another developmental context where DER/Ras signaling plays an important role. For that purpose, we generated *spen* clonal patches in the adult wings and found that wing veins are missing or sometimes become thinner within *spen* clones (Figure 11). Thus, our phenotypic analysis of *spen* in both eyes and wings supports our hypothesis that *spen* is a new positive regulator of the RTK/Ras signal transduction pathway.

DISCUSSION

We isolated *split ends*(*spen*) as a dominant enhancer of a constitutively active form of the Ras pathway antagonist Yan. The direction of interaction suggests that *spen* opposes *yan*

function, perhaps by serving as a positive regulator in the RTK/Ras signal transduction pathway. In order to investigate this possibility, we first cloned and characterized the *spen* gene. *spen* encodes a novel putative RNA binding protein with three N-terminal RRMs and a highly conserved C-terminal SPOC domain. Spen-like proteins exist from worms to humans. However, the sequence similarity between Spen orthologues is limited to the RRMs and SPOC domain. The function of the SPOC domain is not known for any Spen family member. Blast searches reveal that the SPOC domain only exists in proteins that also have the Spen-like N-terminal three RRMs. Similarly, all proteins containing the Spen-like RRMs also have a C-terminal SPOC domain. In other words, the SPOC domain appears structurally linked with the N-terminal RRMs. Such structural association between two domains usually suggests a functional interdependency. The functional importance of the SPOC domain will be addressed in Chapters 3 and 4.

Molecular analysis of *spen* did not suggest an obvious mechanistic link between Spen and the RTK/Ras signaling pathway. To further establish its role as a potential positive regulator of the RTK/Ras signaling pathway, we have studied the biological requirement for *spen* function in contexts where RTK signaling is known to be critical for normal development.

During *Drosophila* eye development, the DER signaling pathway is used reiteratively for both neuronal photoreceptor differentiation and non-neuronal cone cell fate determination (Freeman, 1996). Sevenless RTK mediated signaling events controls R7 photoreceptor development (Zipursky and Rubin, 1994). The fact that *spen* was initially isolated based on modification of an eye phenotype suggests a potential role of *spen* in eye development. *spen* clonal analysis in the adult retina reveals that *spen* function is essential for normal eye development. Loss of *spen* results in a disorganized retina, malformed rhabdomeres and a

combination of extra and missing photoreceptors. The complexity of the *spen* phenotype in the adult retina suggests that *spen* might be involved in multiple contexts during eye development. Consistent with this idea, *spen* was also isolated as an enhancer of the rough eye phenotype associated with dE2F and dDP overexpression, implying that *spen* might be involved in cell cycle regulation during retinal development (Staehling-Hampton et al., 1999). It is interesting to note that *yan* also has been implicated in cell cycle regulation during eye development. Cells within *yan* null mutant clones overproliferate and fail to differentiate as neurons (Rogge et al., 1995). Thus *yan* appears to be a key coordinator between cell proliferation and differentiation. The fact that *spen* was isolated as a strong modifier of both Yan-based and dE2F and dDP based screens strongly suggests that *spen* might function to mediate crosstalk between RTK/Ras pathway and the cell cycle regulatory pathways. Further experiments are needed to address this possibility.

Compared to its function during photoreceptor development, *spen* function during non-neuronal cone cell fate determination appears more specific. Loss of *spen* results in a failure of cone cell fate specification indicated by the reduction or absence of expression of the early cone cell fate marker Cut. However, *spen* mediated regulation of cone cell specification does not appear to act via regulation of *prospero*, a downstream target of both Yan and Pointed known to be involved in cone cell fate determination (Kauffmann et al., 1996). Our results suggest that *spen* might function either downstream or in parallel of *prospero*. Further experiments are required to dissect *spen*'s role during cone cell differentiation.

Our analysis of *spen* clones during eye development support our hypothesis that *spen* is a potential positive regulator of RTK/Ras signaling pathway. To find out whether *spen* is an eye specific factor or a more general regulator, we also studied *spen* clones in the wings. DER

signaling pathway plays an important role in controlling fly wing vein development. Reduced DER signaling leads to missing or thinner wing veins. Consistent with this notion, loss of *spen* resulted in thinner and missing wing veins.

Our phenotypic analysis of *spen* mutant clones during both eye development and wing vein formation strongly suggest that *spen* is a new positive regulator of RTK/Ras signaling pathway. However, additional experiments will be needed to determine where and how *spen* integrates into the RTK/Ras signal transduction pathway. Our results also do not exclude the possibility that *spen* might function in an independent or parallel pathway which indirectly interacts with the RTK signaling pathway.

MATERIALS AND METHODS

Drosophila strains and genetics

The following fly strains were used: *spen*^{AH393}, *spen*^{XFM911}, *spen*^{U284}, *spen*$^{XLE-1796}$, *spen*$^{XIT-V813}$ were isolated in the Yan^{ACT} screen (Rebay et al., 2000); the mapping strain *b pr c px sp* and the *Df(2L)net-PM47c* that fails to complement *spen* were obtained from the Bloomington Stock Center.

Potential secondary lethals were removed from the *spen* chromosomes by first outcrossing to *b pr c px sp*, isolating a *spen b pr c px sp* recombinant, outcrossing this to w^{1118} and then re-isolating the *spen* mutation without any of the markers. While we cannot rule out the presence of closely linked secondary lethals, the fact that similar phenotypes are observed with multiple alleles argues that the chromosomes carry mutations only in *spen*. All phenotypic analysis was done using these "cleaned" chromosomes. Generation of *spen-FRT* chromosomes was as described by (Xu and Rubin, 1993).

spen^{AH393} and *spen*^{XFM911} were determined likely to be null alleles based on a comparison of the embryonic lethal phenotype of *spen/spen* versus *spen/Df(2L)net-PM47c*. Specifically, 200 embryos were counted from matings of outcrossed heterozygous *spen/+* or *Df/+* flies. The number of embryos that did not hatch was determined and the cuticle phenotypes were examined. For these two alleles, both *spen/spen* or *spen/Df* homozygotes died as embryos (scored as an ~25% failure to hatch) and had similar cuticular phenotypes, consistent with them being null alleles. Several other *spen* alleles tested in this manner were clearly hypomorphic mutations (data not shown). In addition, using the monoclonal antibodies we generated against Spen, we find Spen protein expression is undetectable in embryos lacking maternal and zygotic *spen*^{AH393} and *spen*^{XFM911}, suggesting that they appear to be protein nulls.

Scanning electron microscopy and histology

Flies were prepared for scanning electron microscopy by fixation for 2 hr in 1% glutaraldehyde, 1% paraformaldehyde in 1 M cacodylate buffer followed by dehydration through an ethanol series and critical point drying. Fixation and sectioning of adult eyes was as described by Tomlinson et al. (Tomlinson and Ready, 1987).

Generation of spen mutant clones

spen mutant clones were generated using the FLP-FRT system as described (Xu and Rubin, 1993). For clones in the adult retina, *eyeless* FLPase and *white* FRT40 chromosomes were used. For clones in the larval eye discs, *eyeless* FLPase and *armadillo*-lacZ FRT40 chromosomes were used. For wing clones, *hs* FLPase and *yellow* FRT40 chromosomes were used. Heat shock were performed at 38°C for 1 hour at the first instar larva stage.

Immunostaining and microscopy

For cone cell staining, 3rd instar larval eye discs were dissected, fixed in 4% paraformaldehyde and stained with primary secondary antibodies. The stained discs were imaged by confocal microscopy. Anti-Cut antibody was used at a 1:50 dilution. Anti-Prospero was used at a 1:1000 dilution. Rabbit-anti-β-gal antibody (Jackson Laboratory) was used at a 1:20,000 dilution. Cy3- or FITC- conjugated secondary antibodies (Jackson Laboratory) were used at a 1:1000 dilution.

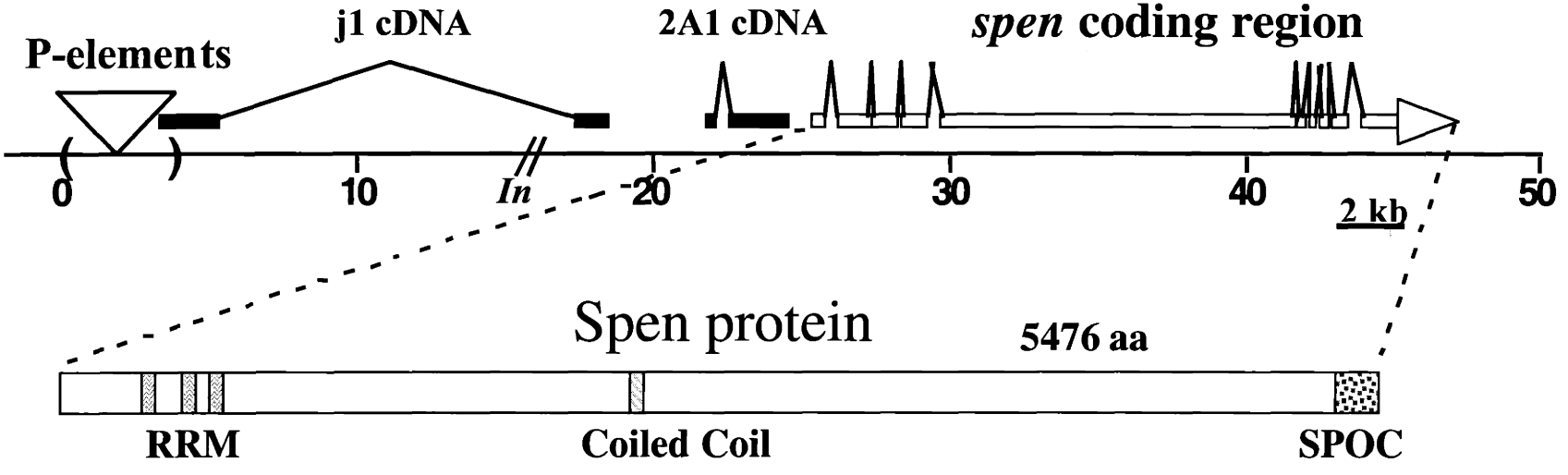
For photoreceptor staining, the 3rd instar larval eye discs were dissected, fixed as described above, and processed for X-Gal staining to mark the *spen* clones. The discs were then

stained with anti-Elav antibody and developed with a DAB reaction. The stained discs were imaged by Normarski microscope. The anti-Elav antibody was used at a 1:50 dilution.

Figure 1 *spen* enhances the rough eye phenotype caused by overexpression of activated Yan (Yan^{ACT}). (A-C) Scanning electron micrographs of the external morphology of adult fly eyes. (D-F) Tangential sections of the adult retinas. (A, D) Wild type (+/+). (B, E) A typical eye from a transgenic fly expressing Yan^{ACT} under control of the Sevenless promoter (+/Sev-Yan^{ACT}). (C, F) A typical eye of a Sev-Yan^{ACT} transgenic fly that is also heterozygous for a *spen* mutation. (*spen*/Sev-Yan^{ACT}). In (D) the arrow points to the pigment lattice that surrounds each ommatidium. In (E), the arrowheads highlight three of the ommatidia that have reduced numbers of photoreceptors.

A.
B.
C.
D.
E.
F.

Figure 2. Molecular characterization of *spen*. *spen* spans an ~50 kb genomic region (black line). The P-element lines that fail to complement *spen* are inserted in an ~4 kb region indicated by the bracket. CDNA j1 and 2A are non-coding cDNAs we isolated that might be 5' untranslated region of the spen cDNA or alternatively spliced exons (Kuang et al, 2000). The predicted Spen protein contains 5476 amino acids. Recognized motifs are indicated by boxes. RRM: RNA Recognition Motif; SPOC: Spen Paralog and Ortholog C-terminal domain.
(This figure is adapted from Rebay et al 2000).



RRM: RNA Recognition Motif SPOC: Spen Paralog and Ortholog C-terminal domain

Figure 3 The alignment of Spen RRMs with the RRMs of Spen-like proteins from different species. The conserved residues that define the RRM motif are indicated with asterisks. The three RRM motifs are marked with a circled number 1, 2 and 3. RNP: RNA-binding Protein (This figure is adapted from Rebay et al 2000).

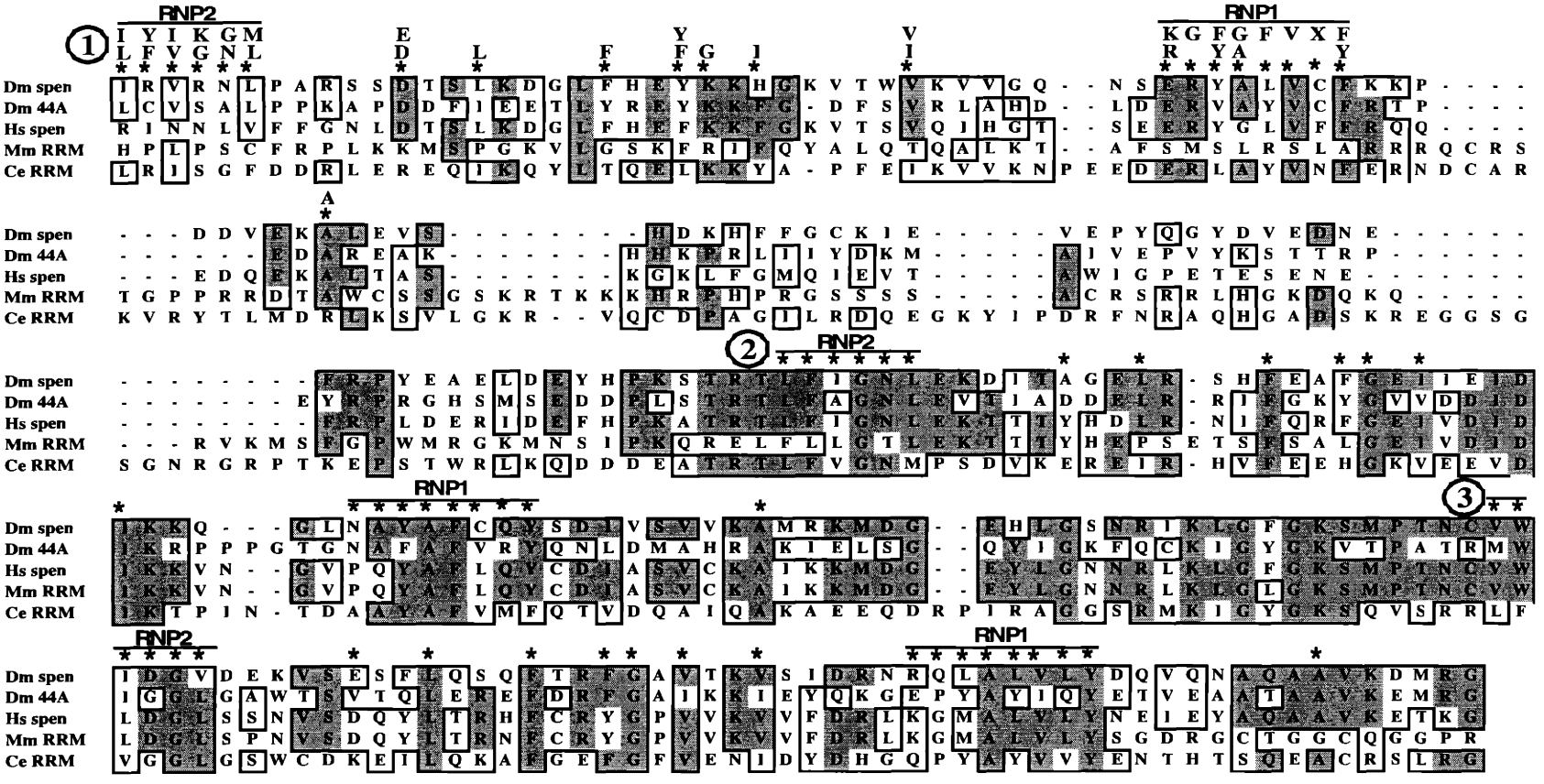

RNP2
RNP1
Dm spen
Dm 44A
Hs spen
Mm RRM
Ce RRM

Figure 4 The alignment of C-terminal SPOC domains. Identical amino acids are boxed and highlighted in dark gray, similar amino acids are boxed and highlighted in light gray. (This figure is adapted from Rebay et al 2000).

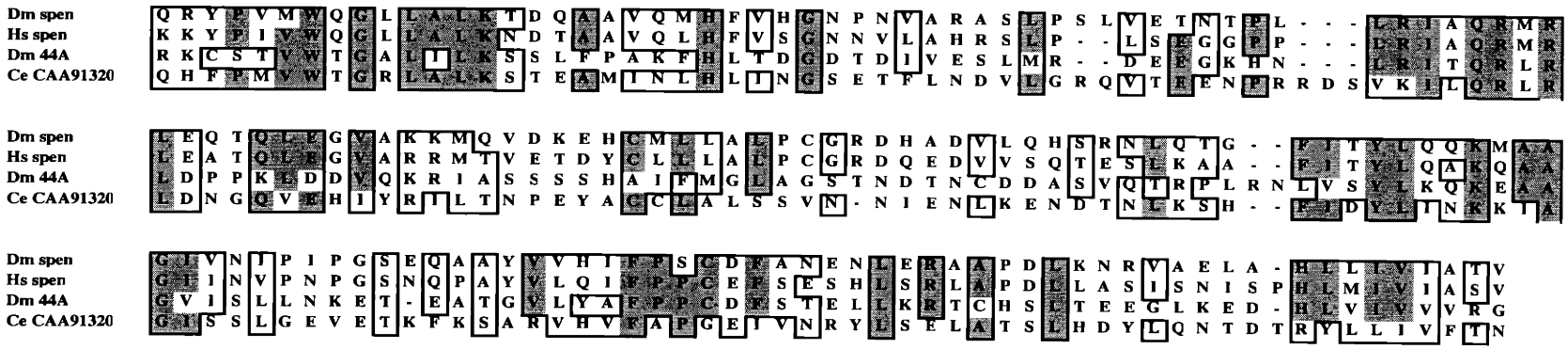

Dm spen
Hs spen
Dm 44A
Ce CAA91320

Figure 5 Spen is a founding member of a new family of putative RNA binding proteins defined by three N-terminal RRMs and a C-terminal SPOC domain. The numbers shown over the SPOC domain represents the percent amino acid identity between Spen and the Spen orthologs in other species.

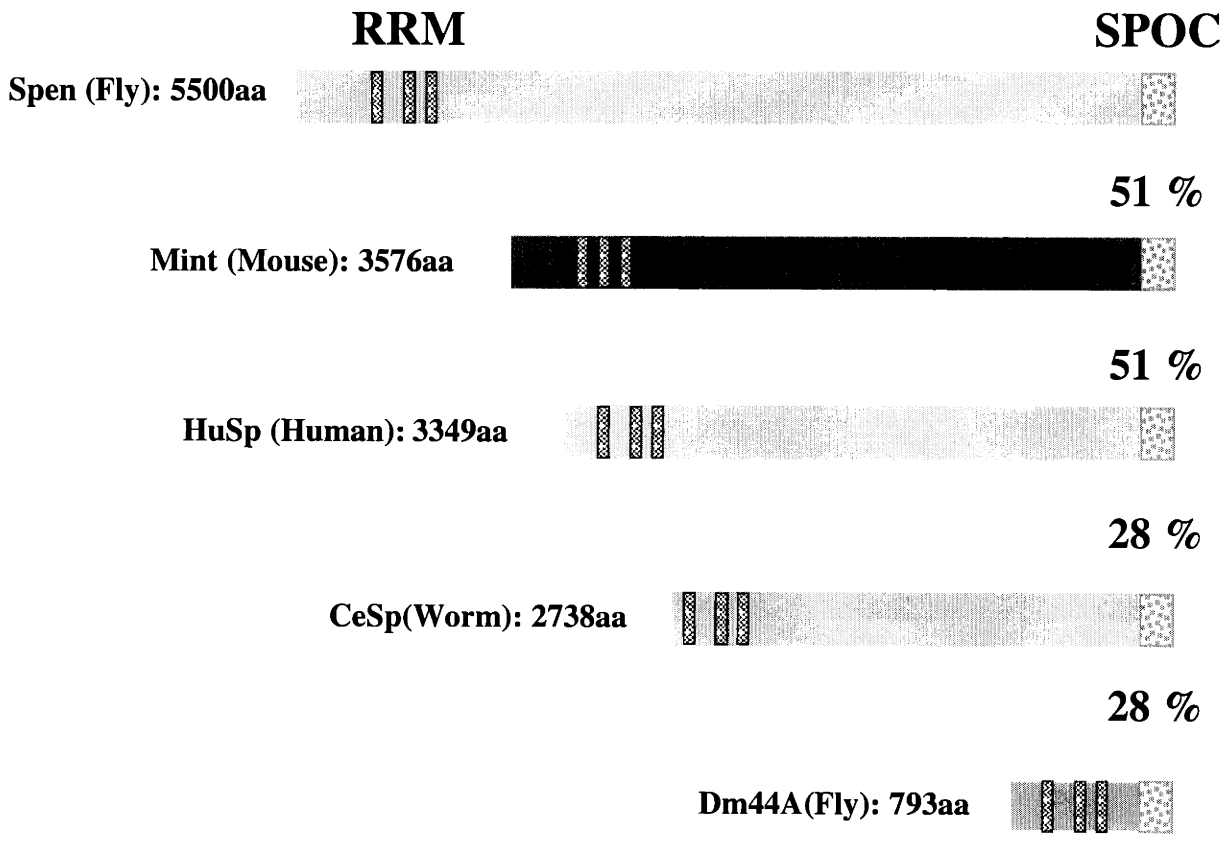

RRM
SPOC
Spen (Fly): 5500aa
51 %
Mint (Mouse): 3576aa
51 %
HuSp (Human): 3349aa
28 %
CeSp(Worm): 2738aa
28 %
Dm44A(Fly): 793aa

Figure 6 *spen* is required for normal *Drosophila* eye development. (A-C) Tangential sections of *spen* mutant clones. The wild-type region is pigmented, while the *spen* mutant clone is unpigmented. (A) $spen^{XFM911}$ clone. The ommatidia within the *spen* clone are greatly disorganized, and the rhabdomeres are elongated. Arrow points to an ommatidium missing photoreceptors; arrow head points to an ommatidium containing extra photoreceptors. (B) $spen^{AH393}$ clone. Phenotype is similar to that of $spen^{XFM911}$. (C) $spen^{U284}$ clone. The ommatidia are disorganized. The shape of rhabdomeres are generally normal, although occasional elongated rhabdomeres are found(arrow). Arrow heads point to ommatidia with missing photoreceptors.

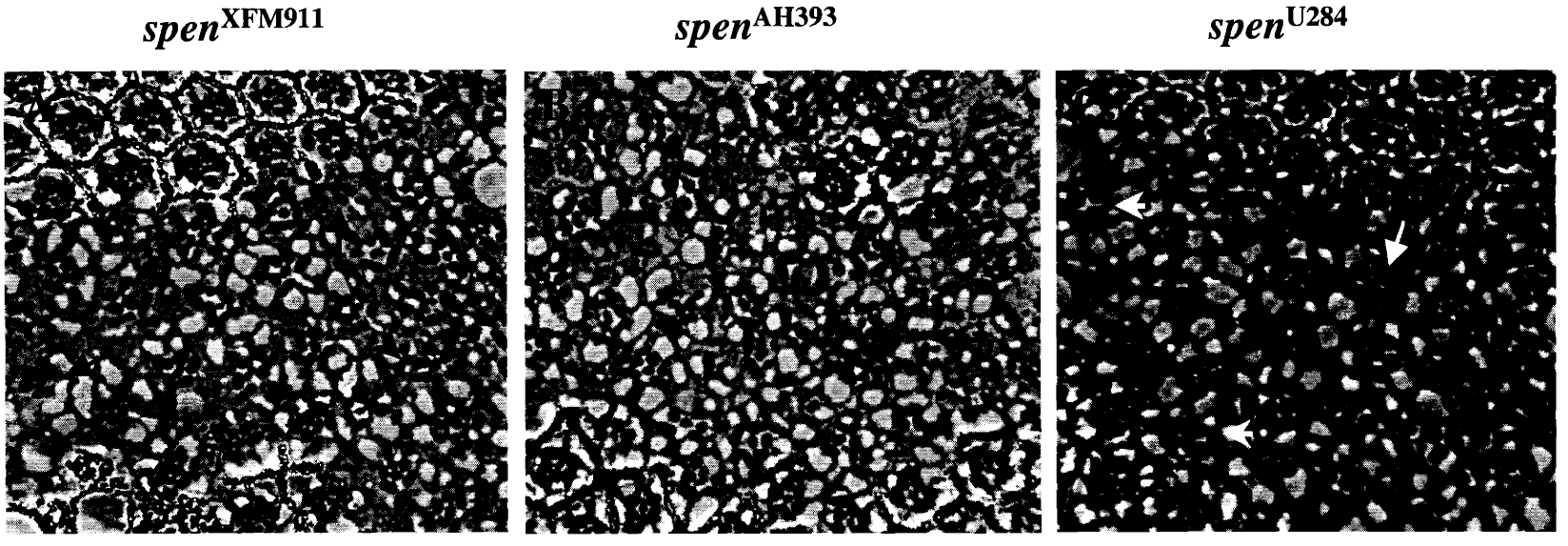

*spen*XFM911
*spen*AH393
*spen*U284

Figure 7 Rhabdomeres are degenerated, twisted and split in *spen* mutant clones. (A-C) Longitudinal sections of *spen* mutant clones. The wild-type regions are pigmented (dark black granules marked by white asterisk) while the *spen* mutant clones are unpigmented (gray tissue). (A) *spen*$^{XLE\text{-}1796}$. High magnification view of a longitudinal section (100X objective lens). Arrow points to the twisted rhabdomeres; arrow head points to an area with fused rhabdomeres. (B) *spen*^{U284}. High magnification view of a longitudinal section (100X objective lens). Black asterisks point to the degenerated rhabdomeres. (C) spen^{XFM911}. Lower magnification view of a longitudinal section (40X objective lens). Black asterisks point to the degenerated rhabdomeres.

A. spen$^{XLE-1796}$ **100X**

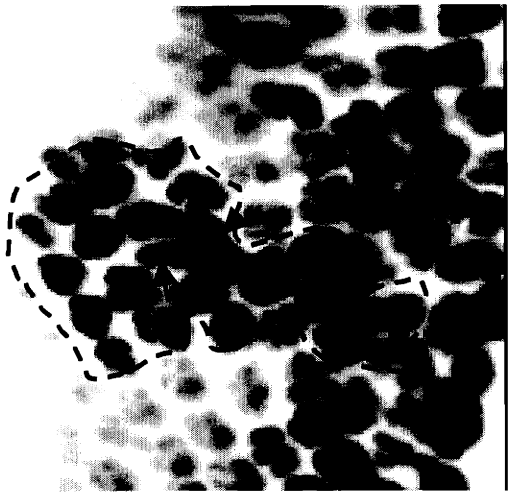
B. spen^{U284} **100X**

C. spen^{XFM911} **40X**

Figure 8 *spen* is required for early photoreceptor development. Elav staining of 3rd instar eye imaginal discs carrying *spen* mutant clones. The clones are marked with a lacZ marker that masks the elav staining in the wild type tissue resulting. The region of the *spen* mutant clonal patch is indicated by the dashed lines. (A) $spen^{AH393}$. (B) $spen^{XIT\text{-}V813}$. The discs are oriented anterior to the left and posterior to the right. Arrows point to developing ommatidial rosettes with missing photoreceptors. Arrow head points to the fused and irregularly spaced ommatidia.

spen^{AH393}

A



spen$^{XIT\text{-}V813}$

B

Figure 9 *spen* function is essential for cone cell fate determination. (A, B) Confocal image of 3rd instar larval eye imaginal disc. (A) Cone cell fates are indicated by an early cone cell fate marker anti-Cut. Cut staining is greatly reduced, if not completely gone, within *spen* clones (marked by white broken lines). (B) *spen* clones are marked by absence of anti-β-gal staining (marked by white broken lines). (C, D) Confocal image of 3rd instar larval antennal disc. (C) Cut staining is not affected by loss of *spen* in the antennal disc (*spen* clone was marked by white broken lines). (D) *spen* clones are marked by absence of anti-β-gal staining (marked by white broken lines).

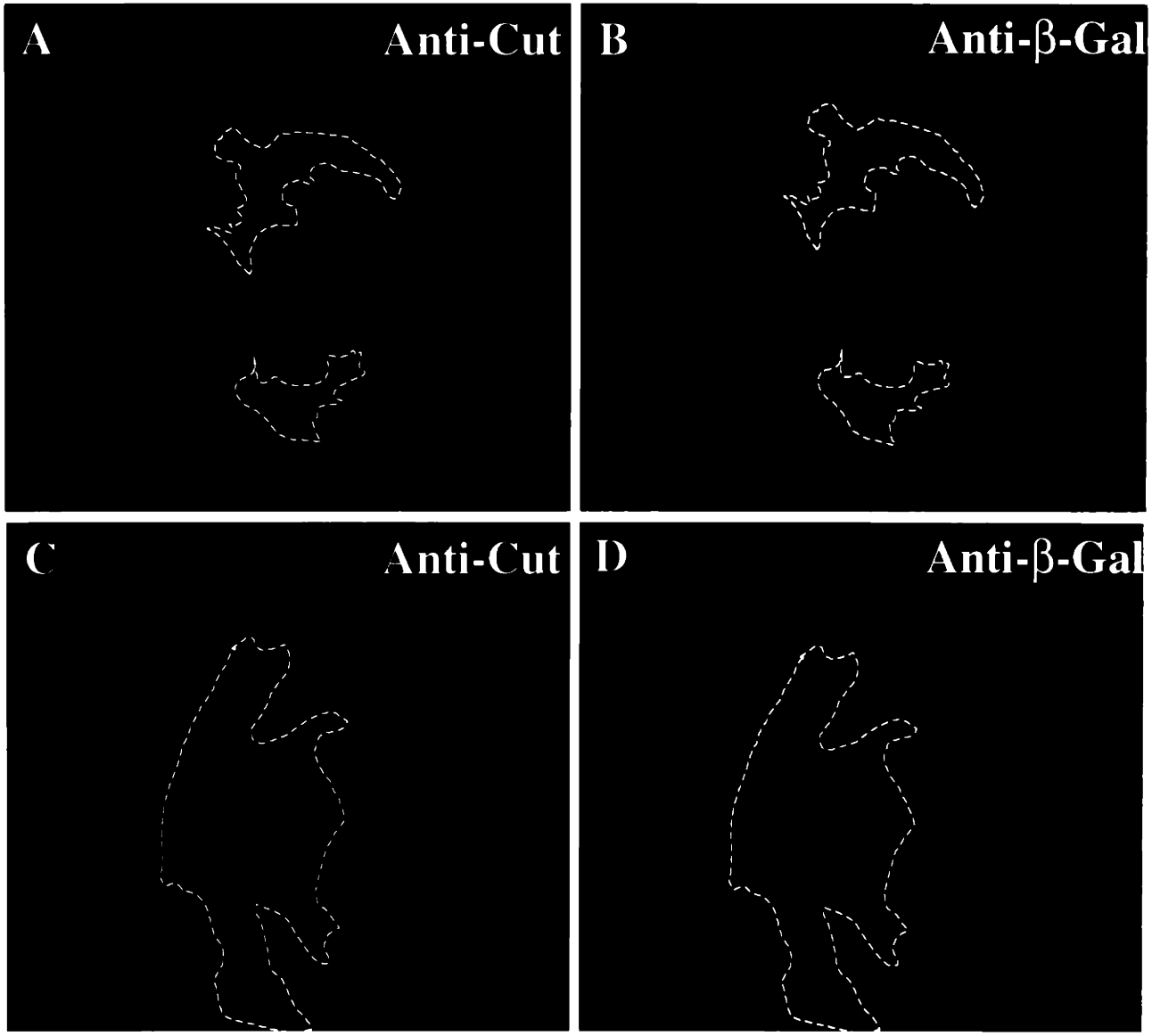

A
Anti-Cut
B
Anti-β-Gal
C
Anti-Cut
D
Anti-β-Gal

Figure 10. Prospero expression in cone cells is not affected by *spen* mutation. (A) Prospero expression appears normal in *spen* clones. (B) *spen* clones are marked by absence of anti-β-gal staining. The area of the *spen* mutant clone is indicated by white dashed lines.

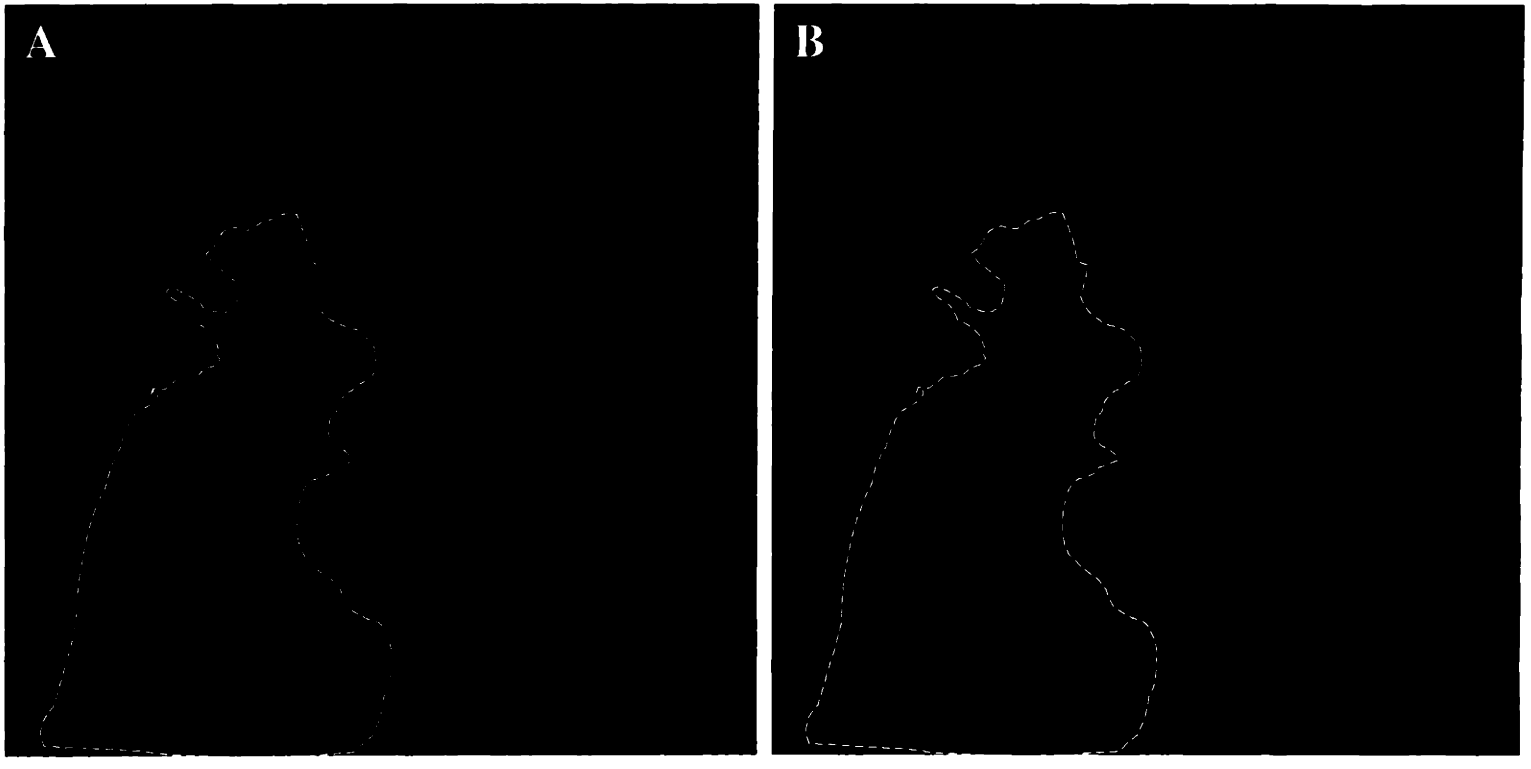

A
B

Figure 11 *spen* is required for Drosophila wing vein formation. High magnification view of part of the wing showing the marginal regions of wing veins L3 and L4. (A)$spen^{AH393}$ (B) $spen^{BU720}$ Arrow head points to the missing wing vein (L4) ; arrow points to the thinner vein (L4).

A
B

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Chapter 3

split ends, a new component of the *Drosophila* DER signaling pathway, regulates development of midline glial cells

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Note

* Ilaria Rebay made the SpenΔC construct and Francis Hsiao and Andrina Williams helped to generate the transgenic flies.

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Abstract

The central nervous system (CNS) of both vertebrates and invertebrates is a bilaterally symmetric structure whose two halves are separated and connected by a specialized set of midline neurons and glia. In Drosophila, the epidermal growth factor receptor tyrosine kinase (DER/RTK)-mediated signaling pathway regulates the migration and survival of the midline glial cells (MGCs). We recently reported the molecular characterization of a gene called *split ends(spen)* which we isolated in a genetic screen designed to identify new components of the RTK/Ras pathway. *spen* encodes an evolutionarily conserved putative RNA-binding protein. To investigate *spen* function in the context of RTK signaling, we have examined the consequences of *spen* loss-of-function mutations on embryonic CNS development. We find that *spen* is required for normal migration and survival of the MGCs and that embryos lacking *spen* have CNS defects strikingly reminiscent of those seen in mutants of several known components of the DER/RTK pathway. In addition, *spen* interacts synergistically with the RTK effector *pointed.* Using MGC targeted expression, we demonstrate that increased DER/Ras pathway signaling can rescue the lethality associated with expression of a dominant negative *spen* construct. We conclude that *spen* encodes a novel positively acting component of the EGFR/Ras signaling pathway.

Introduction

Receptor tyrosine kinases (RTKs) transmit extracellular signals through the Ras/MAPK cascade. RTK/Ras signaling controls a variety of developmental events. One particular context in which RTK-mediated signaling has been extensively studied is the development of the midline

glial cells (MGCs) in the embryonic central nervous system (CNS) of Drosophila (Hummel et al., 1997; Jacobs, 2000). The midline of the CNS consists of a set of specialized neurons and three pairs of glia (referred to as MGP, MGM and MGA, for midline glia posterior, middle and anterior, respectively) that are derived from the mesectoderm, which is a distinct region of the ventral neuroectoderm (reviewed by Jacobs, 2000). MGM and MGA play important roles in organizing the commissural axons that connect the two longitudinal branches of the CNS. Initially, the glial cells provide guidance cues for the pioneer commissures by expressing Commissureless and Netrin (Harris et al., 1996; Mitchell et al., 1996; Tear et al., 1996), both attractants for growth cones expressing the Frazzled/DCC receptor(Kolodziej et al., 1996). Once the pioneer axons have crossed the midline, the MGMs migrate posteriorly past the MGAs and insert themselves between the growing commissure axons, effectively separating anterior commissures (AC) from posterior commissures (PC). Finally, the MGA and MGM cells elongate and wrap around the AC and PC axon bundles to prevent any additional axons from wandering in between (reviewed by Jacobs, 2000). The midline glia also provide repulsive guidance cues by expressing the secreted protein Slit that in turn binds to the Robo receptor on the growth cones of the longitudinal axons, thereby preventing them from crossing the midline(Brose et al., 1999; Jacobs, 2000; Kidd et al., 1999). Given these critical roles, defects in the midline invariably result in disorganization and malfunction of the CNS. The consequences of abnormal MGC development can include fusion of the AC and PC axon bundles, loss of separation between the two longitudinal branches of the CNS, and inappropriate longitudinal axons crossing the midline (Klambt et al., 1991; Seeger et al., 1993).

Studies from several labs have suggested that proper differentiation, migration and survival of the midline glia requires DER-mediated signaling (reviewed by Hummel et al., 1997; Jacobs,

2000). DER is activated in the midline upon binding a secreted form of the ligand Spitz and transduces its signal via the conserved Ras/MAPK cascade. A primary consequence of MAPK activation in Drosophila is activation of Pointed and inactivation of Yan, both members of the ETS family of transcription factors (O'Neill et al., 1994). Inactivation of Yan in the midline cells, as in many other tissues, is necessary for their differentiation(Rebay and Rubin, 1995; Scholz et al., 1997). Pointed plays dual positive and negative roles, functioning as a key inducer of midline glial cell differentiation(Klambt, 1993), while simultaneously restricting the total number of glial cells by activating an Argos-mediated inhibitory feedback loop that attenuates DER signaling(Scholz et al., 1997). A third nuclear protein implicated in both glial cell development and RTK-mediated signaling events is Tramtrack (TTK). Tramtrack represses neuronal differentiation in the midline, thereby promoting the glial fate(Giesen et al., 1997). Although Tramtrack has been shown to be a downstream target of RTK signaling in the developing eye (Li et al., 1997; Tang et al., 1997), the mechanisms whereby Tramtrack activity may be coordinated with DER signaling in the midline are unknown.

In the course of a genetic screen designed to identify novel components in the Ras/MAPK signaling pathway, we recovered mutations in the gene *split ends* (*spen*) (Rebay et al., 2000). Recovery of *spen* as an enhancer of the rough eye phenotype associated with a constitutively active form of the RTK pathway antagonist Yan (Rebay and Rubin, 1995), suggests a possible function for Spen as a positively acting factor in, or in parallel to, the RTK pathway. *spen* encodes an unusually large protein of ~5500 amino acids, and is a novel member of the RNA recognition motif (RRM) family of RNA-binding proteins (RNPs) (Kuang et al., 2000; Rebay et al., 2000; Wiellette et al., 1999). Spen-like proteins are found in *C.elegans*, *M. musculus* and *H. sapiens*, although little is known about their function in these organisms. In addition to a

characteristic set of 3 RRMs, these proteins also have a highly conserved C-terminal domain of unknown function, termed the SPOC domain, that to date is found only in Spen family members (Kuang et al., 2000; Rebay et al., 2000; Wiellette et al., 1999). In order to investigate the requirements for *spen* during Drosophila development and to further establish its role as a positive regulator of the RTK/Ras signaling pathway, we have studied *spen* function in the context of embryonic midline glial cell development.

Results

Spen is a nuclear protein with enriched expression in the ventral midline and embryonic central nervous system.

In addition to its role in Drosophila eye development, RTK/Ras/Yan signaling controls a variety of events during embryogenesis (reviewed by Schweitzer and Shilo, 1997). We were therefore interested in determining whether *spen* function was required only in the eye or whether it might play a more general role in RTK/Yan signaling events in other developmental contexts.

To address this question, we first asked whether Spen was expressed during embryogenesis. Using cDNA fragments as probes for *in situ* hybridization, we found that *spen* mRNA is abundant in early syncytial blastoderm embryos, suggesting an important maternal contribution (Figure 1A). During gastrulation, *spen* mRNA is ubiquitously expressed but is enriched in the mesectoderm and the adjacent neurectoderm (Figure 1B). Expression of *spen* transcripts in the developing CNS remains prominent throughout embryogenesis (Figure 1C).

To confirm that the pattern of *spen* mRNA expression accurately reflects the protein distribution during development, monoclonal antibodies specific to Spen were generated. In

embryos lacking maternal and zygotic *spen*, no staining is detected, indicating the antibodies are specific to Spen (data not shown). Immunostaining in wild-type embryos reveals that the profile of Spen protein expression is identical to that of the transcript, with the highest levels of expression in the developing CNS (Figure 1D-F). In addition, Spen localizes to the nuclei of all cell types in which it is expressed (Figure 1D,E).

The midline glial cells differentiate normally in *spen* mutants but later exhibit aberrant migration and die, resulting in a collapsed and disorganized CNS

To examine the consequences of complete loss of *spen* function, we generated *spen* germline clones using the ovo^{D}-FLP/FRT system (Chou and Perrimon, 1996). We will refer to embryos lacking both maternal and zygotic *spen* as "*spen* mutants". Because Spen expression is strongly enriched in the developing CNS (Figure 1C), we first stained *spen* mutants with the pan-neuronal marker anti-Elav to look for any abnormalities in overall organization of the nervous system. Moderate defects in CNS morphology were observed. In a stage 16 wild type embryo, the CNS consists of two longitudinal stripes of neural cells that are distinctly separated at the ventral midline. Within this midline space, clusters of midline-specific neurons are evident (Figure 2A). In a similarly staged *spen* mutant embryo, the space separating the two halves of the CNS is reduced, and in some segments the two sides are collapsed into each other (Figure 2B, C). Because of this collapse, the midline neurons are difficult to detect by Elav staining; however, labeling with the antibody 22C10 reveals that the ventral midline neurons are present and have no obvious defects in their projections (data not shown).

To determine whether the collapsed CNS phenotype might be associated with defects in the midline, *spen* mutant embryos were examined for expression of the MGC-specific marker Slit

(Rothberg et al., 1990). Using the Slit-lacZ enhancer trap line to count the number of MGCs in *spen* versus wild type embryos, we found no significant difference in number in Stage 12/13 embryos (Table 1, Figure 3A, B). At these stages, the primary defect associated with *spen* mutants appears to be aberrant MGC migration. This defect manifests itself as a failure of the MGCs to migrate anteroposteriorly in a tightly packed configuration along the dorsal surface of the ventral nerve cord (Figure 3C). Instead, the MGCs in *spen* mutants migrate more laterally and become spread out in a scattered and disorganized pattern along the ventral nerve cord (Figure 3D). The migration defect gives the initial appearance that there may be more MGCs in *spen* mutants than in wild type; however, careful counting of the Slit-lacZ positive nuclei suggests this is not the case (Table 1). By Stage 17 however, the number of MGCs is significantly reduced in *spen* mutants relative to wild type (Table 1). To make sure that our analyses were not somehow biased by using only Slit-lacZ to mark the MGCs, we examined expression of another MGC marker, Singleminded (Sim) (Nambu et al., 1991). Double labeling experiments revealed an almost precise overlap of the Sim and Slit markers in both wild type (data not shown) and *spen* mutants (Figure 4 A, B, C). Specifically, at stage 13, there is no indication of Sim-positive MGCs that do not also express Slit-lacZ. By Stage 17, both markers reveal a reduction in MGC number in *spen* mutants relative to wild type (Figure 3E, F; data not shown). Thus it appears that in *spen* mutants, normal numbers of MGCs are initially specified, but later migrate aberrantly, thereby compromising their ability either to survive or to maintain the MGC fate.

By stage 16, in a wild-type embryo, the Slit-positive MGCs have migrated and elongated to ensheathe the AC and PC axons thereby maintaining proper separation and bundling (Figure 5A). In similarly staged *spen* mutant embryos, the MGCs have not properly migrated or wrapped

themselves around the commissure bundles (Figure 5B). In addition, while apoptosis reduces the number of MGCs in wild type embryos from ~8 per segment at stage 13 down to only ~3 per segment by stage 16-17, in *spen* mutants this reduction is even more drastic, leaving only 1-2 MGCs per segment (Table 1 and Figure 3E, F, 5C, D). Thus in *spen* mutants, although initiation of MGC differentiation appears normal, the later aspects of glial development, including migration, wrapping, and survival, are defective.

To confirm that Spen is expressed in the MGCs, embryos carrying the MGC specific enhancer trap line AA142, were double labeled with anti-β-Gal and anti-Spen antibodies (Figure 6). Although nuclear Spen staining is detected throughout the CNS, the highest level of expression is seen is in a group of midline cells located between the AC and PC axon bundles. These cells also express lacZ, confirming their identity as MGCs (Figure 6).

Because defects in glial cell development are likely to perturb organization of the CNS, *spen* mutant embryos were labeled with the antibody BP102 that highlights all axon tracts in the CNS (Figure 7A-C). As predicted, the AC and PC axon bundles are not properly organized or separated, and in some segments are completely fused (Figure 7B, C). In addition, the two longitudinal connectives appear closer together than normal and are occasionally fully collapsed across the midline (Figure 7B, C). Staining with the anti-FasII antibody, which highlights a distinct set of three axon bundles in each longitudinal branch, further clarifies this phenotype. These longitudinal axon tracts never cross the midline in a wild-type embryo (Figure 7D). In contrast, the FasII positive axons cross and recross the midline in *spen* mutants, producing a fragmented and disorganized longitudinal axonal array (Figure 7E, F).

Although strong Spen expression is detected throughout the CNS (Figure 1E, F), lack of *spen* does not appear to impede the normal differentiation and development of most neurons, as

judged by staining with either anti-Elav (Figure 2B, C), 22C10, anti-engrailed or anti-even-skipped (data not shown). Thus our results are most consistent with a primary defect in MGC development. However, because of the general disorganization of the CNS, we can not rule out the possibility that there may be subtle neuronal defects contributing to the *spen* phenotypes.

spen interacts with the RTK effector gene *pointed* during MGC differentiation

The migration and survival of the MGCs is controlled in part by the Drosophila epidermal growth factor receptor (DER) signaling pathway (Hummel et al., 1997; Jacobs, 2000). Given the similarity in phenotype between *spen* mutants and mutations in the DER signaling pathway genes *pointed(pnt)*, *spitz*, *rhomboid* and *Star* (Klambt et al., 1991), together with the fact that we isolated *spen* as an enhancer of activated Yan in an RTK pathway based genetic screen, it seemed plausible that *spen* might function as a positively acting component of the DER pathway. To explore this possibility we first asked whether we could detect synergistic interactions between *spen* and the RTK pathway effector *pointed (pnt)*.

The expectation is that a reduction in activity of a proven positive effector of the DER pathway such as *pnt*, should dominantly enhance the *spen* phenotype. Embryos lacking maternal *spen* can be partially rescued by zygotic *spen* expression from a paternally inherited wild-type allele (we refer to this genotype as *spen/+*). Stage 15-17 *spen/+* embryos appear phenotypically wild-type with only ~4% of the embryos exhibiting defects in the CNS axon tracts as revealed by anti-Fas II staining (Table 2). Embryos heterozygous for a *pnt* loss-of-function mutation (*pnt/+*) have no apparent dominant defects (Table 2). Reducing the *pnt* dosage in the *spen/+* background increases the frequency of axonal defects to ~25% (Table 2). The predominant phenotype is reduced separation between the two longitudinal axon pathways and a single inappropriate

crossing of the midline by one of the FasII positive axon tracts (Figure 8). This dose-sensitive interaction between *pnt* and *spen* strongly supports a role for *spen* as either a positively acting component of the DER pathway or as a component of a parallel pathway synergizing with DER during MGC development.

Midline glial cell specific activation of the DER/Ras pathway rescues the lethality associated with expression of a dominant negative *spen* truncation mutant

To investigate further the connection between *spen* and the DER/Ras signaling pathway in the MGCs, we generated a putative dominant negative *spen* transgene that truncates the C-terminal ~1500 amino acids, including the highly conserved SPOC domain. When transfected into S2 cultured cells, this construct, referred to as SpenΔC, is expressed at high levels and localizes to the nucleus just as we have found for the endogenous wild type Spen protein (data not shown). Ubiquitous expression of SpenΔC is unable to rescue the lethality or phenotypes associated with *spen* mutants (data not shown), implying an essential function for the conserved C-terminal SPOC domain.

To determine whether SpenΔC might behave as a dominant negative mutation, we used the Slit-Gal4 driver to induce high levels of expression specifically in the MGCs in which our phenotypic studies suggest *spen* plays a key role. MGC-specific expression of SpenΔC results in completely penetrant lethality (Figure 10). In contrast, and consistent with the lack of primary neuronal defects associated with *spen* mutants, pan-neural expression of SpenΔC using the Elav-Gal4 driver does not compromise the viability or patterning of the fly (data not shown). These results are consistent with our phenotypic analysis of *spen* mutants, where despite high levels of protein expression throughout the CNS, the primary defects appear specific to the MGCs.

MGC-specific overexpression of SpenΔC results in completely penetrant lethality. The prediction is that if SpenΔC acts as a dominant negative mutant, then overexpression of the transgene should result in defects in the MGCs similar to those seen in *spen* mutants. To address this question, the phenotypes associated with SpenΔC overexpression were examined. Overexpression of SpenΔC specifically in the midline glial cells causes ~50% embryonic lethality. The remaining animals die at later stages, with at least some dying late during pupariation. None are able to eclose. Anti-FasII staining of these embryos did not reveal any obvious defects in the CNS (data not shown). We reasoned that the lack of CNS defects associated with SpenΔC expression might be due to high levels of endogenous Spen. In order to test this hypothesis, SpenΔC was overexpressed in the *spen* zygotic null mutant background. *spen* zygotic null mutants are embryonic lethal but lack major CNS defects because of the heavy maternal contribution (Figure 9A). Overexpression of SpenΔC in the *spen* zygotic null background causes severe CNS defects very similar to those seen in *spen* maternal and zygotic nulls (Figure 9B). Thus spenΔC appears to function as a dominant negative mutant *in vivo*.

To determine whether this lethality might be due to insufficient RTK/Ras pathway signaling, we asked whether increasing the level of DER/Ras pathway signaling, specifically in the MGCs, could compensate for the reduction in *spen* function associated with expression of the putative dominant negative SpenΔC transgene. Whereas Slit-Gal4 driven expression of either an activated Ras^{V12} or the SpenΔC transgene results in lethality, flies expressing both Ras^{V12} and SpenΔC in the MGCs are viable and appear normally patterned. The mutual suppression is extremely penetrant as over 50% of the expected class of flies is recovered (Figure 10). Similar, but less penetrant rescue is obtained when SpenΔC and a secreted form of the DER ligand Spitz are coexpressed in the MGCs. Together these results strongly suggest that *spen* functions

autonomously in the MGCs, acting either downstream of or in parallel to Ras as a positive effector or regulator of RTK signaling (Figure 11).

DISCUSSION

In Drosophila, the genes *rhomboid*, *Star*, *pointed* and *spitz*, all positively acting components of the DER pathway and referred to collectively as the "Spitz group", share a characteristic CNS phenotype similar to that we have described in *spen* mutants. Specifically, whereas the proper number of MGCs is initially specified, they later migrate abnormally and eventually degenerate and die (Hummel et al., 1997). The phenotypic similarities between *spen* and the Spitz group genes as well as our isolation of *spen* as an enhancer of an activated Yan allele, are consistent with the hypothesis that *spen* may be a positively acting factor in the DER/Ras signaling pathway (Figure 11).

The results of our analyses differ from a recent report that excessive numbers of MGCs are initially specified in *spen* mutants (Kuang et al., 2000). Using the Slit-lacZ nuclear enhancer trap marker to count the MGCs, we detect comparable numbers of MGCs in wild type and *spen* mutants up until stage 13 and a reduction in MGC number in *spen* mutants beginning at stage 14. Thus in our *spen* mutants, which behave as genetic and protein nulls, normal numbers of MGCs are initially specified, a phenotype consistent with what has been reported for other DER pathway mutants (Stemerdink and Jacobs, 1997). While the explanation for the differences between our and Kuang et. al.2000's results is unclear, it is not likely to be due to allele specific differences as the alleles used in both studies appear to be protein nulls.

Spen encodes a novel member of a large family of RNA binding proteins whose members are defined by the presence of one or more RNA recognition motifs (RRMs) (Kuang et al., 2000;

Rebay et al., 2000; Wiellette et al., 1999). Post-transcriptional regulation of gene expression allows quick responses to external or developmental signals, and RRM family members have been shown to mediate many different cellular processes including mRNA splicing, stabilization, localization and transport (Siomi and Dreyfuss, 1997). It has been proposed that RRM proteins might function as effectors of critical signaling pathways by binding signal-responsive transcripts and either releasing them for immediate translation or regulating differential splicing in response to activation of the pathway (Siomi and Dreyfuss, 1997).

Although the molecular mechanisms underlying Spen function in the RTK/Ras pathway remain to be elucidated, given it's membership in the RRM family, one possibility is that Spen might directly regulate the processing and/or stability of specific transcripts to generate functionally distinct protein isoforms in response to or required for Ras signaling events. Two attractive potential targets of such activity in the CNS are the Ras pathway effector *pointed* and the zinc finger transcription factor *tramtrack.* Both genes produce alternatively spliced transcripts and are required in the MGCs(Giesen et al., 1997; Klambt, 1993; Read and Manley, 1992). The synergistic interactions we have detected between *spen* and *pointed* make *pointed* a particularly appealing candidate. A third likely possibility, given that we isolated *spen* as an enhancer of an activated *yan* allele, is that Spen might function to destabilize *yan* transcripts in response to RTK-initiated signals. In this model, *spen* would contribute a second level of post-translational regulation that would reinforce the transient MAPK signal that downregulates Yan protein, thereby stabilizing the cell's release from the Yan-mediated block to differentiation. In all these scenarios, *spen* could either function in parallel to the Ras/MAPK cascade, or could itself be directly regulated or activated by the pathway. Further biochemical and genetic studies

are required to elucidate the molecular mechanism underlying *spen* function in the RTK/Ras signal transduction pathway.

MATERIALS AND METHODS

Drosophila strains and genetics

The following fly strains were used: *spen*^{AH393}, *spen*^{XFM911}, *pointed*^{AJ613}and *pointed*^{A0435} were isolated in the Yan^{ACT} screen (Rebay et al., 2000); *hsFLP; Sco/CyO* and *P{w*$^{+}$*, ovo*^{D}*}FRT40/CyO* were obtained from T. Laverty and are as described in (Chou and Perrimon, 1996); UAS-Ras^{V12}, UAS-Sspi, P[*slit* 1.0 lacZ], and *slit*-Gal4 lines were obtained from A. Bergman.

Embryonic stages are as described by Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

Generation of germline spen/spen clones

Maternal germline *spen/spen* clones were generated using the FLP-FRT system as described by Chou et al. (Chou and Perrimon, 1996). The progeny from the cross of *spen,FRT 40/CyO* virgin females and *hsFLP; P{w+, ovoD} FRT 40/CyO* males were heat shocked for 1 hour at 38 °C 4, 5 and 6 days after the eggs were laid. Virgin *spen, FRT 40/P{w+, ovoD} FRT 40* females were crossed to males of the desired genotype, for example *spen/CyO*. lacZ expressing balancer chromosomes were used to allow accurate genotyping of the collected embryos.

Immunohistochemistry

To generate antibodies specific to Spen, a GST-Spen fusion protein was created by subcloning a PCR amplified fragment (Spen primers 5'AGCTAGAACTCGAGGATTGG3' and 5'TCCCATCAGACAAGCTCGGC3'). This fusion protein was purified as described in (Rebay

and Fehon, 2000) and injected into mice. Upon obtaining a high titer response, monoclonal antibodies were made following standard protocols(Harlow and Lane, 1988).

For the stainings, embryos were collected, dechorionated and fixed for 20 minutes at room temperature in 4% paraformaldehyde. The anti-Spen monoclonal antibody IR25 was used at a 1:500 dilution, and detected by HRP conjugated goat anti-mouse secondary antibody (Jackson Laboratories) using a standard DAB reaction. Other antibodies were used at the following dilutions: anti-elav (provided by G. Rubin) at 1: 50, anti-fasciculin II monoclonal antibody 1D41H9 (provided by C. Goodman) at 1: 10 , BP102 (obtained from the Developmental Studies Hybridoma Bank) at 1: 10; anti-slit (provided by S. Artavanis-Tsakonas) at 1 : 50. Anti-Sim (provided by) at 1: 100. Rabbit anti-β-Gal (Jackson Laboratory) at 1: 20,000. After staining, the embryos were dissected to reveal the expression in the CNS more clearly. For confocal imaging, Cy3- or FITC- conjugated secondary antibodies (Jackson Laboratory) were used at 1: 1000.

X-gal staining was used to distinguish the embryonic genotypes when necessary. Embryos were collected, dechorionated and fixed as described above. The embryos were then air-dried and rehydrated in 0.7% NaCl, 0.04% Triton X-100. The rehydrated embryos were washed with X-gal staining solution (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$ and 0.3% TritonX-100). The color reaction was carried out at 37 °C in 1 ml of X-gal staining solution with 20 ul of 10 % X-gal. After sufficiently intense color was obtained, the embryos were devitellinized in a 1:1 mixture of warm methanol and heptane, washed with 100% ethanol and subjected to the antibody staining protocol described above.

In situ hybridization

DNA templates used to make probes were amplified by PCR from genomic DNA using the following primer pairs: for probe #1, 5'AAATCTGCTTCAGTGCCAGG3' and 5'ATCTGTGTCGTCAGAGTCTGC3'; for probe #2, 5'ATGGTAAGCCAACCATCACC3' and 5'TCTGGTTACAGGAGCTGTTACGGG3'. DIG-labeled DNA probes were prepared by the standard random priming method. In situ hybridization procedure was carried out as described (Tautz and Pfeifle, 1989) with some modifications. Embryos were collected, dechorionated, fixed and devitellinized as described for immunostaining. The devitellinized embryos were post-fixed for 20 minutes in 5% formaldehyde in PBT (0.1% Tween in PBS) at room temperature. After being washed 3X with PBT, the embryos were treated with 50 ug/ml proteinase K in PBT for 5 minutes at room temperature. The treated embryos were postfixed for 20 minutes in 5% formaldehyde in PBT and then incubated in 1ml hybridization solution (50% formamide, 5XSSC, 100 ug/ml salmon sperm DNA, 100 ug/ml tRNA, 50 ug/ml heparin and 0.1% Tween) at 48 °C for at least 1 hr, followed by hybridization at 48 °C overnight. The embryos were washed with hybridization solution, a 1:1 mix of hybridization solution and PBT, and twice with PBT at 48 °C for 15 minutes each. The embryos were incubated in Alkaline Phosphatase (AP)-coupled anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:2000 in PBT at room temperature for at least 2 hrs. After thorough washing in PBT, the embryos were rinsed with AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2 and 0.1% Tween), and the color reaction was carried out by adding 3.375 ul of 100 mg/ml NBT (nitro blue tetrazolium) and 3.5ul of 50 mg/ml BCIP (5-bromo-4 chloro- 3 indolyl phosphate) into 1 ml of AP buffer at room temperature.

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Table 1. *spen* is required for midline glial cell survival

	Wild type		*spen*^{AH393}	
	#MGC/Segment	n	#MGC/Segment	n
Stage 13	8.0 ± 0.9	18	8.1 ± 0.4	24
Stage 15	4.8 ± 0.1	24	3.5 ± 0.3	24
Stage 17	3.1 ± 0.2	44	1.9 ± 0.1	39

n refers to the numbers of segments counted.

Table 2. *spen* interacts synergistically with *pointed*.

Genotypes	Percentage of embryos with axon guidance defects	n
$spen^{AH393}$(M)/+(P)	4.2 ± 3.1%	101
pnt^{A0435}/+	0	95
$spen^{AH393}$(M)/+(P); pnt^{A0435}/+	23.8 ± 2.2%	145

The axon guidance defects were detected by anti-fas II staining. Only stage 15-17 embryos were examined and counted.

Figure 1. Spen is abundantly expressed in early embryos and is later enriched in the ventral midline and central nervous system. (A-C) The expression pattern of *spen* mRNA detected by *in situ* hybridization. (D–F) The expression pattern of Spen protein detected by whole mount immunostaining. (A, D) *spen* is abundantly expressed in stage 3 blastoderm embryos. Spen protein localizes to the nuclei (D). (B, E) At stage 7, *spen* is ubiquitously expressed but is enriched in the ventral midline precursor mesectoderm. White arrow indicates the ventral furrow. (C. F) By stage 17, *spen* is almost exclusively expressed in the central nervous system (marked with an asterisk). Embryos are oriented with anterior to the left. (A, D, C, F) lateral view. (B, E) ventral view.

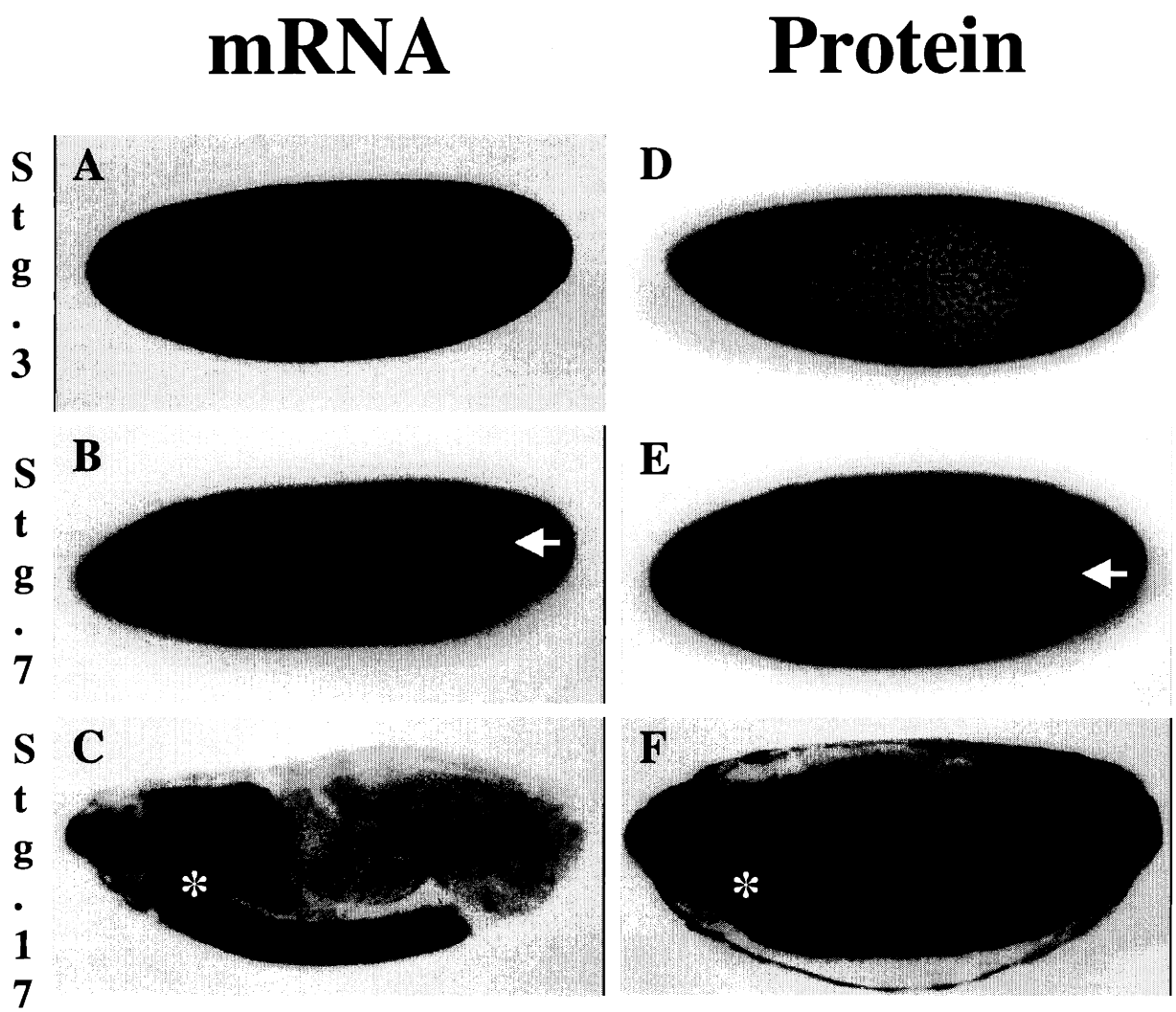

mRNA
Protein
Stg.3
Stg.7
Stg.17
A
B
C
D
E
F
*
*

Figure 2. Embryos lacking *spen* function exhibit a collapsed and disorganized central nervous system. (A-C) Ventral view highlighting the central and peripheral nervous systems using the pan-neuronal marker anti-Elav. Embryos are oriented with anterior to the left. (A) wild type. (B) $spen^{AH393}/spen^{AH393}$. (C) $spen^{XFM911}/spen^{XFM911}$. (B,C) Note the partial collapse and irregular morphology of the two longitudinal branches of the CNS.

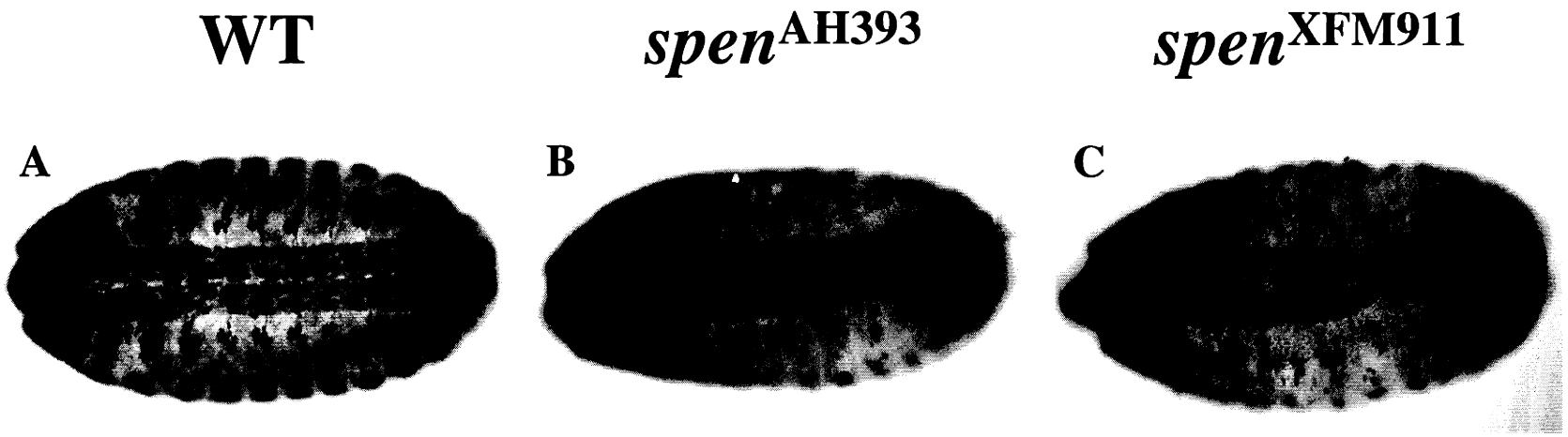
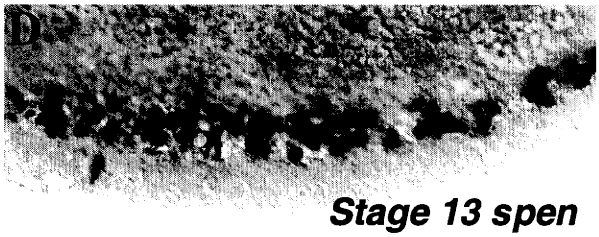

WT
spenAH393
spenXFM911
A
B
C

Figure 3 *spen* is not required for the initial midline glial cell specification, however, it is required for normal migration and survival of the midline glial cells. Embryos carrying midline glia specific enhancer trap P[*slit* 1.0 lacZ] were stained with anti-β-gal antibodies to highlight midline gial cells. (A, C, E) wild type. (B, D, F) $spen^{AH393}/spen^{AH393}$. (A, B) Lateral view of stage 12 embryonic CNS. At stage 12, the midline glial cells start to differentiate. At this stage, *spen* mutants are indistinguishable from wild type. (C, D) Lateral view of stage 13 embryonic CNS. At stage 13, the midline glial cells normally initiate their migration. In *spen* mutants, this migration is aberrant, resulting in a broad scattering of glia over the CNS. (E, F) Lateral view of stage 17 embryonic CNS, the last stage of embryogenesis. At this stage, wild type embryos have 2-3 midline glia per segment arranged in a characteristic pattern whereas *spen* mutants have only 1-2 glia per segment. All the embryos are oriented anterior to the left and dorsal to the top.

Stage 12 wt

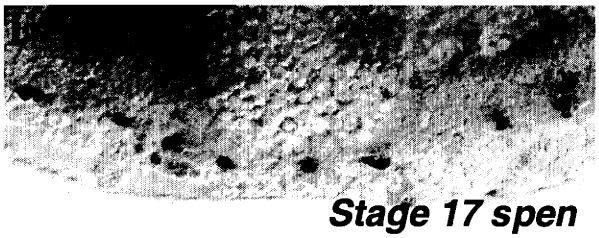
Stage 12 spen

Stage 13 wt



Stage 13 spen

Stage 17 wt



Stage 17 spen

Figure 4 There are equal numbers of Sim-positive and Slit-lacZ-positive cells in *spen* mutants. A stage 13 *spen* embryo expressing the Slit-lacZ MGC-specific enhancer trap double labeled with anti-Sim (green) and anti-β-gal (red). (**A**). Anti-β -gal staining highlights the MGCs expressing Slit-lacZ enhancer trap. (**B**). Anti-Sim staining of the same embryo highlights the same population of MGCs. (**C**). The merge shows almost perfect overlap.

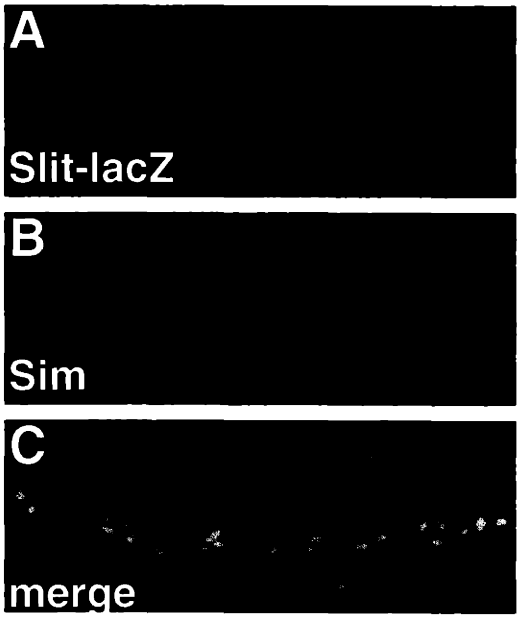

A
Slit-lacZ
B
Sim
C
merge

Figure 5 Midline glial cells are reduced in number and are malformed in *spen* mutants. (A, B) Stage 16 embryos were stained with anti-Slit antibodies and the CNS was dissected. (A) In wild type, there are about two Slit-positive cells in each segment and they are both elongated to ensheath the commissure axon bundles. (B) In $spen^{AH393}/spen^{AH393}$ mutants, there is one Slit-positive cell in most of the segments and it is not properly elongated and thus does not wrap around the commissure axon bundles. (C, D) Stage 16 embryos carrying the midline glia specific enhancer trap P[slit 1.0 lacZ] were stained with anti-β-gal antibodies. (C) Wild type. There are about three midline glial cells in each segment. (D) $spen^{AH393}/spen^{AH393}$ mutant. There is only one midline glial cell in many segments. Segments are occasionaly found with more than one midline glial cell, however, these cells are positioned abnormally. Dissected embryos are oriented anterior to the top.

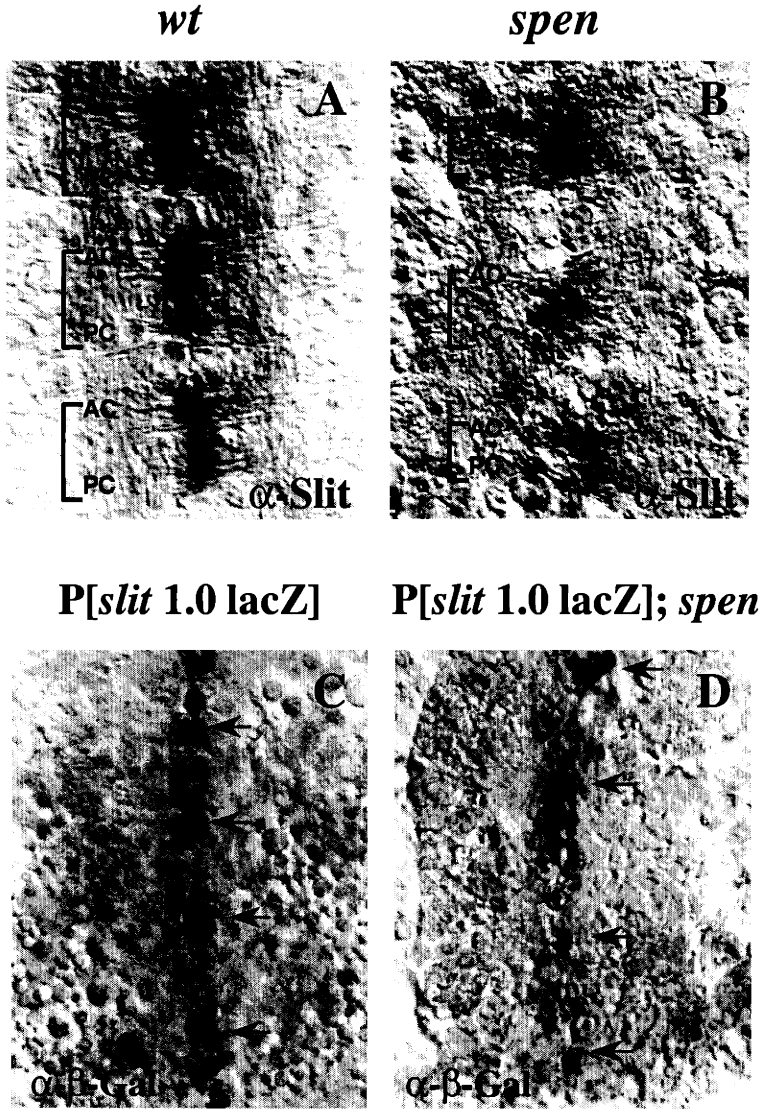

wt
spen
A
B
AC
PC
AC
PC
α-Slit
α-Slit
P[slit 1.0 lacZ]
P[slit 1.0 lacZ]; spen
C
D
α-β-Gal
α-β-Gal

Figure 6 Spen protein is strongly expressed in the midline glia cells.

A stage 13 AA142 enhancer trap embryo double labeled with anti-Spen (red) and anti-β-gal (green) antibodies. (**A**). Spen expression is strongest in the MGCs. (**B**). Anti-β-gal staining marks the MGCs. (**C**). The merge shows almost perfect overlap.

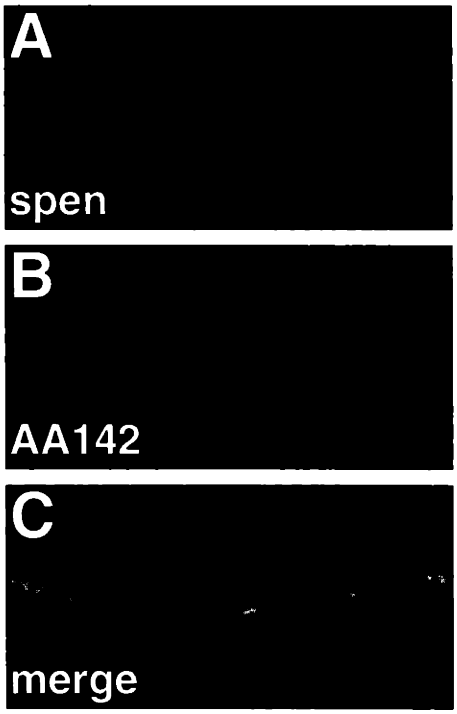

A
spen
B
AA142
C
merge

Figure 7: *spen* mutants exhibit severe axon guidance defects. Dissected CNS from Stage 16 embryos oriented anterior to the top. (**A-C**) Immunostaining with BP102. (**D-F**): Immunostaining with anti-Fas II. (**A,D**) Wild type. (**B,E**): $spen^{AH393}$ mutants have collapsed commissures, reduced separation between the two longitudinal branches, and gaps and inappropriate crossings of the midline by the longitudinal axons. (**C,F**) similar phenotypes are observed in $spen^{XFM911}$ mutants.

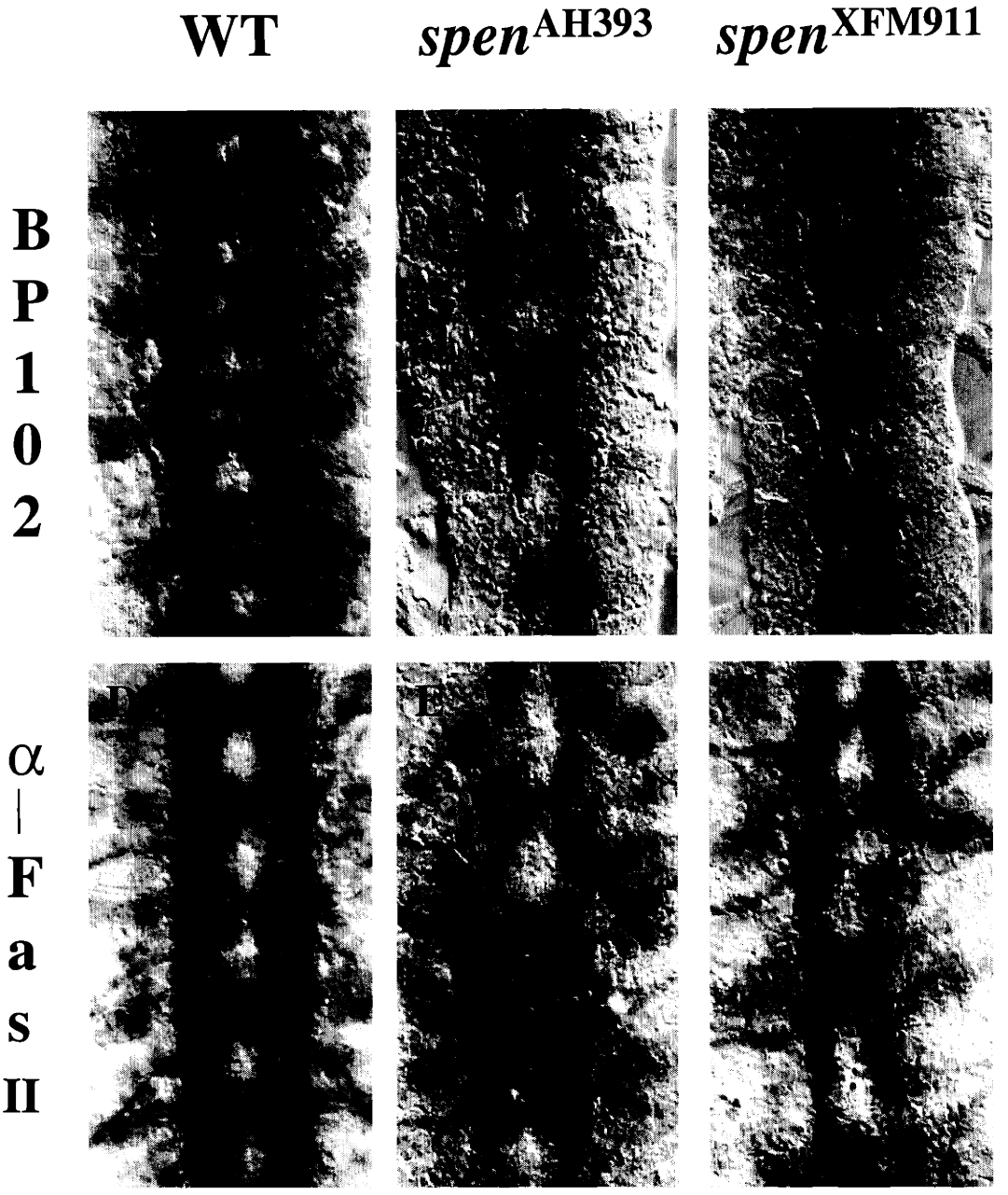

WT
spenAH393
spenXFM911
BP102
α-FasII

Figure 8. *pointed* interacts synergistically with *spen*. (A) Embryos carrying only one copy of *pointed* (*pnt*/+) have wild type looking longitudinal axon tracts as detected by the anti-Fas II antibody. (B). Reducing *pnt* dosage significantly enhances the phenotype associated with spen^{AH393}(M)/+ (Table 1). The predominant phenotype of double heterozygotes (*spen*^{AH393}(M)/+; *pnt*/+) is reduced separation between the two longitudinal axon bundles and a single inappropriate crossing of the midline by one of the FasII positive axon tracts. The arrow points to the inappropriate crossover event. The dissected embryos are oriented anterior to the top.

Pnt / + *Spen / +, pnt / +*

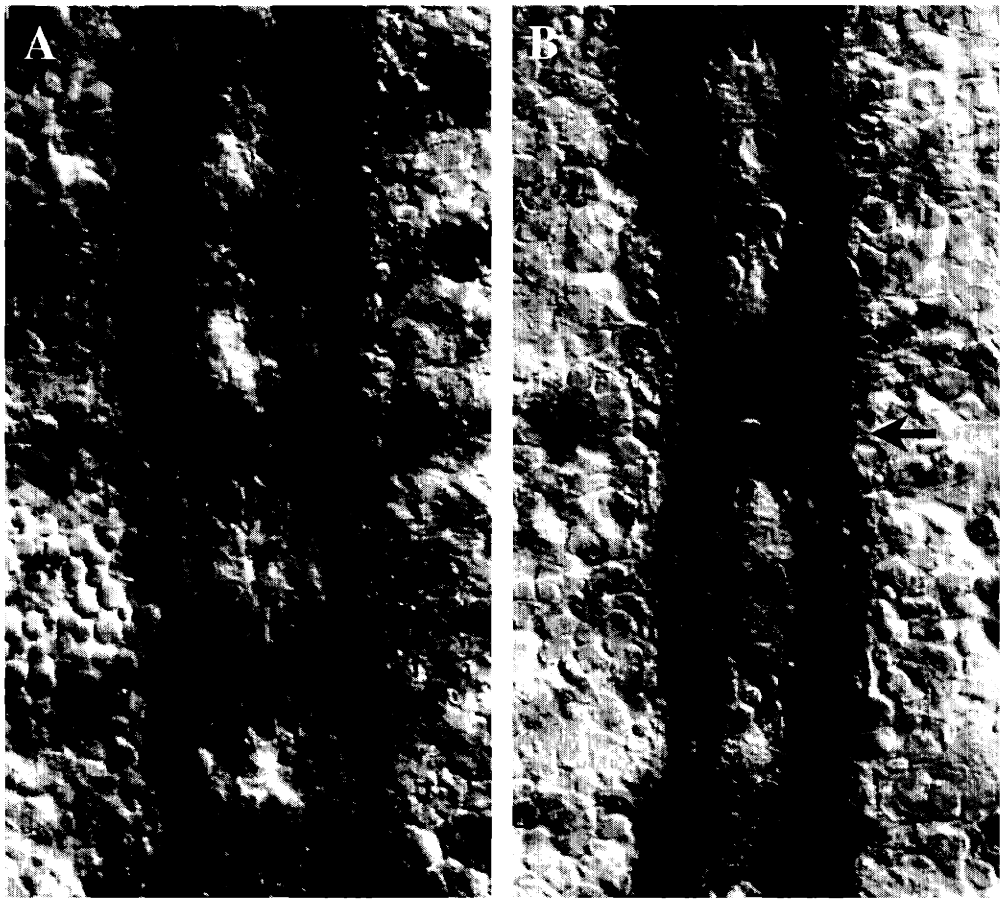


Figure 9 SpenΔC functions as a dominant negative mutant *in vivo*.

Stage 16 embryos labeled with anti-FasII. (A) *spen*^{XFM911} zygotic null embryos appear phenotypically normal. (B) MGC-specific overexpression of SpenΔC in the *spen*^{XFM911} zygotic null background results in severe axon guidance defects.

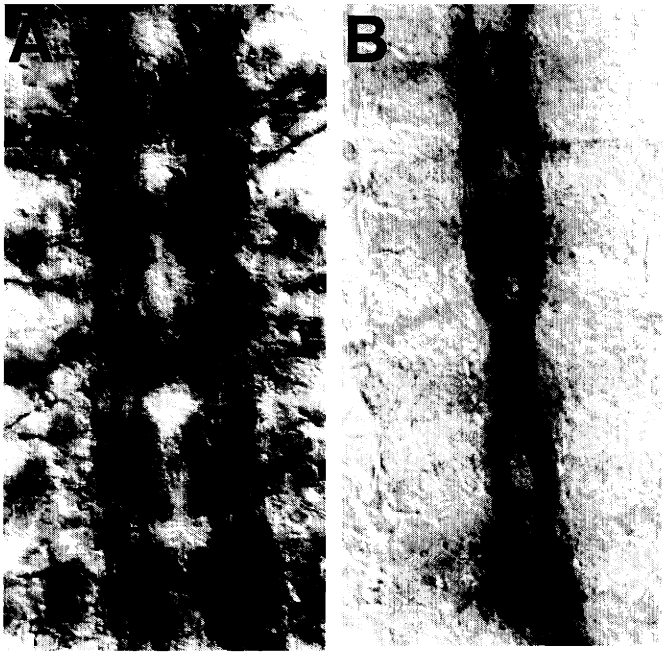
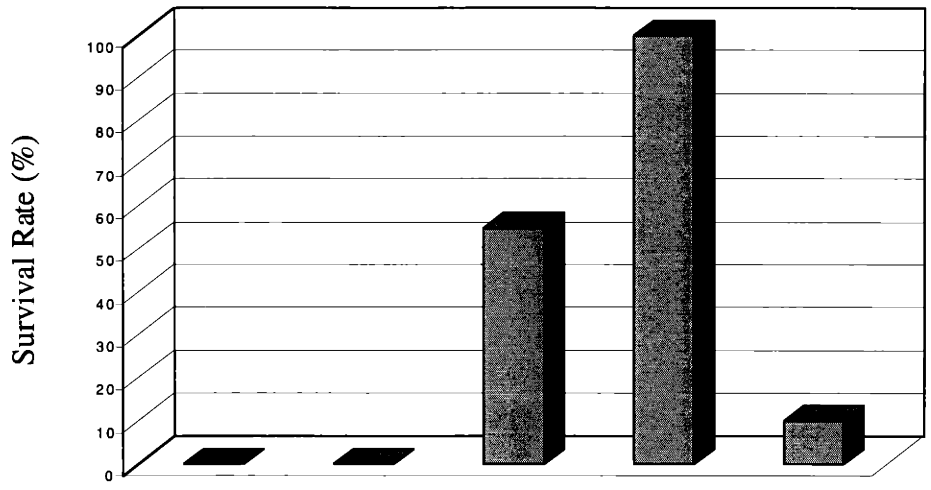

A
B

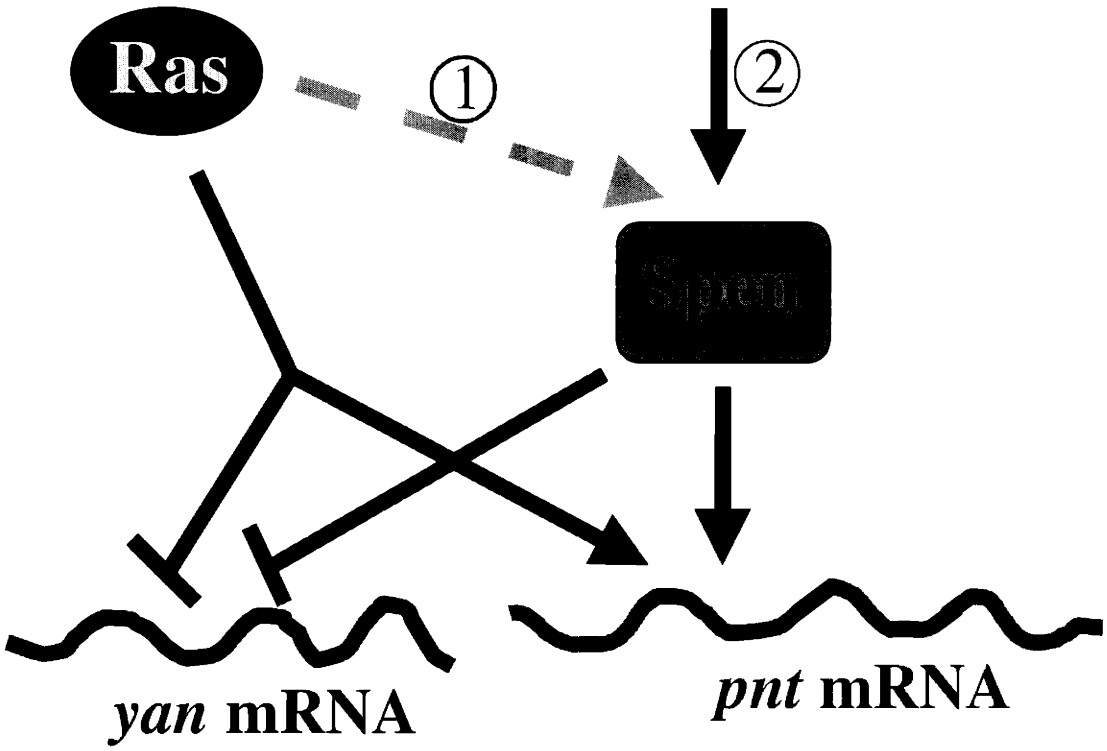
Figure 10 Increased DER signaling in the midline glia suppresses the lethality associated with overexpression of the putative dominant negative Spen, SpenΔC. SpenΔC, Ras^{V12} and Sspi are expressed as UAS transgenes under control of the MGC-specific Slit-Gal4 driver. Survival rate was calculated based on the ratio between the actual number of adult flies recovered and the expected number of flies of each particular genotype. A minimum of 100 flies were counted in each experiment.



	SpenΔC	Ras^{V12}	Ras^{V12}; SpenΔC	Sspi	Sspi; SpenΔC
Survival Rate (%)	0	0	55	100	10

Figure 11 Model. Scenario 1, Spen is likely to function downstream of Ras. Scenario 2: Spen might also function in a pathway parallel to Ras. This model is based on the mutual suppression between Ras^{V12} and SpenΔC. Since Spen is a putative RNA-binding protein, I propose that Spen might directly regulate the RNA levels of downstream Ras effectors such as *yan* and *pointed.* The arrows represent direct or indirect interactions. The steps between Ras, Yan and Pointed have been omitted for simplicity.

Models



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Chapter 4

A Genetic screen designed to isolate *spen*-interacting genes

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Abstract:

To elucidate the molecular mechanisms underlying Spen function in the RTK/Ras signaling pathway, we took a genetic approach to isolate *spen*-interacting genes. To that end, we overexpressed the nuclear-localization-sequence(NLS)-tagged Spen C-terminus (CspenNLS) that contains the highly conserved SPOC domain specifically in the eye. Overexpression of CspenNLS caused a dominant rough eye phenotype. Retinal sections of strong insertion lines revealed a disorganized retina, elongated rhabdomeres and a combination of missing and extra photoreceptors. In addition, eye-specific overexpression of CspenNLS leads to diminished anti-Cut expression in cone cells in the larval imaginal disc. The phenotypes caused by CspenNLS are strikingly reminiscent of *spen* null clones, suggesting that CspenNLS functions as a dominant negative mutant *in vivo*. We have further confirmed this hypothesis by demonstrating that reducing the endogenous level of *spen* enhances the rough eye phenotype caused by CspenNLS.

Using the CspenNLS rough eye phenotype as the starting genetic background, we screened through the deficiency kit, which uncovers ~80% of the fly genome, for dominant modifiers. From this screen we isolated 23 enhancer and 27 suppressor regions. Among the known components and regulators of the RTK pathway, we recovered regions uncovering *Draf*, *prospero*, *Ras*, *pointed*, *vein*, *seven-up* and *mts* (PP2A). Most interestingly, we have isolated multiple overlapping deficiencies as modifiers of CspenNLS, suggesting that the overlapping regions might contain genes directly interacting with Spen. Possible candidate genes within those regions are discussed further.

Introduction

Cell-cell signaling controls development through many different mechanisms. One of the best-studied mechanisms is transcriptional regulation. In the RTK pathway, activated MAP kinase translocates from the cytoplasm to the nucleus to directly modulate the activity of transcription factors (Treisman, 1994). The phosphorylated transcription factors then regulate the expression of target genes that will eventually specify cell fates. However, it seems likely that post-transcriptional regulatory mechanisms may also mediate downstream responses to the RTK pathway.

RTK signaling is highly dynamic, regulating cell fate determination in both a qualitative and quantitative manner. Thus the strength of the signal appears to directly affect the specific cell fate decision (Raz and Shilo, 1993). Because transcriptional responses usually take longer, in certain contexts post-transcriptional regulation is likely to be needed for quick and precise responses to dynamically changing signals.

Our identification of Spen, a putative RNA binding protein, as a novel component of the RTK signaling pathway, makes it tempting to speculate that regulation of splicing, stabilization, translation or localization of specific transcripts could play an important role in potentiating RTK-initiated signals. Interestingly, two genes that function downstream of the RTK pathway, *pointed* and *tramtrack*, are known to be alternatively spliced. *pointed* encodes two alternatively spliced mRNAs, referred to as P1 and P2 (Klambt, 1993). The two proteins share a common carboxyl-terminal half that includes the Ets DNA binding domain, but have distinct amino terminal sequences. Both isoforms are transcriptional activators, but whereas P1 is constitutively active, P2 becomes active only when it is phosphorylated by MAPK in response to RTK/Ras signaling (O'Neill et al., 1994). The expression of the two isoforms is restrictively regulated.

For example, in the embryonic midline, Pointed P1 is expressed in the longitudinal glial cells and the glial cells supporting the ventral unpaired midline (VUM) neurons (Klaes et al., 1994), whereas P2 is restricted to the midline glia and has been shown to function as a DER effector in these cells (Scholz et al., 1997).

tramtrack, also encodes two alternatively spliced isoforms, termed P69 and P88 (Read and Manley, 1992). P69 and P88 have identical amino terminal halves but have distinct zinc finger DNA binding domains in their carboxy-terminal halves. Both Tramtrack proteins act as transcriptional repressors and function in development to block neuronal differentiation (Xiong and Montell, 1993). Similar to Pointed, the two isoforms of Tramtrack are specifically regulated. P88 is dispensable for embryogenesis but is required during imaginal disc development (Lai et al., 1996). In the embryo, P69 is expressed strongly in the midline glia, and loss of P69 leads to a fused commissure phenotype similar, but not identical, to that of the Spitz group genes (Giesen et al., 1997). Despite the similarity in phenotype, Tramtrack has not yet been implicated in DER signaling events in the midline. However in the developing photoreceptors of the eye, Tramtrack P88-mediated repression of neuronal differentiation has been shown to be regulated by downstream DER signaling events (Li et al., 1997; Tang et al., 1997). Although the regulation of alternative splicing in both cases is unclear, it remains an attractive hypothesis that RTK signaling is directly responsible. Our demonstration that Spen, a putative RNA binding protein, functions downstream of Ras is the first indication that such post-transcriptional gene regulation mechanisms might exist in this pathway.

RNA-binding proteins provide a repertoire of functions in eukaryotic cells (reviewed by Siomi and Dreyfuss, 1997). For example, these proteins may facilitate changes in the RNA conformation, which lead to alterations in its translation and/or stability. RNA binding proteins

may target or transport RNA to specific subcellular locations by their own signal sequences or through interactions with other proteins. RNA binding proteins may also function as a part of the splicesome to regulate pre-RNA processing to generate distinct protein isoforms.

In Drosophila, RNA binding proteins have been implicated in important developmental events. During the first two hours of Drosophila embryogenesis, cell fates are determined by maternally contributed mRNAs and proteins. The spacial and temporal restriction of RNA translation and degradation is particularly important. For example, anterior localization of *bicoid* mRNA and posterior localization of *nanos* mRNA determine the anterior and posterior cell fates, respectively (reviewed by Cooperstock and Lipshitz, 1997). The unlocalized *nanos* mRNA is translationally repressed and later degraded in a process mediated by the RNA-binding protein, Smaug (reviewed by Cooperstock and Lipshitz, 1997). An example of regulation of alternative splicing occurs during sex determination. The RNA-binding protein Sex-lethal directly regulates the pre-mRNA splicing both of itself and of a downstream target gene *transformer* (*tra*) (Valcarcel et al., 1993). The binding of Sex-lethal to the *tra* pre-mRNA results in a female specific splicing event, generating a full-length TRA protein. TRA in turn regulates splicing of *double-sex* to activate female differentiation (Valcarcel et al., 1993). A similar function is found in Elav which is required in all neurons to generate a neural specific isoform of Neuroglian (Koushika et al., 1996). Interestingly, the RNA-binding protein Squid has been implicated in the DER signaling pathway and appears required for targeted localization of the mRNA of the DER ligand *gurken*. The correct localization of *gurken* mRNA is essential for establishing dorsal/ventral polarity during oogenesis (Kelley, 1993; Norvell et al., 1999).

In order to elucidate the mechanism underlying Spen function in the RTK pathway, several questions must be addressed. First, it remains to be confirmed that Spen actually binds

RNA. Although Spen contains 3 tandem RRM repeats at its N-terminus, it has been reported that RRM motifs can bind not only to RNA, but also to single- and double- stranded DNA (Bertolotti et al., 1996; DeAngelo et al., 1995; DeFalco and Childs, 1996). In fact, a mouse Spen-like protein, MINT, has been shown to bind DNA *in vitro* (Newberry et al., 1999). Second, the substrates or targets of Spen remain to be identified. Based on the protein sequence of Spen, it is impossible to predict what kind of targets Spen might have. The three RRM motifs of Spen-like proteins are more similar to each other than to RRM motifs found in other proteins (Kuang et al., 2000; Rebay et al., 2000; Wiellette et al., 1999). Thus the homology is limited to the consensus residues that define the RRM motif. Therefore, Spen is likely a founding member of a novel subclass of RRM proteins, whose substrates are at present unknown. Finally, it remains to be determined how Spen function is regulated by the RTK pathway. Many RNA-binding proteins regulate RNA translation, localization and stability through interactions with other proteins (Siomi and Dreyfuss, 1997). The highly conserved Spen C-terminal SPOC domain might mediate such interactions. In addition, phosphorylation has been implicated as a major mechanism regulating both constitutive and alternative splicing (Stojdl and Bell, 1999). Spen contains multiple MAPK consensus sites, indicating that Spen might be a potential target of MAPK. To address all these questions, we decided to take advantage of the powerful genetic tools and well-characterized developmental biology that Drosophila offers as an experimental system. Here we describe a genetic screen we have designed to isolate Spen-interacting genes and report the preliminary results from the deficiency kit screen.

Results

Overexpression of an NLS-tagged Spen C-terminus causes dominant rough eye phenotypes

In order to design a genetic screen to isolate *spen*-interacting genes, we first tried to generate visible phenotypes by overexpression of either full-length or segments of Spen in the fly. Since Spen is an unusually large gene, we were unable to make a full-length Spen cDNA construct. The C-terminal SPOC domain of Spen is highly conserved, and, as described in Chapter 3, a C-terminal truncated protein (SpenΔC) functions as a dominant negative mutant *in vivo*. SpenΔC causes lethality when overexpressed in the embryonic midline glial cells. However, SpenΔC fails to produce any visible phenotypes when it is overexpressed in the eye. Because an eye phenotype would be highly desirable as a genetic background in which to conduct a modifier screen, we decided to overexpress the Spen C-terminus alone to find out whether it might generate a visible phenotype in the eye.

Two types of Spen C-terminal constructs were made, one with a nuclear localization sequence (NLS) at the N-terminus (CspenNLS), the other without (Cspen) (Figure 1). When CspenNLS is transfected into the S2 cells, it is localized to the nucleus just like the endogenous Spen protein and the dominant negative SpenΔC. In contrast, Cspen is localized in the cytosol in S2 cells (data not shown). When CspenNLS is overexpressed specifically in the eye under the *sevenless* promoter, it generates rough eye phenotypes. 10 different insertion lines display a range of phenotypes from mild to very rough. In contrast, 7 different lines of transgenic flies carrying the Cspen construct did not generate any obvious eye phenotypes upon overexpression (Table 1).

Tangential sections of the strong CspenNLS lines reveal that the retinal defects are strikingly reminiscent of those seen in *spen* mutant clones. Specifically, the ommatidia are greatly disorganized, the rhabdomeres are elongated, and there is a combination of extra and missing photoreceptors (Figure 2). In addition, eye-specific overexpression of CspenNLS results

in diminished anti-Cut staining in the cone cell precursors, a phenotype similar to what has been observed in *spen* mutant clones (Figure 3). The striking phenotypic similarity between CspenNLS overexpression and *spen* mutant clones suggests that like SpenΔC, CspenNLS might also function as a dominant negative mutant *in vivo*.

CspenNLS functions as a dominant negative mutant *in vivo*

Before initiating a genetic modifier screen, we wanted to confirm that CspenNLS indeed functions as a dominant negative mutant *in vivo*. The prediction is that if CspenNLS functions as a dominant negative mutant *in vivo*, then reducing the endogenous level of *spen* should enhance the eye phenotypes associated with CspenNLS. When one particular insertion line, CspenNLS383, is overexpressed in eyes under a weak *sevenless* Gal4 driver (2B-Gal4), it causes moderate roughness with random black spots in the eyes. Reducing *spen* dosage strongly enhances this phenotype resulting in very rough eyes with patches of black at the anterior region of the retina (Table 1). This result was confirmed using another insertion line, CspenNLS381, driven by both weak and strong *sevenless* Gal4 lines (Table 1, Figure 4). Our results strongly suggest that CspenNLS is a dominant negative mutant *in vivo* and we speculate that it might interfere with endogenous Spen function possibly by sequestering Spen-interacting factors. Our results also indicate that the phenotypes associated with CspenNLS are dosage sensitive, which is critical for a successful modifier screen.

Deficiency kit screen

Based on this rough eye phenotype, we first screened through ~200 stocks in the deficiency kit obtained from the Bloomington stock center. This collection of deficiencies

uncovers ~80% of the Drosophila genome. From this screen, we isolated 23 enhancer regions and 27 suppressor regions (Table 3 and Table 4; Figure 5). Among the 23 enhancers, there are deficiencies that, based on the reported cytology, presumably uncover known positive regulators of the RTK pathway, including *Draf*, *sevenless*, *vein*, *Ras*, *prospero*, *seven-up* and *pointed.* Isolation of such mutations is consistent with our postulated function of *spen* as a positive regulator of RTK signaling pathway (Table 3). Among 27 suppressors, we isolated a deficiency uncovering protein phosphatase 2A, also known as *microtubule star* (*mts*), which was isolated as a strong enhancer of activated Ras^{V12} in a previous screen (Wassarman et al., 1996).

We also isolated deficiency (042E; 044C) which potentially uncovers the second *spen*-like gene Dm44A as a strong enhancer of CspenNLS. Since CspenNLS functions as a dominant negative mutant in vivo, the direction of the interaction suggests that Dm44A might be functionally redundant with *spen*.

We also noticed that we have isolated many deficiencies that uncover cell cycle regulators including *cyclin* E, *cyclin* B, *cyclin* A, *roadback* (*rob*1) and *gigas*. *spen* was previously isolated as a strong enhancer of Drosophila dE2F and dDP from an eye-based screen (Staehling-Hampton et al., 1999). Thus our results further support a potential role of *spen* during cell cycle regulation.

Among the regions without obvious candidate genes, we found nine of them particularly interesting since multiple overlapping deficiencies uncovering those regions were isolated as modifiers, increasing the likelihood that those regions contain specific *spen*-interacting genes. Among the nine are 3 enhancing regions (004C-004F, 016A and 072C-072D) and 6 suppressing regions (028B, 036A-036D, 044D, 044F, 087B-087E and 089E-090B) (Figure 5).

Discussion:

To elucidate the molecular mechanisms underlying Spen function in the RTK pathway, we decided to take advantage of the powerful genetic tools of Drosophila to isolate *spen*-interacting genes. We first generated a dominant negative mutant protein by overexpressing a nuclear-localization-sequence (NLS)-tagged Spen C-terminus (CspenNLS). The Spen C-terminus contains a highly conserved SPOC domain indicating its likely functional importance. Supporting this notion, truncating the entire C-terminus produces a dominant negative mutant (see Chapter 3). We have found that overexpression of CspenNLS specifically in the eye causes a rough eye phenotype. Tangential sections revealed very similar cellular defects to what we have been seen in *spen* mutant clones. In addition, overexpression of CspenNLS also causes diminished anti-Cut staining in cone cells. These phenotypes are consistent with Cspen NLS functioning as a dominant negative mutant *in vivo*. We have further confirmed this hypothesis by demonstrating that reducing the endogenous level of *spen* enhances the eye phenotype of CspenNLS.

The strong dominant negative effect of CspenNLS led us to postulate that the Spen C-terminus might be involved in protein-protein interactions. Overexpression of CspenNLS could interfere with the function of endogenous Spen by competing for C-terminal interacting proteins. Because endogenous Spen is nuclear, and presumably therefore interacts with other nuclear factors, the prediction was that the C-terminus of Spen would need to be appropriately localized in order to exert any dominant negative effects. Consistent with this hypothesis, we found that overexpression of the Spen C-terminus alone, which without addition of the nuclear localization sequence accumulates in the cytoplasm, has no effects in the eye. Based on these data, we

predicted that we might be able to enhance or suppress the CspenNLS rough eye phenotype by mutating Spen C-terminal interacting genes.

Our screen of the Bloomington Stock Center deficiency kit isolated 23 enhancers and 27 suppressors. Among those modifiers, there are regions containing known components of the RTK pathway, such as *Draf*, *sevenless*, *vein*, *Ras*, *prospero*, *seven-up* and *pointed*, suggesting the screen is working. Although specific gene mutations remain to be tested, the possibility of direct interactions between *prospero*, *seven-up* and *spen* is very interesting since both *prospero* and *seven-up* have been implicated in cone cell development. Particularly, high-dose overexpression of Seven-up driven by the *sevenless* promoter causes elongated rhabdomeres, a combination of missing and extra photoreceptors as well as missing cone cells, a phenotype strikingly similar to those of both *spen* null clones and overexpression of CspenNLS (Begemann et al., 1995; Kramer et al., 1995). *seven-up* encodes an orphan nuclear receptor with two isoforms whose function is normally required for outer photoreceptor specification (Mlodzik et al., 1990). It has been proposed that activation of DER/Ras pathway is required for Seven-up function (Begemann et al., 1995; Kramer et al., 1995). Further genetic and biochemical experiments will be required to investigate potential molecular interactions between Spen and Seven-up.

Our screen also emphasized a potential role of Spen in cell cycle regulation by recovering strong modifying regions containing a spectrum of cell cycle regulators, including Cyclin A, Cyclin B, Cyclin E, Rob1 and Gigas. The function of *spen* in cell cycle regulation was first suggested when *spen* was isolated as a strong enhancer of the rough eye phenotypes associated with overexpression of dE2F and dDP(Staehling-Hampton et al., 1999). In addition, it has been demonstrated that *spen* suppresses the rough eye phenotypes caused by overexpression of either the human cell cycle inhibitor p21 or its Drosophila homologue Dacapo. These observations

strongly suggest that *spen* might function as a negative regulator of cell cycle progression. Although our phenotypic analysis of *spen* null mutation has not revealed a direct connection between *spen* and cell proliferation defects, the complex retinal defects caused by *spen* mutations might reflect a combinatorial effect of *spen* on both cell proliferation and cell differentiation.

Of the suppressing regions, 089E-090B is particularly interesting since two deficiencies overlapping this region, Df(3R)DG2/TM2 and Df(3R)C4, were both isolated as strong suppressors. There is a candidate gene within this region called *osa* that was also isolated together with *spen* as a strong enhancer of dE2F and dDP. Osa, a DNA-binding protein, is part of the Drosophila SWI/SNF DNA remodeling complex (Collins et al., 1999). It has been implicated downstream of the Wingless pathway to directly regulate gene expression (Collins and Treisman, 2000). Again, genetic interactions using specific *osa* mutations will be required to confirm the result.

Our data from the deficiency kit screen strongly suggests CspenNLS will provide an appropriately sensitized background for performing a genetic modifier screen. From such a screen, we can expect to isolate both suppressor and enhancer mutations. Because deficiencies uncover multiple genes, the results of our pilot experiment will need to be confirmed using specific mutations. However, based on our current understanding of Spen, the candidate genes uncovered by the interacting regions appear likely to shed some important light on Spen function, especially during cone cell development and cell cycle regulation. Future genetic and biochemical experiments will further dissect the *spen*-interacting gene networks and the molecular mechanisms underlying the genetic interactions.

Materials and Methods

Fly Stocks

sevenless Gal4 driver 1B and 2B were generated by Rebay I. These lines contain the Sevenless enhancer and promoter cloned upstream of Gal4. The deficiency kit was obtained from the Bloomington stock center.

Immunocytochemistry

Third-instar larvae were collected and dissected in Schneider's media. Salivary glands were fixed by 4 % paraformaldehyde in PBS for 15 minutes at room temperature. Samples were washed in PBT (PBS + 0.1 % Triton-X-100) and blocked in PNT (PBS + 1 % normal goat serum + 0.1 % Triton-X-100), then were incubated in anti-Spen monoclonal antibody IR25 (1:500 dilution in PNT) at 4 C overnight. Cy3-conjugated goat anti-mouse secondary antibody (Jackson Laboratory) was used at 1: 2000 dilution for immunoflurescence detection.

Generation of transgenic flies

Spen C-terminus was amplified by PCR reaction. The following oligos are used for CspenNLS construct: spenNLS13771: 5' GGA AGA TCT ATG CCG AAG AAG AAG CGC AAG GTG GTT GCC GCC AGT CAT TTG GCA CC 3' and Cspen3XI: CCG CTC GAG TTA GAC AGT AGC GAT GAC AAT CAG. The sequence used as a nuclear localization signal is : PKKKRKV. For Cspen construct: oligo spen13771 was used instead of spenNLS13771: 5' GGA AGA TCT ATG GTT GCC GCC AGT CAT TTG GCA CC 3'

DNA used for injection was prepared by QIAGEN midiprep kit, and a final DNA concentration of 1.0 ug/ul of construct and 0.5 ug/ul of helper plasmid was used. The injection mix was injected into the syncycial stage embryos.

Table 1. CspenNLS functions as a dominant negative mutant *in vivo*

Genotypes	Eye Phenotypes	Genotypes	Eye Phenotypes
CspenNLS381/ +;2B-Gal4/+	very mild rough	CspenNLS381/ *spen*^{XFM911}; 2B-Gal4/+	mild rough
CspenNLS381/ +; 1B-Gal4/+	moderate rough, random fusion of ommatidia	CspenNLS381/ *spen*^{XFM911}; 1B-Gal4/+	strong rough; a lot of fused ommatidia
CspenNLS383/ +; 2B-Gal4/+	rough; random black spots	CspenNLS383/ *spen*^{XFM911}; 2B-Gal4/+	strong rough; patches of black at the anterior region

Table 2. Enhancers from the deficiency kit screen

Stock Number	Break point	Candidate genes
936	**002E01-02;003C02**	**Draf**
940	**003D06-E01;004F05**	
944*	**004C15-16;005A01-02**	
5705	**004F05;005A13**	
3560	**009F;010C03-05**	***Dsh*; *sev***
3217*	**014B13;015A09**	
723	**016A02;016A06**	
4953*	**016A02;16C07-10**	
970	**017A01;018A02**	
3588	**035B04-06;035F01-07**	***su(H)*; *cyclin* E**
167	**038A06-B01;040A04-B01**	
3368#	**042E;044C**	
1150	**051B05-11;051D07-E02**	
3520	**052F05-09;052F10-53A01**	**Rho1**
3909#	**059A01-03;059D01-04**	***cyclin* B**
3096*	**064C;065C**	***vein***
3124	**070C01-02;070D04-05**	
3640	**071F01-04;072D01-10**	
2993#	**072C01-D01;073A03-04**	
3617#	**076B01-02;076D05**	
1962#	**085A02;085C01-02**	**Ras**
3128	**086C01;087B01-05**	***pros*; *svp***
4940#	**095A05-07;095C10-11**	

*: those deficiencies were isolated twice independently
#: indicates strong enhancers

Table3. Suppressors from the deficiency kit screen

Stock number	Break point	Candidate genes
90*	023A01-02;023C03-05	
1357	027C02-09;028B03-04	
4955*	028B02;028D03	*mts* (PP2A)
1469	031C-D;032D-E	
2583	035F-036A;036D	
3180	036A08-09;036E01-02	
198	043F;044D03-08	*patched*
201	044D01-04;044F12	*patched*
3591	044F10;045D09-E01	
4966	045A06-07;045E02-03	
5574*	054C01-04;054C01-04	*roadback*1 (*rob*1)
3064	054E08-F01;055B09-C01	
543*	056F05;056F015	
282*	058D01;059A	
1682	059D05-10;060B03-08	
3649	063C02;063F07	
1420	065F03;066B10	
2611	068A02-03;069A01-03	*cyclin* A
5126*	076B04;077B	*gigas*
1893	085D11-14;085F06	
3007	087B11-13;087E08-11	
1467*	089B07-08;089E07-08	
4431*	089E01-F04;091B01-B02	*osa*
3071*	089E03-04;090A01-07	
3012*	091F01-02;092D03-06	
2363	095F07;096A17-18	
430	098E03;099A06-08	

* indicates strong suppressors

Figure 1. Spen Constructs. Subcellular localization was characterized in transfected S2 cells. RRM: RNA Recognition Motif. CC: Coiled-Coil. SPOC: Spen Paralog and Ortholog C-terminal domain. NLS: Nuclear Localization Signal sequence (represented by a red box upstream of the C-terminal Spen).

Spen Constructs

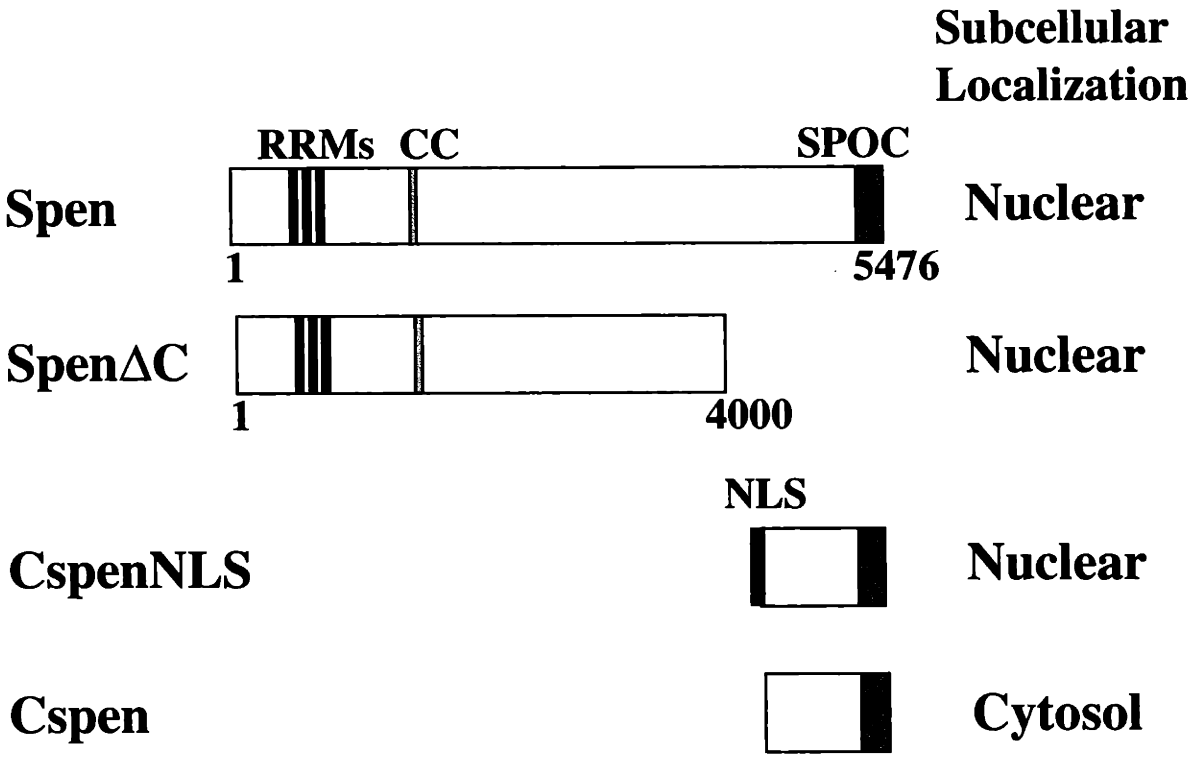


Figure 2. Overexpression of CspenNLS specifically in eye causes rough eye phenotypes strikingly similar to spen mutant clones. (A) *spen*^{XFM911} mutant clones. The unpigmented region corresponds to *spen* null tissues. (B) overexpression of CspenNLS383 driven by a weak *sevenless* promoter 2B-Gal4. Note the elongated rhabdomeres and the combination of missing and extra photoreceptors in both genotypes.

spen^{XFM911}

CspenNLS383;
2B-Gal4

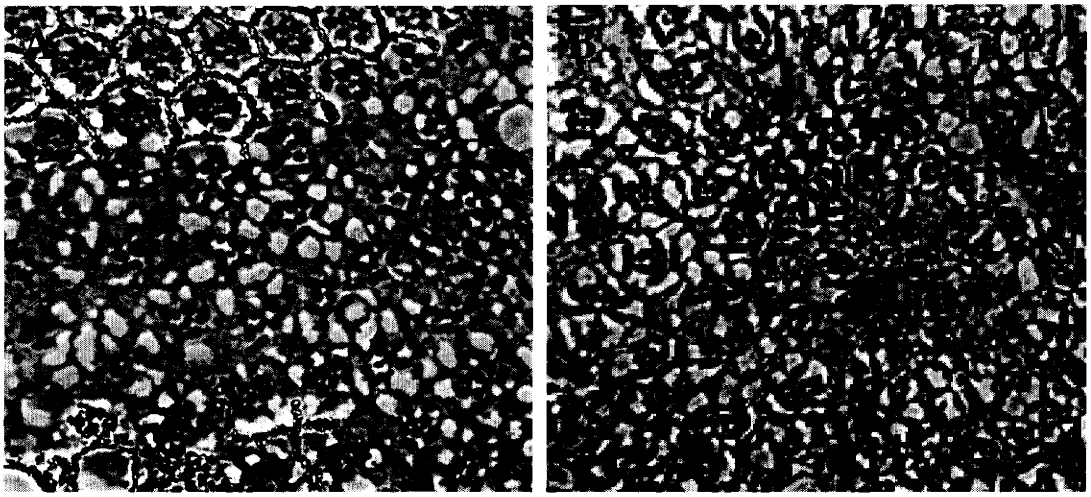


Figure 3. Overexpression of CspenNLS diminishes the Cut expression in cone cell precursors.

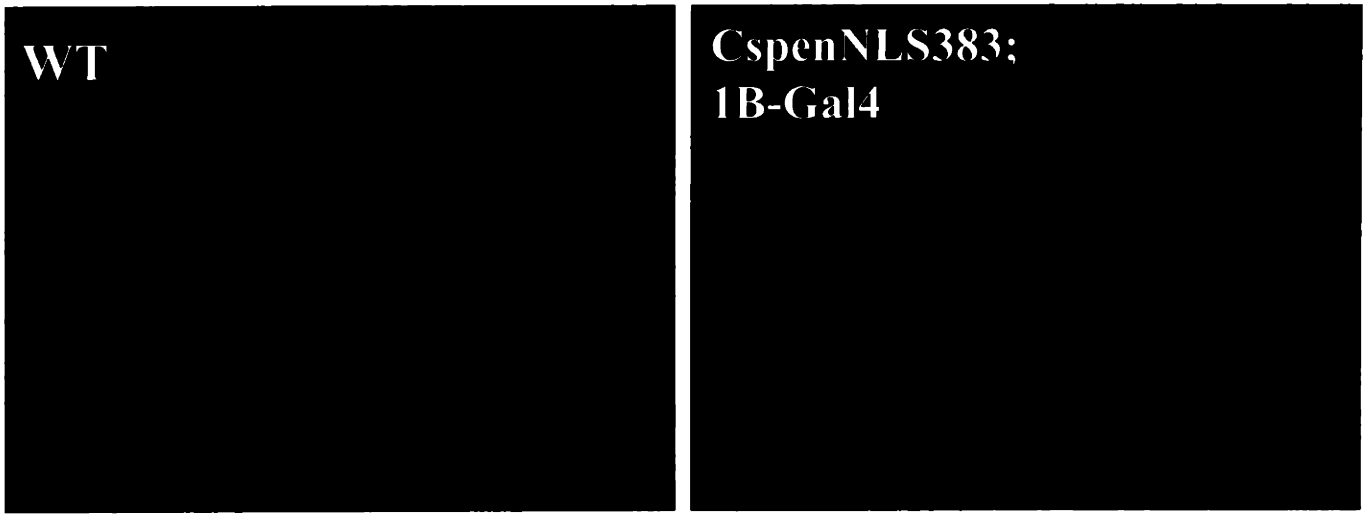

WT
CspenNLS383;
1B-Gal4

Figure 4. CspenNLS functions as a dominant negative mutant in vivo. (A) Mild disorganization in the adult retina caused by expression of CspenNLS381 driven by 2B-Gal4, a weaker sevenless promoter line. (B) Reducing *spen* dosage obviously enhances the phenotype. (C) Loss of photoreceptors and polarity in the adult retina caused by expression of CspenNLS381 driven by 1B-Gal4, a stronger *sevenless* promoter line. (D) Reducing *spen* dosage greatly enhances the overall disorganization and causes rhabdomere malformation.

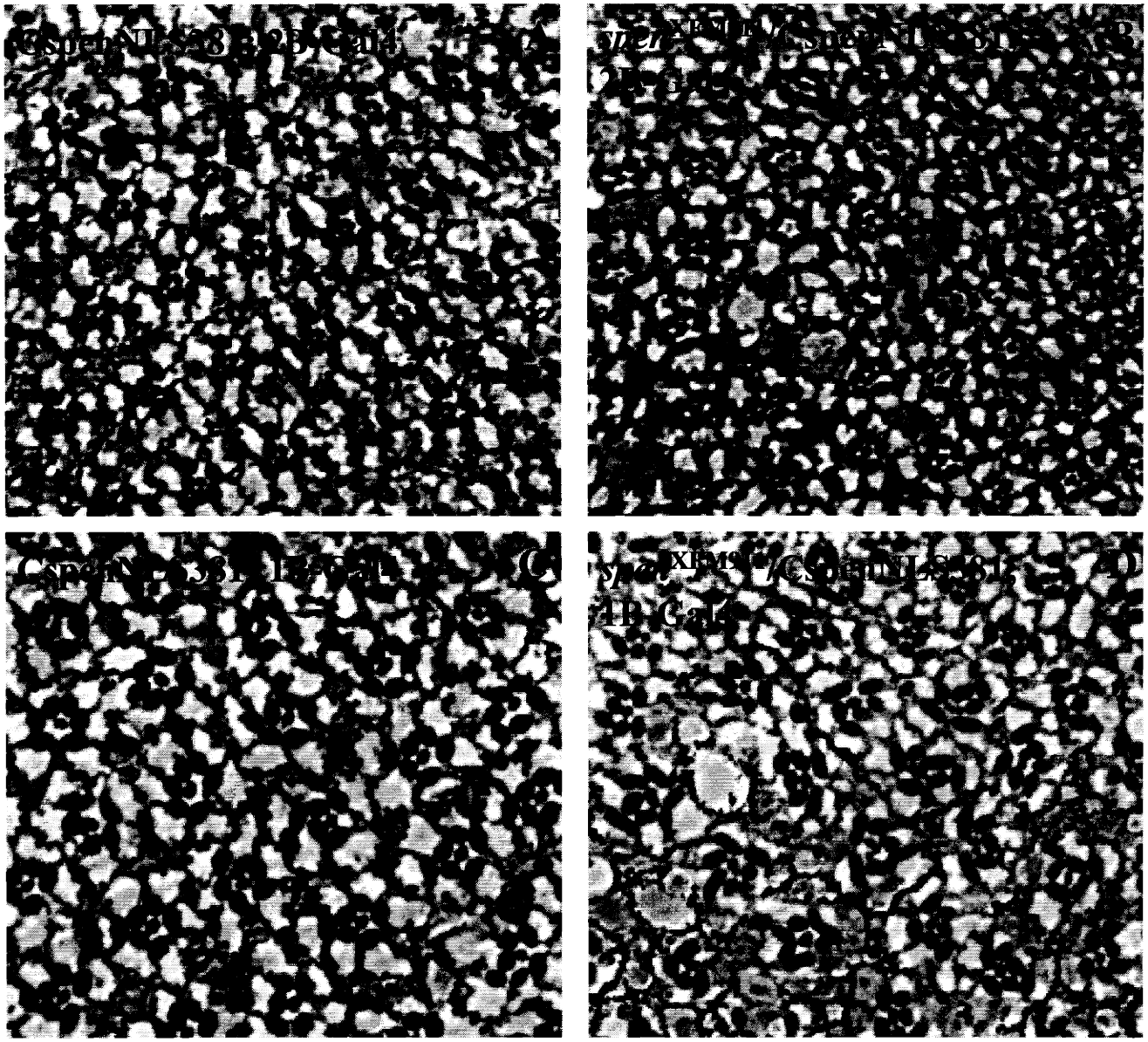
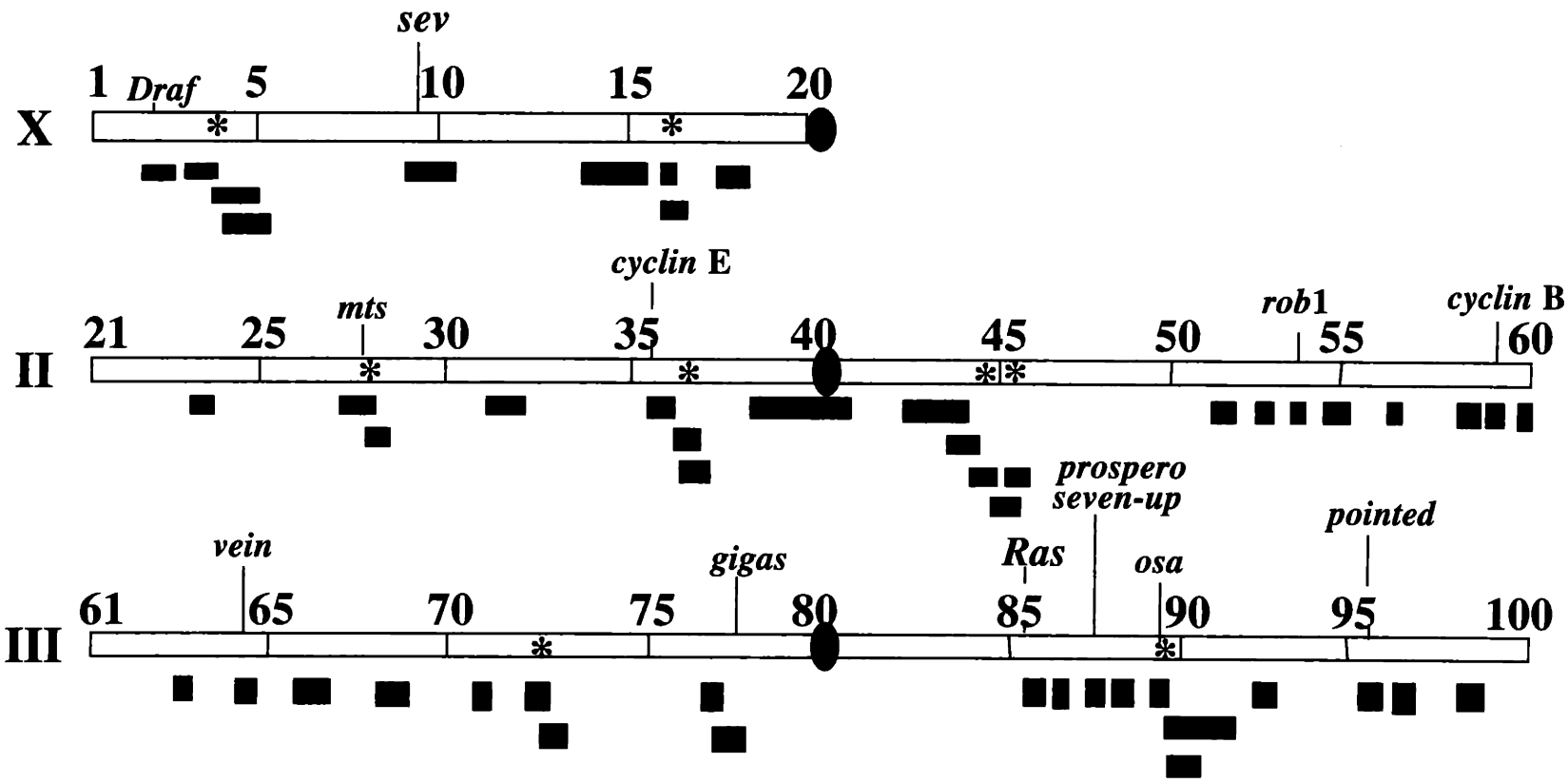


Figure 5. Summary of the deficiency kit screen. A graphic depiction of the X, 2nd and 3rd chromosomes is shown. The numbers indicate cytological position along chromosomes. Centromeres are indicated by gray ovals. The red blocks represent enhancer regions of CspenNLS, while the blue blocks represent the suppresser regions. Asterisks indicate the regions where overlapping deficiencies were isolated as modifiers.

Summary of the screen



enhancer suppressor * regions isolated by overlapping defs.

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Chapter 5

Conclusions and Discussion

Conclusions:

We have isolated *split ends* as a strong enhancer of the rough eye phenotype associated with constitutively active Yan (Yan^{ACT}), suggesting that *spen* may be a positive regulator or effector of the RTK/Ras signaling pathway. Molecular characterization of Spen reveals that Spen contains 3 RNA Recognition Motifs (RRMs) in tandem at its N-terminus, suggesting that Spen is a putative RNA-binding protein. Spen also contains a highly conserved C-terminal SPOC domain. Spen-like proteins exist from *C. elegans* to humans, and they likely define a novel subfamily of RNA-binding proteins based on the RRM sequence similarities. Characterization of *spen* mutant phenotypes in the context of RTK signaling suggests that *spen* is required for Drosophila eye development and wing vein formation, both tissues in which RTK, and especially DER, signaling plays essential roles.

We have focused our analysis on embryonic midline glial development and have demonstrated that *spen* function is required for the normal midline glial cell migration and survival. Loss of *spen* function leads to aberrant migration and as a result, a reduced number of midline glial cells. Consequently, *spen* mutants exhibit severe CNS morphology and axon guidance defects. This phenotype is strikingly reminiscent of those seen in *spitz* group genes, consistent with our hypothesis that *spen* is a positive regulator of the DER signaling pathway. Further supporting this hypothesis, we demonstrated that *spen* synergistically interacts with *pointed*. We have also showed that the C-terminal truncated Spen (SpenΔC) functions as a dominant negative mutant *in vivo*, and that overexpression of SpenΔC specifically in the midline glia causes lethality. Furthermore, we have demonstrated that the lethality associated with SpenΔC can be rescued by overexpression of activated Ras^{V12} and activated ligand Spitz. Since SpenΔC mutually suppresses the lethality caused by Ras^{V12}, *spen* is likely to function genetically

downstream of or in parallel to Ras. The implication of a putative RNA binding protein downstream of Ras suggests that there might be post-transcriptional gene regulation mechanisms downstream of the RTK pathway to allow cells to quickly and precisely response to extracellular signals.

In order to elucidate the mechanisms underlying *spen* function in the RTK pathway, we designed a genetic screen to isolate *spen*-interacting genes. The Spen C-terminus contains a highly conserved SPOC domain, which is we have shown to be functionally important. Overexpression of an NLS-tagged Spen C-terminus (CspenNLS) specifically in the eye causes a dominant rough eye phenotype. Reducing endogenous *spen* dosage enhances this phenotype, suggesting that CspenNLS functions as a dominant negative mutant *in vivo*. It also indicates that the eye phenotype is dosage sensitive, suggesting it may be a good background in which to perform a genetic modifier screen designed to isolate *spen*-interacting genes. Using this rough eye phenotype as starting background, we have screened through the deficiency kit which uncovers most of the Drosophila genome and isolated 23 enhancing and 27 suppressing regions. Among the modifiers, there are regions uncovering known RTK pathway components, including *Draf*, *sevenless*, *Ras*, *vein*, *seven-up* and *pointed*, consistent with *spen* as a component of the RTK pathway. Most importantly, we have isolated multiple overlapping deficiencies as modifiers of *spen*, suggesting that those overlapping regions might contain genes specifically interacting with *spen*. Good candidate genes were suggested and discussed, and further genetic and biochemical analysis of the candidate genes will shed important light on the molecular mechanisms underlying Spen function.

Discussion:

Possible redundancy and complexity of *spen* function

Despite the overall similarity in phenotype between *spen* and DER pathway mutants, there are differences. *spen* mutant phenotypes are less severe than those of *pointed*, *rhomboid*, *Star* and *spitz*. In the Spitz group mutants, the majority of the midline glia die by stage 16, while in *spen* mutants, the percentage of missing midline glia, as judged by either Slit or enhancer trap P[slit 1.0 lacZ] expression, is at most ~50%. Consequently, the axon guidance defects associated with loss of *spen* function are less severe. One explanation for these differences is possible redundancy of Spen function. Blast searches of Drosophila genomic sequence predict the existence of a second Spen-like gene, referred to as Dm44A(Rebay et al., 2000; Wiellette et al., 1999). Although the predicted protein is smaller than Spen, the overall structure is similar. Both proteins contain 3 RRM motifs that are ~33% identical and ~50% similar at the amino acid level as well as a highly conserved C-terminal SPOC domain (~60% amino acid identity, ~70% similarity). Although there is currently no biological characterization of this second Spen-like gene, it is plausible that it might be functionally redundant with Spen during development.

Our preliminary characterization of Dm 44A expression during embryogenesis supports this possibility since Dm44A exhibits a very similar expression pattern to *spen* (data not shown). Even if the usual functions of these two genes were not completely overlapping, there are an increasing number of examples where if the function of a critical gene is lost, a related family member may be able to partially compensate for the loss by performing functions it would not normally carry out under wild-type conditions (for example, MAPK redundancy in yeast(Madhani et al., 1997)). Thus in *spen* loss-of-function mutants, it is possible that even if the Dm44A gene product is not normally required for midline glial development, it might be

recruited to function in the place of Spen. In either scenario, the end result would be partial rescue, thereby explaining the relatively less severe midline defects in *spen* mutants. Redundancy between *spen* and Dm44A could also explain why *spen* phenotypes appear restricted to the CNS midline despite ubiquitous expression throughout embryogenesis. Functional analysis of the Dm44A Spen-like gene will be required to explore these possibilities.

Another possible explanation for the difference in phenotype observed between *spen* and DER pathway mutations is that *spen* may not have a simple linear function in the DER pathway. Supporting this idea, it has been reported previously that *spen* function in Ras signaling may be complicated(Dickson et al., 1996). We recovered *spen* as an enhancer of Yan^{ACT}, suggesting a positive function for *spen* in the pathway. However, *spen* alleles were also isolated as enhancers of an activated Raf transgene, Raf^{torY9}, implying an antagonistic function in the pathway(Dickson et al., 1996). To further complicate the issue, *spen* also enhances the *Raf* loss-of-function phenotype (Rebay, I. unpublished result; Dickson et al., 1996).

Consistent with these apparently contradictory genetic interactions, *spen* null clones in the adult retina contain both missing photoreceptors and extra photoreceptors, phenotypes commonly associated with loss of function mutations in positive and negative regulators of RTK signaling, respectively (Dickson et al., 1996); chapter 2). One possible explanation for these opposite phenotypes is that Spen might be involved in a feedback regulatory loop associated with the DER/Ras/Yan pathway, providing both positive and inhibitory functions. Alternatively, Spen could function in a parallel pathway whose output is integrated with RTK-mediated signals at multiple levels. Because of the complexity of the *spen* phenotypes in the larval and adult tissues, and the necessity to remove both maternal and zygotic function for embryonic studies, epistasis experiments to determine where *spen* functions within the hierarchy of the RTK

signaling cascade will not be trivial. Understanding the biochemical function of Spen and its interaction partners will be of critical importance to determining its role in the RTK/Ras/Yan pathway.

Spen may mediate post-transcriptional gene regulation downstream of the RTK/Ras signaling pathway

The molecular mechanisms underlying Spen function in the RTK/Ras pathway are unclear. Given its membership in the RNA binding protein superfamily, Spen might mediate post-transcriptional regulation of gene expression in the RTK pathway. Post-transcriptional regulation, including RNA splicing, RNA transport, RNA stabilization, RNA degradation and RNA translation, has been recognized as an important regulatory mechanism during development since such regulation allows cells to quickly and precisely respond to extracellular stimuli (Siomi and Dreyfuss, 1997). Increasing evidence supports the idea that such post-transcriptional regulatory mechanisms play important roles in mitogen-activated signaling pathways. For example, in Drosophila, the RNA-binding protein Squid is required for the targeted localization of mRNA of the DER ligand *gurken*, a process essential for DER signaling during oogenesis (Norvell et al., 1999). In mammalian systems, mitogen-activated signaling pathways induce expression of the immediate early genes c-*jun* and c-*fos* (Wolfes et al., 1989). Interestingly, the RNA levels of both genes are restrictively regulated by a unique adenylate/uridylate (AU)-element in their 3' untranslated regions (UTR) (Chen and Shyu, 1995). A number of AU-rich sequences in the 3' UTR of both genes function as RNA destablizing elements. It has been shown that deletion of those regions from the c-*fos* 3' UTR greatly increases the stability of the c-*fos* RNA, resulting in conversion of the c-*fos* proto-oncogene into

an oncogene(Chen and Shyu, 1995). Although the mechanism underlying c-*jun* and c-*fos* RNA destablization is far from understood, the existence of such phenomena downstream of mitogen-activated signaling pathways suggests that post-transcriptional RNA regulation is an important aspect of gene regulation.

In order to dissect the molecular basis underlying Spen function in the RTK signaling pathway, we have taken a genetic approach to isolate Spen-interacting genes, as described in Chaper 4 of this thesis. However, biochemical experiments should also be considered. For example, *in vitro* RNA or DNA binding assays will help to first distinguish whether Spen is an RNA-binding protein or a DNA-binding protein since the mouse Spen orthologue MINT has been shown to bind DNA in vitro. Once the nucleic acid binding preferences of Spen are known, these assays can be used as screens to isolate potential Spen targets. In addition to identifying DNA and/or RNA sequences of Spen targets, it will be equally important to determine what proteins physically associate with Spen. Yeast two-hybrid screens using different domains of Spen as bait, in particular the RRMs, the coiled-coil region, and the C-terminus, can be used to isolate interacting proteins. Top priority in such a screen should be given to the highly conserved Spen C-terminal SPOC domain that we have shown to be functionally important. The proteins isolated from such screens can be compared to the genes isolated from the CspenNLS genetic modifier screen, and the ones isolated from both screens should be the focus for further studies.

Spen may mediate cross-talk between Ras, Hox and cell cycle pathways

Spen mutations have been recovered in a variety of genetic screens, implying a potentially complex and pleiotropic function during development. *spen* was originally recovered as a

mutation affecting axon growth in the embryonic CNS (Kolodziej et al., 1995). In addition to isolation of *spen* in Ras pathway based screens, *spen* alleles have also been identified because of interactions with the Hox gene *deformed* in the embryo and with the cell cycle genes dE2F and dDP in the eye(Staehling-Hampton et al., 1999; Wiellette et al., 1999). In the embryo, *spen* functions in parallel with the head patterning genes *Deformed* and *empty spiracles* to promote sclerite formation, while in the thorax, it acts in concert with *Antennapedia* and *teashirt* to repress head development. In the eye, *spen* dominantly enhances phenotypes associated with GMR driven expression of *dE2F* and *dDP*, while it suppresses the phenotypes of GMR driven expression of the cell-cycle inhibitor p21. These results suggest that *spen* functions to antagonize cell-cycle progression in Drosophila eye development.

While Spen could play completely distinct roles in these three processes, it is tempting to speculate that Spen might somehow serve to mediate cross-talk between the RTK/Ras signaling pathway, Hox gene regulation of tissue identity, and the cell-cycle machinery. Increasing evidence suggests that such crosstalk mechanisms must exist between these pathways to coordinate normal development. For example, in humans, upregulation of certain Hox genes, such as HoxA9, results in overproliferation of hematopoietic stem cells and has been implicated in leukemia formation (Chiba, 1998). In Drosophila, the *proboscipedia* homeotic phenotypes are enhanced by reduction of Ras activity (Boube et al., 1997). In *C. elegans* vulval development, the normal vulval cell fates are determined by coordinated regulation of the *lin-39* Hox gene activity with both activation of the Ras signaling pathway and suppression of the Rb/E2F/DP pathway (Lu and Horvitz, 1998; Sengupta and Bargmann, 1996). Because Spen homologues exist in all these organisms, and at least in Drosophila *spen* function appears important for these three processes, *spen* may be a good candidate as a coordinator of cell cycle regulation, the RTK

signaling pathways and Hox-mediated activation of specific developmental pathways. More detailed genetic and biochemical analyses of *spen* function in flies, worms and mammals will be important to help us understand how these three pathways are inter-regulated during animal development.

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Appendix A:

Identification of missense mutations in Son-of-sevenless suggests potential novel functions of this protein

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Note:

*** Andrina Williams helped map EY2-3 and did the retinal sections in Figure 2.**

 # Xun Zhao in Peter Kim's laboratory helped with the structure simulation in Figure 5.

Abstract

EY2-3 was isolated as a strong dominant enhancer of the rough eye phenotype caused by overexpressing a constitutively active form of Yan (Yan^{ACT}). Yan is an ETS-domain containing transcription repressor which functions downstream of the MAPK cascade as a negative regulator of the Ras signal transduction pathway. EY2-3 mutations are homozygous lethal, although certain transheterozygous allelic combinations give rise to adult escapers with rough eyes. Retinal sections of these eyes reveal missing photoreceptors, mostly R7s. Analysis of genetic interactions between EY2-3 and *yan* shows that EY2-3 suppresses the eye phenotype associated with the hypomorphic yan^{1} allele. Conversely, reduction of *yan* activity suppresses both the lethality and the eye phenotypes of EY2-3. EY2-3 maps to the cytological region 33 on the left arm of the second chromosome. All the alleles of EY2-3 fail to complement null alleles of *son of sevenless* (*sos*) suggesting that EY2-3 is allelic to *sos*. The one exception is M98, which is the EY2-3 allele that shows the strongest enhancement of Yan^{ACT}. However, sos^{null} alleles are not good enhancers of Yan^{ACT}. Consistent with its role as a Ras guanine nucleotide exchange factor, sos^{null} alleles strongly suppress the rough eye phenotype caused by the activated mutation Ras^{V12}. In contrast, the alleles of EY2-3 which strongly enhance Yan^{ACT}, hardly suppress Ras^{V12}. Sequencing the allele M98, which dramatically enhances Yan^{ACT}, identifies a conserved D296N mutation at the N-terminal Dbl Homology (DH) domain in the SOS protein. The DH domain of SOS is proposed to function as a guanine nucleotide exchange factor of Rho family small GTPases. Our results led us to suggest that in addition to its function as a Ras GEF, SOS may mediate a Ras-independent signaling pathway connecting to Yan.

Introduction

Members of the Ras superfamily of small GTPases function as molecular switches by constantly cycling between active GTP-bound and inactive GDP-bound states (Bourne et al., 1991). The GTP-bound state is promoted by guanine nucleotide exchange factors (GEFs) by stimulating the exchange of GDP for GTP. Distinct GEFs are used for members of different subfamilies of small GTPases. For instance, Son-of-sevenless is a Ras GEF (Bonfini et al., 1992), while proteins containing Dbl homology (DH) domains function as GEFs for the Rho/Rac/CDC42 subfamily small GTPases (reviewed by Cerione and Zheng, 1996). Because of their unique molecular features, members of the Ras superfamily are important signal transducers in a variety of signaling pathways. For example, Ras mediate signals from receptor tyrosine kinases (reviewed by van der Geer et al., 1994). Rho/Rac/CDC42 subfamily members are involved in signaling pathways regulating cytoskeleton reorganization (reviewed by Hall, 1998).

One of the key questions in the signal transduction field is how the signaling pathways crosstalk with each other. Both genetic and biochemical data suggest that the RTK/Ras signal transduction pathway is not a simple linear pathway, but is instead part of a complex signaling circuitry. One important aspect of this crosstalk network is the interaction between Ras and other small GTPase mediated signaling pathways. It has been proposed that Rho/Rac/CDC42 are downstream effectors of Ras based on biochemical studies in mammalian systems (Qiu et al., 1995). However, the link between activated Ras and Rho/Rac/CDC42 is poorly understood. One candidate for coupling the Ras and Rho family signals is Son-of-sevenless. Son-of-sevenless contains a CDC 25 homologous region in the C-terminus, conferring its Ras GEF activity (Simon et al., 1991). Interestingly it also contains DH and PH domains in tandem at the

N-terminus, which is characteristic of Rho family GEF structure (Soisson et al., 1998). It has been shown that the N-terminus of mouse Sos can function as a GEF specifically for Rac in cultured cells (Nimnual et al., 1998). Nothing is known about the *in vivo* function of Sos as a GEF for the Rho subfamily small GTPases.

In order to better understand RTK signaling, a genetic screen was designed to isolate new components of RTK pathway based on modification of the eye phenotype associated with Yan^{ACT} (Rebay et al., 2000). One strong enhancer group isolated from this screen, EY2-3, turned out to be allelic to *son-of-sevenless*. Here we show that this Yan-based screen selected special alleles of *sos*, whose interaction with Yan^{ACT} appears to be Ras-independent. Sequencing of two alleles of EY2-3 reveals that they both contain missense mutations in the conserved N-terminal Dbl Homology (DH) domain and the linker region between the DH-PH domain and CDC25 homology domain. We suggest that further study of these EY2-3 alleles of *sos* may lead to discovery of a new function of Sos.

Results

EY2-3 was isolated as a strong enhancer of Yan^{ACT} and its function is required for normal eye development

EY2-3 was isolated as a strong enhancer of Yan^{ACT}. It enhances the rough eye phenotypes caused by both Sev-Yan^{ACT} and GMR-Yan^{ACT}(Figure 1 and Rebay et al., 2000). It also enhances the eye phenotype of a gain-of-function yan^{S2382} allele (Rebay et al., 2000). These results suggest that EY2-3 normally functions to antagonize *yan* function. While *yan* is a negative regulator of RTK/Ras signal transduction pathway, EY2-3 is likely a positive regulator of the pathway.

EY2-3 is homozygous lethal, but transheterozygous allelic combinations occasionally give rise to adult escapers with moderate rough eyes. We notice that one particular allele M98 gives rise to such escapers when it is transheterozygous with every other EY2-3 allele (Table 1). Interestingly, M98 happens to be the strongest enhancer of Yan^{ACT} among this group. Retinal sections of the eyes of the escaper flies revealed missing photoreceptors (Figure 2). The percentage of ommatidia with missing photoreceptors ranges from 27 % to 82 % depending on the allelic combinations (Table 1). Our results strongly suggest that EY2-3 plays an important role during photoreceptor development.

EY2-3 and *yan* mutually suppress each other

In order to further investigate the relationship between EY2-3 and *yan*, we conducted a series of genetic experiments. We first examined the effect of reducing *yan* dosage on EY2-3 phenotypes and found that reduction of *yan* partially suppresses the lethality of EY2-3 (Table 2). For example, the number of escapers from $E2\text{-}3^{UV349}/E2\text{-}3^{M98}$ increases from 4 out of 86 to 12 out of 46 with yan^1 in the background. Similar results were observed in $EY2\text{-}3^{XHL777}/EY2\text{-}3^{M98}$ transheterozygotes (Table 2). In addition, we found that reducing *yan* activity suppresses EY2-3 eye phenotypes (Table 3). For example, in escapers from $EY2\text{-}3^{UV349}/EY2\text{-}3^{XHL777}$ transheterozygotes, the percentage of ommatidia with missing photoreceptors decreases from 27 % to 10 % when yan^1 is in the background. Similar results were observed in escapers from $EY2\text{-}3^{UV349}/EY2\text{-}3^{M98}$ transheterozygotes (Table 3). Conversely, we found that EY2-3 suppresses yan^1 eye phenotypes. Since *yan* is a negative regulator of RTK signaling pathway, 95 % of ommatidia in yan^1/yan^1 escapers contain extra photoreceptors. Reducing EY2-3 activity by introducing $EY2\text{-}3^{UV349}$ into the background reduces the abnormal ommatidia to 73 % (Table 4).

Furthermore, when we combined the yan^1/yan^1 homozygous mutation with the EY2-3^{UV349}/EY2-3^{XHL777} transheterozygous mutation, we found that *yan* and EY2-3 mutually suppress each other. In the double mutant flies, the percentage of ommatidia with extra photoreceptors reduces to ~24 % from ~96 % in yan^1/yan^1 flies, and the percentage of ommatidia with missing photoreceptors reduces to ~5 % from ~27 % in EY2-3^{UV349}/EY2-3^{XHL777} escapers (Table 5). Our genetic interaction data strongly suggest that EY2-3 and *yan* antagonize each other, which is consistent with our hypothesis that EY2-3 is a positive regulator of RTK pathway.

EY2-3 is allelic to *son-of-sevenless*

During the process of cloning EY2-3, we mapped it to the cytological region 33 on the 2nd chromosome, which contains a known component of the Ras signaling pathway, *son-of-sevenless*(*sos*). Complementation tests between alleles of EY2-3 and *sos* reveal that most of the EY2-3 alleles, except M98, fail to complement *sos*. EY2-3^{M98}/Df(*sos*) transheterozygous flies are viable with mild rough eyes. Our results suggest that EY2-3 is allelic to *sos*. The behavior of EY2-3^{M98} suggests that it is a special allele.

***sos*^{null} alleles and EY2-3 alleles interact differently with Yan^{ACT} and Ras^{V12}**

Since *sos* encodes a guanine nucleotide exchange factor of Ras, the most obvious mechanism for *sos* to enhance Yan^{ACT} is through its direct interaction with Ras. So the prediction from this would be that the *sos* null alleles, which have totally lost the Ras GEF function, should be the strongest enhancers of Yan^{ACT}. However, we noticed interesting discrepancies between the EY2-3 alleles and the existing *sos* alleles. First we found the existing *sos* null alleles, including the deficiency uncovering *sos*, only weakly enhance Yan^{ACT}, while

some of the EY2-3 alleles, and especially EY2-3^{M98}, strongly enhance Yan^{ACT} (Table 6). In addition, consistent with its role as a Ras GEF, the *sos* null alleles strongly suppress the rough eye phenotype associated with Ras^{V12}. However, none of the EY2-3 alleles isolated from Yan screen suppresses the Ras^{V12} phenotype strongly. In fact, strong enhancers of Yan^{ACT} like EY2-3^{M98} and EY2-3^{UV349} slightly enhance Ras^{V12} phenotype (Table 6, Figure 3). Our results led us to hypothesize that some of the EY2-3 alleles isolated from the Yan^{ACT} modifier screen are special alleles of *sos* , which might reveal a novel Ras-independent function of *sos*.

Sequencing of EY2-3^{M98} identified a D296N substitution in a highlyconserved DH domain region at the N-terminus of Sos

As a first step to test our hypothesis, we sequenced two alleles of EY2-3, EY2-3^{M98} and EY2-$3^{Xmn1025}$. In both alleles, we identified single amino acid substitutions outside of the predicted Ras GEF domain. In EY2-3^{M98}, there is a D296N missense mutation in a highly conserved DH domain located in the N-terminus of Sos. In EY2-$3^{Xmn1025}$, there is a D622V missense mutation located in a linker region between the N-terminal DH-PH domains and Ras GEF domain (Figure 4). Structure simulation of EY2-3^{M98} mutation reveals that the D296N mutation is located in a highly negatively charged protein surface, which is likely to be involved in protein-protein interactions (Figure 5).

Discussion

One of our strong enhancer groups of Yan^{ACT}, EY2-3, turned out to be allelic to *son-of-sevenless*. However, our genetic interaction data suggests that the alleles of EY2-3 behave differently than the existing *sos* alleles, most of which were isolated as enhancers of *sevenless*

(Simon et al., 1991). This difference is noted when comparing their interactions with both Yan^{ACT} and Ras^{V12}. While the EY2-3 alleles strongly enhance the rough eye phenotype associated with Yan^{ACT}, the sos^{null} alleles only weakly enhance it. While the sos^{null} alleles strongly suppress the rough eye phenotype caused by Ras^{V12}, the EY2-3 alleles hardly suppress it. These results led to a hypothesis that the enhancement of Yan selects special alleles of *sos* which might reveal a potential Ras-independent function of Sos. Sequencing of two EY2-3 alleles EY2-3^{M98} and EY2-$3^{Xmn1025}$ supports our hypothesis by identifying, in both alleles, single amino acid substitutions in regions outside of the predicted Ras-GEF domain of Sos.

EY2-3^{M98} is especially interesting to us for the following reasons. First, EY2-3^{M98} dramatically enhances the rough eye phenotype of Yan^{ACT}. Its enhancement of Yan^{ACT} is much stronger than any other alleles of EY2-3. However, it fails to suppress eye phenotype associated with Ras^{V12}. Second, EY2-3^{M98} complements a Df(*sos*). The flies of EY2-3^{M98}/Df(*sos*) are viable but have a mild rough eye phenotype. Furthermore, transheterozygotes of EY2-3^{M98} over every other EY2-3 allele give rise to escapers with rough eyes. Finally, sequencing of EY2-3^{M98} identified a D296N substitution in a highly conserved DH domain at the N-terminus of Sos. Our results strongly suggest that the D296N mutation in EY2-3^{M98} might disrupt a specific function of Sos, which connects to *yan* in a Ras-independent manner.

The DH domain is shared by proteins which can function as guanine nucleotide exchange factors of the Rho/Rac/CDC42 family of small GTPases (Cerione and Zheng, 1996). The function of DH domains is highly specific. It has been demonstrated that the N-terminal DH domain of mouse Sos functions as a GEF for Rac but not for Rho and CDC42 in cultured cells (Nimnual et al., 1998). Although the function of the N-terminus of fly Sos remains to be determined, it is highly possible that it might function similarly. It is intriguing to think that Sos

is a protein with two GEF domains with distinct functions. Both Ras and Rho/Rac/CDC42 GTPases are important molecular switches that function in a variety of signaling events. Ras signaling is involved in cell proliferation, differentiation and apoptosis, while Rho families are most important for cytoskeleton regulation during cell migration and morphogenesis. One of the most fascinating questions in current biology is how cells coordinate the different signal transduction pathways during development. Sos is obviously a potential coordinator between Ras and Rho/Rac/CDC42 signaling pathways. The mutation identified in EY2-3^{M98} provides us a unique opportunity to study this new function of Sos.

Materials and Methods

Drosophila strains and genetics

The following fly strains were used: EY2-3^{EK1069}, EY2-3^{D120}, EY2-3^{M98}, EY2-3^{UV349}, EY2-3^{XER522}, EY2-3^{XEQ410}, EY2-3^{XGL710}, EY2-3^{XHL777}, EY2-3^{XLY814}, EY2-3^{Xmn1025} were isolated from Yan modifying screen; *sos*^{e2H}, *sos*^{123}, *sos*^{2240} were obtained from M. Simon; *yan*^{1}, *yan*^{S2382}, CR2 (Cyo-sev-Ras^{V12}), T2B (sev-Ras^{V12}TM3) were obtained from G. Rubin; Deficiency 3138 was obtained from the Bloomington Stock Center.

Scanning electron microscopy and histology

Flies were prepared for scanning electron microscopy by fixation for 2 hr in 1% glutaraldehyde, 1% paraformaldehyde in 1 M cacodylate buffer followed by dehydration through an ethanol series and critical point drying. Fixation and sectioning of adult eyes was as described by Tomlinson et al. (Tomlinson and Ready, 1987).

Acknowledgement:

We thank Xun Zhao in Peter Kim's laboratory for helping with structure simulation.

Table 1. Transheterozygotes of EY2-3 alleles have rough eye phenotype

Transheterozygotes	% of ommatidia with missing Rs
M98/EK1069	60% (n = 244)
M98/XER522	71% (n = 165)
M98/XGL710	63% (n = 191)
M98/XLY814	47% (n = 266)
M98/XEQ410	82% (n = 159)
M98/D120	56% (n = 102)
M98/UV349	85% (n = 345)
UV349/XHL777	27% (n = 177)

n: the total number of ommatidia counted.

Table 2. Reduction of *yan* activity suppresses the EY2-3 lethality

Genotype	#Escapers/#Total	Genotype	#Escapers/#Total
UV349/M98	4/86	yan^{1} UV349/ M98	12/46
XHL777/M98	2/65	yan^{1} XHL777/ M98	13/63

Table 3. Reducing *yan* activity suppresses EY2-3 eye phenotype

Genotype	% of ommatidia with missing Rs	Genotype	% of ommatidia with missing Rs
UV349/M98	85 % (n= 345)	*yan*[1] UV349/ M98	1 % (n = 227)
UV349/ XHL777	27 % (n = 177)	*yan*[1] UV349/ XHL777	10 % (n = 314)

Table 4. EY2-3 suppresses *yan*^{1} eye phenotype

Classes of ommatidia	*yan*^{1}/*yan*^{1} (n = 418)	*yan*^{1}UV349/ *yan*^{1} (n = 278)
Wild type	3.6 %	25.5 %
Extra Rs	95.5 %	73.3 %
Missing Rs	1.0 %	1.1 %

Table 5. *yan* and EY2-3 mutually suppress each other

Genotypes	*yan*^{1}/*yan*^{1}	*yan*^{1}UV349/ *yan*^{1}XHL777	UV349/ XHL777
% of wt ommatidia	3.6 %	70.7 %	73 %
% of ommatidia with extra Rs	95.5 %	23.8 %	0 %
% of ommatidia with missing Rs	1 %	5.5 %	27 %

Table 6. sos^{null} alleles strongly suppress Ras^{V12}, while the alleles of EY2-3 which strongly enhance Yan^{ACT} hardly suppress Ras^{V12}

	CR2 (Cyo-Ras^{V12}) modification	T2B (sev-Ras^{V12}TM3) modification	Scy (sev-Yan^{ACT}) enhancement
W1118	- -	- -	- -
M98	enhance +	enhance ±	++++
Xmn1025	-	-	++
UV349	enhance ±	enhance +	++
sos^{123}	suppress +++	suppress +++	+
sos^{2240}	suppress ++++	suppress ++++	+
Df#3138	suppress +++++	suppress ++++±	-
sos^{e2H}	suppress ++++±	NA	-

Figure 1. EY2-3 was isolated as a strong enhancer of the rough eye phenotypes caused by overexpression of Yan^{ACT}. Scanning electron micrographs of adult eyes. (A) *sev*-Yan^{ACT}/+ (B) *sev*-Yan^{ACT}/EY2-3. Note the striking increase in severity of the rough eye phenotype.

sev-Yan^{ACT}/+

sev-Yan^{ACT}/EY2-3

Figure 2. Retinal sections of escapers from transheterozygotes of EY2-3 alleles reveal missing photoreceptors. (A) WT (B) M98/XGL710. Arrows point to examples of two ommatidia missing both R7 as well as an outer photoreceptor.

WT

M98/XGL710

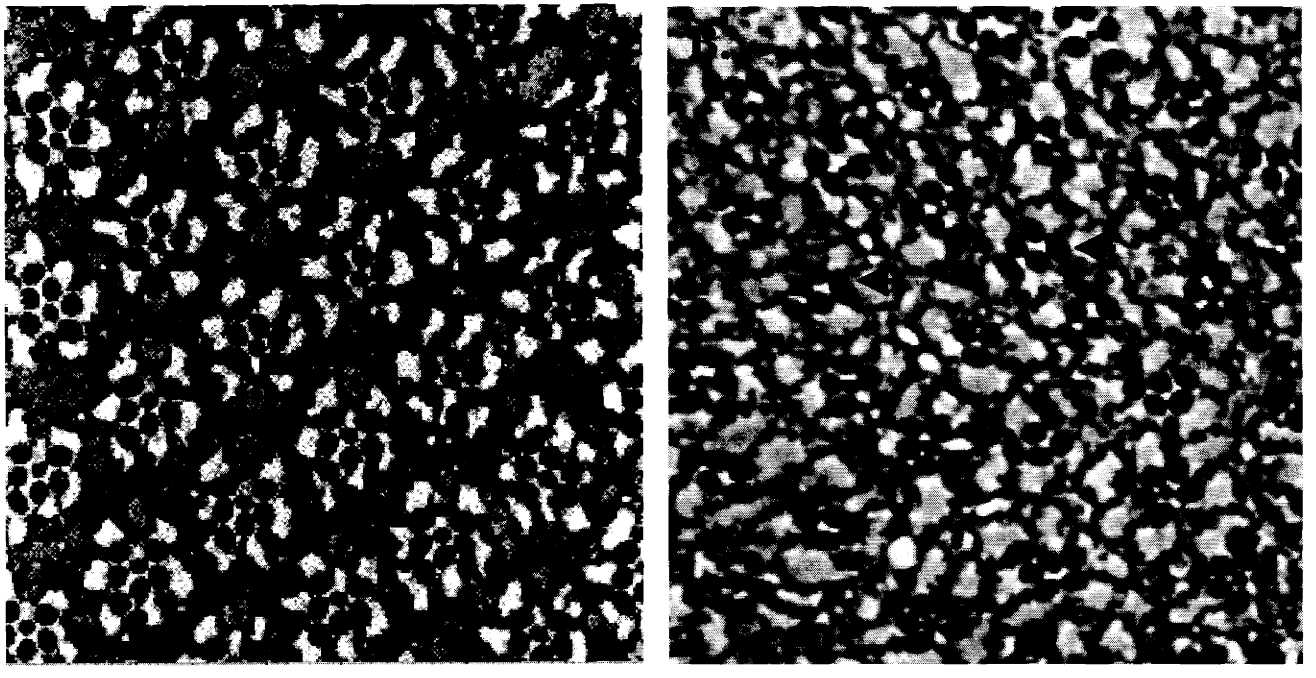


Figure 3. *sos*^{null} alleles strongly suppress the rough eye phenotypes caused by Ras^{V12}, while EY2-3^{M98} hardly suppresses Ras^{V12}. (A) CR2/+. CR2: Cyo-sev-Ras^{V12}. (B) CR2/3138. 3138: Df(*sos*). (C) CR2/M98 (D) CR2/*sos*^{e2H}. *sos*^{e2H} is a null allele of *sos*.

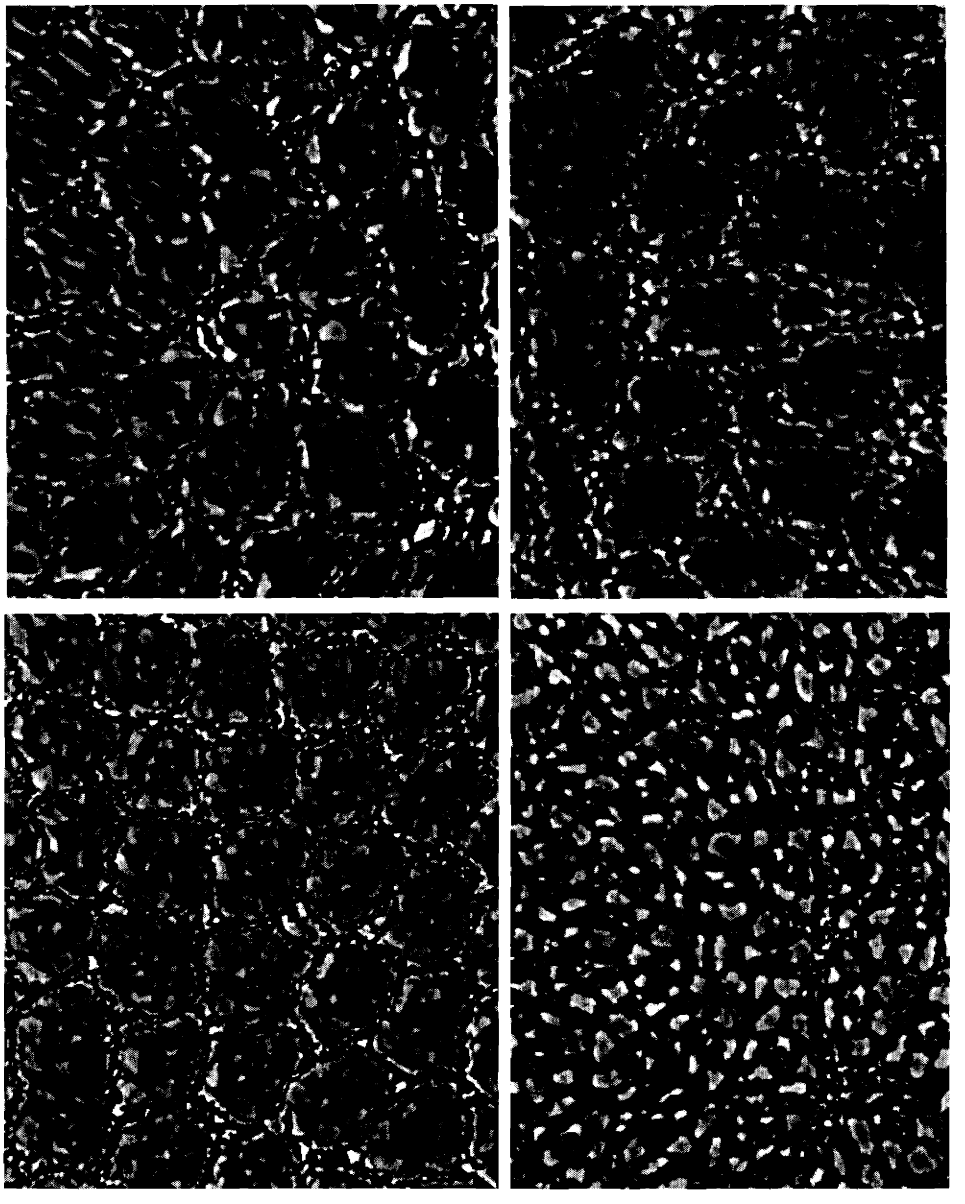


Figure 4. Schematic illustration of the mutations identified in EY2-3^{M98} and EY2-3^{Xmn1025}. M98 contains a single D296N missense mutation in the conserved Dbl Homology region at the N-terminus of the protein. Xmn1025 contains a D622V missense mutation in the linker region between the PH domain and the Cdc25 homology region. DH: Dbl Homology. PH: Plekstrin Homology.

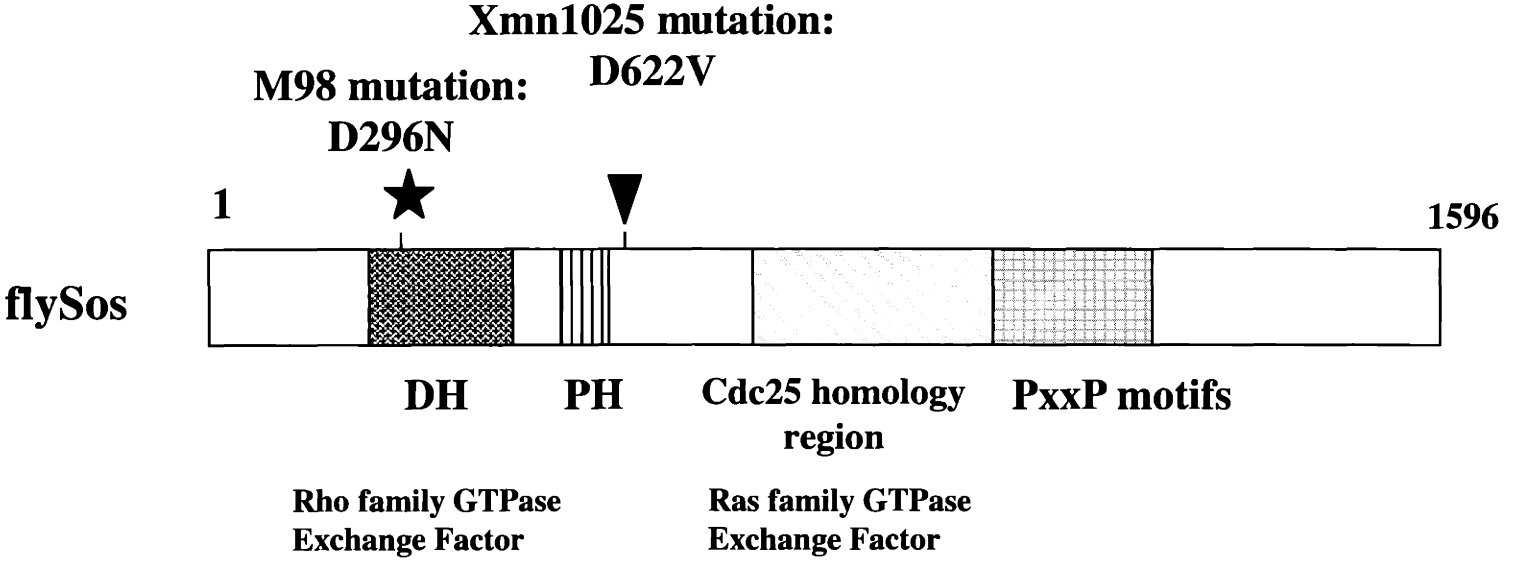
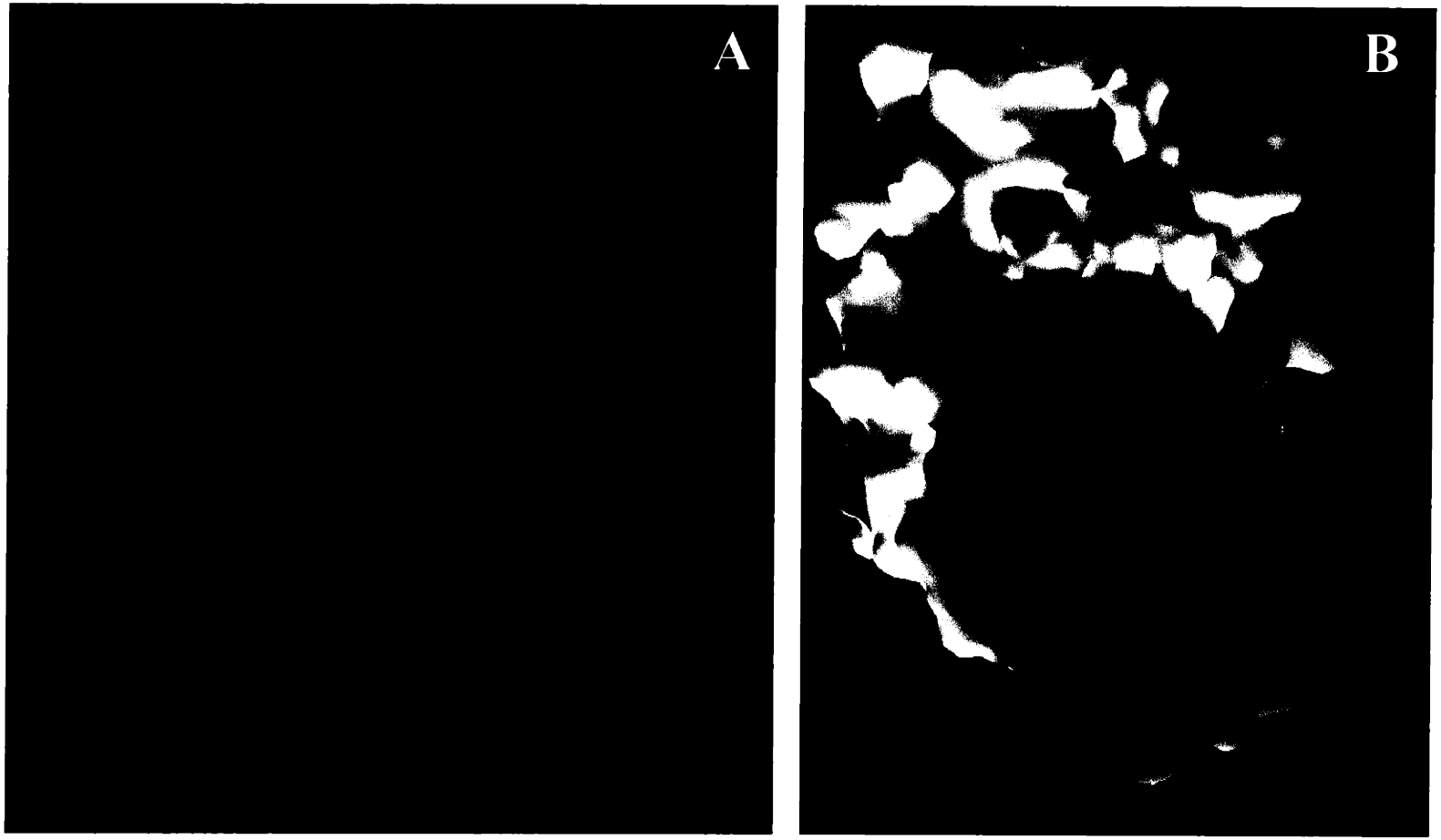

Xmn1025 mutation:
D622V
M98 mutation:
D296N
1
1596
flySos
DH
PH
Cdc25 homology region
PxxP motifs
Rho family GTPase Exchange Factor
Ras family GTPase Exchange Factor

Figure 5. Structure simulation of EY2-3^{M98} mutation (D296N) suggests that D296N is mapped to a highly negatively charged protein surface. (A) Ribbon model of the Dbl Homology domain of SOS. The mutated residue is indicated by ball-and-stick. (B) Stereo view of the Dbl Homology domain. Red indicates negative charge. Arrow points to the approximate location of the mutation.


A
B

References:

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Appendix B

Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death

Fangli Chen*, Bradley Hersh*, Barbara Conradt, Zheng Zhou, Dieter Riemer, Yosef Gruenbaum, H. Robert Horvitz

Note:
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* These authors contributed equally

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9. The Arizona site was at 34°N in high-elevation (2500 m) mixed conifer forests. This site is in the center of an extensive tract of forest with minimal human impact and containing large predators, including black bears, mountain lions, bobcats, coyotes, and foxes [T. E. Martin, *Ecology* **79**, 653 (1998)]. The Argentina site was at 26°S in the center of El Rey National Park, a large park that contained large predators, including all large cats such as jaguars, mountain lions, and ocelots. The presence of large predators is important because their loss can allow increases in mesopredators that can increase nest predation rates (*19*).
10. Nests were located using parental behavior and checked every 1 to 4 days to determine the fate of clutches and whether parents were successful in fledging at least one young or failed because of predation or other causes, following the method of T. E. Martin and G. R. Geupel [*J. Field Ornithol.* **64**, 507 (1993)].
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13. Incubation was used for this test, because parents and young do not make noise during this period, allowing clear tests of the influence of parental activity. During the nestling period, the begging noise of young could influence predation rates independently of parental activity [D. Haskell, *Proc. R. Soc. London Ser. B* **257**, 161 (1994); J. Briskie, P. R. Martin, T. E. Martin, *Proc. R. Soc. London Ser. B* **266**, 2153 (1999)].
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25. From 1993 to 1998, birds were videotaped during both incubation and nestling periods with video cameras for the first 6 hours of the day, beginning 0.5 hours before sunrise, as described in (*22*). This protocol standardized both time of day and sampling duration. All video recordings during the nestling period were made within 1 to 2 days of the time when primary feathers broke their sheaths to control for stage of development. The number of trips per hour was averaged over the 6 hours of monitoring for each nest (*22*).
26. This study was designed to allow paired comparisons of traits between latitudes (Figs. 1 and 4) using paired sample *t* tests. Paired comparisons are a strong way to compare between latitudes because they can control for both phylogeny and ecology (Table 1). Paired comparisons use contrasts between extant species that do not require estimates of branch lengths and make no assumptions about modes of character evolution (*12*, *23*). When phylogenetic paths cross, the average for nodes that do not cross is used (*24*). As a result, the two *Basileuterus* species are averaged and compared to the average of the two *Vermivora* species for all paired comparisons, yielding six paired comparisons.
27. Visitation rates were quantified as described in (*25*). The number of trips per hour was calculated for each nest and then averaged across all nests within a species to obtain the mean for each species. A minimum of six nests (6 hours each) was used (*22*), but many more nests were sampled per species in most cases.
28. Daily predation rates represent the probability per day that a nest is depredated [G. L. Hensler and J. D. Nichols, *Wilson Bull.* **93**, 42 (1981)]. Only species with $n > 20$ nests were used.
29. Relationships among species were examined while controlling for phylogeny by means of independent contrasts (*3*, *12*). Controlling for phylogeny is important because behaviors may be similar in closely related species (*12*). A phylogeny was constructed using recent phylogenetic information (*3*). We calculated linear contrasts for each node in the phylogeny using the Comparative Analysis by Independent Contrast program [A. Purvis and A. Rambaut, *Comp. Appl. Biosci.* **11**, 247 (1995)]. These independent contrasts were used to examine correlations that were forced through the origin (*12*).
30. Food loading was measured as the size of visible food in the bills of parents arriving at the nest of nestlings that had broken their primary feather sheaths within 1 to 2 days. A small (4 cm) remote telephoto camera lens (MicroVideo) was placed within 1 m of nests to allow high-resolution closeup video images and measurement of food loading. The load size was estimated by measuring bill size and using it to calibrate the area of digital video images of load size obtained from video footage using GRABIT!!. Area was used to estimate load size.
31. Hole-nesting species typically have lower predation rates and larger clutches than do open-nesting birds (*3*). Five species in Argentina that nested in holes or in complex protected nests (*Piculus rubiginosus*, *Synallaxis superciliosa*, *Syndactyla rufosuperciliata*, *Troglodytes aedon*, and *Troglodytes solstitialis*) had daily predation rates from 0 to 0.018 ($\bar{x} = 0.0066 \pm 0.003$), which is much lower than rates for the open-nesting species (Fig. 3B). However, we lacked clutch size data for three of these species.
32. We thank C. Ghalambor, J. McKay, J. Tewksbury, K. Marchetti, T. Price, and two anonymous reviewers for helpful comments; many field assistants for their help in collecting the field data; the Arizona Game and Fish Agency and Coconino and Apache-Sitgreaves National Forests for their logistical support of the Arizona work; and the Laboratorio de Investigaciones Ecologicas de las Yungas, M. Rouges, P. Marconi, and El Rey National Park staff for logistical support of the Argentina work. Supported by grants from NSF (DEB-9527318, DEB-9707598, and DEB-9900343), the U.S. Geological Survey Biological Resources Division, and the International Program of the U.S. Fish and Wildlife Service.

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Translocation of *C. elegans* CED-4 to Nuclear Membranes During Programmed Cell Death

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The *Caenorhabditis elegans* Bcl-2–like protein CED-9 prevents programmed cell death by antagonizing the Apaf-1–like cell-death activator CED-4. Endogenous CED-9 and CED-4 proteins localized to mitochondria in wild-type embryos, in which most cells survive. By contrast, in embryos in which cells had been induced to die, CED-4 assumed a perinuclear localization. CED-4 translocation induced by the cell-death activator EGL-1 was blocked by a gain-of-function mutation in *ced-9* but was not dependent on *ced-3* function, suggesting that CED-4 translocation precedes caspase activation and the execution phase of programmed cell death. Thus, a change in the subcellular localization of CED-4 may drive programmed cell death.

Programmed cell death is important in regulating cell number and cell connections and for sculpting tissues during metazoan development (*1*). When misregulated, programmed cell death can contribute to various disease states, including cancer, autoimmune disease, and neurodegenerative disease (*2*). Many of the central components of the cell death machinery have been identified through genetic studies of the nematode *Caenorhabditis elegans* (*3*). Loss-of-function mutations in any of the genes *egl-1*, *ced-3*, or *ced-4* or a gain-of-function mutation in the gene *ced-9* block programmed cell death. Loss-of-function mutations in *ced-9* cause sterility and maternal-effect lethality as a consequence of ectopic cell death and can be suppressed by *ced-3* and *ced-4* mutations but not by *egl-1* mutations, suggesting that *ced-9* acts upstream of *ced-3* and *ced-4* and downstream of *egl-1*. CED-9 is a member of the Bcl-2 family of cell-death regulators (*4*), and the EGL-1 protein contains a BH3 (Bcl-2 homology 3) domain and can physically interact with CED-9 (*5*). *ced-3* encodes a caspase (*6*), while CED-4 is similar to mammalian Apaf-1, an activator of caspases (*7*). CED-4 can bind

CED-9 and CED-3 in vitro, in yeast, and in mammalian cells (*8*), and the interaction of CED-9 and EGL-1 may influence CED-4 activity (*9*). These observations suggest a model (*3*) in which CED-3 causes programmed cell death; CED-4 activates CED-3; CED-4 is directly inhibited by CED-9 (*10*); and EGL-1 initiates cell death by directly inhibiting CED-9. To determine when and where these cell-death proteins act, we have explored physical interactions among them using immunohistochemistry.

To study the expression and subcellular localization of CED-9 and CED-4 in *C. elegans*, we generated polyclonal antibodies that recognize these proteins (*11*). Affinity-purified antibodies to CED-9 (anti–CED-9) specifically recognized bacterially expressed CED-9 and a 32-kD protein corresponding to CED-9 on a Western blot of wild-type (WT) (N2) embryo lysates; this protein was absent in *ced-9(n2812)* embryo lysates (*12*, *13*). The *ced-9(n2812)* allele contains an amber stop mutation at codon 46 and is probably a molecular and genetic null allele (*4*). Fixed embryos stained with anti–CED-9 revealed that CED-9 was present in all cells during *C. elegans* embryogenesis (Fig. 1A), beginning as early as the two-cell stage. CED-9 levels peaked at approximately the 200-cell stage and slowly diminished, becoming undetectable around the time of hatching. CED-9 protein was not observed in larvae or adults. On the subcellular level, CED-9 exhibited a weblike, cytoplasmic staining pattern. CED-9 staining was highly similar to the staining of Mitotracker Red (*14*), which specifically labels mitochondria (Fig. 1).

Anti–CED-4 recognized bacterially expressed CED-4 and detected a 63-kD protein on Western blots of N2 embryo lysates; this protein was absent in *ced-4(n1162)* embryo lysates (*12*). The *ced-4(n1162)* allele contains an ochre stop mutation at codon 79 and is probably a molecular and genetic null allele (*15*). Embryos stained with anti–CED-4 displayed a weblike pattern in all cells (Fig. 1D), very similar to the patterns of CED-9 and Mitotracker Red. CED-4 staining appeared at approximately the 100-cell stage, before the first programmed cell death, persisted through embryogenesis, and like CED-9, was not detected in larvae and adults. Of the 131 developmental cell deaths in *C. elegans* hermaphrodites, 113 occur during embryogenesis and the remainder occur during larval development. Although we have not detected CED-4 or CED-9 in larvae, *ced-4* and *ced-9* mutants are defective in larval programmed cell deaths, suggesting that the CED-4 and CED-9 proteins act postembryonically.

We examined whether the expression and localization of CED-9 and CED-4 were affected by mutations that disrupt programmed cell death. Loss-of-function mutations in *ced-3*, *ced-4*, and *egl-1*, genes required for programmed cell death, did not affect either the expression pattern or mitochondrial localization of CED-9 protein. The expression and localization of CED-4 protein was also unaffected by loss-of-function mutations in *ced-3* and *egl-1*. To determine the expression pattern and localization of CED-4 in the absence of functional CED-9 protein, we stained *ced-9(n2812)*; *ced-3(n717)* double-mutant embryos with anti–CED-4. Because *ced-9(n2812)* embryos derived from homozygous *ced-9(n2812)* hermaphrodites arrested before the appearance of visibly recognizable corpses and before CED-4 expression, these embryos could not be studied directly for CED-4 localization. Because *ced-3(n717)* did not affect the localization of CED-4 but does suppress the lethality of *ced-9(n2812)*, we instead used this double mutant to analyze CED-4 in the absence of CED-9. In *ced-9(n2812)*; *ced-3(n717)* embryos, CED-4 was not localized to mitochondria but rather was associated with nuclear membranes (Fig. 2, A to C), as visualized by double staining embryos with anti–CED-4 and antibodies directed against *C. elegans* lamin (*16*). We obtained similar results using the *ced-9* loss-of-function alleles *n1950 n2161* or *n1950 n2077* in combination with *ced-3(n717)*. Mitotracker Red staining was not altered in *ced-9(n2812)*; *ced-3(n717)* embryos, indicating that the shift in CED-4 localization represents a movement of CED-4 protein rather than a change in the morphology and/or localization of mitochondria (*17*).

To confirm this localization of CED-4 protein to nuclear membranes in *ced-9(lf)* embryos, we performed subcellular fractionations of embryo lysates (*18*). Both CED-9 and CED-4 were present predominantly in the organelle and membrane fraction, which includes the mitochondria [for example, (*19*)], in WT embryos (Fig. 3). By contrast CED-4 was present almost exclusively in the nuclear fraction in *ced-9(n2812)*; *ced-3(n717)* embryos. Thus, in WT embryos, in

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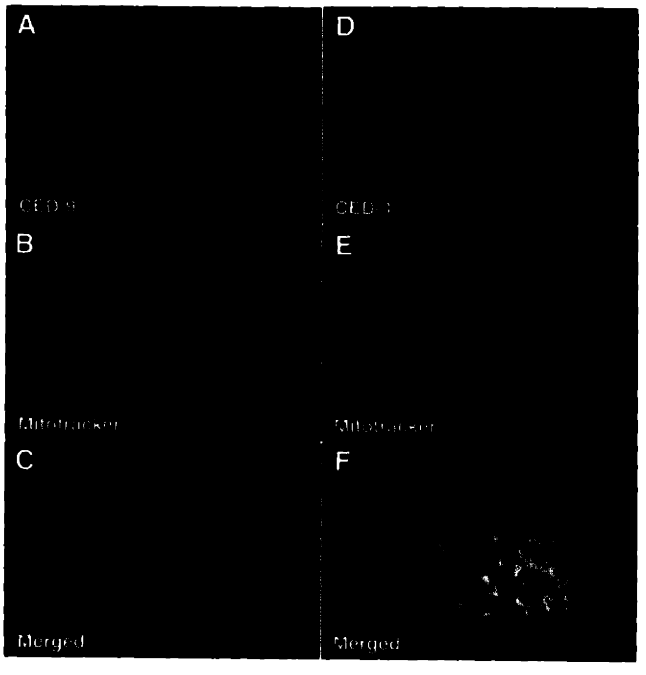

Fig. 1. CED-9 and CED-4 are localized to mitochondria in WT embryos. (**A**) CED-9 expression in a WT embryo of ~ 30 to 50 cells. (**B**) Mitotracker Red localization in the same embryo as in (A). (**C**) Merged image of (A) and (B). (**D**) CED-4 expression in a WT embryo of ~200 cells. (**E**) Mitotracker Red localization in embryo in (D). (**F**) Merged image of (D) and (E).

which most cells survive, both CED-9 and CED-4 appeared to be predominantly mitochondrial. However, in *ced-9(n2812); ced-3(n717)* embryos, in which ectopic cell death was presumably initiated but blocked by the *ced-3* mutation, CED-4 was redistributed from mitochondria to nuclei. Thus, CED-9 protein is necessary to localize CED-4 to mitochondria.

These data suggest that stimuli that induce programmed cell death would induce a redistribution of CED-4 to nuclear membranes and that it might be possible to block programmed cell death by blocking CED-4 relocalization. We tested these predictions by ectopically inducing programmed cell death in embryos.

The binding of EGL-1 protein to CED-9 may directly inhibit CED-9 function and trigger programmed cell death by releasing CED-4 from a CED-9–CED-4 complex (*5*, *9*). To determine whether EGL-1 protein can affect the localization of CED-9 or CED-4, we expressed EGL-1 protein globally from an *egl-1* cDNA under the control of two *C. elegans* heat-shock promoters (P_{hsp}*egl-1*) (*20*) in the presence of the *ced-1(e1735)* mutation, which reduces cell-corpse engulfment and allows the quantification of cells that have undergone programmed cell death (*21*). Animals carrying heat-shock vectors without the *egl-1* cDNA insert developed normally, but transgenic animals carrying P_{hsp}*egl-1* arrested during embryogenesis after heat-shock treatment. The few hatched L1 larvae contained many more cell corpses than vector-only animals, indicating extensive programmed cell death (Table 1). Localization of CED-9 was unaffected in these animals. By contrast, overexpressed EGL-1 triggered the translocation of CED-4 from mitochondria to nuclei (Fig. 4A).

We next introduced the extrachromosomal array carrying P_{hsp}*egl-1* into two strains in which programmed cell death is blocked. The *ced-3(n717)* mutation suppressed programmed cell death induced by EGL-1 overexpression (Table 1) but did not affect CED-4 translocation from mitochondria to nuclear membrane (*22*). This observation supports the idea that the release of CED-4 is not merely a consequence of cell death but rather precedes the execution of programmed cell death. Like *ced-3(n717)*, the *ced-9(n1950)* gain-of-function mutation blocked the ectopic death induced by *egl-1* overexpression (Table 1). However, unlike *ced-3(n717)*, *ced-9(n1950)* also blocked the translocation of CED-4 (Fig. 4B), suggesting that this mutant form of CED-9 either is unable to interact with EGL-1 or is unable to release CED-4. We tested the interaction of CED-9(G169E) protein, which is encoded by the *ced-9(n1950)* mutation, with EGL-1 protein both in vitro and in yeast two-hybrid experiments and were unable to detect any difference between the interactions of the CED-9 and CED-9(G169E) proteins with the EGL-1 protein (*23*). It is possible that these in vitro studies failed to reveal a defect in the interaction between EGL-1 and CED-9 sufficient to produce the gain-of-function phenotype observed in vivo in *ced-9(n1950)* animals. Alternatively, in *ced-9(n1950)* animals, EGL-1 may form a ternary complex with CED-9 and CED-4 without causing the release of CED-4. We also generated an *egl-1* heat-shock construct bearing the *egl-1(n3082)* mutation, which results in a truncated EGL-1 protein, a disruption of CED-9 binding, and a strong cell-death defective phenotype. Transgenic animals carrying this construct had significantly fewer corpses than animals bearing the WT *egl-1* construct (Table 1). CED-4 localization was predominantly mitochondrial, but in occasional animals a few cells displayed nuclear CED-4 localization. Thus, overexpression of this *egl-1(n3082)* gene resulted in a

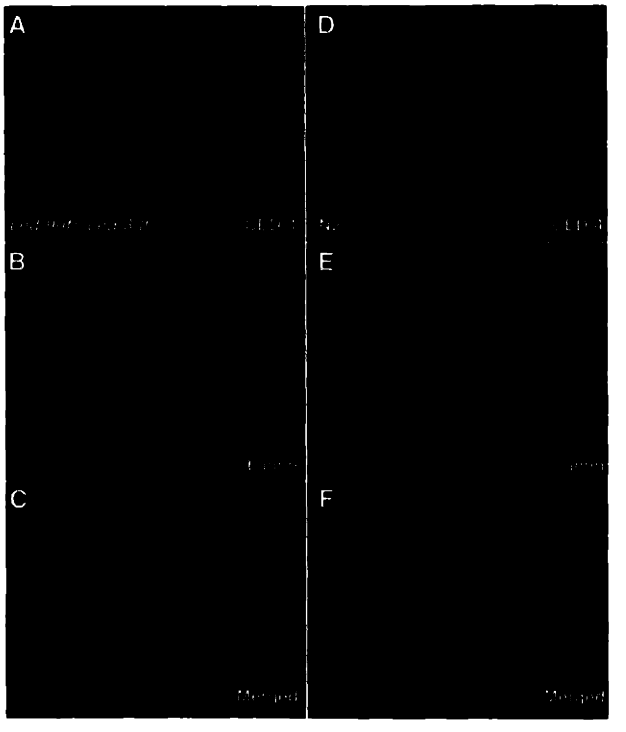

Fig. 2. CED-9 is required for the localization of CED-4 to mitochondria. (**A**) CED-4 expression in a *ced-9(n2812); ced-3(n717)* loss-of-function (*lf*) embryo of ~150 cells. (**B**) Lamin localization in the same embryo as in (A). (**C**) Merged image of (A) and (B). (**D**) CED-4 expression in WT embryo of ~200 cells. (**E**) Lamin staining of embryo in (D). (**F**) Merged image of (D) and (E).

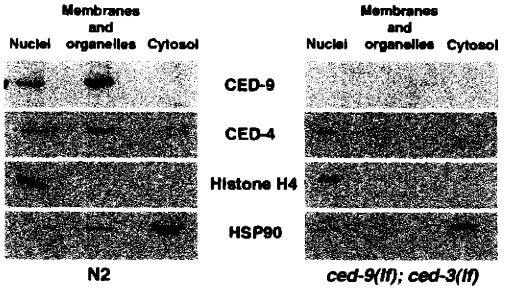

Fig. 3. CED-4 fractionates primarily with membranes and organelles from WT embryos and with nuclei from *ced-9(lf)* embryos. Western blot of subcellular fractionation (*18*) of lysates from WT and *ced-9(n2812); ced-3(n717)* double-mutant embryos, separated into nuclear, organelle and membrane, and cytosolic fractions. Histone H4, a nuclear fraction marker. HSP90, *C. elegans* heat-shock protein, a cytosolic marker.

weak partial induction of both programmed cell death and CED-4 translocation.

Overexpression of *egl-1* was sufficient to trigger both cell death and CED-4 translocation. Is *egl-1* necessary for the CED-4 translocation that occurs in the absence of CED-9? We stained *ced-9(n2812)*; *ced-3(n717)*; *egl-1(n1084 n3082)* embryos and determined that CED-4 protein was nuclear, just as in the *ced-9(n2812)*; *ced-3(n717)* embryos. Thus, in the absence of CED-9 protein, EGL-1 is not required to release CED-4 from mitochondria to nuclei, indicating that EGL-1 promotes CED-4 translocation by antagonizing the activity of CED-9.

Thus, we observed that CED-4 was mitochondrial in living cells and nuclear in cells that had initiated programmed cell death, so that the subcellular localization of CED-4 appeared to correlate with the cell-death status of a cell. We next studied the localization of CED-4 in six *ced-4* missense mutants: *n2860*, *n2879*, *n3040*, *n3043*, *n3100*, and *n3141*. In five of the six mutants, CED-4 was mitochondrially localized in the presence of CED-9 and was associated with the nuclear membrane in the absence of CED-9, as in the WT. In *ced-4(n3040)* embryos, however, CED-4 displayed a diffuse, cytoplasmic localization both in the presence and in the absence of CED-9 (Fig. 4C), distinct from the weblike mitochondrial pattern of WT CED-4. *ced-4(n3040)*, which causes a proline-to-leucine substitution at amino acid 23 (P23L) in a region that lacks any known protein motifs, results in as strong a cell-death defect as does *ced-4(n1162)*, which contains an early ochre nonsense mutation. This P23L substitution reduces the interaction between CED-9 and CED-4 by about 75% in the yeast two-hybrid assay (*24*). The failure of CED-4(P23L) to associate with either mitochondria or nuclear membranes suggests that CED-4 is actively recruited not only to mitochondria (presumably through interaction with CED-9) but also to the nucleus. Alternatively, CED-4 may first have to interact with CED-9 to be competent to translocate to nuclear membranes. That WT CED-4 associated with nuclear membranes in the absence of CED-9 argues against this latter model.

CED-9 localization to mitochondria in *C. elegans* embryos is not surprising, given that the mammalian CED-9–like cell-death protectors Bcl-2 and Bcl-X_L both localize to mitochondria (*25*). Although Bcl-X_L and the CED-4–like protein Apaf-1 have been reported to physically interact (*26*), Moriishi *et al.* (*27*) recently reported that they could find no interaction between Apaf-1 and any known anti-apoptotic Bcl-2 family member. Furthermore, there is no evidence for the localization of Apaf-1 to mitochondria. Apaf-1 was isolated as a cytosolic activator of caspases (*7*), and overexpressed CED-4 is cytosolic in mammalian cells (*8*). Therefore, the mitochondrial localization of CED-4 is unexpected.

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The death-promoting proteins Bax and BAD, which like EGL-1 contain BH3 domains, translocate to mitochondria and bind anti-apoptotic Bcl-2 family members in response to apoptotic signals (*30*). Whether and how this translocation promotes cell death is unknown. Our results suggest that Bax and BAD may act to release Apaf-1 or another CED-4–like protein, allowing it to activate caspase processing. Some caspase precursors, specifically procaspases-2, and -3, are present in mitochondria and upon activation translocate to nuclei (*31*). It is possible that this movement of caspases involves the translocation of a complex that includes a CED-4–like protein. By analogy, the translocation of a CED-4–CED-3 complex from mitochondria to the nuclear envelope could provide access for the active caspase to both the nucleus and the cytosol, thereby fulfilling the roles of the multiple, differentially localized mammalian caspases.

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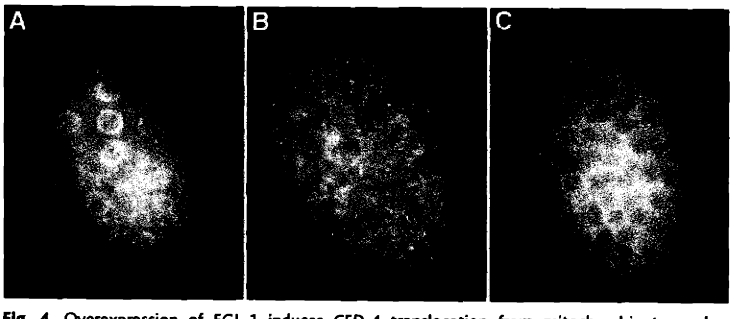

Fig. 4. Overexpression of EGL-1 induces CED-4 translocation from mitochondria to nuclear membranes in *ced-9(+)* embryos but not in *ced-9(n1950)* embryos. (**A**) CED-4 localization after heat shock in a *ced-9(+)* embryo carrying P_{hsp}*egl-1*. (**B**) CED-4 localization after heat shock in a *ced-9(n1950)* embryo carrying P_{hsp}*egl-1*. (**C**) CED-4 localization in a *ced-4(n3040)* embryo was diffusely cytoplasmic.

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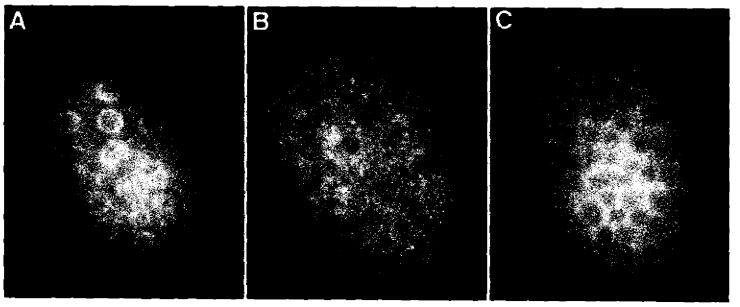

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4(P23L) mutant protein was diffusely cytoplasmic, suggests that CED-4 is recruited to nuclear membranes, possibly by interacting with another protein or protein complex. The identification of such a CED-4 receptor should help us understand the mechanism of action of CED-4 in the execution of programmed cell death.

References and Notes

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11. A partial *ced-9* cDNA was cloned into vector pET19b to generate a His-10–CED-9 fusion protein lacking the COOH-terminal 29 amino acids of CED-9. The fusion protein was purified on Ni^{2+}–nitrilotriacetic acid–agarose (Qiagen) and injected with RIBI adjuvant into rabbits. The antiserum was affinity-purified against His-10–CED-9 immobilized on nitrocellulose. For the generation of anti–CED-4, full-length *ced-4S* cDNA was cloned into pXHA [S. J. Elledge *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2907 (1992)]. The hemagglutinin–CED-4 fusion was purified from inclusion bodies of *Escherichia coli* and injected into rabbits and rats. The rabbit antiserum was used without further purification, and rat antiserum was affinity-purified against glutathione-S-transferase–CED-4 immobilized on nitrocellulose. Embryos were collected by bleaching mixed-stage worms in a 0.8 N NaOH, 8% hypochlorite solution. Embryos were fixed and permeabilized essentially as described [C. Guenther and G. Garriga, *Development* **122**, 3509 (1996)]. Embryos were incubated in a 1:100 dilution of primary antibody, washed four times, incubated for 2 hours in a 1:25 dilution of secondary antibody, washed four times, washed once in phosphate-buffered saline plus 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/ml), and resuspended in an equal volume of VECTASHIELD mounting medium (Vector Labs). Embryos for mitochondrial colocalization experiments were collected from worms grown in the dark on NGM agar plates containing MitoTracker Red CMXRos (2 μg/ml, Molecular Probes).
12. Supplementary data can be found in Web figure 1 at www.sciencemag.org/feature/data/1046764.shl.
13. Mutant strains carrying the following alleles of cell-death genes were used in this study: *ced-1(e1735)*, engulfment-defective; *ced-3(n717)*, splice acceptor mutation, exon 7; *ced-9(n2812)*, Q46amber; *ced-9(n1950)*, G169E; *ced-9(n1950 n2161)*, *ced-9(n1950 n2077)*, loss-of-function mutations; *ced-9(n1653)*, Y149N; *ced-4(n1162)*, Q79ochre; *ced-4(n2860)*, E263K; *ced-4(n2879)*, E276K; *ced-4(n3040)*, P23L; *ced-4(n3043)*, D20N; *ced-4(n3100)*, S339P; *ced-4(n3141)*, R53K; *egl-1(n1084)*, G-to-A nucleotide transition at nucleotide +5631; *egl-1(n1084 n3082)*, *n1084* lesion plus 5–base pair (bp) deletion in *egl-1* coding region.
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17. Supplementary data can be found in Web figure 2 at www.sciencemag.org/feature/data/1046764.shl.
18. Embryos for fractionation were collected as described (*11*). Embryos were resuspended in five volumes of cold hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose) and homogenized in a 1-ml Dounce tissue grinder. The homogenates were centrifuged at 40*g* briefly to remove worm debris. The supernatant was centrifuged twice at 750*g* for 10 min, and the resulting pellets were pooled as the nuclear fraction. The supernatant was further centrifuged at 100,000*g* for 1 hour. The pellet was designated the organelle and membrane fraction and the supernatant the soluble cytosolic S100 fraction. The pooled nuclear fraction was washed once with homogenization buffer. One-fifth of each fraction was used for immunoblotting analysis. Rabbit polyclonal antibody directed against human acetylated histone H4 (Upstate Biotechnology) was used as a marker for the nuclear fraction. Monoclonal anti–Ce HSP90 was used as a marker for the cytosolic fraction.
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20. P_{hsp} *egl-1* (*5*) was injected at a concentration of 2 ng/μl, along with p76-16B [L. Bloom and H. R. Horvitz, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3414 (1997)] at 50 ng/μl, into a *ced-1(e1735)*; *egl-1(n1084 n3082)* *unc-76(e911)* strain as described [C. Mello and A. Fire, *Methods Cell Biol.* **48**, 451 (1995)]. For immunofluorescence, mixed-stage transgenic worms were incubated at 33°C for 1 hour followed by a 2-hour recovery at 20°C. Embryos were then collected as in (*11*). For analysis of cell corpses, transgenic adults were allowed to lay eggs at 20°C for 2 hours, subjected to a 1-hour heat shock at 33°C, allowed to lay eggs for an additional 2 hours at 20°C, and then removed from the plates. Hatched L1 transgenic animals were examined by Nomarski microscopy for corpses in the head.
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Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF

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The molecular control of self-renewal and differentiation of stem cells has remained enigmatic. Transgenic loss-of-function and overexpression models now show that the dosage of glial cell line–derived neurotrophic factor (GDNF), produced by Sertoli cells, regulates cell fate decisions of undifferentiated spermatogonial cells that include the stem cells for spermatogenesis. Gene-targeted mice with one *GDNF*-null allele show depletion of stem cell reserves, whereas mice overexpressing GDNF show accumulation of undifferentiated spermatogonia. They are unable to respond properly to differentiation signals and undergo apoptosis upon retinoic acid treatment. Nonmetastatic testicular tumors are regularly formed in older GDNF-overexpressing mice. Thus, GDNF contributes to paracrine regulation of spermatogonial self-renewal and differentiation.

The stem cells for spermatogenesis are single cells in the periphery of seminiferous tubules. The stem cells either self-renew by forming single stem cells or they become interconnected pairs of cells destined to differentiate. Such cells divide further into syncytial chains of usually not more than 16 cells that enter mitosis and apoptosis synchronously (*1*, *2*). Stem cells, pairs, and chains are collectively called undifferentiated spermatogonia, which subsequently become differentiating spermatogonia, spermatocytes, spermatids, and sperm cells. All types of undifferentiated spermatogonia are morphologically and molecularly alike, but they can be distinguished by the absence or presence of synchronized mitotic