Requirement of Split ends for Epigenetic Regulation of Notch Signal-Dependent Genes during Infection-Induced Hemocyte Differentiation^{∇†}

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Received 5 August 2008/Returned for modification 17 October 2008/Accepted 30 December 2008

***Drosophila* producing a mutant form of the putative transcription coregulator, Split ends (Spen), originally identified in the analysis of neuronal development, display diverse immune defects. In order to understand the role of Spen in the innate immune response, we analyzed the transcriptional defects associated with *spen* mutant hemocytes and their relationship to the Notch signaling pathways. Spen is regulated by the Notch pathway in the lymph glands and is required for Notch-dependent activation of a large number of genes involved in energy metabolism and differentiation. Analysis of the epigenetic marks associated with Spen-dependent genes indicates that Spen performs its function as a coactivator by regulating chromatin modification. Intriguingly, expression of the Spen-dependent genes was transiently downregulated in a Notch-dependent manner by the Df activated upon recognition of pathogen-associated molecules, demonstrating the existence of cross talk between hematopoietic regulation and the innate immune response. Our observations reveal a novel connection between the Notch and Toll/IMD signaling pathways and demonstrate a coactivating role for Spen in activating Notch-dependent genes in differentiating cells.**

In response to microbial infection, *Drosophila* generates a battery of innate immune responses to inactivate and remove the invading microbes. *Drosophila* blood cells, or hemocytes, stand at the center of this defense: they produce diverse antimicrobial peptides against infection and consume the microbes by phagocytosis (10, 52). In response to invading microbes, the hemocytes activate several signaling pathways that cross-check each other's activity to maintain a balanced immune response (26, 42, 45, 49, 50), which seems to call for integration of complex signaling pathways. On top of this, the hematopoietic progenitor cells appear to be influenced by the innate immune response and control its development, adding another layer of regulatory interactions between signaling pathways (26). Upon contact with a PAMP, some hematopoietic stem cells differentiate into immune effector cell types, indicating that developmental signals work together with the innate immune signals generated by specific PAMP receptors (52). To understand the regulatory network behind this important process, many laboratories are seeking to identify gene products connecting the regulatory networks of different signaling pathways during innate immune responses.

Notch is one of the key molecules required for development of the immune system. It regulates cell-fate determinations during hematopoiesis in metazoans, and this process is accompanied by activation of cell type specific transcription (1, 12,

39). In *Drosophila*, Delta or Serrate binds to the Notch receptor on neighboring cells, which results in cleavage of Notch and translocation of the Notch intracellular domain (NICD) to the nucleus. In the nucleus, NICD associates with the DNA-binding protein Su(H) (RBP-J in mammal), recruits histone acetyltransferase containing complexes, and triggers the transcription of Notch target genes such as E(spl)-C, needed for cell fate specification (3, 21, 24). When Notch is not activated, most of the Notch target genes are tightly repressed by Hairless and a corepressor protein complex (4, 6, 16, 31). In mammals, no Hairless homolog has been identified, but it has been suggested that MINT (for Msx-2 interacting nuclear target) performs the role of corepressor of Hairless (23). Both in *Drosophila* and in mammals, Notch activation of target genes results in replacement of a repressing chromatin modifier associated with Hairless or MINT with an activating modifier. Hence, transcriptional coregulators, such as Hairless and MINT, appear to play a pivotal role in Notch signaling, and their impact on the chromatin status of the target genes may be the key factor in cell fate determination.

MINT belongs to the evolutionarily conserved SPEN gene family, together with human SHARP and *Drosophila* Spen. SPEN family members contain an RNA recognition motif (RRM) and a SPOC (for Spen paralogous and orthologous C-terminal) domain (22, 53). Structural analysis of SHARP revealed that it associates with histone deacetylase through a C-terminal repression domain, whereas it interacts with the RNA coactivator SRA via the RRM located in its N-terminal domain (48), thus demonstrating its role as both corepressor and coactivator. Deletion of MINT in mice resulted in embryonic lethality around embryonic day 14.5 due to multiple abnormalities, and analysis of hematopoiesis in MINT^{-/-} pre-

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† Supplemental material for this article may be found at <http://mcb.asm.org/>.

∇ Published ahead of print on 12 January 2009.

cursors revealed a defect in B-cell development that could be attributed to defects in Notch signaling (23). Recently, Raffel et al. proposed that a mouse SPEN family member OTT1 is required for B lymphopoiesis and plays an inhibitory role in the myeloid, megakaryocytic, and progenitor compartments (40). It is possible that misregulation of the OTT1-dependent hematopoietic developmental pathway causes abnormal Notch signaling, which contributes to OTT1-MAL-associated acute megakaryocytic leukemia. *Drosophila* Spen was initially identified as an antagonist of Notch signaling during *Drosophila* retinal development (8). As shown in vertebrates, Notch signaling in *Drosophila* also has an early role in the proliferation of hematopoietic cells and plays a critical role in differentiation of crystal cells and lamellocytes (9, 27). Therefore, the SPEN family of proteins appears to play regulatory roles in cell fate specification during both neurogenesis and hematopoiesis, possibly by regulating Notch signaling pathways. Furthermore, the Toll pathway appeared to interact with Notch signaling during *Drosophila* hematopoiesis. Defects in Toll signaling resulted in misregulation of *Drosophila* hematopoiesis, followed by inappropriate hemocyte proliferation and melanotic tumor formation (11, 29, 38). These results indicate a possible link between the Toll and Notch signaling pathways, but the underlying mechanism remains unclear.

We previously isolated a mutant allele of *spen* (*spen*^{GE10359}) that results in defective immunity to *Beauveria bassiana* infection and inappropriate hemocyte development (15). In view of the role of Spen as a key regulator of Notch signaling in *Drosophila* neural development and mammalian hematopoiesis, the compromised immunity shown in the *spen* mutant flies appears to reflect defects in the immune cells caused by inappropriate development. In the present study, we examined the developmental defects of *spen* hemocytes and compared them to those of mutants defective in regulatory signaling, in order to understand the molecular function of Spen in hemocyte development. The expression of Spen in hematopoietic cells of the larval lymph gland is dependent on Notch activity, whereas Notch expression is not affected by *spen* mutation. Genome-wide transcriptome analyses of *spen* and *Notch* pathway mutant hemolymphs and their chromatin modification profiles revealed that Spen mediates Notch signaling by modifying the histone methylation patterns of a large group of genes mostly involved in energy metabolism and formation of specific types of cell. Intriguingly, we also found that the Notch signal was transiently downregulated in a Dif-dependent manner during infection, demonstrating the presence of cross talk between the innate immune response mediated by pathogen recognition receptors and hematopoiesis regulated by Notch signaling. Notch and Spen expression in the lymph glands recovered several hours after infection, with especially strong expression in several patches of hemocytes in the lymph glands; these eventually differentiated into lamellocytes. Here, we present evidence for chromatin regulation by Spen in mediating Notch signaling during hematopoiesis and for cross talk between Notch and the innate immune response required for the development of hemocytes.

MATERIALS AND METHODS

***Drosophila* stocks.** *Drosophila melanogaster* strains were cultured on a standard cornmeal-yeast medium at 25°C and 60% humidity. The *spen*^{GE10359} mutant with

a P-element in an untranslated region of the *spen* gene was purchased from GenExel (Daejeon, South Korea). Revertants for P-element mutant were generated via precise excision of the P-element by crossing with flies containing Δ2-3 transposase, as described by Robertson et al. (43). The *E(spl)^{rv1}*, *Su(H)¹(417)*, and *Tl²* lines were obtained from the Bloomington Stock Center. The *Dif²* was a gift from Kwang-Min Choe, and *Rel^{E20}* and *Imd* were gifts from Won-Jae Lee. *N^{rs1}* and *N^{Mcd8}* were kindly provided by Marie Meister. *STAT* RNAi mutant was performed as described previously (18).

Immunostaining of hemocytes. Third-instar larvae were bled in 20 μl of phosphate-buffered saline (PBS), and the hemocytes were transferred to glass slides and incubated for 30 min at room temperature. The cells were then fixed with 4% paraformaldehyde in PBS and blocked in PBS with 0.3% Triton X-100 and 1% bovine serum albumin. The fixed hemocytes were incubated with antigen affinity-purified anti-Spen rat polyclonal antibody overnight in a humid chamber at 4°C. They were then washed, incubated with appropriate fluorescein isothiocyanate-conjugated secondary antibody (Sigma), and analyzed with a Zeiss LSM 510 META confocal microscope system (Carl Zeiss).

Microarray analysis. In vivo experiments, total RNA (1.0 to 1.3 μg) was isolated from the dissected hemocytes (from 200 to 300 third-instar larvae) with TRIzol (Invitrogen). An antisense RNA was generated by in vitro amplification with a RiboAmp RNA amplification kit (Arcturus kit 0201). To identify genes whose expression changed significantly in mutant flies, RNAs from wild-type, *spen*, *E(spl)*, and *Su(H)* mutant flies were analyzed by using an Affymetrix *Drosophila* Genome 2.0 array with 18,880 probe sets. The Affymetrix arrays were run in the DNA Link (Seoul, South Korea). The data were processed by using GeneChip operating software 1.4 from Affymetrix, Inc., and microarray data were analyzed by using GenMAPP2.1. Up- and downregulated (threefold) genes in each data set were identified by using MAPFinder (7).

In order to define gene expression levels in hemocytes in response to fungal infection, live *B. bassiana* organisms were injected into the posterior end of third-instar larvae using a tungsten needle, and the larvae were incubated for 1 h at 25°C. Total RNA (1.0 to 1.3 μg) was isolated from dissected hemocytes (from 200 to 300 third-instar larvae with TRIzol) and amplified with a RiboAmp RNA amplification kit. Microarray experiments were performed as described previously (19). All of the microarray experiments were done with twin array cDNA 6K chips from Digital Genomics (Seoul, South Korea) as described previously (20). Cy5-labeled RNAs from fungal infected wild-type hemocytes were cohybridized with Cy3-labeled RNAs from uninfected hemocytes. The microarray data were analyzed by significance analysis of microarrays in a one-class response format.

Immunohistochemistry of the lymph gland. Lymph glands were dissected as described in Lebestky et al. (28). They were dissected in PBS by cutting at seven-eighths of their length along the larval body. Using fine tweezers, the inner tissue was inverted and the lymph glands were separated from the surrounding tissue. They were fixed for 30 min in 4% paraformaldehyde in PBS at room temperature and washed for 5 min three times in TNBT (80 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100). After permeabilization, the glands were blocked for 30 min in TNBT-5% goat's serum at room temperature. Mouse anti-Notch C17.9C6 (Hybridoma Bank; 1:50), antigen affinity-purified rat anti-Spen antibody (1:100), and anti-L1 (gift of Istvan Ando, 1:30) were then diluted in TNBT, followed by incubation with lymph glands overnight at 4°C. Several rinses in TNBT were followed by 1 h of incubation in TRITC (tetramethyl rhodamine isothiocyanate)-conjugated anti-mouse immunoglobulin G (IgG; 1:200; Sigma) and fluorescein isothiocyanate-conjugated anti-rat IgG (1:250; Sigma) in a humidified chamber. After four washes for 10 min each time with PBST (0.1% Tween 20 in PBS), the lymph glands were finally mounted in Vectashield fluorescent mounting medium (Vector Laboratories) and analyzed with a Zeiss LSM 510 META confocal microscope system.

Analysis of ChIP-chip and expression data for Kc cells. We downloaded chromatin immunoprecipitation (ChIP)-chip data for activating histone marks (H3K4me3, H3K79me2, H3Ac, and H4Ac) in *Drosophila* Kc cells from <http://www.fmi.ch/groups/schubeler.d/web/data.html> (47). The binding profiles of the Polycomb proteins (Pc, Esc, and Scf) and their associated histone mark (H3K27me3) in *Drosophila* Kc cells (51) were obtained from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) (GSE4564). Kc cell mRNA expression data was generated by comparative hybridization against genomic DNA (36) and available under the accession number GSE5089 of the GEO.

In situ hybridization. The cDNA fragment spanning *sut4* (*CG1380*) nucleotides 481 to 1111 and *CG32064* nucleotides 601 to 1200 were subcloned from transcripts (between BamHI and EcoRI sites) into the pSPT19 vector. Digoxigenin-labeled riboprobes were prepared with SP6 RNA polymerase by using a DIG RNA labeling kit (Roche). Lymph glands were prepared from third-instar

FIG. 1. *Spen* mutant flies are highly susceptible to fungal infection. (A) The survival rates (%) of wild-type (*wt*) flies, flies carrying several alleles of *spen*, and *spen*^{rev2} adult flies, were measured after subjecting them to septic injury using *B. bassiana*. Infections were performed in at least four replicates. *spen*^{rev2}, a P-element excision derivative of *spen*^{GE10359}. (B) Determination of the protein level of Spen in circulating plasmatocytes. Immunostaining was performed in wild-type (*wt*), *spen*^{GE10359}, and *spen*^{rev2} hemocytes with rat anti-*spen* antibody. Scale bars, 10 μ m. Experiments were repeated four times with similar results.

larvae, fixed with 4% paraformaldehyde in PBS for 30 min, and washed in PBST (0.1% Tween 20 in PBS). They were prehybridized with 100 μ g of salmon sperm DNA/ml in hybridization buffer (5 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 100 μ g of salmon sperm DNA/ml, 50 μ g of heparin/ml, 0.1% Tween 20) at 55°C for 1 h, and hybridizations were performed at 55°C overnight after addition of *sut4* or *CG32064* probe (150 ng). After a wash with PBST, the glands were incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche, Mannheim, Germany) for 1.5 h at room temperature, and hybridization signals were visualized with BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium. Reactions were performed until hybridization signals accumulated to a sufficient level and were stopped by washing in PBST. The lymph glands were finally mounted in Vectashield mounting medium (Vector Laboratories), followed by analysis with a Zeiss Axioplan 2 microscope.

ChIP. Hemocytes were dissected separately from approximately 10,000 wild-type and *spen* mutant larvae, and ChIP experiments were performed as described previously (19). The antibodies used in these reactions were: rabbit IgG (20 μ l, sc2027; Santa Cruz), rabbit polyclonal anti-trimethylated H3-K4 (20 μ l, ab8580; Abcam), rat polyclonal affinity purified anti-Spen antibody (25 μ l). A total of 600 μ g of protein-DNA complexes (260 nm) for IgG and anti-trimethylated H3-K4 or 100 μ g for anti-Spen (or IgG) was immunoprecipitated using specific antibodies. The dissociated DNA fragments were recovered with a MiniElute purification kit (Qiagen). The eluted DNA and DNA obtained from 0.8% (anti-trimethylated H3-K4) or 0.08% (anti-Spen) of the ChIP input samples were used in the PCR amplification. A typical reaction was subjected to 30 cycles (anti-trimethylated H3-K4) or 33 cycles (anti-Spen) consisting of 94°C for 30s, 50°C for 1 min, and 72°C for 1 min, followed by one cycle of 72°C for 5 min. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis. Primer pairs were as follows: *sut4* promoter, positions -300 to -281 and -150 to -127; *CG32064* promoter, positions -312 to -292 and -196 to -176; *CG14718* promoter, positions -206 to -185 and -70 to -49; and *Hex-2* promoter, positions -216 to -196 and -70 to -44.

Lamellocyte counting. Spores were injected into the posterior end of second-instar larvae by using a Picospritzer III injector (Parker Hannifin) and incubated for 48 h at 25°C. Late wandering third-instar larvae were rinsed well in PBS (137 mM NaCl, 2.7 mM KCl, 6.7 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and blotted on Kimwipes to remove excess PBS before bleeding, and the larval cuticles were ripped gently near the posterior end while submerging the larvae in 20 μ l of PBS. The hemocytes were transferred to a Neubauer improved hemacytometer (Marienfeld) to count lamellocytes.

Microarray accession numbers. The microarray data has been deposited in the GEO (<http://www.ncbi.nlm.nih.gov/geo>) database with accession numbers GSM275363, GSM275364, GSM275365, GSM275366, and GSM279948.

RESULTS

***Spen* is required for the innate immune response of hemocytes.** In a previous study, we identified a *spen* allele that showed defects in defense against microbial infection and in wound healing (15). To confirm the requirement of *Spen* for the innate immune response, we analyzed the survival of flies harboring several *spen* mutant alleles (*spen*^{GE10359}, *spen*^{poc361},

and *spen*⁰³³⁵⁰), as well as of flies of the precise excision derivative (*spen*^{rev2}) of *spen*^{GE10359} after fungal infection. The survival of all flies with *spen* mutant alleles was strongly reduced after fungal infection (survival rates of 49.11, 24.14, and 6.12% in *spen*⁰³³⁵⁰, *spen*^{poc361}, and *spen*^{GE10359} alleles, respectively), and precise excision of the P-element from the *spen* gene completely reversed the survival defect of the *spen*^{GE10359} mutant (Fig. 1A). In addition, we found that the level of Spen protein in the *spen*^{GE10359} mutant hemocytes was dramatically reduced compared to that of wild-type and precise excision line (*spen*^{rev2}) (Fig. 1B). These results confirm that *Spen* plays an essential role in *Drosophila* immunity. The requirement for *spen* for proper immune development was also shown by the formation of melanotic tumors in *spen*^{GE10359} mutant flies (see Fig. S1 in the supplemental material), as well as by increased proliferation of plasmatocytes, and reduced differentiation of crystal cells (15). Together, these results indicate that *Spen* is required for *Drosophila* innate immunity by regulating hemocytes development.

***Spen* is required in hemocytes for expression of genes involved in energy metabolism and development.** In a previous study, we showed that *spen* mutant flies have abnormal numbers of hemocytes, phagocytic defects, and melanotic tumors, pointing to a major defect in *spen* hemocytes (15). In order to identify the molecular defects in the mutant hemocytes, we collected hemocytes from 200 to 300 third-instar wild-type and mutant larvae and analyzed their transcript profiles using Affymetrix arrays. Microarray analysis of the *spen* mutant hemocytes revealed transcriptional defects mainly in activation of genes. Of 18,880 genes analyzed, 921 genes, including antimicrobial peptide genes (*attD* and *cecC*) and genes involved in energy metabolism, were downregulated more than threefold in the mutant. The microarray results were confirmed by quantitative real-time PCR analysis of several hemocyte transcripts (see Fig. S2 in the supplemental material). Gene ontology (GO) terms analysis of the downregulated genes using MAPPFinder (7) showed that many genes involved in diverse aspects of energy metabolism and spermatogenesis were strongly represented (Table 1). Less than one-tenth as many genes were upregulated more than threefold (91 genes). GO term analysis of the upregulated genes showed specific enrichment of genes involved in mesoderm development (Table 2). In addition, genes involved in cell adhesion (*Sgs3*, *Sgs4*, *Sgs5*, *Sgs7*, *Sgs8*, *Lac*, *Eig71Ee*, and *CG16857*), chitin-based cuticle

TABLE 1. GO groups enriched in the downregulated genes of each mutant hemocytes^a

GO term and biological process	Count ^b	% ^c	z score ^d
<i>spen</i> down			
Malate metabolic process	6	75.000	5.727
Galactose metabolic process	6	66.667	5.282
Glycolysis	13	38.235	5.012
Spermatogenesis	17	36.170	4.787
Tricarboxylic acid cycle	8	40.000	4.091
Carbohydrate transport	9	50.000	3.94
Spermatid development	8	34.783	3.805
Glycerol-3-phosphate metabolic process	5	45.455	3.607
Microtubule-based movement	13	26.000	3.445
Carbohydrate metabolic process	12	10.619	3.164
<i>E(spl)</i> down			
Glycolysis	13	38.235	4.966
Tricarboxylic acid cycle	8	40.000	4.055
Response to unfolded protein	9	34.615	3.757
Spermatogenesis	16	34.043	3.654
ATP synthesis coupled proton transport	13	26.531	3.373
ATP biosynthetic process	5	21.739	3.206
Proton transport	5	16.667	3.094
<i>Su(H)</i> down			
Mitochondrial transport	5	62.500	4.377
Tricarboxylic acid cycle	8	40.000	4.107
Carbohydrate transport	9	50.000	3.956
Glycolysis	10	29.412	3.393
Spermatogenesis	14	29.787	3.365
Nucleotide catabolic process	5	62.500	3.363
Response to unfolded protein	8	30.769	3.186

^a All terms with a z score of 3 or a count of at least five genes.
^b That is, the number of downregulated genes (>3-fold).
^c That is, the percentage of downregulated genes within the GO term.
^d That is, the z score (standardized difference score) using the expected value and standard deviation of the number of genes meeting the criterion of a GO term under a hypergeometric distribution.

(*Lcp1* and *Lcp4*), and glycosylation (*fringe*) were highly expressed (see Table S1 in the supplemental material). This transcription profile indicates that the *spen* mutant hemocytes are defective as immune cells, with low metabolic activity, and that they produce adhesion molecules, which may promote association of the hemocytes to form melanotic tumors as observed in the mutant larvae (see Fig. S1 in the supplemental material).

Spen is required for activation of Notch-dependent genes. The defects that we observed in the *spen* mutant hemocytes are often seen in association with inappropriate hemocyte development (15). The Notch pathway plays an instructive role in the development of hemocytes, and mammalian homologs of Spen (SHARP and MINT) have been proposed to be negative regulators of the Notch signaling pathway (9, 23, 34, 35). Therefore, we explored the possibility that the defects observed in the *spen* mutants might result from inappropriate regulation of the Notch pathway. In order to examine whether the genes regulated by Spen are affected by Notch signaling, we compared the transcriptional defects in the hemocytes of the *spen* mutant to those in mutants that affect downstream mediators of the Notch signaling pathway, namely, *Su(H)* (Suppressor of Hairless) and *E(spl)* (Enhancer of split).

As expected from their epistatic relationship, *Su(H)* and *E(spl)* caused similar transcriptional defects (Fig. 2A). GO

TABLE 2. GO groups enriched in the upregulated genes of each mutant hemocytes^a

GO term and biological process	Count ^b	% ^c	z score ^d
<i>spen</i> up			
Mesoderm development	8	13.33333	5.408
<i>E(spl)</i> up			
DNA replication initiation	8	53.33333	8.347
DNA replication	11	22.44898	6.834
Cell division	9	18.75	5.099
Cell cycle	14	14.14141	3.947
Cytokinesis	10	18.18182	3.94
Compound eye development	9	19.14894	3.718
Mesoderm development	12	20	3.673
Sensory organ development	5	15.625	3.288
Mitosis	9	14.7541	3.002
<i>Su(H)</i> up			
DNA replication initiation	7	46.66667	6.835
Carbohydrate transport	7	38.88889	5.774
Mesoderm development	14	23.33333	5.433
DNA replication	13	26.53061	4.934
Proteolysis	58	11.83673	3.69
Cytokinesis	10	18.18182	3.529
Cell cycle	11	11.11111	3.502
Mitosis	8	13.11475	3.333
Muscle development	5	23.80952	3.238
Heart development	6	19.35484	3.083
Cell division	7	14.58333	2.871
Open tracheal system development	9	13.84615	2.647

^a All terms with a z score of 3 or a count of at least five genes.
^b That is, the number of upregulated genes (>3-fold).
^c That is, the percentage of upregulated genes within the GO term.
^d That is, the z score (standardized difference score) using the expected value and standard deviation of the number of genes meeting the criterion of a GO term under a hypergeometric distribution.

term analysis of the differentially regulated genes in the two sets of mutant hemocytes revealed that the same classes of genes were highly enriched among the genes differentially regulated in the *E(spl)* and *Su(H)* mutant hemocytes (Tables 1 and 2). In both, genes involved in DNA replication, the cell cycle and cell division were highly upregulated, while genes involved in energy metabolism and spermatogenesis were significantly downregulated. Comparison of the genes differentially regulated in the *spen* mutant hemocytes with those differentially regulated in the Notch pathway mutants, revealed a strong correlation, but only with regard to the downregulated genes (Fig. 2A and B). This result indicates that Spen is required to activate genes regulated by Notch but not for Notch-mediated repression of genes involved in cell proliferation.

Spen expression in lymph glands is regulated by Notch signaling. Because Notch signaling is mainly required during development, most of the transcriptional defects observed in the *spen* mutant hemocytes could result from the loss of Spen activity during hematopoiesis in the lymph glands. In order to detect a possible regulatory relationship between Spen and Notch signaling during *Drosophila* hematopoiesis, we investigated the levels of Spen and Notch expression in lymph glands by immunohistochemistry. We observed that the expression of Notch in wild-type lymph glands was restricted to the primary and secondary lobes (Fig. 3A). By comparison, Spen protein was found throughout the lymph gland, but especially in the pericardial cells. Expression of Notch in lymph glands was lost

FIG. 2. Transcriptome analysis of the circulating hemocytes in *spen* and *Notch* pathway mutants. (A) Bivariate scatter plots of $\log_2$ -transformed microarray data comparing *E(spl)* versus *Su(H)* and *spen* versus *Su(H)*. Those genes downregulated in *spen* hemocytes are denoted by blue dots. The Pearson correlation coefficient (r) is shown for each plot. (B) Microarray analysis of hemocytes in *E(spl)*, *Su(H)*, and *spen* mutant flies. Columns correspond to the different mutants, and rows to different genes. The expression levels of genes up (red)- and down (green)-regulated more than threefold (at least one observation with that absolute value) compared to that of wild-type hemocytes are shown (left). Representative GO groups belonging to groups I and II are shown on the right, together with functional annotations and gene symbols.

at the restrictive temperature in *N^{ts1}* flies but was not affected by mutations in *E(spl)*, the downstream mediator of Notch signaling, or in *spen*. However, Spen expression was severely decreased in the lymph gland and pericardial cells of the *N^{ts1}* and *E(spl)* mutants (Fig. 3A). This result indicates that Spen expression is regulated by the Notch pathway via *E(spl)*. Next, we analyzed Spen expression in the Notch gain of function mutant, *N^{Mcd8}*. Notch protein levels were increased in the *N^{Mcd8}* mutant and there was a dramatic increase in Spen levels (Fig. 3A). In addition, we observed that the lymph glands of *N^{Mcd8}* flies were much larger than those of the wild type. Taken together, these data indicate that Notch signaling positively regulates Spen expression in the lymph glands of *Drosophila*.

In order to confirm that the transcriptional defects observed in the circulating hemocytes reflected defective Notch signaling during hemocyte differentiation, we performed in situ hybridization to monitor the lymph gland for expression of *sut4* and *CG32064*, two representative Spen-dependent genes. As shown in Fig. 3B, high levels of *sut4* and *CG32064* transcripts were visible in the cortical zone of wild-type lymph glands. However, transcript levels were greatly diminished in *spen* and *N^{ts1}* mutant lymph glands, suggesting that Notch regulates expression of its putative targets by controlling the expression of Spen during lymph gland differentiation.

Spen activates its target promoters by histone methylation.

Having established a requirement for Spen in Notch signaling during hemocyte development and the innate immune response, we wondered how Spen could regulate such a large number of genes. The presence of a SPOC domain in Spen suggested that the Spen-dependent genes might be regulated at the chromatin level. Therefore, we attempted to relate the Spen-dependent genes to the epigenetic signatures seen in ChIP-chip experiments. To this end, we obtained, from public ChIP-chip data, the activating (H3K4me3, H3K79me2, H3Ac, and H4Ac) (47) and repressing (H3K27me3) histone modification patterns, and binding profiles of the Polycomb proteins (Pc, Esc, and Sce) (51) in *Drosophila* Kc cells, a cell line derived from a hematopoietic ancestor (2). We then compared the expression levels and epigenetic signatures of the Spen-dependent genes to those of the rest of the genes. The expression levels of the Spen-dependent genes in Kc cells were significantly lower than those of the rest of the genes, indicating that Notch-dependent gene expression is turned off in Kc cells (Fig. 4A). When we examined the association between the histone marks and the genes underexpressed in the *spen* mutant, we observed an absence of activating histone marks, (H3K4me3, H3K79me2, H3Ac, and H4Ac), and increased binding of Pc and Esc, which are associated with the repressing

FIG. 3. Notch-dependent expression of Spen in lymph glands. (A) Immunohistochemical analysis of the lymph glands of third-instar larvae of the indicated genotypes with anti-Notch (red) and anti-Spen (green) antibodies, along with DAPI (blue) staining. Scale bar, 200 μm. (B) In situ hybridization analysis of *sut4* and *CG32064* transcripts in lymph glands. The genotypes of the lymph glands are indicated on the top. The cortical zone (CZ) and medullary zone of the lymph glands are marked with gray and yellow lines, respectively. Scale bar, 100 μm. The *Nts1* mutant was raised at 18°C. To obtain the *Notch* mutant phenotype, we incubated third-instar larvae at 29°C for 12 h and analyzed the lymph glands after further incubation of the larvae for 18 h at 18°C.

histone mark H3K27me3 (Fig. 4B). Therefore, the Spen-dependent genes in Kc cells appear to be mainly regulated by modification of histones.

To confirm that Spen plays an important role in histone modification of target gene promoters during hemocyte development, we examined the binding of Spen to the promoters of the Spen-dependent genes, and the presence of trimethylated H3K4, by ChIP with anti-Spen and anti-H3K4me3 antibodies, respectively. Wild-type and *spen* mutant hemocytes were dissected from approximately 10,000 larvae for each ChIP analysis. We observed strong binding of Spen to the target gene promoters in wild-type hemocytes, along with association of trimethylated H3K4 with the promoters, and these effects were reduced in the Spen-dependent promoters of the mutant hemocytes (Fig. 4C). Therefore, the Notch signal appears to maintain the metabolic activities and developmental potential of progenitor cells by regulating histone modification of the promoters of target genes in a Spen-dependent manner.

Spen-dependent genes are downregulated in hemocytes after fungal infection. In addition to the developmental defects, *spen* mutant hemocytes were defective in their response to fungal infection. Lamellocytes were barely detectable in untreated flies, but infection induces lamellocyte differentiation such that more than 150 lamellocytes were present in the circulation of third-instar larvae of wild-type and *spen^{rev2}* (a precise P-element excision revertant of the *spen^{GE10359}*). However, only one-third of this number of lamellocytes was detected in the hemolymph of *spen^{GE10359}* mutant larvae after infection (Fig. 5A). Similarly, lamellocyte differentiation induced by bacterial infection was defective in the *spen* mutant (see Fig. S3 in the supplemental material). In order to confirm that Spen is required for lamellocyte differentiation, we compared the levels of differentiating lamellocytes in the lymph

glands of wild type and *spen* mutants. We infected third-instar larvae with *B. bassiana* spores and examined the expression of lamellocyte-specific antigen L1 in differentiating lymph glands cells. Immunostaining of the lymph glands with lamellocyte-specific L1 antibody revealed patches of strong L1 staining in the cortical zone of wild-type lymph glands 18 h after infection, with only very weak L1 staining in the lymph glands of the *spen* mutant (Fig. 5B). Coimmunostaining with anti-Spen antibody showed that Spen protein was highly expressed in several patches in the cortical zone of wild-type lymph glands, most of which overlapped with the L1 expressing patches. These results indicate that Spen also plays an important role in regulating lamellocytes differentiation in response to infection (Fig. 5C).

In order to understand the regulatory mechanism behind infection-induced lamellocyte differentiation, we examined the expression profiles of wild-type hemocytes with or without fungal infection. Third-instar wild-type larvae were subjected to septic injury with live *B. bassiana* for 1 h, and blood cells were isolated from 300 larvae. Comparison of the scatter plot analyses of the transcriptional changes in hemocytes induced by fungal infection and those affected by *spen* mutation revealed that, in addition to activation of genes involved in the innate immune responses, all of the Spen-dependent genes were downregulated strongly by infection (Fig. 5D). Quantitative reverse transcription-PCR analysis of the hemocytes confirmed that *sut4* and *CG32064* transcripts (representative Spen-dependent genes) were drastically reduced in the wild type after 1 h of infection (see Fig. S4A in the supplemental material). We also measured *sut4* transcripts in the lymph glands after spore infection and found that they were almost completely eliminated from wild-type lymph glands 6 h after infection. We were puzzled by this unexpected downregulation of Spen-dependent genes despite their being required for la-

FIG. 4. Epigenetic regulation of Spen-dependent genes. The statistical status of the Spen-dependent genes (S) and Spen-independent genes (I) for expression levels (A) and epigenetic patterns (B) are shown. (A) The expression levels of Spen-dependent genes are significantly downregulated compared to other genes in Kc cells. The statistical significance of the downregulation is shown at the bottom. (B) Epigenetic status of Spen-dependent genes compared to Spen-independent genes. Of 921 genes that were repressed more than threefold in our full genome *Drosophila* Affymetrix microarray analysis, only 198 are matched to specific probes of the ChIP-chip analysis, which includes probes for 5,186 *Drosophila* genes. This cohort of genes was compared to the rest of the genes, and box plots comparing the two groups with respect to activating histone modifications (H3K4me3, H3K79me2, H3Ac, and H4Ac), the repressing epigenetic mark (H3K27me3), and the binding strength of polycomb group proteins (Pc, Esc, and Sce) are shown. The green boxes indicate the levels of activating histone marks in the Spen-dependent genes; the red boxes indicate the levels of repressing histone marks or binding strength of polycomb group proteins in the Spen-dependent genes; the white boxes represent the status of the Spen-independent genes. (C) ChIP assays of wild-type (*wt*) and *spen* mutant hemocytes. Immunoprecipitation was performed with IgG, anti-Spen, or anti-trimethylated H3K4 antibodies. Coimmunoprecipitated DNA fragments were amplified with specific primers for the promoter regions of the genes indicated on the left.

mellocyte differentiation. In order to address this question, we monitored the temporal expression of *sut4* after infection. In situ analysis revealed that *sut4* transcripts were detectable again in lymph glands 12 h after infection (Fig. 5E), and this was confirmed by quantitative reverse transcription-PCR analysis (see Fig. S4B in the supplemental material). These results indicate that fungal infection can trigger transient downregulation of Spen-dependent genes in wild-type hemocytes and lymph glands; this appears to be required for the differentiation of precursor hemocytes to lamellocytes.

Notch signaling is downregulated after fungal infection. The transient downregulation of Spen-dependent genes hints that Notch signaling may be regulated by infection. To test this idea, we examined Notch expression in the lymph gland following infection. Lymph glands were dissected from third-instar larvae 6 and 12 h after injection with *B. bassiana* spores and analyzed with anti-Notch and anti-Spen antibodies. Interestingly, Notch expression was drastically reduced 6 h after infection in the wild type but returned to normal by 12 h. In agreement with the observation that Spen expression is dependent on Notch, Spen levels in the lymph glands followed a pattern similar to that of Notch (Fig. 6A). Therefore, the transient downregulation of Notch may allow some precursor cells to cease being stem cells and differentiate into effector immune cells.

The temporary downregulation of Notch in the lymph gland

after infection suggests that Notch expression is regulated by pathogen recognition pathways. In response to fungal infection, *Drosophila* defense systems are mainly activated via Toll signaling. In order to test whether activation of the Toll receptor is specifically required for the downregulation of Notch expression during fungal infection, we examined expression of Notch in the lymph glands of *Toll* and *Imd* mutants after fungal infection. Notch expression was strongly reduced in the *Imd* mutant, but no reduction was observed in *Toll* mutant flies, indicating that the Notch downregulation is mediated by the Toll pathway (Fig. 6A). In order to narrow down the specific signal required for Notch downregulation, we performed similar experiments with mutants defective for downstream mediators of the innate immune signaling such as Dif, Relish, and dSTAT. Intriguingly, downregulation of Notch triggered by fungal infection turned out to require Dif specifically. Therefore, the Dif activated by fungal infection appears to evoke the downregulation of Notch. Similar bacterial infection experiments revealed that downregulation of Notch in the lymph glands occurs. However, the response to *E. coli* required Imd and Dif, but neither Toll nor Relish (Fig. 6B). Because Relish is the main transcription factor downstream of Imd induced by bacterial infection and is responsible for activating important immune effector functions, the specific requirement for Dif after bacterial infection suggests a specific role of Dif in Notch

FIG. 5. Requirement of Spen for lamellocyte differentiation. (A) Counts of circulating lamellocytes in third-instar larvae after fungal infection. The numbers of lamellocytes in the circulation of third-instar larvae with (+) or without (–) spore infection are shown. The means and standard deviations of five independent experiments are shown. In each experiment, at least six larvae of each genotype were counted, and the results were averaged. (B) Immunohistochemical analysis of the lymph glands of wild-type (*wt*) and *spen* mutant third-instar larvae with lamellocyte-specific anti-L1 antibody (red) at the indicated times after infection with live *B. bassiana* spores. DAPI staining patterns are shown. The cortical zones (CZ) are marked by white lines. Scale bar, 50 μ m. (C) Immunohistochemical analysis of wild-type lymph glands with anti-L1 (red) and anti-Spen (green) antibodies, along with the DAPI (blue) staining 18 h after infection with live *B. bassiana* spores. Scale bar, 10 μ m. The data are representative of three independent experiments. (D) Microarray analysis of wild-type hemocytes 1 h after fungal infection. Bivariate scatter plots of $\log_2$ -transformed microarray data compare fungal infection of wild-type versus *spen* mutant hemocytes. Genes whose expression decreased in the *spen* hemocytes relative to wild-type (*wt*) hemocytes are indicated by blue dots. The Pearson correlation coefficient (r) is shown. (E) In situ hybridization analysis of *sut4* transcripts in lymph glands after spore infection. Wild-type larvae were subjected to septic injury with *B. bassiana* spores, and lymph glands were dissected from third-instar larvae at 6-h intervals. Scale bar, 100 μ m. The results are representative of three independent experiments.

downregulation. Together, these results demonstrate cross talk between the pathogen recognition and Notch pathways during infection-induced hematopoiesis.

DISCUSSION

Spen was initially identified as a coactivator protein required for guidance of a subset of axons in *Drosophila* (22). *spen* mutation causes cell fate determination defects in neurons and affects the expression of Su(H) and Yan, the key transcriptional effectors of Notch signaling (22, 41). Thus, *spen*-related defects in nervous system development are thought to result from inappropriate regulation of the Notch signaling pathways. The defects observed in *spen* mutant hemocytes, viz. inappropriate development of plasmocytes, and poor development of crystal cells and lamellocytes (15), indicate that the Notch signal pathways also play important roles in hematopoiesis. In particular, our observations suggest the idea that Spen is the transcriptional effector protein of Notch signaling during hematopoiesis. Transcriptome analysis of *spen* and Notch path-

way mutant hemocytes demonstrated that Notch signaling is required for the expression of a large number of genes involved in energy metabolism through Spen-dependent epigenetic regulation, while repressing genes involved in cell proliferation in a Spen-independent manner. This provides the correct environment for most of the hemocytes in lymph glands to maintain “stem cellness” (Fig. 7A). Upon infection, innate immune signaling not only activates the defense response but downregulates Notch in a Dif-dependent manner. Notch inhibits the genes involved in cell proliferation, along with the silencing of the genes required for energy metabolism. The temporal transition of the expression patterns of hemocytes by infection may allow some progenitor cells to differentiate into functional immune effector cells (Fig. 7B). It is not clear whether Spen functions in the actual Notch expressing cells to relay the Notch signal directly to the target genes or whether it acts in neighboring cells in response to Notch. The fact that Spen is highly expressed in pericardial cells, in which Notch is barely detectable, favors, but not decisively, the latter possibility.

Activation of lineage-specific transcription factors has been

FIG. 6. *Dif*-dependent downregulation of Notch signaling. Immunohistochemical analysis of lymph glands with antibodies against Notch (red) and Spen (green). Third-instar larvae of the indicated genotype were infected with live *B. bassiana* spores (A) or *E. coli* (B), and lymph glands were dissected at 6-h intervals as indicated on the left. *TP³* flies were obtained from the embryo collection, grown at 25°C for 30 h and shifted to 29°C for 72 h. Scale bar, 200 μm. The results are representative of three independent experiments.

suggested as the major function of the Notch signaling required for hematopoiesis (44). Notch signaling controls the expression of lineage-specific transcription factors, which in turn influences the fate of a bipotential progenitor cell. However, the list of genes regulated by Spen, which is under the control of Notch signaling, reveals another interesting aspect of hemocyte development. In addition to genes required for specific types of differentiation, *spen* mutation caused downregulation of a large number of genes, including most of the genes involved in energy metabolism. This result indicates that many genes involved in energy metabolism are coregulated epigenetically to ensure the coexpression of functionally interacting genes under a specific physiological condition. In particular, the transition from a progenitor to a differentiated blood cell may require a chromatin structure enabling a differentiation into a new cell type. This restructuring of chromatin during hematopoiesis may induce the epigenetic silencing

of genes involved in energy metabolism. It is not known who initiates this epigenetic regulation, but the fact that these genes are coregulated epigenetically by Spen-mediated chromatin modification is intriguing.

Our biochemical data demonstrate that Spen acts as a coactivator downstream of Notch signaling. However, many previous genetic observations point to a corepressor role for Spen. In fact, several observations indicate that Spen may function as both a coactivator and a corepressor protein. First, the SPOC and RRM domains of Spen have been shown to interact with histone deacetylase and nuclear receptor coactivator, respectively (48). Second, mammalian Spen homologs interact with transcriptional activators or corepressors depending on environmental conditions. Su(H) in *Drosophila* and RBP-J, which are known to interact with MINT, have been found to have both activator and repressor roles in mammals, depending on the presence or absence of Notch signaling (13, 16, 17, 23, 24,

FIG. 7. Novel connection between the Notch and Toll signal pathways. Genes (signals) with active or inactive status are marked with solid lines and boxes with colors or dashed lines and boxes without colors, respectively. (A) Chromatin regulation by Spen mediating Notch signaling in hematopoiesis in the absence of infection was determined. Spen expression in the *Drosophila* lymph glands was completely dependent on Notch activity and appeared to be regulated downstream of E(spl). Spen functions as a coactivator by regulating the chromatin status of a large group of genes mostly involved in energy metabolism and development. Notch inhibits the expression of genes involved in cell proliferation. The expression pattern may favor the maintenance of stem cell phenotypes. (B) Cross talk between the Toll and Notch pathways during infection-induced hematopoiesis. In *Drosophila* lymph glands, activated Dif is required for transient downregulation of Notch levels, and activated Dif may stimulate the degradation pathways mediated by ubiquitination or endocytosis. Notch downregulation inactivates genes involved in energy metabolism while releasing the inhibition of genes involved in proliferation. This expression pattern may favor the differentiation of hemocytes.

25, 35). Therefore, Spen may interact with both types of transcription factor depending on the conditions, as shown for MINT and RBP-J in mammals. However, the transient degradation of Spen along with Notch upon the hemocyte differentiation indicates that Spen might be used differently in the lymph glands. It may associate only with the activated promoters together with a coactivating histone modifying enzyme.

In order to achieve an effective immune response against acute infections, innate immune processes and immune cell development must be coordinated. The transient downregulation of Notch in the lymph glands reveals cross talk between innate immunity and hematopoiesis represented by the Toll and Notch pathways, respectively. Several observations already indicated the involvement of Toll pathway in hemocyte differ-

entiation. A dominant, gain-of-function mutation of Toll causes overproliferation of hematopoietic precursors and their differentiation into lamellocytes (38). The activated Dorsal was suggested to increase the mitotic activity of hemocytes, but the underlying mechanism used by Toll pathway to regulate hematopoiesis is not known. Therefore, the specific requirement of activated Dif for the downregulation of Notch during hemocyte differentiation is intriguing and suggests the degradation of Notch as a potential target of the Dif-mediated regulation. Recently, ubiquitin- or endocytosis-mediated degradation has been proposed as the mechanism of Notch downregulation. Several E3 ligases, including Sel-10, Itch, c-Cbl, and Deltex, ubiquitinate Notch, triggering its degradation (14, 32, 33, 37). On the other hand, the negative regulator Numb causes endocytosis of Notch via α -adaptin, followed by its proteasome-mediated degradation (5, 30, 46). Therefore, activated Dif may stimulate these degradation pathways to downregulate Notch. However, it is not known what is the direct target of the activated Dif. Elucidation of the putative cross talk mechanisms involving the two pathways should contribute to our understanding of the complex regulatory network involved in cell differentiation.

ACKNOWLEDGMENTS

We thank Won-Jae Lee, Kwang-Min Choe, and Marie Meister for providing mutant flies; Istvan Ando for providing L1 antibody; the Developmental Studies Hybridoma Bank for antibody; and the Bloomington Stock Center and GenExel for stock.

This study was supported by a grant from the Global Research Laboratory Program of the Korean Ministry of Education, Science, and Technology to Y.-J.K.

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