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split ends encodes large nuclear proteins that regulate neuronal cell fate and axon extension in the *Drosophila* embryo

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SUMMARY

***split ends (spen)* encodes nuclear 600 kDa proteins that contain RNA recognition motifs and a conserved C-terminal sequence. These features define a new protein family, Spen, which includes the vertebrate MINT transcriptional regulator. Zygotic *spen* mutants affect the growth and guidance of a subset of axons in the *Drosophila* embryo. Removing maternal and zygotic protein elicits cell-fate and more general axon-guidance defects that are not seen in zygotic mutants. The wrong number of chordotonal neurons and midline cells are generated, and we identify defects in precursor formation and EGF receptor-dependent inductive processes required for cell-fate specification. The number of neuronal precursors is variable in embryos that lack Spen. The levels of Suppressor of Hairless, a key transcriptional effector of Notch required for precursor formation, are reduced, as are the nuclear levels of Yan, a transcriptional repressor that regulates cell fate and proliferation downstream of the EGF receptor. We propose that Spen proteins regulate the expression of key effectors of signaling pathways required to specify neuronal cell fate and morphology.**

Key words: Spen, RRM, Su(H), Ras, Notch, EGF receptor

INTRODUCTION

A complex interplay between signaling pathways and transcription factors regulates neuronal development in the *Drosophila* embryo (Jan and Jan, 1994). Positional signals induce the formation of proneural clusters, groups of cells that are competent to become neurons, in the neuroectoderm. Cells in these clusters express proneural helix-loop-helix transcription factors such as members of the Achaete-scute Complex (ASC-C) (Campos-Ortega, 1995; Skeath and Doe, 1996) and Atonal (Jarman et al., 1993). Neuroblasts, cells that are destined to generate neurons and support cells, are produced by several mechanisms. Most commonly, neuroblasts are cells that retain proneural gene expression and activate the Notch receptor and Suppressor of Hairless (Su(H))-dependent transcription in adjacent cells (Artavanis-Tsakonas et al., 1999). This process of lateral inhibition prevents neighboring cells from maintaining proneural gene expression and becoming neuroblasts. In other cases, proneural precursors recruit neighboring cells to assume neuronal fates by activating EGF receptor (EGFR) signaling in these cells (Okabe and Okano, 1997; zur Lage and Jarman, 1999). EGFR signaling through the MAP kinase (MAPK) cascade inactivates Yan, a transcriptional repressor, and activates Pointed, a transcriptional activator that binds to the same DNA sites (O'Neill et al., 1994; Rebay and Rubin, 1995). The opposing activities of these MAPK cascade targets regulate cell fate and proliferation. After neuronal cell fate has been determined, terminal differentiation ensues. As part of this program, neurons elaborate axons and dendrites and establish synaptic connections with target cells. Though interactions between extracellular cues and receptors in the growth cone are central to this process (Tessier-Lavigne and Goodman, 1996), transcription factors also control the expression of cues and receptors, and can therefore regulate morphology and targeting (Landgraf et al., 1999; Siegler and Jia, 1999).

Mutations in *split ends (spen)* affect the growth and guidance of subsets of sensory, motor and CNS axons in the *Drosophila* embryo (Kolodziej et al., 1995). We show here that *spen* encodes novel nuclear 600 kDa proteins that contain three predicted RNA recognition motifs (RRMs) (Burd and Dreyfuss, 1994). We identify a family of Spen-like proteins in worms, flies and vertebrates that contain RRMs and a similar C-terminal sequence.

We have identified additional alleles of *spen* that disrupt the open reading frame, and behave genetically as nulls with respect to axon guidance. Cell fate appears to be normally specified in embryos homozygous for these mutations, but maternally contributed protein can be detected in *spen* mutant embryos until the onset of terminal neuronal differentiation and axon outgrowth. When maternally contributed protein is also removed, cell fate is abnormal in several tissues and the axon defects are more widespread.

In embryos that lack maternal and zygotic Spen, variable

numbers of neurons and midline glia are observed. In this paper, we identify defects in neuronal precursor formation and analyze their molecular basis. First, proneural clusters in these mutant embryos yield variable numbers of precursor cells, suggesting a defect in lateral inhibition and Notch signaling. We find that the levels of Su(H), a key transcriptional effector of Notch, are reduced in these embryos. Su(H) activates the transcription of *Enhancer of Split Complex* (*En(Spl)-C*) genes (Bailey and Posakony, 1995), and the levels of En(Spl)-C proteins are also lower in the absence of Spen. Second, the recruitment of ectodermal cells into neuronal or midline fates by EGFR signaling is probably defective. The nuclear levels of Yan are lower in many cells in these embryos. Thus, Spen is required to express key nuclear targets of Notch and EGFR signaling in the nervous system and other tissues. We propose that Spen, a nuclear protein, regulates the expression of genes critical for determining neuronal cell fate and morphology.

MATERIALS AND METHODS

Drosophila strains

The original diepoxybutane (DEB)-induced *split ends* allele (*spen*^{1}) was isolated (Kolodziej et al., 1995) in an Oregon R background. *spen*^{2}, *spen*^{3}, *spen*^{4} and *spen*^{5} were similarly generated and backcrossed to an *al dp b pr px sp* mapping strain. Recombinants with *dp b pr px sp* were backcrossed to *yw; OreR* to remove these markers. Deficiency strains were obtained from the Bloomington Stock Center. *spen*^{P1} was identified in a collection of kinesin-lacZ enhancer trap lines (Giniger et al., 1993b) by complementation testing as an allele of *spen*. *spen*^{P2}, *spen*^{P3} and *spen*^{P4}: *l(2)k06805, l(2)k08102* and *l(2)k03350* were identified by complementation testing P element alleles mapped to polytene division 21B4-6 by the Berkeley *Drosophila* Genome Project. *hsFLP1; spen*^{poc361} *40A FRT* and *hsFLP1*; *spen*^{poc231} *40A FRT* stocks were obtained from Dr William McGinnis (University of California, San Diego, USA), and *hsFLP1; spen*^{3} *40A FRT* and *hsFLP1; spen*^{5} *40A FRT* stocks were constructed by standard methods.

Molecular cloning

Genomic DNA fragments flanking the *spen*^{P1} insertion site were obtained by plasmid rescue and used to probe a λgt10 12-24 hour embryonic cDNA library (Clontech). The resulting cDNA clones were used to reprobe the λgt10 cDNA library, a λgt11 cDNA library (Zinn et al., 1988) and a plasmid 12-24 hour embryonic cDNA library (Brown and Kafatos, 1988). cDNAs were sequenced using a Perkin Elmer Dye-terminator sequencing kit (Perkin Elmer) and samples processed at the HHMI Duke University Microchemistry Facility. The GenBank accession number for *spen* cDNA sequence is AF221715. Related proteins were discovered by BLAST searching NCBI databases, and RRM motifs compared using Pfam (http://pfam.wustl.edu/index.html). Human Spen sequences were deduced from genomic DNA sequence using the FGENES and FGENEH program (http://dot.imgen.bcm.tmc.edu: 9331/seq-search/gene-search.html).

Single-stranded conformation polymorphism (SSCP)

Primers (sequence available on request) were designed to generate 280-330 bp PCR fragments spanning the *spen* coding region. Genomic DNA from wild-type and heterozygous mutant flies was PCR amplified, and DNA fragments isolated on 3% MetaPhor agarose gels (FMC Corporation) and then electrophoresed on MDE™ gels according to the manufacturer's directions (FMC Corporation). The MDE™ gels were stained with SYBR Gold (Molecular Probes) and DNA fragments imaged on a Hitachi fluorescence scanner.

Immunohistochemistry and antibody production

cDNA fragments encoding Spen amino acids 3050-3919 or 4925-5554 were cloned into pGEX6P1 (Pharmacia) vectors, and the GST-fusions were purified on glutathione-Sepharose 6B beads (Pharmacia) according to the manufacturer's instructions. The 3050-3919 fusion was cleaved from the beads by Precision Protease (Gibco). Purified proteins were injected into rats at Cocalico Biologicals Inc. (Reamstown, PA, USA). The antisera were used at dilutions of 1:1,000. The 3050-3919 antibody was used in the experiments shown in Figs 4-8; the 4925-5554 antibody also recognizes Spen protein in embryos.

Immunohistochemistry with mouse monoclonal antibodies (mAbs) 1D4, 22C10, BP102, 4D9 (anti-engrailed,), 9F8A9 (anti-elav), 3C10 (anti-even skipped), 5C8 (anti-myosin heavy chain), C1.427 (anti-connectin), anti-En(Spl)-C, anti-yan, anti-diphosphoMAPK (Sigma), mouse anti-Su(H), rabbit polyclonal antibodies against Rhomboid and Atonal and rat anti-Spen, was performed as described (Mitchison and Sedat, 1983). Confocal fluorescence microscopy was performed on a Zeiss LSM 410 (Vanderbilt Confocal Facility). Wild-type and mutant embryos were processed and examined in parallel using confocal microscopy, and image processing was performed equivalently on the data. Embryos labeled using HRP-immunohistochemistry were staged (Campos-Ortega and Hartenstein, 1985), filleted and mounted in 90% glycerol for photography. Multiple focal planes were composited using Adobe Photoshop. A *lacZ*-containing *CyO* second chromosome balancer (Lee et al., 2000) or the Spen antibody were used to distinguish mutant from wild-type embryos.

RESULTS

spen encodes a member of a new family of proteins that contain RNA recognition motifs and a novel C-terminal domain

spen affects the growth and guidance of a subset of sensory and CNS axons (Kolodziej et al., 1995). To clone the gene, we mapped the *spen*^{1} mutation genetically to 21B, and obtained the P element allele *spen*^{P1} by complementation screening a collection of lethal P element insertions. We later obtained three other lethal P element insertions (*spen*^{P2}, *spen*^{P3} and *spen*^{P4}) from the Berkeley Drosophila Genome Project (Fig. 1). Mobilization of the *spen*^{P1} element (Robertson et al., 1988) yielded viable revertants, indicating that the P element insertion was responsible for the lethality.

We used DNA fragments flanking the *spen*^{P1} insertion to obtain genomic clones for *spen* and to map the P element insertions and transcripts in the region. The four P element insertions are inserted in DNA encoding the 5′ UTR of the 15-18 kb *spen* mRNAs (Figs 1, 2). The sequence of overlapping cDNA clones indicates that *spen* encodes novel proteins of approx. 600 kDa (Fig. 2). These proteins vary slightly at their N termini and also in the internal protein sequence due to alternative splicing (Fig. 2); otherwise, they are identical and contain three RNA recognition motifs (RRMs) (Figs 2, 3A).

BLAST searches of GenBank (Altschul et al., 1990) identified proteins that contain related C-terminal sequences in worms, flies and vertebrates (Fig. 3B). These proteins all contain RRMs and are predicted to have relative molecular masses of >300 kDa or <95 kDa. Different genes in these organisms produce the large and shorter Spen-like proteins. The RRMs of the large Spen-like proteins are more related to each other than to the RRM consensus, suggesting that they share similar nucleic acid binding properties (Fig. 3A). The 360 kDa

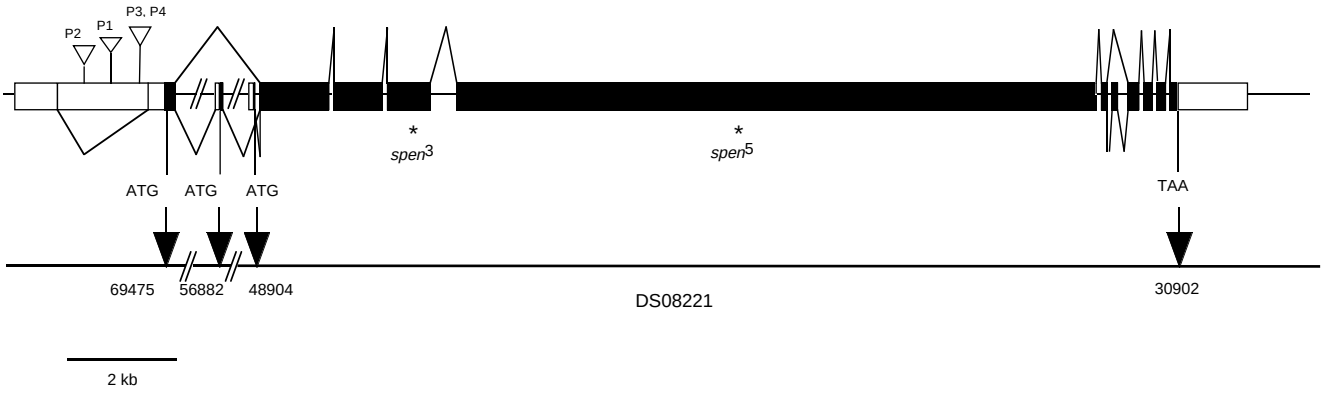

Fig. 1. The *spen* locus at 21B4-6 on chromosome II. The approximately 60 kb P1 clone DS08221 (accession number AA005334) contains the *spen* gene and has been completely sequenced by the Berkeley Drosophila Genome Project (BDGP). The P-element insertion site of $spen^{P1}$ was identified by DNA sequencing of the plasmid rescue construct. The P-element insertion sites of $spen^{P2}$, $spen^{P3}$ and $spen^{P4}$ were identified through inverse PCR by the BDGP. Boxes, exons; black boxes, coding region exons; ATG, start site (three alternative sites); TTA, stop codon; angles connecting boxes, cDNA splices; asterisks, locations of $spen^{3}$ and $spen^{5}$ mutations. A stop codon in the alternative exon that includes the second ATG probably prevents usage of the first ATG in this class of transcripts. In the $spen^{3}$ mutant, nucleotides 45655-45690 are replaced by GGCG. In the $spen^{5}$ mutant, nucleotides 39548-39601 are deleted. The resulting frame shifts are predicted to cause premature termination of the Spen polypeptides.

mouse Spen-like protein, MINT, contains three RRMs that bind to GT-rich dsDNA in vitro (Newberry et al., 1999). RRMs are found in many proteins involved in RNA processing, but also function as dsDNA binding motifs in several transcription factors (Bertolotti et al., 1996; DeAngelo et al., 1995).

The predicted C-terminal 168 amino acids of the Spen protein are 51% identical and 67% similar in sequence to the corresponding domains of the >300 kDa predicted vertebrate proteins (Fig. 3C). The <95 kDa Spen-like proteins contain C-terminal sequences that are more distantly related to Spen than those in longer forms (Fig. 3C). Thus, Spen identifies a new family of RRM-containing proteins that also contain a distinctive C-terminal domain.

We have found that *spen* is allelic to *polycephalon (poc)*, a gene required for head development and an enhancer of mutations in the *Deformed* homeobox gene (Gellon et al., 1997). *spen* is also allelic to *En(raf)2A*, an enhancer of activated *raf* (Dickson et al., 1996) and to $En(yan^{ACT})2\text{-}7$, an enhancer of activated *yan* (Rebay et al., 2000). *spen* also enhances eye phenotypes associated with ectopic expression of the E2F transcription complex (Staehling-Hampton et al., 1999).

Virtually identical sequence results have been obtained for the *poc* cDNAs (accession no. AF188205) (Wiellette et al., 1999), with the exception of an additional start site and the internal splice reported here (Figs 1, 2). Point mutations in the conserved C-terminal domain have been identified among alleles of *spen/poc* (Wiellette et al., 1999). We therefore refer to the C-terminal domain as the SPOC domain.

To confirm the gene's identity, we obtained four other diepoxybutane (DEB) alleles by complementation screening. We mapped two of these mutations by single stranded conformational polymorphism (SSCP) (Orita et al., 1989) and DNA sequencing of PCR products derived from mutant chromosomes. $spen^{3}$ and $spen^{5}$ are small deletions in the *spen* ORF that cause premature truncation of the Spen proteins (Fig. 2). $spen^{3}$ is predicted to produce a protein approximately one-sixth the size of wild type and $spen^{5}$, a protein one-half the size of wild type. Thus, we conclude that *spen* encodes a family of 600 kDa, RRM-containing proteins.

spen encodes widely expressed nuclear proteins

RRM-containing proteins regulate mRNA transcription, splicing, stability, localization, and translation, and are located in the nucleus (Robinow and White, 1991), the cytoplasm (Gao and Keene, 1996) or both cellular compartments. To investigate Spen's roles in development, we determined Spen's subcellular localization and expression in embryos. Antibodies against two different C-terminal epitopes reveal that Spen proteins are nuclear (Fig. 4A-E) and expressed in most, if not all, cells. Spen proteins are not detected with these antisera in stage-12 or later $spen^{3}$, $spen^{5}$ or $spen^{poc361}$ mutant embryos (Fig. 4F and data not shown). Thus, these antisera are specific for Spen and these alleles do not produce detectable full-length proteins.

The Spen proteins are detectable as early as cellular blastoderm and are ubiquitously nuclear during early development (Fig. 4A). Spen expression in the CNS appears to be at higher levels than in the surrounding epidermis after stage 15 (Fig. 4D). Spen is also expressed in non-neuronal cells, most likely glia, within the CNS. Spen proteins are also detected in muscle nuclei (data not shown).

Maternally contributed Spen protein is required for the specification of cell fate during neuronal and midline development

Maternal protein persists in *spen* mutant embryos until the onset of terminal neuronal differentiation (see above). To investigate the developmental role of maternally contributed Spen, germ line clones of *spen* alleles ($spen^{5}$, $spen^{poc231}$ and $spen^{poc361}$) were generated in virgin females by standard methods (Chou and Perrimon, 1996) and the females crossed to heterozygous $spen^{3}$ mutant males. Females containing germ line clones of $spen^{3}$ were crossed to heterozygous $spen^{5}$ mutant males. Since these females can only lay eggs that lack Spen in the germ line, half of the resulting embryos lack both maternally and zygotically

Fig. 2. The predicted primary structure of the Spen proteins. The longest isoform contains (5554 amino acids) has a predicted molecular mass of 599 kDa. Three possible N-terminal sequences were deduced from cDNA cloning. The second and the third N-terminal isoforms, not shown here, start with MLIVM and MRR, respectively. Open arrow, the first residue (Ser) common to all N-terminal sequences. Gray boxes, the three RRM domains. Black arrows, the points of truncation in *spen*^{3} and *spen*^{5} proteins, respectively. Black, the conserved C-terminal domain (SPOC). The underlined residues close to the C terminus are absent in some cDNAs. The accession number for the sequence of the longest isoform is AF221715.

contributed Spen. Most experiments were performed on embryos derived from *spen*^{3} germline clone × heterozygous *spen*^{5} or *spen*^{poc361}germline clone × heterozygous *spen*^{3} crosses, since these alleles produce no detectable full-length Spen protein. We were therefore able to identify embryos that lack both maternal (M) and zygotic (Z) Spen protein by the absence of Spen staining. We refer to such embryos as *MZspen* embryos (*MZspen* in Figs 5-8); embryos that lack only zygotic *spen* expression are referred to as *Zspen* mutant embryos and still express Spen protein until late stage 12 (*Zspen* in Figs 4-8). The molecular basis of the *spen*^{poc231} and *spen*^{poc361} EMS-generated alleles has not been determined, but we find that *spen*^{poc231} produces detectable, though not functional, protein and *spen*^{poc361} does not (data not shown). The severity of mutant phenotypes was comparable among protein null alleles, and was only slightly weaker in embryos derived from *spen*^{poc231} germline clones (Table 1 and data not shown).

In *MZspen* embryos, the number of many PNS and CNS cell types is altered, and the development of other organs is affected. In order to analyze the molecular basis of these defects, we focused on the well-marked development of the lateral chordotonal (lch) sensory neurons. Wild-type embryos contain a cluster of five lch neurons in each abdominal hemisegment (Fig. 5A). In *MZspen* embryos, this number varies from none to six, and is typically four (Table 1, Fig. 5B-C). Clusters containing the normal number are often disorganized.

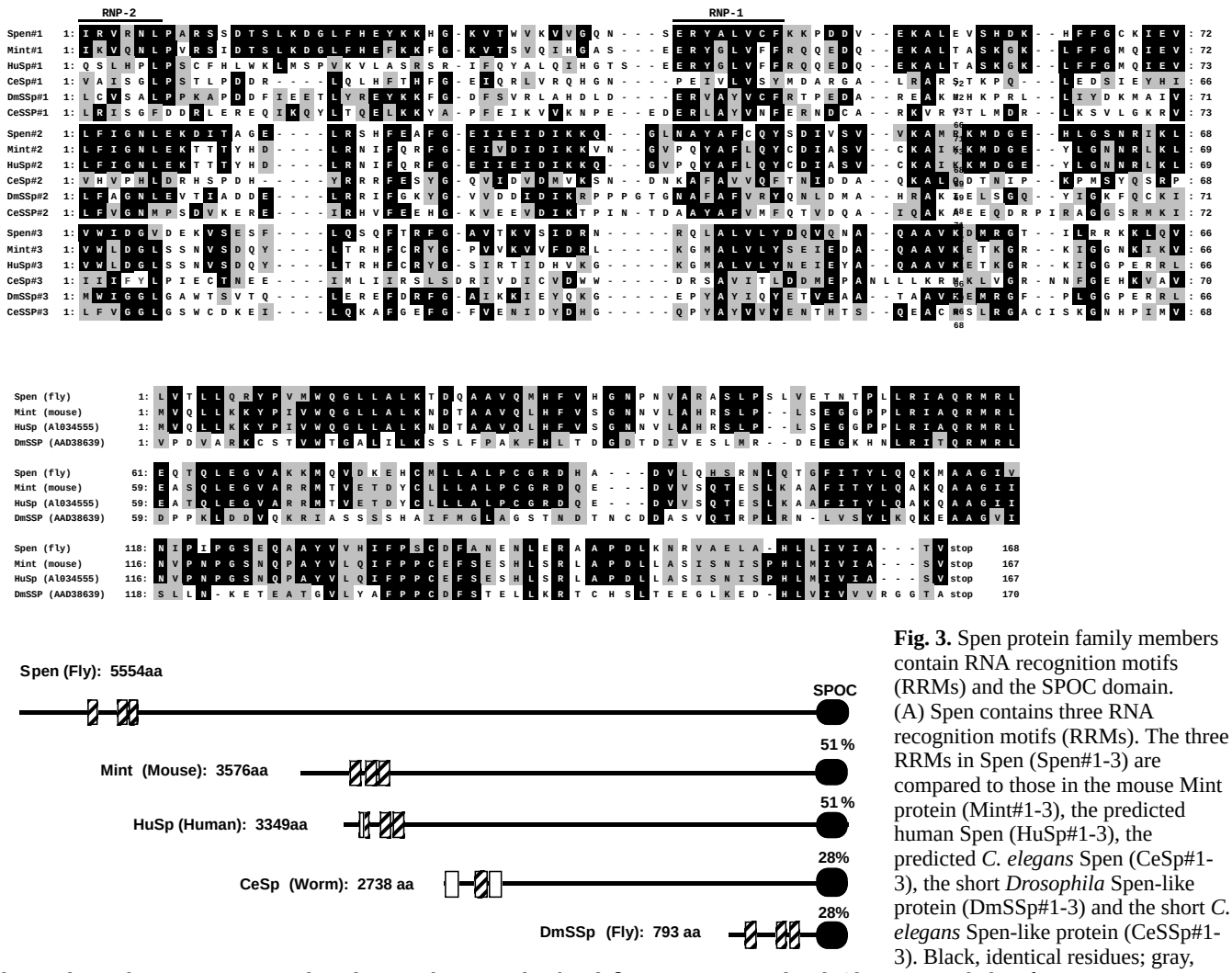

Fig. 3. Spen protein family members contain RNA recognition motifs (RRMs) and the SPOC domain. (A) Spen contains three RNA recognition motifs (RRMs). The three RRMs in Spen (Spen#1-3) are compared to those in the mouse Mint protein (Mint#1-3), the predicted human Spen (HuSp#1-3), the predicted *C. elegans* Spen (CeSp#1-3), the short *Drosophila* Spen-like protein (DmSSp#1-3) and the short *C. elegans* Spen-like protein (CeSSp#1-3). Black, identical residues; gray, similar residues. The RNP-1 octapeptide and RNP-2 hexapeptides that define RRMs are overlined. Alignment with the Pfam RRM consensus introduces additional gaps and a lower level of sequence identity. Accession numbers: Spen, HuSp (AL034555; genomic sequence that overlaps with cDNA clone KIAA0929), CeSp (CAA91320), DmSSp (AAD38639), CeSSp (AAC19192). Partial sequence is also available for a zebrafish Spen-like protein (AI793898) and a human short Spen-like protein (L13434). (B) The Spen C-terminal domain (the SPOC domain) in Spen, Mint and Human Spen isoforms (HuSp), and *Drosophila* short Spen-like protein (DmSSp). Black, identical residues; gray, similar residues. (C) Schematic drawing of Spen-like proteins. Hatched boxes, good matches to the RRM consensus; clear boxes, weaker matches. *Drosophila* Spen and short Spen-like proteins contain three strong RRMs recognized by Pfam; other RRMs are better matches to Spen than to the consensus. The percentages of identical residues in the SPOC domains (black oval) of HuSp, CeSp, and DMSSp proteins with Spen are indicated. The SPOC domains of CeSp and DmSSp share 28% identity.

Table 1. The distribution of lch neuron numbers in wild-type embryos and embryos lacking Spen

Allele	Total number*	Number of lch neurons in embryos (% of total)						
		0	1	2	3	4	5	6
Wild type	42	0	0	0	0	0	100	0
spen^{poc231}	76	0	2.6	0	14.5	51.3	30.3	1.3
spen^{3}	103	1	4.9	10.7	19.4	38.9	23.3	1.9
spen^{poc361}	117	0	6.0	5.1	19.7	37.6	26.5	5.1

*The total number of abdominal hemisegments examined.

Other mutations have been previously shown to affect lch neuron number by affecting either precursor formation or EGF receptor signaling. In wild-type development, three Atonal-expressing cells become lch precursors (Lage et al., 1997). These cells activate EGFR signaling in two neighboring ectodermal cells, recruiting these cells to the lch neuronal fate

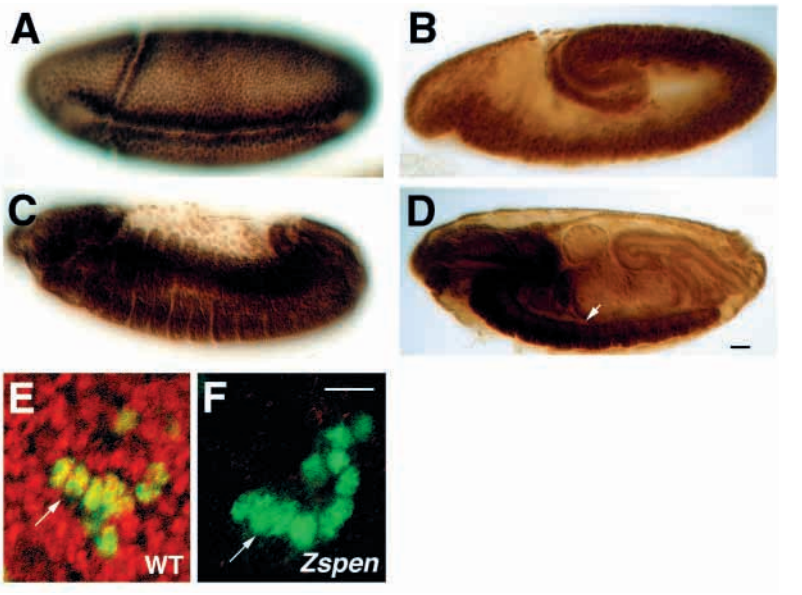

Fig. 4. Spen are ubiquitous nuclear proteins. (A) A stage-6 embryo stained with anti-Spen. Spen is detected in all nuclei. (B) Anti-Spen labels the nuclei in all germ layers at stage 10. (C) Spen is detected in all epidermal nuclei at stage 12. (D) In a stage-17 embryo, Spen is enriched in the CNS (arrow). (E) The lateral chordotonal (lch) neurons (arrow) and surrounding epidermal cells in a stage-15 wild-type embryo, immunostained with anti-ELAV (green) and anti-Spen (red). Elav is expressed in the nuclei of all neurons (Robinow and White, 1991). The yellow color indicates overlap between Spen and Elav; Spen appears present in all cell nuclei. (F) A section similar to E showing the lch neurons and surrounding cells in a stage-15 *spen*^{3} mutant embryo (*Zspen*). Spen staining is absent. Anterior, left; dorsal, up. Bars, 25 μm (A-D); 5 μm (E,F).

(Okabe and Okano, 1997). Mutants that block either precursor formation or recruitment therefore produce characteristic numbers of lch neurons. *atonal*, which blocks lch proneural precursor formation, produces occasionally one lch neuron (Jarman et al., 1995). Mutations in the *EGF/Spitz* group, which block the recruitment process, produce only three lch neurons (Bier et al., 1990; Rutledge et al., 1992). Gain-of-function mutations in the EGF pathway yield 6-7 lch neurons (Okabe and Okano, 1997). By contrast, the lch neuron phenotypes in *MZspen* embryos spans the range observed in loss-of-function mutations that affect precursor formation or recruitment and gain-of-function mutations in EGFR signaling.

The variable numbers of lch neurons could therefore reflect defects in precursor formation, EGFR signaling or another

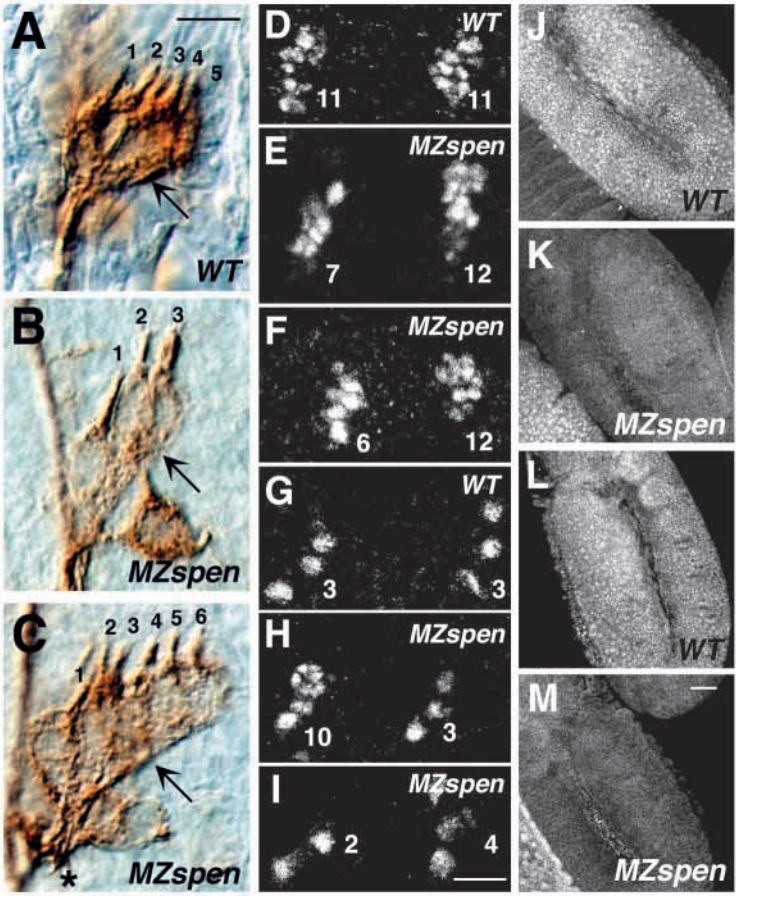

Fig. 5. Variable numbers of lch neurons and precursor cells are produced in *MZspen* embryos. (A) The cluster of five lch neurons found in abdominal hemisegments in a stage-15 wild-type embryo. (B) A cluster of three lch neurons in a stage-15 *spen*^{poc231}/*spen*^{3} embryo derived from a *spen*^{poc231} germ line clone. (C) A cluster of six lch neurons in a stage-15 *spen*^{poc231}/*spen*^{3} embryo derived from a *spen*^{poc231} germ line clone. The lch axons stall prematurely (asterisk). (D-M) 1 μm optical sections; atonal staining was used to compare wild-type to mutant stages. (D-I) Lch precursor formation in wild-type and *spen*^{3}/*spen*^{5} embryos derived from *spen*^{3} germ line clones. (D-F) A relatively early stage in ch proneural cluster refinement. (D) The number of Atonal-expressing cells (11) is relatively constant in each abdominal hemisegment in a wild-type embryo. (E,F) Variable numbers of Atonal-positive cells are observed in adjacent abdominal hemisegments in two different embryos that lack Spen. (G-I) A later stage in proneural ch cluster refinement. (G) Three Atonal-positive cells per hemisegment are observed in a wild-type embryo. (H,I) Variable numbers of Atonal-positive cells in adjacent abdominal hemisegments in similarly staged *spen*^{3}/*spen*^{5} embryos derived from *spen*^{3} germ line clones. (J-M) The expression of Notch effectors is defective in *spen*^{3}/*spen*^{5} embryos derived from *spen*^{3} germ line clones. (J) Su(H) is widely expressed in cell nuclei in a wild-type embryo during neurogenesis. (K) Su(H) expression is dramatically reduced in a similarly staged *MZspen* embryo. All stage-10-11 embryos that lack Spen show this level of Su(H) expression. (L) Widespread nuclear En(Spl)-C expression in a stage-11 wild-type embryo near the end of proneural cluster refinement. (M) En(Spl)-C expression is reduced in a *MZspen* embryo. Anterior, left top in J-M. Bars, 5 μm (A-C); 10 μm (D-I); 25 μm (J-M).

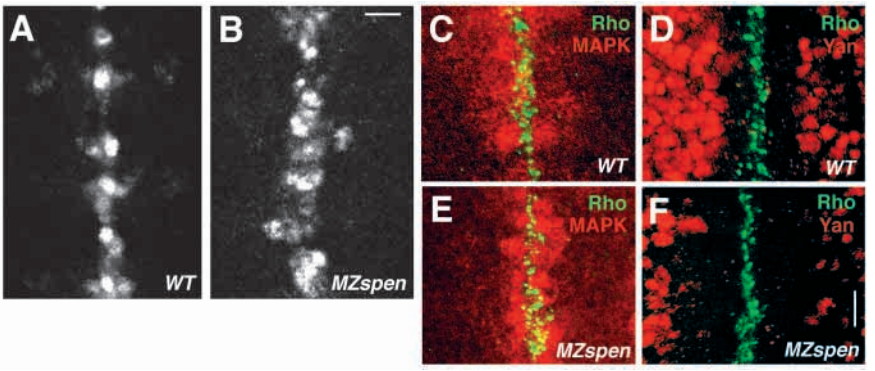

Fig. 6. Midline cell fate is altered in *MZspen* embryos. All panels show 1 μm confocal sections. (A) The Sim-labeled midline cells in a stage-13 wild-type embryo. (B) There are approximately twofold more Sim-positive cells in the midline of a stage-13 $spen^{poc361}/spen^{3}$ embryo derived from a $spen^{poc361}$ germ line clone. (C) The expression of Rhomboid (green) in midline cells and activated-MAPK (red) in midline and flanking cells in a stage-10 wild-type embryo. (D) Rhomboid expression (green) at the midline and Yan expression (red) in the nuclei of CNS cells in a stage-10 wild-type embryo. Cell nuclei in a wide domain outside the midline and the zone of high activated MAPK all contain Yan. (E) Rhomboid expression and MAPK activation appears similar to wild-type in a stage-10 $spen^{3}/spen^{5}$ embryo derived from a $spen^{3}$ germ line clone. (F) Rhomboid expression (green) at the midline and Yan expression (red) in the nuclei of CNS cells in a stage-10 $spen^{3}/spen^{5}$ embryo derived from a $spen^{3}$ germ line clone. Rhomboid expression appears normal, but Yan is present in fewer nuclei in mutant embryos, and the domain flanking the midline of nuclei that lack Yan is wider and less well defined. Bars, 5 μm (A,B); 7.5 μm (C-F).

pathway. We followed the formation and refinement of the chordotonal (ch) proneural cluster in wild-type and *MZspen* embryos using an antibody directed against Atonal, the helix-loop-helix transcription factor that specifies ch neuronal fate (Jarman et al., 1993). Atonal is initially expressed in the nuclei of a patch of ectodermal cells in each abdominal hemisegment, the proneural cluster (Jarman et al., 1993). Atonal expression becomes progressively restricted to the precursor cells that contribute to both lateral and ventral chordotonal clusters (Jarman et al., 1993). At any stage of this dynamic process, abdominal hemisegments in wild-type embryos contain similar numbers of Atonal-expressing cells (Fig. 5D,G). By contrast, in *MZspen* embryos, abdominal hemisegments contain variable numbers of these ch precursor cells (Fig. 5E,F,H,I).

The variability in precursor number suggested possible defects in Notch signaling because Notch is required to generate the appropriate numbers of precursors from proneural clusters. We determined that Notch expression is normal in embryos lacking Spen (data not shown), but that expression of Su(H), the key known transcriptional effector of Notch signaling (Fortini and Artavanis-Tsakonas, 1994), is dramatically reduced throughout the embryo (Fig. 5J,K). Not surprisingly, the expression of Enhancer of Split Complex (En(Spl)-C) proteins, which depends on Su(H) (Bailey and Posakony, 1995), is also reduced in the embryos that lack Spen (Fig. 5L).

Midline development is also defective in *MZspen* embryos. The Singleminded (Sim) transcription factor is expressed by midline glial cells and their precursors (Nambu et al., 1991; Thomas et al., 1988) (Fig. 6A). *MZspen* embryos have approximately twice as many Sim-positive cells (Fig. 6B).

Embryos that lack maternal and zygotic Su(H) are neurogenic, and are missing some midline cells (Lecourtois and Schweisguth, 1995), but *MZspen* embryos are not neurogenic, and produce extra midline cells. These differences suggest that Spen affects another pathway required for neuronal and midline cell fate. EGFR/Ras signaling is required for ch neuron and midline development (Gabay et al., 1996; Okabe and Okano, 1997), ectopic activation of *ras1* produces extra midline cells (Scholz et al., 1997), and *spen* enhances mutations in the Ras pathway (Rebay et al., 2000). We therefore investigated whether cells can send and receive EGF signals in the absence of Spen. The cells at the midline

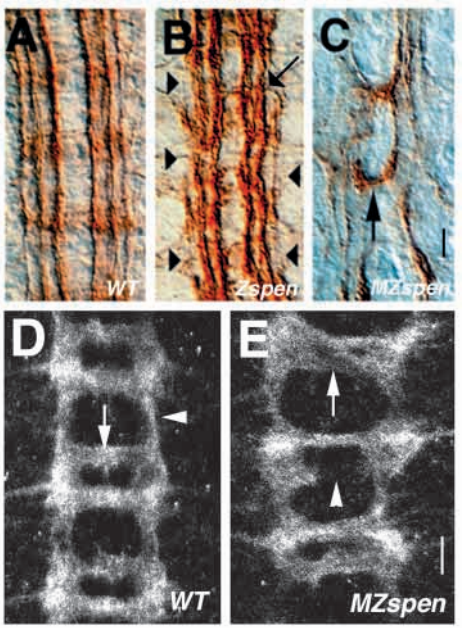

Fig. 7. CNS axon guidance and extension defects in zygotic (Z) *spen* and *MZspen* embryos. (A) mAb 1D4 reveals three longitudinal axon fascicles on each side of the midline in a late stage-16 wild-type embryo. (B) In a stage-16 *spen* mutant embryo, the outermost longitudinals stall (arrowheads) and are misrouted (arrow). Axons also appear less well fasciculated than in wild type. Similar defects in other fascicles are also observed using a mAb directed against connectin (Gould and White, 1992), which labels a smaller axon subset than mAb 1D4 (data not shown). (C) In a stage-16 $spen^{poc361}/spen^{3}$ embryo derived from a $spen^{poc361}$ germ line clone, all of the longitudinal axon tracts are disrupted and axons inappropriately cross the midline (arrow). (D) Frazzled is expressed on commissural (arrow) and longitudinal axons (arrowhead) in a stage-15 wild-type embryo. (E) In a stage-15 $spen^{poc361}/spen^{3}$ embryo derived from a $spen^{poc361}$ germ line clone, Frazzled expression appears normal, but commissures are missing (arrowhead) or poorly separated (arrow). Anterior is at the top of each panel. Bars, 5 μm (A-C); 5 μm (D,E).

Table 2. Motor axon development in wild-type and *spen* mutant embryos

Genotype	ISN phenotype[a] WT	ISNb phenotypes[b] WT	Missing 1	Missing 2	Missing 3	Misrouted	SNa phenotypes[c] WT	Stall	Bif. stall	L stall	D stall	Misrouted
WT	100(104)	74(100)	19	0	7	0	97(92)	0	0	0	3	0
spen^{3}	100(69)	17(82)	12	1	64	6	29(93)	25	20	8	10	8
spen$^{3/Df}$	99(77)	6(79)	6	5	80	3	25(71)	13	35	4	17	6
spen^{5}	97(131)	4(145)	11	6	72	7	27(143)	17	14	5	27	10
spen$^{5/Df}$	100(64)	18(80)	15	0	54	14	18(72)	14	7	11	22	28
spen$^{3/5}$	97(65)	4(79)	3	0	87	6	20(75)	42	9	4	18	8

[a]The wild-type (WT) ISN forms three characteristic arborizations over the dorsal muscles. The first number represents the percentage of abdominal hemisegments that appear wild type and the number in parentheses, the total number of hemisegments scored in late stage-16 or -17 embryos.

[b]The WT ISNb forms three connections with ventral muscles. The first number represents the percentage of hemisegments that appear wild type and the number in parentheses, the total number of hemisegments scored. The percentage of mutant hemisegments in which the ISNb motor axons are missing one, two or all three connections are indicated. In *spen* mutants, the connections formed are also often smaller than those observed in wild type, reflecting either a lag in innervation, or a defect in synapse formation. Misrouted ISNb axons are those that fail to defasciculate from the ISN and bypass the ventral muscles or those that innervate ventral muscles in the adjacent hemisegment.

[c]The WT SNa bifurcates just above the dorsal edge of muscle 12 and extends a lateral (L) and a dorsal (D) branch. The first number represents the percentage of hemisegments that appear wild type and the number in parentheses, the total number of hemisegments scored. Stall: SNa motor axon bundles that either did not reach muscle 12, or more commonly, did not bifurcate. Bifurcate stall: SNa motor axon bundles that bifurcated, but did not extend branches of wild-type length. L or D stall: SNa motor axon bundles that extended either an apparently normal dorsal or an apparently normal lateral branch. Misrouted: SNa motor axon bundles that bifurcated prematurely, fasciculated with the ISN, or formed three branches.

normally express Rhomboid, a membrane protein that potentiates EGF signaling (Fig. 6C) (Bier et al., 1990). Consequently, the EGFR, Ras and the MAPK cascade are activated in midline cells and cells immediately flanking the midline, as assayed with an antibody that detects activated MAPK (Gabay et al., 1997) (Fig. 6C). Rhomboid expression at the midline and MAPK cascade activation in adjacent cells (Fig. 6E) appear normal in the absence of Spen. In the absence of Spen, Rhomboid expression in the ch proneural cluster, and MAPK signaling in the cluster and neighboring cells also appears normal (data not shown). Thus, Spen does not appear to affect early steps in EGFR signaling.

Nonetheless, defects in MAPK interactions with nuclear targets such as the Yan transcriptional repressor could contribute to the cell-fate phenotypes. *spen* mutants enhance activated *yan* (Rebay et al., 2000), whose protein product is resistant to MAPK inactivation (Rebay and Rubin, 1995). We therefore examined Yan protein levels and localization *MZspen* embryos. In stage-10 wild-type embryos, Yan protein is detected in most nuclei in the developing CNS, but not in those of midline cells or in cells immediately adjacent to the midline (Scholz et al., 1997) (Fig. 6D). Levels of Yan appear to be relatively uniform outside this domain, and the boundary between cells that express Yan and the midline region appears relatively sharp. However, in *MZspen* embryos, Yan is absent or present at reduced levels in many cells that would normally express Yan (Fig. 6F). Abnormal reductions in Yan levels are also observed surrounding ch proneural clusters (data not shown). These results suggest that Spen is required to maintain or establish the nuclear levels of Yan in many cells in the embryo.

Mutations in *spen* affect axon growth and guidance

The cell-fate changes in *MZspen* embryos are not observed in embryos that lack only zygotically contributed Spen. Maternally contributed Spen can be detected in zygotic mutant embryos until late stage-12 (data not shown), at which point most cell-fate decisions have been made. We detected no defects in zygotic *spen* mutant embryos with respect to neuronal cell fate as assayed with mAbs that recognize CNS neuronal subsets (anti-eve, anti-engrailed, anti-ftz) (Doe et al., 1988a,b; Patel et al., 1989), PNS neurons (22C10) (Fujita et al., 1982), glial development (anti-repo and anti-Sim) (Xiong et al., 1994; Nambu et al., 1991), and muscle development (anti-myosin heavy chain and anti-connectin) (data not shown).

spen^{1} affects the growth and guidance of a subset of CNS and sensory axons (Kolodziej et al., 1995), but whether the apparent specificity of these phenotypes reflects residual protein function has not been determined. *spen*^{3} *and spen*^{5} are predicted to produce proteins one-sixth and one-half the size of Spen respectively (Fig. 1), and so could be nulls. We investigated whether residual Spen function is present in zygotic mutant embryos by comparing CNS and motor axon development in wild-type, *spen*^{3} and *spen*^{5} zygotic mutant embryos, and in embryos that lack both maternal and zygotic Spen. In zygotic mutant embryos, we identified defects in the elongation and pathfinding of axons in a subset of longitudinal CNS axon tracts, in the intersegmental nerve b (ISNb) and segmental nerve a (SNa) motor axon pathways, in the transverse nerve (TN), but not in the commissural CNS axon tracts, nor in the intersegmental nerve (ISN) motor axon pathways (Table 2). The frequency of motor axon defects is similar in *spen*^{3} homozygous embryos and embryos heterozygous for *spen*^{3} and a chromosomal deficiency that removes the 21B region (Table 2), suggesting that *spen*^{3} is a null allele. Defects in axon extension and guidance are more pronounced when maternally contributed Spen is also removed, indicating that maternally contributed Spen provides residual function in zygotic mutants (see below).

mAb 1D4 reveals three parallel longitudinal axon tracts that extend continuously in the CNS on each side of the ventral midline in wild-type late stage-16 or stage-17 embryos (Seeger et al., 1993) (Fig. 7A). In *spen* mutant embryos, the outermost fascicle is discontinuous in some segments, and these axons occasionally invade the middle fascicle (Fig. 7B). Axon defects are more severe, and extend to all axon tracts in the CNS in

spen mutant embryos derived from *spen* germ line clones (Fig. 7C,E).

mAb 1D4 also labels motor axons (Van Vactor et al., 1993). In each abdominal hemisegment of the *Drosophila* embryo, approximately 30 motor axons innervate 30 muscle fibers in a stereotyped pattern (Landgraf et al., 1997; Sink and Whitington, 1991). We examined the development of the intersegmental nerve (ISN), which innervates the dorsal muscles, the ISNb, which innervates ventral muscles, and the SNa, which innervates the lateral muscles (Table 2). The ISN appears normal in zygotic *spen* mutants. In late stage-16 wild-type embryos, the ISNb has defasciculated from the ISN and formed three connections with ventral muscles: at muscles 12 and 13 and the cleft between muscles 6 and 7 (Table 2, Fig. 8A). In late stage-16 zygotic *spen* mutant embryos, most ISNb motor axons defasciculate from the ISN, but stall short of their ventral muscle targets (Fig. 8B). In the 4-18% of cases where three connections can be detected in *spen* mutant embryos (Table 2), they appear generally smaller than wild type. The SNa motor axons are also abnormal in *spen* mutant embryos (Table 2). In stage-16 wild-type embryos, the SNa motor axons have traversed the ventral muscle field and bifurcated just above the ventral muscles (Fig. 8A). The dorsal branch extends away from the ventral muscles, and the lateral branch extends posteriorly, roughly parallel to the dorsal edge of muscle 12. In stage-16 zygotic *spen* embryos, most SNa motor axons either stall near the initial entry into the lateral muscle field, or make shorter than wild-type dorsal or lateral extensions (Fig. 8B).

In *MZspen* embryos, the development of all motor axon pathways is defective (Fig. 8C-D). Motor axons exit the CNS, pick the correct pathways, but fail to innervate their muscle targets. In the cases of the SNa and ISNb motor axons, these axons must first cross the midline, and the occasional absence of these axon tracts may reflect midline defects. However, in hemisegments where distinct ISN, SNa and ISNb fascicles are observed, the motor axons still fail to reach target muscle (Fig. 8C), and even occasionally cross over segmental boundaries (Fig. 8D). Some of these defects may also reflect the disorganized and missing muscle fibers in these embryos (data not shown). Thus, Spen appears not to be required for motor axons to distinguish dorsal, lateral and ventral muscles, but is required for later steps in motor axon development in the neuron, muscle or both.

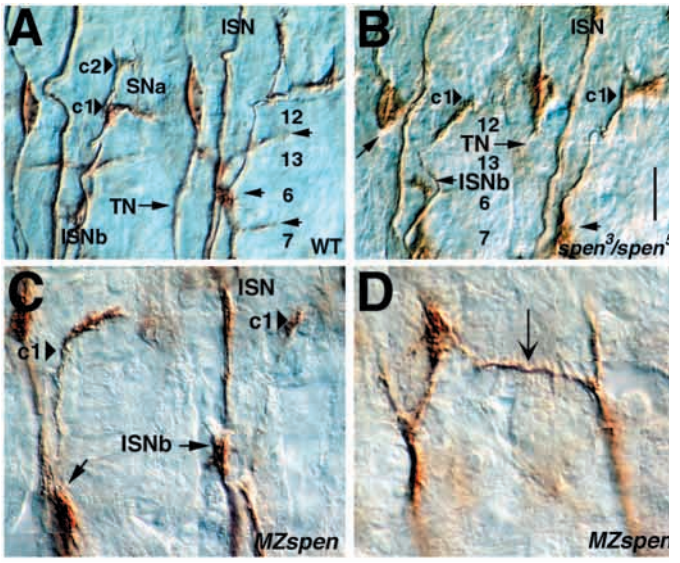

Fig. 8. *spen* affects motor axon innervation of target muscles. (A) Two hemisegments from a late stage-16 wild-type embryo stained with mAb 1D4. The SNa motor axons innervate the lateral muscles. They bifurcate at choice point 1 (c1), and the dorsal branch bifurcates again at choice point 2 (c2). The ISNb motor axons innervate ventral muscles 12, 13, 6 and 7, and form a stereotyped pattern of neuromuscular connections (short arrows). The ISN innervates more dorsal muscles; its terminal connections are not shown. The transverse nerve (TN) extends parallel to the ISN. (B) Two hemisegments from a late stage-16 $spen^{3}/spen^{5}$ embryo stained with mAb 1D4. The SNa motor axons fail to extend beyond c1 (left hemisegment), or bifurcate, and then stall shortly thereafter (right hemisegment). The ISNb fails to make the normal pattern of three connections with ventral muscles (short arrows) and does not extend fully into the ventral muscle field (left hemisegment) or fails to enter it (right hemisegment). The TN also fails to form (long arrows). (C) The SNa (arrowheads) and ISNb (arrows) motor axons stall before reaching target muscles in $spen^{poc361}/spen^{3}$ embryos derived from a $spen^{poc361}$ germ line clone. (D) ISN motor axons cross segmental boundaries (arrow) in a stage-14 $spen^{poc361}/spen^{3}$ embryo derived from a $spen^{poc361}$ germ line clone, rather than migrating straight dorsally as in wild type. Anterior is to the left, dorsal is at the top of each panel. Bar, 10 μm (A,B); 8 μm (C,D).

DISCUSSION

We have molecularly characterized the *spen* gene, analyzed its roles in specifying cell fate and neuronal morphology, and identified alterations in gene expression that likely underly its role in cell-fate specification. The $spen^{3}$ and $spen^{5}$ mutations disrupt the open reading frame of novel 600 kDa nuclear proteins. Zygotically contributed Spen is required for the growth and guidance of subsets of CNS, motor and sensory axons; residual maternal protein likely explains the apparent cell-type specificity of these phenotypes. Maternally contributed Spen is required to specify neuronal cell fate. The wrong numbers of lch neurons and midline cells are produced in embryos that lack Spen. We identify alterations in the number of lch neuron precursors, and identify general molecular defects in two key processes that regulate cell fate: lateral inhibition and inductive signaling. *spen* is required for the normal expression of Su(H), the main known transcriptional effector of Notch, the receptor that mediates lateral inhibition (Artavanis-Tsakonas et al., 1999). Moreover, we show that the ability of cells to respond to inductive signals may be altered. *spen* is required for the normal expression of Yan, a key target and antagonist of EGFR signaling (Rebay and Rubin, 1995). Thus, defects in Notch and EGFR signaling are likely to be at least partially responsible for the cell-fate defects. Spen is therefore the first nuclear link between these two signaling pathways.

Spen is a member of a new, evolutionarily conserved protein family

Worms, flies and vertebrates contain distinct genes that encode either large (>300 kDa) or smaller (<95 kDa) Spen-like proteins. These proteins contain RNA recognition motifs

(RRMs) and a conserved C-terminal sequence, the SPOC domain, but little other homology. RRMs were identified originally in RNA binding proteins involved in mRNA splicing, stability and translation (Burd and Dreyfuss, 1994), and more recently as DNA binding motifs in several transcription factors (Bertolotti et al., 1996; DeAngelo et al., 1995; Newberry et al., 1999).

These transcription factors include MINT, which binds via the RRMs to GT-rich DNA sequences (Newberry et al., 1999) and contains the C-terminal SPOC domain. The MINT protein is proteolytically processed in vivo into 110 kDa N-terminal and 250 kDa C-terminal fragments (Newberry et al., 1999). The MINT RRM-containing N-terminal domain both suppresses FGF activation of the rat osteocalcin promoter and activates the HSV thymidine kinase promoter (Newberry et al., 1999). Thus, depending on the promoter, the RRM-containing fragment can act as a repressor or an activator of transcription. These data and structural similarities between the MINT and Spen RRM domains suggest that Spen may function as a transcription factor, rather than as an RNA binding protein. Spen could be required for *Su(H)* and *yan* transcription, or affect the levels of Su(H) and Yan proteins by another mechanism.

Mutations in *spen* enhance mutations in the *Deformed* homeobox gene, and these include point mutations in the SPOC domain (Wiellette et al., 1999). Intriguingly, the C-terminal fragment of MINT binds to the Msx2 homeobox protein in vitro, though not via the SPOC domain, and may coregulate the expression of genes required for craniofacial development (Newberry et al., 1999). Thus, the C-terminal domain of Spen is likely to be important for interactions with transcription factors and key signaling pathways regulating cell fate.

spen mutants produce the wrong numbers of neuronal precursors and alter Su(H) expression

Embryos that lack Spen are defective in the specification of neuronal and midline cell fate. The correct cell types are generally made at the appropriate positions in the embryo, but in the wrong numbers. In the case of the lch neurons, whose precursors are molecularly well marked, these defects at least partially reside in precursor specification.

Both lch and midline development are similar in that Notch signaling is required to generate precursors committed to a particular fate from an initial group of developmentally equivalent cells (Martin-Bermudo et al., 1995; Menne and Klambt, 1994; Sun et al., 1998). Su(H) is a key transcriptional effector of Notch signaling. We have shown that *spen* is required for normal Su(H) expression throughout the embryo during proneural cluster refinement. Consequently, En(Spl)-C proteins, repressors of neuronal differentiation whose expression is Su(H)-dependent (Bailey and Posakony, 1995), are not detectably expressed in the absence of Spen. Thus, general defects in Notch signaling likely contribute to the defects in lch precursor formation.

Embryos that lack both maternal and zygotic Su(H) produce excess neuroblasts, and exhibit a neurogenic phenotype (Lecourtois and Schweisguth, 1995). However, *MZspen* embryos are not neurogenic. In *MZspen* embryos, variable numbers of neuronal precursors are produced, but these may not all become neurons. Refinement of proneural clusters still occurs in the absence of Spen, but occurs less reproducibly. These differences in phenotype suggest that in the absence of Spen, maternally contributed Su(H) allows some Notch signaling to occur or refinement is Su(H)-independent. Spen could also be involved in specifying initial proneural cluster size independent of its effect on Su(H); variable initial cluster size in *MZspen* embryos may not have been detected in our analysis, and may affect refinement. Though further experiments will be required to distinguish among these possibilities, our results clearly show that refinement of lch proneural clusters is defective in embryos lacking maternal and zygotic Spen.

spen affects the nuclear levels of Yan, a transcriptional target of the MAPK cascade that controls cell fate and proliferation

Midline and lch neuron development require precursors to activate the EGFR/Ras pathway in adjacent cells (Gabay et al., 1996; Okabe and Okano, 1997). Mutations in *spen* enhance mutations in the *ras* pathway and affect cell fate (Dickson et al., 1996; Rebay et al., 2000), suggesting that Spen could be in the Ras pathway or regulate its activity. We investigated whether Spen affects the ability of cells to send or receive the EGF/Spitz signal. In embryos that lack Spen, precursors at the midline and in the lch proneural cluster express Rhomboid at levels comparable to wild type, and MAPK is activated in adjacent cells. These data suggest that wild-type and mutant cells are similar in their ability to send an EGF/Spitz signal and to activate MAPK in response.

In light of Spen's nuclear location, and *spen*'s interactions with activated *yan* (Rebay et al., 2000), we examined Yan levels in embryos that lack Spen. Alterations in the levels or activity of this key target of MAPK signaling alter cell fate and proliferation in the eye imaginal disc and embryo (Lai and Rubin, 1992; Rebay and Rubin, 1995; Rogge et al., 1995). The loss of Yan triggers the inappropriate entry of cells into S phase, and causes defects in head development (Rogge et al., 1995) that overlap with *spen* phenotypes (Wiellette et al., 1999). We found that in the absence of Spen, nuclear levels of Yan are widely reduced. Thus, Spen is required to maintain or establish normal levels of Yan, and *spen* may enhance activated *yan* by reducing the levels of the endogeneous protein. Our data suggests that Spen functions downstream of the MAPK cascade or in parallel to regulate Yan, rather than upstream.

Spen regulates the expression of Notch and EGF receptor nuclear targets

Taken together, our data suggest that Spen affects cell-fate specification by disrupting at least two key signaling pathways: Notch and EGFR. First, Spen is required for normal Su(H) expression throughout the embryo during neurogenesis. As a result, Notch-mediated lateral inhibition is defective and abnormal numbers of neuronal precursors are specified in embryos that lack Spen. These precursors likely retain their correct identity; the ch precursors still express Atonal and form ch neurons. Second, cells that lack Spen often do not express normal nuclear levels of Yan, which antagonizes EGFR signaling to control cell proliferation and fate (Lai and Rubin, 1992; Rebay and Rubin, 1995; Rogge et al., 1995). Hence, some cells that lack Spen may respond aberrantly to an EGF signal. Though Spen is ubiquitous, other cells achieve

apparently normal levels of Yan in the absence of Spen, suggesting that not all Yan expression requires Spen.

Defects in Su(H) and Yan protein expression occur in embryos that lack Spen and can themselves cause the number of neurons and midline cells to vary (Lecourtois and Schweisguth, 1995; Rogge et al., 1995; Scholz et al., 1997). Whether combination of these two effects completely explains the observed variability remains to be tested; loss of either Su(H) or Yan causes modest reductions in midline glia, rather than the increase observed in embryos that lack Spen. However, reductions in Notch levels can partially suppress hypomorphic Yan phenotypes during eye development (Rogge et al., 1995). In the embryo, reducing Notch activity may lead to forming extra lch precursors; lowering Yan levels may affect the recruitment of other cells to become neurons, or the ability of precursors to become neurons. Reduced Su(H) expression in embryos that lack Spen may therefore partially counter the effect of lowering Yan.

Notch and Ras/MAPK signaling are also required for the development of other tissues (Hartenstein et al., 1992). The dependence of Su(H) and Yan expression on Spen is not restricted to the nervous system, and may contribute to defects in muscle and tracheal development observed in *MZspen* embryos (B. Kuang and P. Kolodziej, unpublished data). The muscle and trachea phenotypes may parallel those observed in the nervous system, in that many muscle- and trachea-specific markers are normally expressed in the absence of Spen, but the number of cells and their morphology in these tissues is likely altered. Defects in head and cuticle development observed in *MZspen* embryos may also reflect these defects in signaling pathways. *Notch* and *spen* mutants are both enhancers of *Deformed* mutants (Florence and McGinnis, 1998), and *yan* and *spen* head development defects overlap. Further study of *spen*'s role in the development of these other tissues will clarify whether these phenotypes result from defective Notch and Ras/MAPK signaling.

Our data do not exclude other possible explanations for the cell-fate defects such as effects on cell division or fate transformation within a lineage. *spen* mutants enhance the rough eye phenotype caused by the ectopic expression of dE2F and its heterodimeric partner dDP and suppress the rough eye phenotype caused by $p21^{CIP1}$(Staehling-Hampton et al., 1999). dE2F/dDP ectopic expression causes extra cell divisions and $p21^{CIP1}$ has opposite effects. Effects on Yan levels or activity probably do not explain the genetic interactions between *spen* and these cell-cycle regulators because the dE2F/dDP phenotype is not sensitive to *yan* gene dosage (Staehling-Hampton et al., 1999). Thus, Spen may act on other molecules that regulate the decision to proliferate or differentiate. These possibilities notwithstanding, the expression of many highly specific markers for cell fate appears relatively normal in *MZspen* embryos, once defects in Su(H) and Yan expression are considered.

spen affects the growth and guidance of CNS, motor and sensory axons

In zygotic *spen* mutant embryos, cell fate appears normal, though subsets of CNS, motor and sensory axons are defective in growth and guidance. The requirement for Spen in axon development is likely broader than the zygotic mutant phenotype suggests. When maternally contributed Spen protein is also removed, motor axons that would innervate muscles in zygotic mutants fail to innervate target muscles. As in zygotic mutants, later stages of motor axon development are affected rather than early or intermediate stages.

Mosaic analysis will be required to determine whether these defects reside in the neuron, other cells, or both, given Spen's ubiquitous expression and role in cell fate. In embryos that lack maternal and zygotic Spen, the cell-fate phenotypes could be mechanistically linked to the axon guidance defects, though altering lch neuron number by blocking EGFR signaling does not itself cause defects in axon development (P. Kolodziej, unpublished observations). However, the cell-fate defects in embryos that lack Spen are more complex, and affect the nuclear outputs of at least two signaling pathways not previously known to be directly linked. One of these, Notch, regulates both neuronal cell fate (Artavanis-Tsakonas et al., 1999) and morphology (Giniger, 1998; Giniger et al., 1993a; Sestan et al., 1999). Its role in regulating neuronal morphology in a culture system correlates with Su(H) activity (Sestan et al., 1999), suggesting that transcriptional responses to Notch signaling may also be important for axon development. Spen can alter the expression of Su(H), and may, via this mechanism or an independent nuclear effect, regulate morphology.

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