DOI: 10.1534/genetics.106.055558

Characterization of the *split ends*-Like Gene *spenito* Reveals Functional Antagonism Between SPOC Family Members During Drosophila Eye Development

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Manuscript received January 6, 2006
Accepted for publication March 7, 2006

ABSTRACT

The novel family of SPOC domain proteins is composed of broadly conserved nuclear factors that fall into two subclasses, termed large and small, based on protein size. Members of the large subgroup, which includes Drosophila SPEN and human SHARP, have been characterized as transcriptional corepressors acting downstream of a variety of essential cell signaling pathways, while those of the small subclass have remained largely unstudied. Since SPEN has been implicated in Drosophila eye development, and the small SPOC protein NITO is also expressed in the developing eye, we have used this context to perform a structure–function analysis of NITO and to examine the relationship between the two SPOC family subclasses. Our results demonstrate that the phenotypes obtained from overexpressing NITO share striking similarity to those associated with loss of *spen*. Dosage-sensitive genetic interactions further support a model of functional antagonism between NITO and SPEN during Drosophila eye development. These results suggest that large and small SPOC family proteins may have opposing functions in certain developmental contexts.

CONSERVED signaling pathways are used reiteratively throughout development to specify the cell and tissue types composing an adult organism. Since these pathways do not function independently of each other, cells must receive and respond to multiple interconnected signals. One critical strategy for information integration occurs at the level of the nuclear effectors of these signal transduction cascades that act in a concerted fashion to regulate expression of target genes required for proper development. While in most cases the underlying molecular mechanisms remain poorly understood, the use of large-scale, unbiased genetic screens in model systems such as Drosophila has proven to be a powerful approach to identify and dissect these conserved nuclear circuitries.

One potential mediator of nuclear signal integration identified by such screens is *split ends* (*spen*). A role for *spen* as a nuclear effector was first revealed in several independent genetic screens designed to isolate new downstream players in the Drosophila receptor tyrosine kinase (RTK) signaling pathway (Dickson *et al.* 1996; Rebay *et al.* 2000; Therrien *et al.* 2000). From these and subsequent investigations, *spen* has been positioned as a positive regulator and/or effector of RTK-mediated signaling events in multiple developmental contexts, including the eye and the embryonic central nervous system (Chen and Rebay 2000; Rebay *et al.* 2000). Additional studies have implicated *spen* in a diverse spectrum of cellular processes including neuronal cell fate specification and survival, axon guidance, cell cycle, *hox* gene regulation, and cell positioning (Kolodziej *et al.* 1995; Gellon *et al.* 1997; Staehling-Hampton *et al.* 1999; Wiellette *et al.* 1999; Chen and Rebay 2000; Kuang *et al.* 2000; Lane *et al.* 2000; Rebay *et al.* 2000; Brumby *et al.* 2004; Mace and Tugores 2004; Mutsuddi *et al.* 2004).

Importantly, *spen* appears to operate downstream of multiple signaling pathways. In addition to its role in RTK-mediated signaling events, *spen* functions as a context-specific positive regulator of Wingless signaling and as a likely regulator of Notch signaling (Oswald *et al.* 2002; Schreiber *et al.* 2002; Kuroda *et al.* 2003; Lin *et al.* 2003). Together these results suggest a complex role for SPEN as a nuclear effector and potential integrator of multiple signaling pathways.

spen encodes the founding member of a family of proteins characterized by three N-terminal RNA recognition motifs (RRMs) and a novel C-terminal domain, called the SPEN paralog ortholog conserved domain, or SPOC domain (Figure 1A) (Wiellette *et al.* 1999; Kuang *et al.* 2000; Rebay *et al.* 2000). SPEN orthologs have been identified in worms, flies, mosquitos, mice, humans, and other vertebrates, and more recent studies have identified proteins in plants and yeast carrying the

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SPOC domain in conjunction with other functional motifs (Sanchez-Pulido *et al.* 2004). The RRMs suggest a role for SPOC family proteins in RNA or DNA binding and in the case of SPEN are necessary for nuclear localization (I. Rebay, unpublished data), while the SPOC domain of SPEN and its human and mouse orthologs SHARP (SMRT/HDAC1 associated repressor protein) and MINT (Msx2-interacting nuclear target protein) have been implicated in transcriptional regulation and repression (Shi *et al.* 2001; Oswald *et al.* 2002; Kuroda *et al.* 2003; Yang *et al.* 2005).

SPOC family proteins can be further divided into two subclasses on the basis of their size. We will refer to members of these subclasses as "large" SPOC family proteins, which include SPEN, MINT, and SHARP, and "small" SPOC family proteins, which are also well conserved from worms to humans. In contrast to large SPOC family proteins, almost nothing is known about the functions of small SPOC proteins. Thus far, only the human small SPOC family member one twenty two (OTT)/RNA-binding motif protein-15 (RBM15) has been studied. Specifically, chromosomal translocations identified in cases of acute megakaryocytic leukemia revealed a fusion with *MAL* (megakaryocytic acute leukemia)/*MKL1* (megakaryoblastic leukemia-1) that results in a chimeric protein that includes almost the entire coding region of both genes, with *RBM15/OTT* at the N terminus and *MAL/MKL1* at the C terminus (Ma *et al.* 2001; Mercher *et al.* 2001). Recent evidence suggests that the *RBM15–MKL1* fusion may contribute to leukemogenesis through an increased ability to activate serum response factor (SRF) target genes (Cen *et al.* 2003).

As the Drosophila genome encodes both large and small SPOC family proteins, SPEN and SPENITO (NITO), respectively, this provides an opportunity for comparing the two SPOC subfamilies in a genetically tractable system. To explore the relationship between small and large SPOC family proteins we have examined the effects of genetically manipulating NITO levels in the eye, a tissue in which *spen* loss of function results in developmental defects (Dickson *et al.* 1996; Rebay *et al.* 2000). We found that overexpression of *nito* perturbs eye development, resulting in phenotypes similar to those observed in *spen* mutants and suggesting the possibility of functional antagonism between NITO and SPEN in this context. Dosage-sensitive genetic interactions between *spen* and *nito* further support an antagonistic relationship between these two genes during eye development.

MATERIALS AND METHODS

Drosophila stocks and transgenic lines: The UAS-*nito* full-length construct was generated by fusing the GH11110 cDNA sequence, derived from a Berkeley Drosophila Genome Project clone, in frame to a 5′-Myc tag downstream of a UAS promoter (pUAST). UAS-*nito*ΔC was generated by fusing the 5′-fragment of *nito*, corresponding to amino acids 2–593, to a 5′-Myc tag downstream of UAS promoter (pUAST). UAS-*nito*ΔN was made by fusing two 3′-*nito* fragments, corresponding to amino acids 471–793 and generated from GH11100 cDNA by PCR, to a 5′-Myc tag downstream of a UAS promoter (pUAST). The 3′ fragment contains an SV40 NLS inserted by PCR with specific primers.

nito-RNAi was generated against the 5′ end of the *nito* coding region using previously described methodology (Kalidas and Smith 2002). A 606-bp fragment of *nito* genomic DNA was amplified using primers 5′-RI Dm44A G (5′-CAGAATTCGAG TAGTCATCGAGACGGAGCCGG-3′) and 3′-H3 Dm44A G (5′-CTTAAGCTTCTGCAAAGCATCTTAGATTAGCCAAGG-3′), and a 548-bp cDNA fragment, corresponding to the reverse complement of the genomic sequence but lacking the internal intron, was amplified from full length *nito* cDNA using primers 5′-H3 Dm44A cDNA (5′-CTTAAGCTTCTATATTCCGGTCTG GTTGTGG-3′) and 3′-*Kpn*I Dm44A cDNA (5′- CTGGTACC GAGTAGTCATCGAGACGGAGC-3′). The genomic fragment was cut with *Eco*RI and *Hin*dIII and the cDNA fragment cut with *Hin*dIII and *Kpn*I and both fragments were ligated into a pUAST vector digested with *Eco*RI and *Kpn*I. UAS-*myc-nito*-FL, UAS-*myc-nito*ΔC, UAS-*myc-nito*ΔN, and UAS-*nito* RNAi were used to generate transgenic lines as previously described (Rebay *et al.* 1993).

The following fly stocks were used: w^{1118}; sev^{med}-*Gal4, UAS-spen*^{DN} (Lin *et al.* 2003); *sev-Gal4* (weak and medium generated by I. Rebay and strong from Bloomington Stock Center); $spen^{xfm911}$/*Cyo* (Rebay *et al.* 2000).

Western blots: Western blot to examine expression of UAS-*nito* transgenes was carried out by crossing UAS lines to hsp70Gal4, heat-shocking adults for 1 hr, followed by a 1.5 hr recovery. A total of 25 fly heads were collected for each sample, homogenized in 2× SDS buffer, and run on a gel. Protein levels were examined using mouse anti-MYC mAb 9E10 (1:500; a gift from R. Fehon).

Immunohistochemistry: Larval eye discs were dissected in Drosophila S2 cell media, fixed in 4% paraformaldehyde in 1× PBS for 10 min at room temperature, washed 3 times in PT (1× PBS + 0.1% Triton X-100), blocked 1 hr in PNT (1× PBS + 0.1% Triton X-100 + 1% NGS). Primary antibodies were incubated overnight at 4° in PNT on a rotator. Mouse anti-elav (Developmental Studies Hybridoma Bank) was used at 1:50. Samples were then washed in PT and incubated with goat anti-mouse HRP (1:500; Jackson ImmunoResearch, West Grove, PA) in PNT for 2 hr, washed, and developed. For acridine orange staining, larval eye discs were dissected in S2 media, incubated with 1 mm acridine orange diluted 1:500 in S2 media for 10 min, rinsed, and mounted in 1× PBS for immediate viewing.

RT–PCR analysis: Sixty pairs of eye discs were dissected from control (*w:* GMR-GAL4) and GMR-GAL4> *nito*-RNAi third instar larvae. Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and cDNA was synthesized using random primers (Promega) from 1 μg of total RNA. PCR amplification was performed with *nito* primers 5′-AGGTTCCTCTTCTTCAGTTCCCCC-3′ and 5′-TTG GTGTCGTTTGTGGACCCTG-3′ and *Rps17* primers 5′-CGA ACCAAGACGGTG AAGAAG-3′ and 5′-CCTGCAACTTGATG GAGATACC-3′ to compare expression levels. NIH Image was used to quantitate expression levels of *nito* relative to *Rps17*. Each experiment was performed twice.

RESULTS AND DISCUSSION

Sequence conservation defines two distinct SPOC family subclasses: SPOC family proteins fall into two apparent subclasses based on their size. To determine

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DmNITO    629  RKCSTVWTGALILKSSLFPAKFHLTDGDTDIVESLMRD--EEGK---HNL   673
HsRBM15   783   KLCLAWQGMLLLKNSNFPSNMHLLQGDLQVASSLLVEGSTGGK--VAQL   829
MmRBM15   775   KLCLAWQGMLLLKNSNFPSNMHLLQGDLQVASSLLVEGSTGGK--VAQL   821
CeNITO    370  EAYAATWSGKMALKKTDYPVKFYRVYGAERLPVKLLRD---EDD-VPLRL   415
DmSPEN    5314 QRYPVMWQGLLALKTDQAAVQMHFVHGNPNVARASLPS-LVETN--TPLL   5360
HsSHARP   3503 KKYPIVWQGLLALKNDTAAVQLHFVSGNNVLAHRSLP--LSEG---GPPL   3457
MmMINT    3483 KKYPIVWQGLLALKNDTAAVQLHFVSGNNVLAHRSLP--LSEG---GPPL   3527
CeSPEN    2549 QHFPMVWTGRLALKSTEAMINLHLINGSETFLNDVLGRQVTEENPRRDSV   2598

DmNITO    674  RITQRLRLDPPKLDDVQKRI--ASSSSHAIFMGLAGSTNDTN------CD   714
HsRBM15   830  KITQRLRLDQPKLDEVTRRIKVAGPNGYAILLAVPGSSDSRSSSSSAASD   879
MmRBM15   822  KITQRLRLDQPKLDEVTRRIKVAGPNGYAILLAVPGSSDSRSSSSSATSD   871
CeNITO    416  LITQRLSLS-SQNLLFDKLASCS---SKELSLGVITGKKE----------   451
DmSPEN    5361 RIAQRMRLEQTQLEGVAKKMQVD--KEHCMLLALPCGRDH--------AD   5400
HsSHARP   3458 RIAQRMRLEATQLEGVARRMTVE--TDYCLLLALPCGRDQ--------ED   3587
MmMINT    3528 RIAQRMRLEASQLEGVARRMTVE--TDYCLLLALPCGRDQ--------ED   3567
CeSPEN    2599 KILQRLRLDNGQVEHIYRILTNP---EYACCLALSSVNNI--------EN   2637
                  ******
DmNITO    715  DASVQTRPLRNLVSYLKQKEAAGVISLLN---KETEATGVLYAFPPCDFS   762
HsRBM15   880  TATSTQRPLRNLVSYLKQKQAAGVISLPVGGNKDKENTGVLHAFPPCEFS   929
MmRBM15   872  TAASTQRPLRNLVSYLKQKQAAGVISLPVGGNKDKENTGVLHAFPPCEFS   921
CeNITO    452  -----LSDLQPLVNYFTNKEAAGVVTVPG---------GILYIFPFCEFA   487
DmSPEN    5401 VLQHSRNLQTGFITYLQQKMAAGIVNIPIPG--SEQAAYVVHIFPSCDFA   5448
HsSHARP   3588 VVSQTESLKAAFITYLQAKQAAGIINVPNPG--SNQPAYVLQIFPPCEFS   3635
MmMINT    3568 VVSQTESLKAAFITYLQAKQAAGIINVPNPG--SNQPAYVLQIFPPCEFS   3615
CeSPEN    2638 LKENDTNLKSHFIDYLINKKIAGISSLGEVE--TKFKSARVHVFAPGEIV   2685

DmNITO    763  TELLKRTCHSLTEEGLKEDHLVIVVVRGGTA      793
HsRBM15   930  QQFLDSPAKALAKS--EEDYLVMIIVRGAS       957
MmRBM15   922  QQFLDSPAKALAKS--EEDYLVMIIVRAKLVNSG   953
CeNITO    488  LKLLAEFTPQVNVFNENCPFLLGALAVRAPGT     519
DmSPEN    5449 NENLERAAPDLKNRVAELA-HLLIVIATV       5476
HsSHARP   3636 ESHLSRLAPDLLASISNISPHLMIVIASV       3664
MmMINT    3616 ESHLSRLAPDLLASISNISPHLMIVIASV       3644
CeSPEN    2686 NRYLSELATSLHDYLQNTDTRYLLIVFTN       2714
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FIGURE 1.—Alignment of conserved SPOC domain across multiple species. Residues with * are divergent between large and small SPOC proteins. Alignments and similarity/identity comparisons were performed with MacVector's ClustalW program. Accession numbers for GenBank sequences are as follows: Dm NITO, NP_610339; Dm SPEN, NP_722616; Hs SHARP, Q96T58; HsOTT/RBM15, CAC38861.1; Mm MINT, Q62504; Mm RBM15, AAH57038; Ce SPEN, E88320; CeNITO, AAC1912. Abbreviations for species are as follows: Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; and Ce, *Caenorhabditis elegans*. Numbers refer to amino acid residues.

whether such a distinction might be functionally significant, sequence alignments of the conserved C-terminal SPOC motif were performed to compare the level of sequence conservation in the SPOC family in general and subclass members in particular (Figure 1). Analysis revealed only 27% identity and 50% overall similarity between the SPOC domains of SPEN and NITO, the Drosophila representatives of the large and small subfamilies, respectively; however, upon comparison of the SPOC domains of these proteins with those of their respective subclass family members, a higher level of conservation was revealed. Drosophila SPEN and human SHARP exhibit 58% sequence identity and 79% overall sequence similarity, while Drosophila NITO and human RBM15/OTT share 47% sequence identity and 62% overall sequence similarity. Comparable results were obtained by comparing the RRM motifs (data not shown). These results reveal a higher level of sequence conservation within SPOC family subclasses relative to the family in general, raising the possibility that subclasses may have adopted divergent functions.

Overexpression of *nito* perturbs adult eye morphology: To better understand the relationship between large and small SPOC proteins, we were interested in determining if *spen* and *nito* function synergistically or antagonistically *in vivo*. Because the large SPOC family member *spen* is required for Drosophila eye development (DICKSON *et al.* 1996; REBAY *et al.* 2000; LIN *et al.* 2003) and the fly eye provides a uniquely powerful system in which to explore functional relationships between signaling molecules (ZIPURSKY and RUBIN 1994), we focused our analyses on this tissue. RT–PCR confirmed that *nito*, like *spen*, is expressed in the developing eye disc (Figure 4 and data not shown), further validating the approach.

Because no *nito* mutants are currently available, an *in vivo* structure–function analysis was undertaken to investigate *nito* function during eye development. While the phenotypes resulting from overexpression of a gene must be interpreted with caution, such overexpression models frequently result in sensitized genetic systems that can provide powerful tools for investigating *in vivo* relationships between signaling molecules. Myc-tagged full-length NITO (NITO-FL), NITO lacking the N terminus (NITOΔN; an exogenous nuclear localization sequence was added to ensure proper nuclear targeting), and NITO lacking the C terminus (NITOΔC) were cloned downstream of a UAS promoter and the transgenes were expressed in flies using eye-specific GAL4 drivers (Figure 2A). Three different *sevenless*-Gal4 (*sev-Gal4*) drivers, which promote expression in photoreceptors R1, R3, R4, R6, R7, the cone cells, and the "mystery" cells, which are poorly understood interommatidial cells that are never recruited to the ommatidia and ultimately apoptose, were utilized in this study: sev^{strong} couples the *sev* enhancer to the *hsp70* promoter, resulting in the highest levels of expression; sev^{medium} contains both the *sev* enhancer and *sev* promoter and expresses at an intermediate level; sev^{weak} contains the same regulator sequences as sev^{medium} but expresses at lower levels, presumably as a consequence of position effect of the transgene. To avoid unnecessary confusion, we will refer to these collectively as *sev-Gal4* and will identify the specific driver in the figure legends as appropriate.

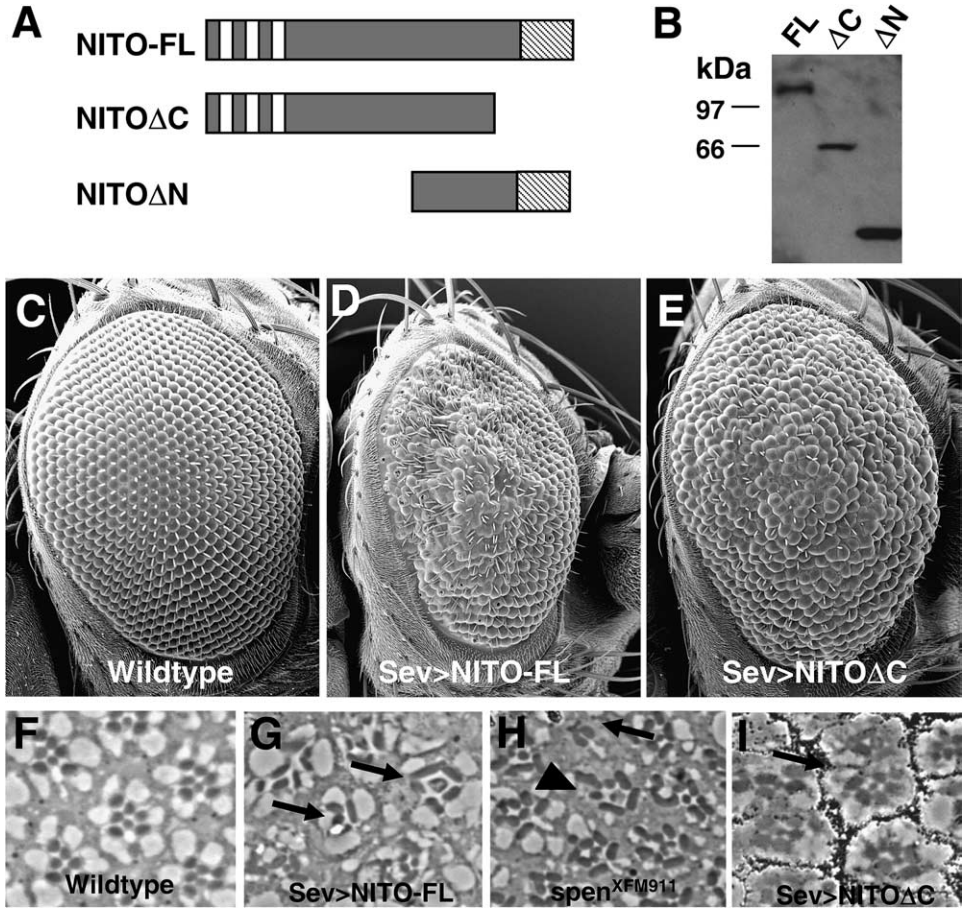

FIGURE 2.—Overexpression of *nito* results in rough eye phenotypes. (A) Overexpression of *nito* constructs. (B) Western blot with anti-myc antibody using head lysates from *hsp70Gal4;UAS-nito* flies. (C–E) SEM of adult eyes. (C) Wild-type (w^{1118}) control. (D) sev^{medium}*-GAL4/UAS-nito-FL.* (E) sev^{strong}*-GAL4/UAS-nito*Δ*C.* (F–H) Sections of adult eyes. (F) Wild-type (w^{1118}) control. (G) sev^{medium}*-GAL4/UAS-nito-FL.* Arrows indicate ommatidia missing photoreceptors. (H) $spen^{XFM911}$/$spen^{XFM911}$ mutant eye clones. Arrow indicates ommatidium missing photoreceptors. Arrowhead indicates disorganized ommatidium. (I) sev^{strong}*-GAL4/UAS-nito*Δ*C.* Arrow indicates ommatidial fusion.

Sev-Gal4-driven overexpression of NITO-FL and NITOΔC yielded dosage-dependent adult rough eye phenotypes (Figure 2, C–E), while overexpression of NITOΔN was indistinguishable from wild type (data not shown). Western blots confirmed the expression of all transgenes (Figure 2B), and immunohistochemistry showed nuclear localization of NITO in all cases (data not shown), indicating that the lack of a NITOΔN phenotype is not due to the absence or mislocalization of protein.

While overexpression of NITO-FL and NITOΔC both perturb eye development, the resulting phenotypes are distinct. These data are not unexpected given previous results for the large SPOC family protein, SPEN, in which overexpression of SPENΔC functions as a dominant negative with respect to *spen* (CHEN and REBAY 2000). We therefore speculate that NITOΔC functions analogously as a dominant negative relative to *nito*, whereas NITO-FL expression simply augments the pool of full-length NITO. Specifically, we observe that overexpression of NITO-FL results in roughening of the posterior part of the eye and an overall decrease in eye size (Figure 2D), whereas overexpression of NITOΔC more uniformly perturbs the external morphology of the eye (Figure 2E).

To distinguish the NITO-FL and NITOΔC rough eye phenotypes at the cellular level, adult eyes were sectioned and examined for defects. In wild-type ommatidia, photoreceptors are arranged in a trapezoidal array with seven of the eight photoreceptors visible in one plane of view (Figure 2F). The regular trapezoidal arrangement of photoreceptors is disturbed in both overexpression systems (Figure 2, G–H). When NITO-FL is overexpressed we see a decrease in the number of photoreceptors per ommatidia, elongated rhabdomeres, as well as a general disorganization of the ommatidia (Figure 2G). These observations suggest that the rough eye phenotype is due to a loss of photoreceptors and possible defects in the accessory cells, which normally provide support for the rhabdomeres in the ommatidia. This phenotype is strikingly reminiscent of that seen in sections of *spen* mutant eye clones (Figure 2H) (DICKSON *et al.* 1996), raising the possibility that overexpressed *nito* may function antagonistically with respect to *spen* in the developing eye.

Eyes overexpressing NITOΔC also appear disorganized compared to wild type, although in contrast to NITO-FL ommatidia, photoreceptor number is not strongly affected. Rather, the most prevalent defect appears to be ommatidial fusions (Figure 2I) suggesting that cone and pigment cells, rather than photoreceptors, are most affected. Given that the Gal4 driver used for these experiments is expressed primarily in a subset of photoreceptors, the cone cells and interommatidial mystery cells, the accessory cell defects we observe upon

nito overexpression may be due in part to indirect effects on pigment cells. Thus, NITO-FL ommatidia have defects in photoreceptor number and ommatidial morphology, while NITOΔC ommatidia have defects in accessory cells required for the spacing of ommatidia.

***nito* overexpression impairs cell survival in the developing eye:** To further investigate the defects caused by overexpressing *nito*, we examined the effects of increasing *nito* expression in early eye development. First, we examined recruitment of the photoreceptor neurons into ommatidia by looking at expression of the pan-neural marker ELAV in the larval precursor to the eye, the eye imaginal disc (Figure 3, A–C). Consistent with the differences observed in the adult phenotypes, the larval phenotypes associated with *sev-Gal4*-driven expression of NITO-FL and NITOΔC are also distinct.

In eye discs overexpressing NITO-FL, initial recruitment of photoreceptors appears normal (Figure 3B). However, approximately seven rows posterior to the furrow there is a decrease in the number of photoreceptors per ommatidium. Thus while NITO-FL expression does not perturb initial photoreceptor recruitment, subsequent development and/or survival are compromised, resulting in the reduced number of photoreceptors observed in the adult eye. The loss of photoreceptors upon overexpression of NITO-FL is also similar to *spen* mutant clones, which have reduced numbers of photoreceptors in mutant ommatidia in the developing imaginal disc (D. Doroquez and I. Rebay, unpublished data), consistent with the observations made in adult eye sections (Figure 2). In contrast to NITO-FL and *spen* mutant clones, and consistent with the ommatidial fusions observed in adult eye sections, overexpression of NITOΔC causes loss of spacing between ommatidia in the larval eye disc, while recruitment of photoreceptors is not affected (Figure 3C).

To examine the possibility that the phenotypes associated with overexpression of NITO-FL and NITOΔC were due primarily to cell death, we stained eye discs with the apoptotic marker acridine orange (Figure 3, D–F). In the wild-type eye disc very little cell death is observed (Figure 3D). In NITO-FL eye discs a stripe of cell death occurs in the posterior part of the differentiating eye disc (Figure 3E), consistent with the loss of photoreceptors observed in the ELAV-probed eye disc (Figure 3B) and similar to the elevated cell death phenotype observed in *spen* mutant clones (D. Doroquez and I. Rebay, unpublished data). However, coexpression of the apoptotic inhibitor p35 or introduction of the H99 deficiency that removes the proapoptotic genes *hid*, *reaper*, and *grim* did not suppress the NITO-FL rough eye phenotypes (data not shown), suggesting that increased apoptotic cell death is unlikely to be the primary factor contributing to the NITO-FL-associated eye defects. In discs overexpressing NITOΔC, increased cell death is observed more anteriorly relative to that for NITO-FL (Figure 3F *vs.* 3E), consistent with the ommatidial spacing defects observed in the ELAV-probed disc (Figure 3C).

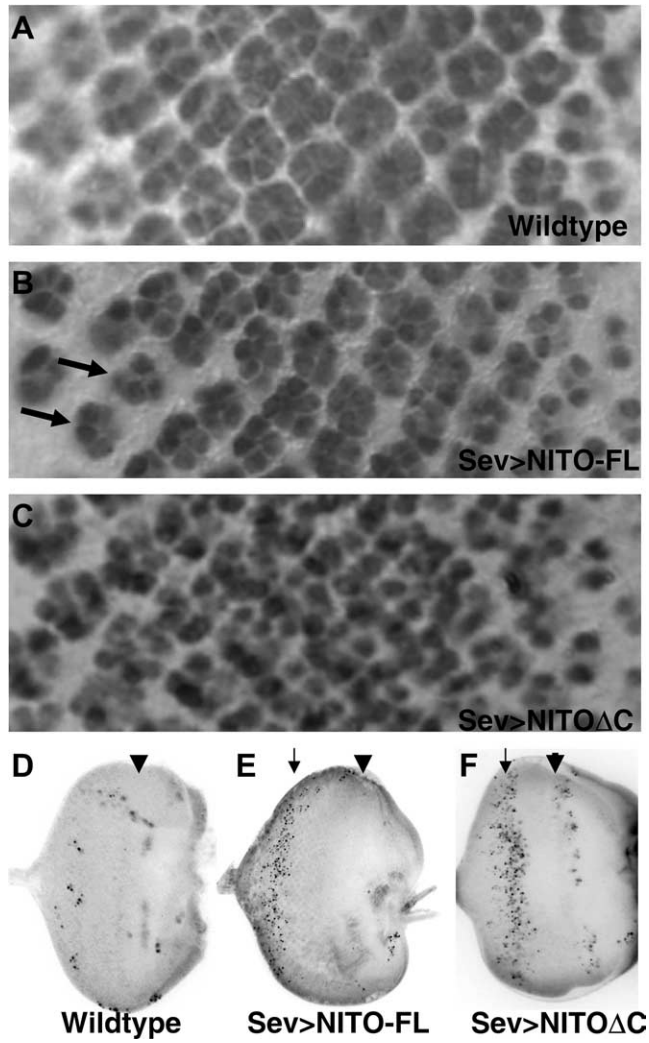

Figure 3.—Developmental defects associated with *nito* overexpression. Eye imaginal discs are oriented posterior to the left. (A–C) Elav staining of third instar larval eye imaginal discs. (A) Wild-type (w^{1118}) control. (B) sev^{strong}-*GAL4/UAS-nito-FL*. Arrows indicate ommatidial clusters missing photoreceptors. (C) sev^{strong}-*GAL4/UAS-nito*Δ*C*. (D–F) Acridine orange staining of third instar larval eye discs. Location of cell death is indicated with arrow; arrowhead indicates the morphogenetic furrow. (D) Wild-type (w^{1118}) control. (E) sev^{strong}-*GAL4/UAS-nito-FL*. (F) sev^{strong}-*GAL4/UAS-nito*Δ*C*.

SPEN and NITO act antagonistically: The potential for functional antagonism between SPEN and NITO was suggested by the similarity of phenotypes observed in adult eye sections overexpressing NITO-FL and in *spen* mutant eye clones. To further investigate this potential antagonism, we performed a series of dose-sensitive genetic interactions between *spen* and *nito*.

First, we examined the effects of reducing *spen* levels in the NITO-FL overexpression background. If NITO-FL antagonizes SPEN function, as suggested by our phenotypic analysis, further reducing *spen* should

exacerbate the NITO-FL overexpression phenotype. An important requirement for such an experiment is the need for dose-sensitive NITO-FL phenotypes. Two observations suggest NITO-FL provides a dose-sensitive phenotype ideal for studying genetic interactions: first, expression of independent transgenic lines with the same *sev-Gal4* driver results in a range of phenotypes (Figure 4, A and B); and second, expression of a given NITO-FL transgene with sev^{weak} results in a mild rough eye phenotype (Figure 4B), whereas expression of the same line at a higher level using the sev^{medium} produces a more severe phenotype (Figure 2D). Consistent with our hypothesis of an antagonistic relationship between *spen* and *nito*, we found that heterozygosity for a null *spen* allele enhanced the rough eye phenotype associated with NITO-FL expression, as demonstrated by an increased number of ommatidia lacking photoreceptors (Figure 4, B–D, I, J, N, and O).

Next, we investigated the consequences of increasing or decreasing *nito* levels in the background of a dominant negative *spen* transgene ($spen^{DN}$), which encodes the C-terminal 936 amino acids of *spen* (Lin *et al.* 2003) and also produces dose-sensitive phenotypes (D. Doroquez and I. Rebay, unpublished results). Because both transgenes are capable of perturbing eye development on their own, to distinguish between additive and synergistic interactions we used a NITO-FL transgenic line that when expressed with sev^{weak} exhibits only very mild perturbations of the adult eye (Figure 4A). As expected given the NITO structure–function analysis, NITO-FL causes an enhancement of the $spen^{DN}$ rough eye phenotype, an increase in necroses in the eye, and a complete loss of organization (Figure 4, A, E, F, K, L, P, and Q). Thus, overexpression of *nito* and overexpression of $spen^{DN}$ appear to act in the same direction, suggesting opposing functions for NITO and SPEN.

As loss-of-function mutations in *nito* have not been isolated, we generated a *nito* transgenic dsRNA construct to investigate the consequences of reducing endogenous *nito* expression levels with respect to *spen* function. RT–PCR from Drosophila eye discs confirmed that this construct mediates partial knockdown of *nito* expression (Figure 4S). *In vivo*, while dsRNA-mediated knockdown of *nito* expression does not perturb eye morphology on its own, *nito*-RNAi partially rescues the rough eye phenotype resulting from overexpression of $spen^{DN}$ (Figure 4, G, H, L, M, Q, and R), again suggesting antagonism between *nito* and *spen*. Eye sections show fewer missing ommatidia in *nito*-RNAi, $spen^{DN}$ adult eyes relative to those overexpressing $spen^{DN}$ alone, as well as fewer missing photoreceptors in ommatidia lacking the full complement of photoreceptors and more normal rhabdomere morphology (Figure 4, Q and R). Together, these dose-sensitive genetic interactions argue for mutual antagonism between the large SPOC family member *spen* and the small SPOC family representative *nito* during Drosophila eye development.

It remains to be determined if the antagonistic relationship between *nito* and *spen* is maintained in developmental contexts outside of the eye. Previous work examining the role of SPEN in Wingless signaling suggested the presence of a redundant partner for SPEN (Lin *et al.* 2003), for which NITO would be a good candidate, given their sequence conservation. *In situ* hybridization for *nito* and *spen* suggests that both are also ubiquitously expressed throughout embryonic development (data not shown), and considering the broad range of embryonic phenotypes attributed to *spen* mutants (Kolodziej *et al.* 1995; Chen and Rebay 2000; Kuang *et al.* 2000), exploration of context-specific interactions between *spen* and *nito* in the embryo will likely improve our understanding of the relationships between these two related proteins. We predict that certain developmental events will require synergism between *nito* and *spen*, whereas others, as we demonstrate in the eye, will require antagonism.

At the cellular level, *spen* is implicated as a positive component of Wingless and RTK/RAS signaling (Chen and Rebay 2000; Rebay *et al.* 2000; Lin *et al.* 2003), and large SPOC family proteins SHARP and MINT are implicated as negative regulators of Notch signaling (Oswald *et al.* 2002; Kuroda *et al.* 2003). Given the ability of *nito* to antagonize *spen* function in the developing eye, it seems reasonable to speculate that NITO also acts as a downstream regulator/effector of some or all of these pathways. Furthermore, the antagonism between *nito* and *spen* may provide a mechanism for differential regulation of output from these pathways.

Mechanistically, how might one envision the mutual antagonism between SPEN and NITO? Large SPOC proteins have been previously shown to serve as transcriptional corepressors (Shi *et al.* 2001; Oswald *et al.* 2002; Kuroda *et al.* 2003; Yang *et al.* 2005). Thus one attractive possibility is that small SPOC proteins might serve as transcriptional activators. In this model, by virtue of their conserved RRM and SPOC motifs, small and large SPOC proteins might compete for access to common binding partners. The resulting complexes, depending on whether they contain SPEN or NITO, would then either repress or activate transcription. In a slight variation of the model, one could propose that SPOC proteins might be able to either repress or activate transcription and so depending on context would act either synergistically or antagonistically. Unfortunately, we have found that Drosophila cultured cells do not provide an appropriate environment in which to assay the activity of SPOC proteins (J. Jemc, D. Doroquez and I. Rebay, unpublished results) so we have not been able to test this model with respect to SPEN and NITO. However, using mammalian COS cells, we have observed that while the SPOC motif of SHARP represses transcription as previously published (Shi *et al.* 2001; Oswald *et al.* 2002), the SPOC motif of

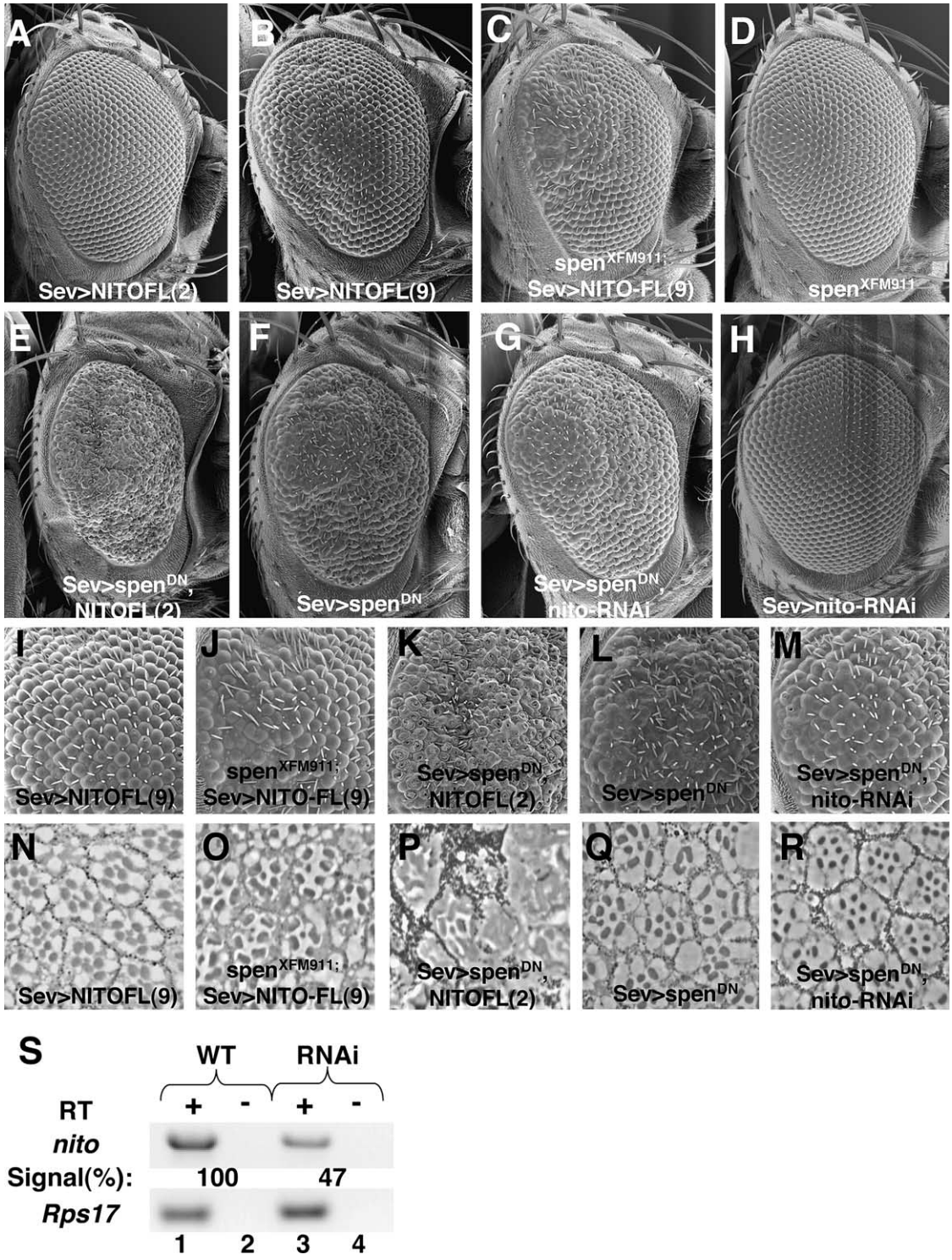

FIGURE 4.—*Nito* and *spen* function antagonistically. (A–H) Scanning electron micrograph (SEM) of adult eyes, oriented posterior to the left. (I–M) Higher magnification view of the posterior region of eyes shown in C–G. (N–R) Eye sections of genotypes indicated in I–M. (A) *UAS-nito-FL(2)/+; sev^{weak}-GAL4/+*. (B, I, and N) *UAS-nito-FL(9)/+; sev^{weak}-GAL4/+*. (C, J, and O) *spen^{XFM911}/UAS-nito-FL(9); sev^{weak}-GAL4/+*. (D) *spen^{XFM911}/CyO*. (E, K, and P) *UAS-nito-FL(2)/+; sev^{weak}-GAL4, UAS-spen^{DN}/TM6B*. (F, L, and Q) *sev^{weak}-GAL4, UAS-spen^{DN}/TM6B*. (G, M, and R) *sev^{weak}-GAL4, UAS-spen^{DN}/UAS-nito-RNAi*. (H) *sev^{weak}-GAL4/UAS-nito-RNAi*. Numbers in parentheses [(2) and (9)] refer to independent transgenic insertions of *UAS-nito-FL*. (S) RNAi efficiently knocks down *nito* expression. RT–PCR with *nito* (top) and *Rps17* primers (bottom) with RT (+; lanes 1 and 3) and without RT control (−; lanes 2 and 4). The percentage of *nito* expression in GMR-GAL4>RNAi discs relative to wild-type eye discs is normalized to expression levels for the *Rps17* control.

RBM15, the human NITO ortholog, strongly activates transcription (J. Jemc and I. Rebay, unpublished data). Thus, perhaps the antagonistic relationship between SPEN and NITO that we report here in the context of Drosophila eye development reflects a conserved antagonistic relationship between large and small SPOC proteins that is manifested at the level of transcriptional output.

In conclusion, we have demonstrated an antagonistic relationship between the large and small SPOC family proteins in the developmental context of the Drosophila eye. The finding that SPOC family proteins function as downstream effectors of a variety of signaling pathways suggests they may act to fine tune transcriptional output downstream of these cascades. Thus, it will be extremely interesting to determine whether the antagonistic relationship we have observed between NITO and SPEN in the eye is a general property of small and large SPOC proteins, or if it is unique to Drosophila eye development. Determination of transcriptional targets and cofactors will be required to understand how SPOC family proteins function to regulate and integrate information from these signaling pathways.

We thank R. Fehon for anti-MYC, the Developmental Studies Hybridoma Bank for anti-ELAV and anti-Cut, and the Bloomington Stock Center for fly stocks. We thank J. T. Littleton, D. Doroquez, and M. Rosenzweig for critical reading of this manuscript and all members of the Rebay lab for advice during the course of this work. Scanning electron microscopy was performed at the W. M. Keck Facility for Biological Imaging. This work was supported by NIH grant RO1 EY-012549 to I.R.

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Communicating editor: K. V. Anderson