Persephone/Spätzle Pathogen Sensors Mediate the Activation of Toll Receptor Signaling in Response to Endogenous Danger Signals in Apoptosis-deficient *Drosophila*[*][S]

Ming Ming[‡], **Fumiaki Obata**[‡§1], **Erina Kuranaga**[¶], and **Masayuki Miura**[‡§2]

From the [‡]*Department of Genetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan, the* [§]*Core Research for Evolutional Science and Technology (CREST), Basic Research Program, Japan Science and Technology Agency, Chiyoda-ku, Tokyo 102-0075, Japan, and the* [¶]*Laboratory for Histogenetic Dynamics, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan*

Background: Apoptosis deficiency causes necrotic cell death that activates the innate immune response in mammals.
Results: Apoptosis-deficient *Drosophila* exhibited constitutive Toll pathway activation mediated by serine protease Persephone.
Conclusion: Apoptosis-deficient *Drosophila* produces damage-associated molecular patterns (DAMPs) leading to canonical Toll signaling through a Persephone-mediated proteolytic cascade that cleaves the Toll ligand Spätzle.
Significance: DAMP-mediated Toll-like receptor signaling is conserved in invertebrates.

Apoptosis is an evolutionarily conserved mechanism that removes damaged or unwanted cells, effectively maintaining cellular homeostasis. It has long been suggested that a deficiency in this type of naturally occurring cell death could potentially lead to necrosis, resulting in the release of endogenous immunogenic molecules such as damage-associated molecular patterns (DAMPs) and a noninfectious inflammatory response. However, the details about how danger signals from apoptosis-deficient cells are detected and translated to an immune response are largely unknown. In this study, we found that *Drosophila* mutants deficient for Dronc, the key initiator caspase required for apoptosis, produced the active form of the endogenous Toll ligand Spätzle (Spz). We speculated that, as a system for sensing potential DAMPs in the hemolymph, the *dronc* mutants constitutively activate a proteolytic cascade that leads to Spz proteolytic processing. We demonstrated that Toll signaling activation required the action of Persephone, a CLIP domain serine protease that usually reacts to microbial proteolytic activities. Our findings show that the Persephone proteolytic cascade plays a crucial role in mediating DAMP-induced systemic responses in apoptosis-deficient *Drosophila* mutants.

Inflammatory responses are activated by determinants that are inherent to infectious agents, known as pathogen-associated molecular patterns, as well as by endogenous factors pro-duced by host cells. These latter signals, analogically termed damage- (or danger)-associated molecular patterns (DAMPs),[3] are inaccessible to the immune system but can be released by severely stressed and damaged cells as in the case of pathogenic infection (1), traumatic injury (2), or spontaneous tumor formation (3).

Physiological responses to DAMPs are functionally governed by the innate immune system, which is evolutionarily conserved and plays a key role in homeostasis. Among the cellular receptors that sense these danger signals, the Toll-like receptor family is reported to be responsive to a variety of DAMPs. DAMP release is associated with cellular damage, in particular the loss of membrane integrity or rupture of the plasma membrane, a hallmark of the necrotic form of cell death. In eukaryote cells, an intracellular family of proteases known as caspases functions to orchestrate apoptotic signaling, leading to the removal of unwanted cells without stimulating the immune system. However, necrotic cell death can arise from apoptotic pathway suppression because of caspase deficiency or pharmacological inhibitors (4, 5).

Dronc, the *Drosophila* ortholog of caspase-9, is thought to be crucial in initiating apoptotic caspase cascades during development as well as in response to stress (6, 7). Animals lacking zygotic *dronc* arrest as early to mid pupae and exhibit a wide range of defects, including impaired apoptosis, random formation of melanotic clots, and severe blood cell hyperplasia (8–11).

In the current study, we demonstrated that the hemolymph of apoptosis-deficient *Drosophila dronc* mutants contains DAMPs that activate the Toll innate immune pathway. We provide substantial evidence that the extracellular protease Persephone is indispensable for sensing the DAMPs derived from

[3] The abbreviations used are: DAMP, damage-associated molecular pattern; RFP, red fluorescence protein.

apoptosis-deficient cells, indicating that Toll-like receptor-mediated DAMP signaling is conserved in *Drosophila*.

EXPERIMENTAL PROCEDURES

Drosophila Strains—*Drosophila* stocks were raised on standard cornmeal-agar media at 25 °C and 60–64% humidity, in either vials or bottles, unless otherwise indicated. Fly stocks used for overexpression experiments were generated through transgene combination or homologous recombination, using standard crosses. The genetic deletion lines *dronc*$^{\Delta A8}$ and *drice*$^{\Delta 2C8}$ were described previously (8). The following strains were obtained from the Bloomington *Drosophila* Stock Center: w^{1118}, *Act5C-Gal4*, and *w;;His2Av-mRFP, FRT80B/TM6B, Tb*. The tissue-specific Gal4 driver for hemocytes (*pxn-Gal4*) was described previously (12).

The stock lines *spz*^{rm7}, *dMyd88*^{Kra1}, *Tl*^{10b}, and *Drs-GFP* were described previously and were kindly provided by Dr. Shoichiro Kurata with the authors' permission. *Dronc Nc*^{51} (9) was a generous gift from Dr. John M. Abrams; *UAS-Dronc*^{wt}*-FLAG* was from Dr. Sally Kornbluth (13); *UAS-Dronc*^{C318A} was from Dr. Pascal Meier (6); *UASp-Spz-HA* was from Dr. David Stein (14); and *pxn-Gal4, UAS-GFP* was from Dr. Michael J. Galko. *dark*^{82} was obtained by the courtesy of Dr. John M. Abrams (15). *psh*^{1} was a kind gift from Dr. Jean-Marc Reichhart (16).

Drosophila Culture—To prepare germ-free animals, wild-type and *dronc*$^{\Delta A8}$ mutant embryos were collected on the surface of grape juice agar and were subjected to consecutive washing with 70% ethanol, 70% ethanol, 2.7% sodium hypochlorite solution (Purelox-X), 70% ethanol, 70% ethanol, and 1× PBS buffer in a clean cabinet. Subsequently, the dechorionated embryos were maintained in sterile food at 25 °C (method adopted from Ref. 17). The removal of bacteria was confirmed by plating assay on LB medium as follows (37 °C, 24 h). The larvae undergo a primary wash with 70% ethanol to remove the microbes attached to the body surface, followed by a hand-prepared homogenation in sterile PBS solution. The total homogenates corresponding to contents in one individual third instar larva were inoculated on LB plate. No bacterial colonies were detected in germ-free condition.

Quantitative Reverse Transcription-PCR—A sample batch containing five or six wandering third instar larvae or the dissected fat bodies from eight or nine individual larvae was homogenized with a Multi-Beads Shocker (Yasui Kikai) set to 1500 rpm, 15 s, and three cycles. The homogenate was used for RNA extraction with TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized with the Takara PrimeScript RT reagent kit with gDNA eraser using 1 μg of total RNA. The cDNA products (5 μl of a 10-fold diluted solution) were then used for PCR amplification with Takara Premix Ex Taq II (Tli RNaseH Plus) in a LightCycler 480 system (Roche Applied Science). The quantitative PCR cycle was as follows: denaturing at 95 °C for 30 s → PCR cycles at 95 °C for 5 s and at 55 °C for 25 s for 45 cycles → melting curve at 95 °C for 5 s, at 65 °C for 1 min, and at 97 °C with acquisition every 5 s → cooling down at 40 °C for 10 s. The housekeeping genes *RNA polymeraseII* and *rp49* were used as internal controls interchangeably. For each set

of data, the value of one wild-type sample was used as a calibrator, and the relative ratios of the other genotypes *versus* the calibrator were calculated accordingly. The results were summarized from three or more independent tests. The primers used are summarized in supplemental Table S1.

Mosaic Clone Analysis—To generate *dronc* null clones in the larval fat bodies, embryos at 6–8 h after egg laying, with the genotype *hs-Flp;; His2Av-RFP, FRT80B/dronc*$^{\Delta A8}$*, FRT80B*, were subjected to a 30-min heat shock pulse at 37 °C to induce FLP-mediated recombination between *cis*-acting allelic *FRT* sites. The heat-treated larvae were kept at 21 °C until they reached the wandering third instar stage. In the dissected larval fat body, cell clones were recognized by loss of the *His2Av-RFP* allele, which directs nuclei-localized red fluorescence protein (RFP) expression. Both the *dronc*$^{\Delta A8}$ mosaic clone and the control fat body clone were subjected to immunohistochemistry staining to examine the localization of Dorsal.

Immunohistochemical Staining and GFP Fluorescence Analysis—Wandering third instar larvae were dissected to collect fat bodies or blood cells. The samples were fixed with 4% paraformaldehyde for 22.5 min at room temperature, followed by five consecutive 10-min washes in PBST (PBS + 0.1% Triton X-100). The fixed samples were blocked for 30 min in 5% donkey serum, followed by overnight incubation at 4 °C with primary antibodies: mouse monoclonal anti-Dorsal (1:200, clone 7A4 (18); Hybridoma Bank), or rabbit polyclonal anti-tRFP antibody (1:250; Evrogen AB232). Washed samples were incubated for 1–2 h at room temperature with the corresponding secondary antibody: Alexa Fluor goat anti-mouse IgG (1:500; Invitrogen) or donkey anti-rabbit-cy3 (1:500; Jackson). F-actin and nuclear DNA were labeled with rhodamine-conjugated phalloidin (1:500) and Höechst 33342 (4 μM), respectively. Samples were mounted in mounting buffer and analyzed by confocal microscopy (TCS SP5; Leica). Images were captured after maximum projection processing of multiple confocal scanning planes. To examine the GFP fluorescence emitted from the larval fat body (*Drs-GFP* reporter) or hemocytes (*pxn-Gal4, UAS-GFP*), the larvae were washed with PBS and submerged in ice-chilled PBS, and then the intact larvae were analyzed under a Leica stereomicroscope equipped with GFP-compatible filters.

Western Blotting of Larval Hemolymph—Wandering third instar larvae were collected from control (*pxn-Gal4, UASp-spz-HA*) and mutant (*pxn-Gal4, UASp-spz-HA; dronc*$^{\Delta A8}$) groups; washed successively in PBS, 70% ethanol, and PBS; and placed on ice until dissection. The dissection was performed by ripping off a strip of the larval skin longitudinally starting from the posterior dorsal end without compromising internal tissues. For both genotypes, hemolymph containing an adequate number of blood cells from five or six larvae was bled into 10 μl of PBS, followed by centrifugation at 5000 × g and 4 °C for 30 min. The supernatant was then further centrifuged at 8000 × g for 10 min to obtain the hemolymph aqueous fraction. The samples were separated on 15% SDS-polyacrylamide gels under denaturing conditions and then semi-dry transferred to an Immobilon-P membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). After blocking

for 40 min in 4% skim milk/TBST (w/v) at room temperature, the membrane was incubated with mouse monoclonal anti-HA tag (clone 12CA5, 1:1000; Roche Applied Science) at 4 °C overnight and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:1000; Promega) for 1 h at room temperature. The blots were visualized using Immobilon Western Chemiluminescence (Millipore). Western blotting images were captured using an LAS4000 MINI (GE Healthcare ImageQuant) and were processed using Photoshop software.

Fat Body ex Vivo Culture Assays—Isolated wild-type fat bodies were incubated in Ringer's solution, with or without hemolymph extracts from flies of various genotypes. Each fat body explant was cultured with hemolymph obtained from four individual donor larvae. The co-incubation was performed for ~7 h in a humid incubator. The fat body explants were then subjected to Dorsal immunostaining analysis to measure the extent of immune activation, and the images were captured with a confocal microscope (TCS SP5; Leica). After recovering the images, we scored the results by classifying the degree of Dorsal activation in the fat body explants according to three distinctive Dorsal staining patterns (see Fig. 3F); fat body cells with minimal, medium, or intense Dorsal staining in the nuclei were designated as levels 0, 1, and 2, respectively. Subsequently, the percentage of cells associated with each of the three classes was calculated for each of the genotypes evaluated (see Fig. 3G).

Statistical Tests—Experimental differences were compared between experimental and control groups, and p values were obtained using the two-tailed Student's t test. The one-way analysis of variance test was used to compare multiple sets of samples.

RESULTS

Dronc Deficiency Leads to Drosomycin Induction in the Fat Body through Dorsal Activation—In *Drosophila*, Dronc is the initiator caspase activated at the onset of apoptosis, and loss of this protein blocks most, if not all of the apoptosis occurring during development and in response to stress (8–11). To study the stress-responsive pathway that is triggered in the *dronc* null mutant (*dronc*[ΔA8]), we measured the expression level of a number of stress signal target genes using quantitative RT-PCR in L3 larvae. The *dronc* mutants exhibited elevated levels of transcripts for *Turandot A* (*TotA*) and *Thor*, the obligate downstream target genes for the JAK/STAT pathway, and the insulin receptor cascade, respectively (Fig. 1, *A* and *B*) (19–21). Turandot proteins are involved in the physical protection and repair of damaged tissue upon stimulation by mechanical, thermal, or infectious stress (22), whereas the expression of the translational initiation factor Thor is induced in response to wounding (21), starvation (20), or oxidative stress (23) through the activation of dFOXO. In contrast, expression of the transcript encoding the phosphatase *Puckered* (*Puc*), a negative regulator of the JNK pathway (24), was unchanged in the mutant larvae (Fig. 1C), suggesting that JNK signaling may not be important for responding to the physiological stress associated with caspase deficiency.

It was previously shown that the *Thor* promoter contains a canonical NF-κB recognition sequence and that Thor plays critical roles in host immune defense (21). We next assessed the activation of immune pathways by quantifying the expression of antimicrobial peptides, an array of immune effectors predominantly regulated by the two central immune pathways in *Drosophila*. *Diptericin* (*Dpt*) and *Attacin A* (*AttA*) are induced via Imd signaling, whereas *Metchnikowin* (*Mtk*) and *Drosomycin* (*Drs*) are preferentially induced following the activation of Toll signaling (25). Quantitative RT-PCR demonstrated that *Mtk* was induced in *dronc*[ΔA8] mutants, whereas there were no changes in *Dpt* or *AttA* expression compared with the controls (Fig. 1, compare *D* and *E*). Remarkably, a strong induction of *Drs* (>10-fold) was observed in the mutant larvae (Fig. 1E), suggesting appreciable immune activation through the Toll signaling pathway.

Further analysis indicated the concurrent up-regulation of *Spätzle* transcripts (Fig. 1F), as well as an enrichment of transcripts encoding the Rel inhibitor *Cactus* and the NF-κB-like protein *Dorsal* in the fat body cells (the insect counterpart of the liver and white adipose tissue in mammals) (Fig. 1G). The increased expression of the transcript encoding *Cactus* suggested that chronic Toll activation in the *dronc*[ΔA8] mutant might induce a negative feedback response. The profound elevation of *Drs* in the fat body cells indicated that this tissue is the primary site of Toll pathway activation (Fig. 1G). This possibility was confirmed by the observation of an intense GFP signal in the fat body of *dronc* mutants carrying the *Drs-GFP*, *Dipt-lacZ* (DD) reporter transgene (25, 26) (Fig. 1H).

These observations led us to examine the intracellular localization of Dorsal. In wild-type fat body cells, Dorsal protein was distributed evenly between the nuclei and cytosol (Fig. 2A), indicating a lack of Toll signaling activation, analogous to *spz*[rm7], the loss of function mutant for the Toll ligand Spätzle (Fig. 2F). However, in *dronc*[ΔA8] fat body cells, the nuclear translocation of Dorsal was observed (Fig. 2B, penetrance: 53%), analogous to *Toll*[10b], a constitutively active mutant of the Toll receptor (Fig. 2G).

The accumulation of Dorsal in the nuclei of *dronc* mutants was readily reversed by the ubiquitous expression of wild-type *dronc* (*Act5C* > *dronc*[wt]; Fig. 2, *C* and *D*). In contrast, *dronc*[C318A], which encodes a catalytically inactive Dronc protein that exhibits impaired autoprocessing and enzymatic activity (6, 7), failed to rescue the *dronc* mutant phenotype when expressed as a transgene (Fig. 2E). Taken together, we conclude that defective Dronc activity leads to a systemic activation of Toll signaling *in vivo*.

The association of the Dorsal nuclear translocation phenotype with apoptotic deficiency was further confirmed by the observation of the same phenotype in mutants of *dark* (the *Drosophila* ortholog for *apaf-1*) (penetrance, 12%) and *drICE* (the predominant effector caspase in *Drosophila*) (penetrance, 21%) (8, 15) and in a transheterozygous *dronc* mutant (*dronc*[ΔA8]/*Nc*[51]) (penetrance, 31%) (Fig. 2H) (9). Thus, impaired apoptotic signaling results in spontaneous Toll signaling in the fat body cells.
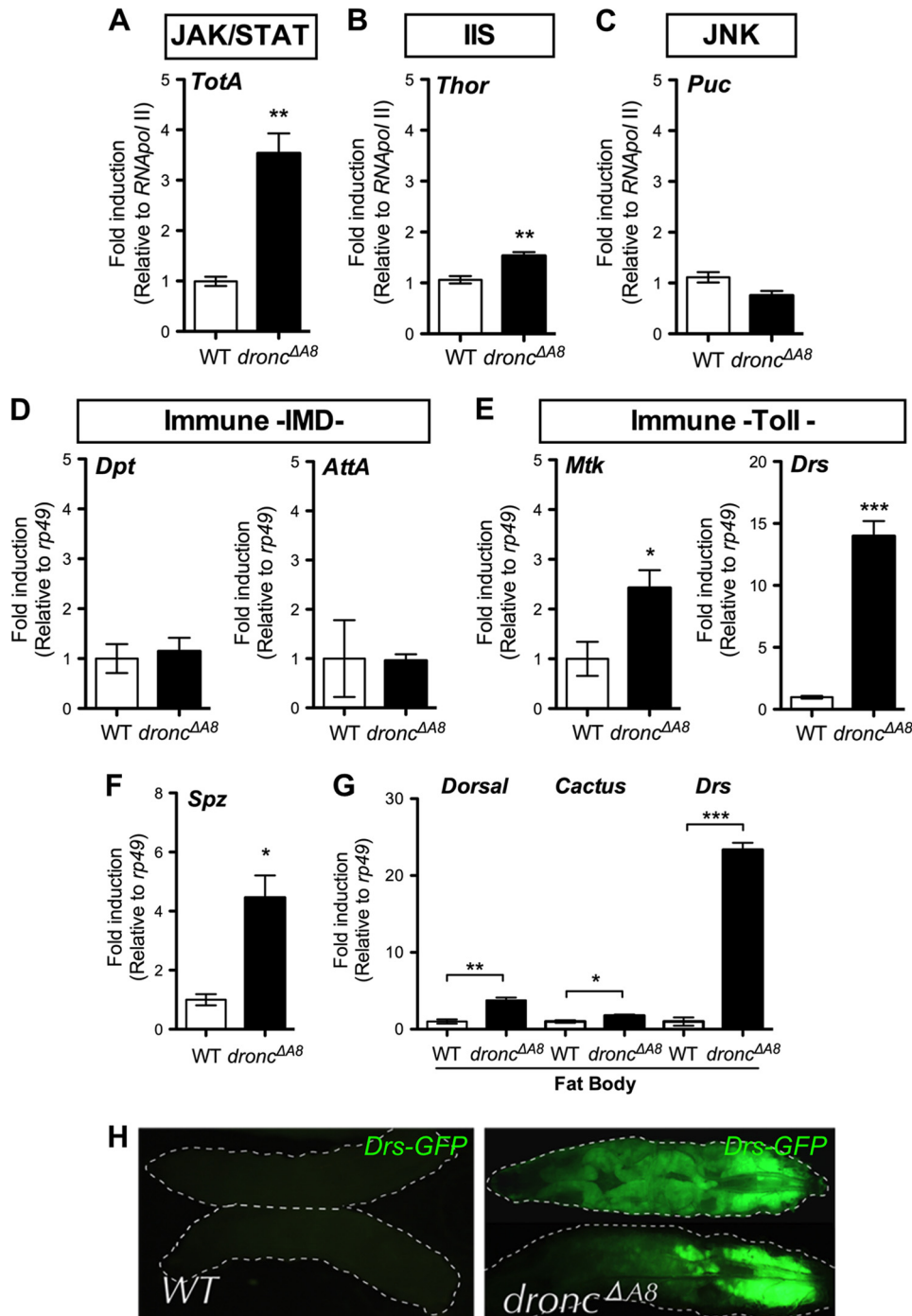
FIGURE 1. **dronc∆A8 mutants exhibit elevated stress responses and constitutive Toll pathway signaling.** *A–E*, transcript levels of genes for stress response and innate immunity were examined using quantitative RT-PCR with total RNA from wild-type and *dronc*$^{\Delta A8}$ L3 larvae. *F*, transcripts encoding extracellular components Spz were significantly induced in the whole body of *dronc*$^{\Delta A8}$ flies. *G*, transcripts encoding the Dorsal/NF-κB, Cactus/IκB, and Drs were induced in the *dronc*$^{\Delta A8}$ fat body. *H*, expression of the *Drs*-GFP fluorescent reporter was induced predominantly in the *dronc*$^{\Delta A8}$ fat body. The genotype was WT, *Drs-GFP, Dpt-lacZ. dronc*$^{\Delta A8}$, *Drs-GFP, Dpt-lacZ;; dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$. Statistics are results of Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

Humoral Factors in the Hemolymph from dronc$^{\Delta A8}$ *Mutants Sustain the Activation of Dorsal in the Fat Body*—The fact that larval fat body cells do not undergo programmed cell death via apoptosis (27) prompted us to investigate the cell autonomy of the *dronc* deficiency-induced Dorsal activation in fat body cells. To this end, we generated a genetic mosaic for *dronc*$^{\Delta A8}$ in an otherwise heterozygous background based on a ubiquitously induced recombination and examined Dorsal localization in the mosaic fat bodies. In *dronc*$^{\Delta A8}$ mosaic clones, Dorsal nuclear translocation could not be detected (Fig. 3*A*), suggesting that *dronc* deficiency does not directly lead to Dorsal activation cell autonomously but may exert its effect via hemolymph-borne soluble factors.

To explore this possibility, we set up a fat body *ex vivo* culture system to examine whether hemolymph from *dronc*$^{\Delta A8}$ mutants contains DAMPs that could lead to Dorsal activation
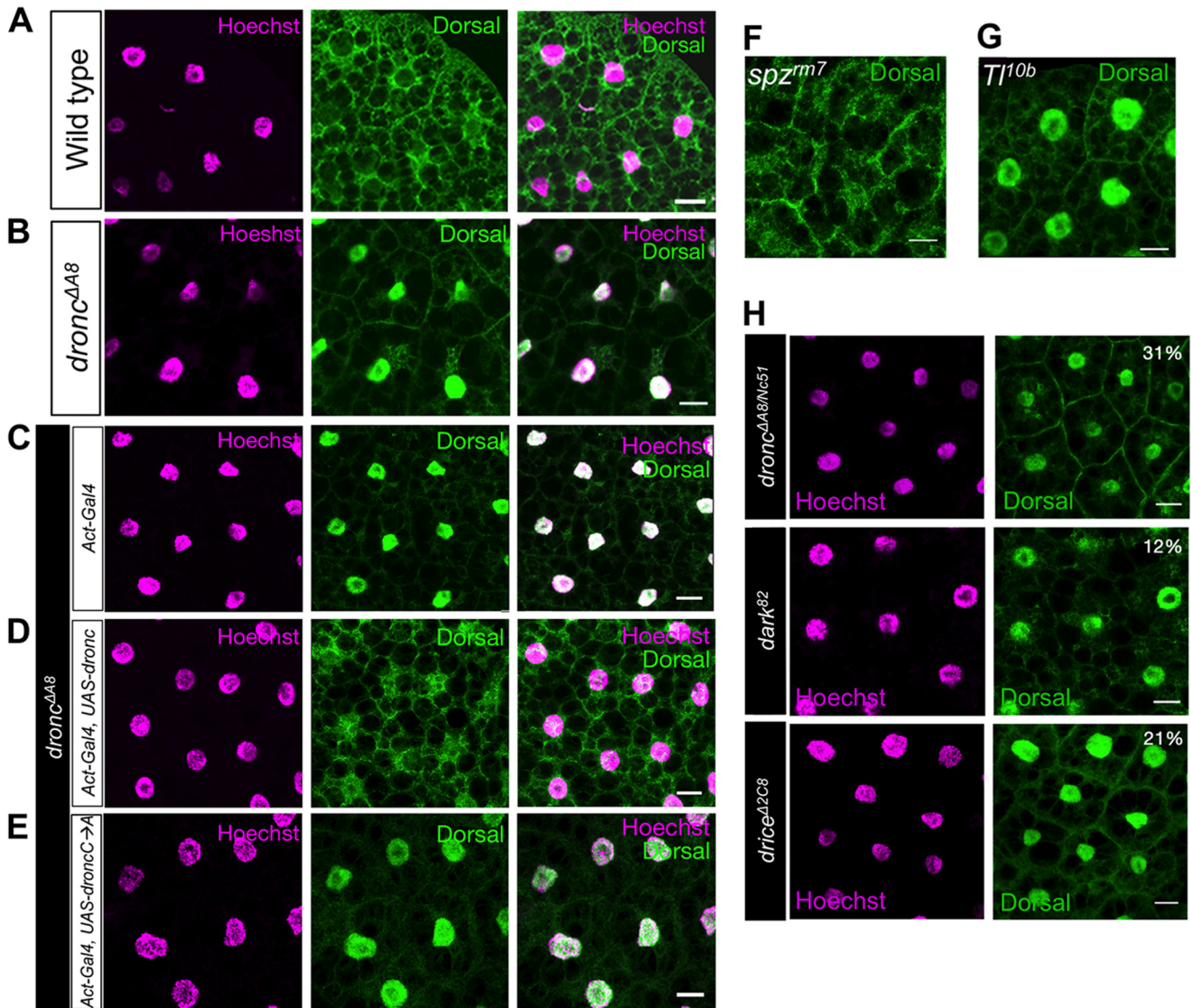
FIGURE 2. **Intracellular distribution of Dorsal in fat body cells.** *A* and *B*, immunohistochemistry staining revealed a substantial nuclear accumulation of Dorsal protein in *dronc*$^{\Delta A8}$ fat body cells. Images show the maximal projection of multiple confocal images. *C–E*, the enzymatic activity of Dronc plays an essential role in repressing spontaneous activation of the Toll/Dorsal cascade. The details are described in the text. The genotypes were *Act5c-Gal4/+; dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$ (*C*); *Act5c-Gal4/UAS-dronc*^{wt}-*FLAG; dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$ (*D*); and *Act5c-Gal4/UAS-dronc*^{C318A}; *dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$ (*E*). *F* and *G*, immunohistochemical staining of Dorsal; *spz*^{rm7} and *Toll*^{10b} (*Tl*^{10b}) mutants were used as negative and positive controls, respectively. *H*, dorsal nuclear translocation in the fat bodies of other apoptosis-deficient mutants was observed to various extents. The penetrance of Dorsal nuclear staining among the tested larvae is indicated as a percentage in the *top right corner* of each panel. *Scale bar*, 20 μm.

in wild-type fat body explants. In controls in which Ringer's solution was used in the co-culture, Dorsal protein was predominantly found in the cytoplasm (Fig. 3*B*), and most cells exhibited staining at level 0 (Fig. 3, *F* and *G*; see also "Experimental Procedures"), suggesting that the *ex vivo* culture procedure itself does not lead to immune activation in the isolated fat body. Addition of wild-type hemolymph to the co-culture system induced a moderate level of Dorsal nuclear translocation (Fig. 3*C*), possibly because of factors released from tissue damage occurring during the course of hemolymph extraction. Thus, wild-type hemolymph induced level 2 Dorsal activation in one-fourth of the fat body cells (Fig. 3*G*). As seen in Fig. 3*D*, the addition of *dronc*$^{\Delta A8}$ hemolymph resulted in a profound increase in the level of Dorsal nuclear translocation. Taken together, these findings support the idea that the constitutive Dorsal activa-

tion observed in *dronc*$^{\Delta A8}$ mutant fat bodies was due to humoral DAMPs circulating in the hemolymph.

In *Drosophila*, Toll receptor activation at the cell surface requires interaction with its cytokine-like ligand Spätzle (Spz) (28). Remarkably, genetic depletion of functional *spz* from the *dronc*$^{\Delta A8}$ hemolymph (*dronc*$^{\Delta A8}$ *spz*^{rm7}) abolished its ability to enhance Dorsal activation in fat body explants and restored the staining pattern to a level where most cells exhibited cytoplasmic Dorsal localization (Fig. 3, *E* and *G*). Taken together, these data suggest that the Toll/Dorsal signaling observed in *dronc*$^{\Delta A8}$ fat bodies is mediated by DAMPs in the hemolymph.

Toll Activation Is Induced by Host Factors—The Toll signaling pathway is activated following the detection by Toll receptors of microbial determinants, predominantly found in fungi and Gram-positive bacteria (29, 30). However, in germ-free
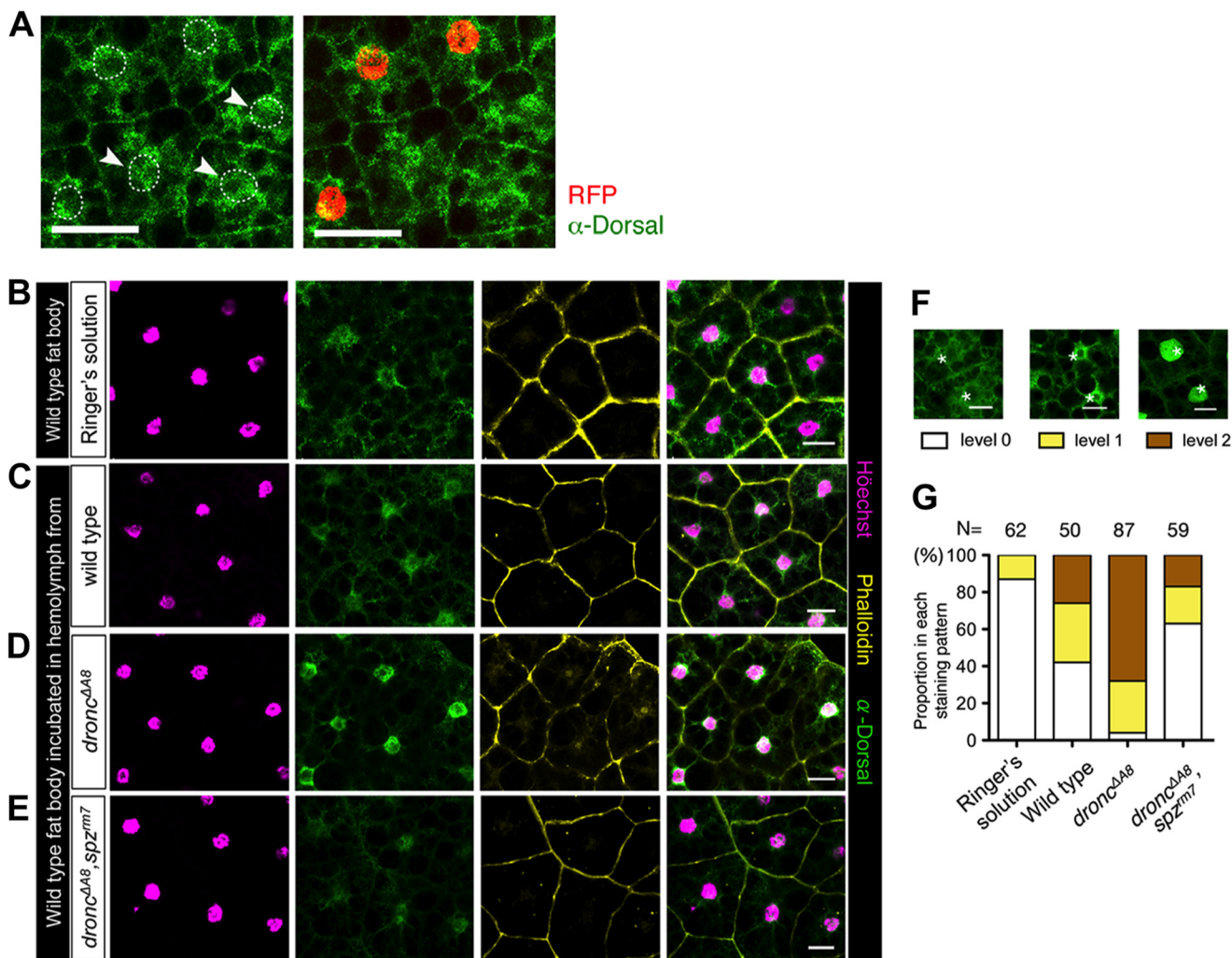
FIGURE 3. **Dorsal activation is triggered by extracellular stimuli.** *A*, dorsal intracellular distribution in *dronc*$^{\Delta A8}$ mosaic clones. RFP negative cells are homozygous *dronc*$^{\Delta A8}$ mutant cells. Nuclear RFP (*red*) and Dorsal protein (*green*) were examined by immunohistochemical staining. The larval genotype was *hs-Flp;;His2Av-RFP,FRT80B/dronc*$^{\Delta A8}$,*FRT80B*. *Scale bar*, 50 μm. *B–E*, immunohistochemistry staining of fat body explants incubated with Ringer's solution (*B*), hemolymph from wild-type (*w*^{1118}, *C*), *dronc*$^{\Delta A8}$ mutants (*D*), or *dronc*$^{\Delta A8}$ *spz*^{rm7} double mutants (*E*). The distribution of DNA (*magenta*), Dorsal (*green*), and F-actin (*yellow*) is shown as maximal projection confocal images. An image overlay illustrates Dorsal nuclear translocation. *Scale bar*, 20 μm. *F*, classification of Dorsal distribution in fat body explants, based on Dorsal nuclear staining patterns. *Scale bar*, 20 μm. *G*, for each culture condition, the percentage of cells associated with each Dorsal staining pattern was calculated. The total cell number counted for each treatment is indicated as *N*. *N* was normalized to 100%.
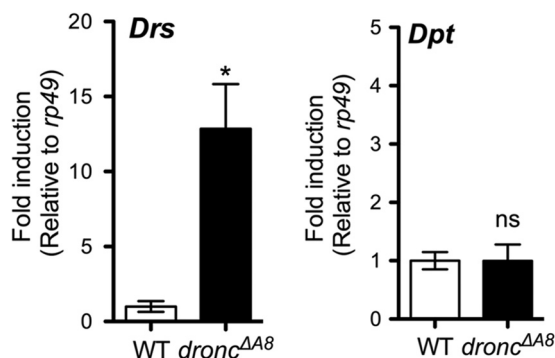

FIGURE 4. **Drosomycin induction in *dronc*ΔA8 mutants cultured under germ-free conditions.** Quantitative RT-PCR analysis showed a sustained elevation of *Drs* in germ-free *dronc*$^{\Delta A8}$ mutants, whereas *Dpt* was not induced under either condition. Statistics are results of Student's *t* test. *, $p < 0.05$. The data show means ± S.E.

dronc$^{\Delta A8}$ mutants that were not exposed to microbial components, *Drs* was induced, whereas *Dpt* remained unaffected (Fig. 4). This finding indicated that in *dronc*$^{\Delta A8}$ mutant larvae, the Toll ligand Spz is probably activated by host factors, rather than by microbial determinants, leading to activation of the canonical Toll signaling pathway. These immunogenic host factors are likely to be DAMPs that are up-regulated in the apoptosis-deficient mutant.

Hyperactivation of Toll Signaling in Circulating Hemocytes— In addition to initiating a systemic immune response, Spz also functions pleiotropically, regulating hematopoietic development and proliferation, as well as malignancy *in vivo*, probably through engaging the Toll receptor in hematopoietic tissues (31). Consistent with this scenario, we detected robust Dorsal nuclear translocation in the circulating hemocytes of *dronc*$^{\Delta A8}$ mutants, which was comparable to that seen in fat bodies and was also dependent on functional Spz (Fig. 5*A*). Hyperactivation of the *Drosophila* Toll pathway could cause hema-
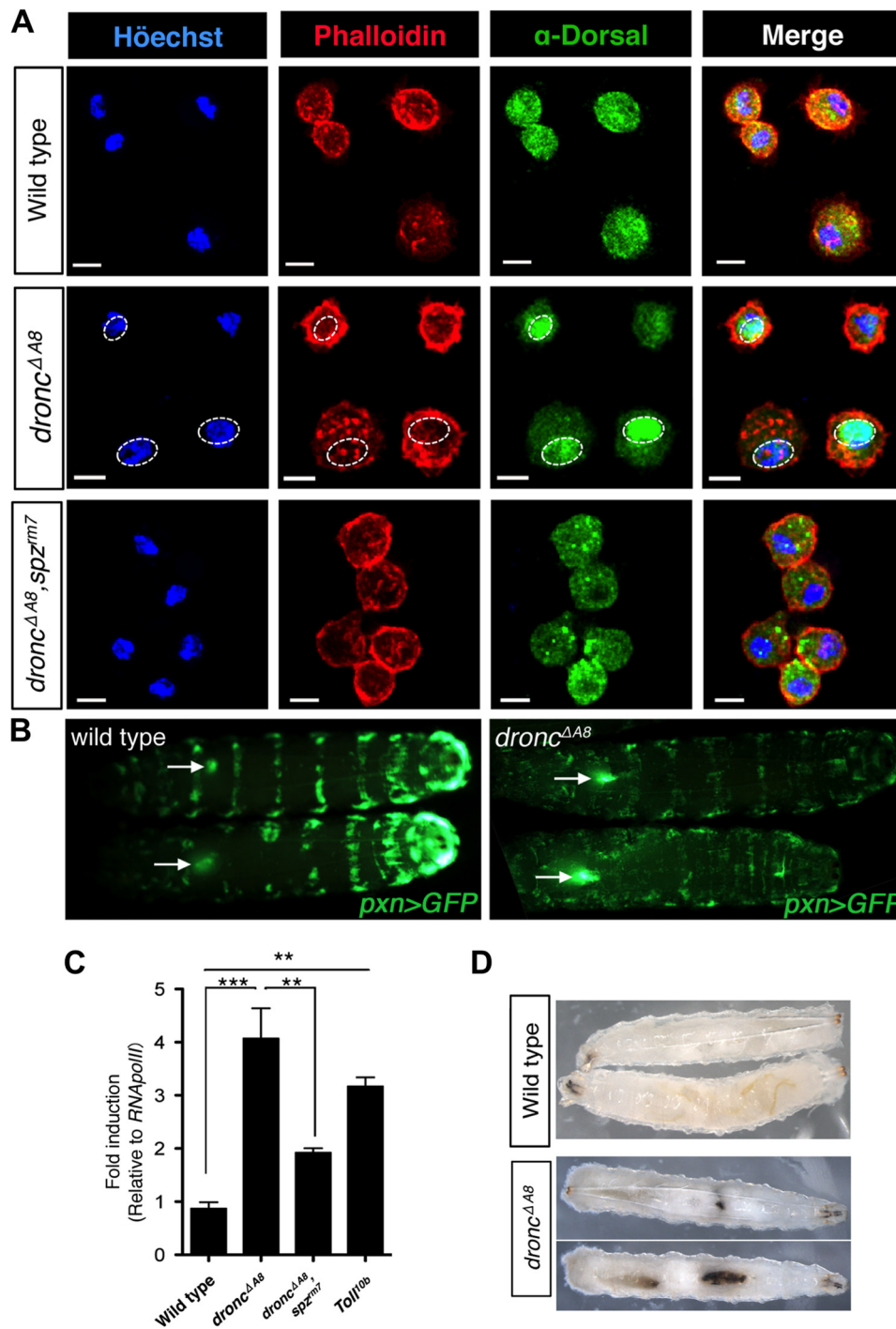
FIGURE 5. **droncΔA8 mutants exhibit hematopoietic disorders and random formation of melanotic clots.** *A*, Spätzle mediates Dorsal nuclear transloca- tion in circulating hemocytes from *dronc*$^{\Delta A8}$ mutants. The nuclei (*cyan*) and cell periphery (*red*) were visualized by staining with Höechst 33342 and rhodamine- conjugated phalloidin, respectively. Immunohistochemical staining showed the nuclear accumulation of Dorsal (*green*) in *dronc*$^{\Delta A8}$ mutants, whereas the loss of *spz* abrogated this phenotype. The genotype for each row is indicated at *left*. *Scale bar*, 5 μm. *B*, visualization of lymph gland hypertrophy. Images show GFP fluorescence from the dorsal side. The *arrows* point to lymph glands containing GFP-expressing hemocytes, with the *arrowheads* oriented toward the posterior end of the larvae. The genotypes were *pxn-Gal4, UAS-GFP* for wild type and *pxn-Gal4, UAS-GFP; dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$ for *dronc*$^{\Delta A8}$ mutants. *C*, quantitative RT-PCR analysis of *hemese*, a pan-hemocyte marker in *Drosophila* (32, 57) indicated that *dronc*$^{\Delta A8}$ mutants have increased total blood cell numbers, comparable to the level observed in *Tl*^{10b} mutants. The data show means ± S.E. Statistics are results of one-way analysis of variance test. **, $p < 0.01$; ***, $p < 0.0001$. *D*, induction of a melanotic tumor in *dronc*$^{\Delta A8}$ flies.

topoietic phenotypes previously associated with dysregu- lated Toll signaling, including lymph gland hypertrophy, blood cell hyperproliferation, and the spontaneous forma- tion of melanotic clots (32, 33). These defects were nicely corroborated in the *dronc*$^{\Delta A8}$ mutants (Fig. 5, *B–D*). Inter-

estingly, increased hemocyte number assessed by *hemese* expression (Fig. 5*C*) and spontaneous melanization were observed in the *dronc* mutants (Fig. 5*D*), as well as in another apoptosis-deficient mutant (*dark*), in previous reports (9, 11, 34). In *Drosophila*, circulating hemocytes function as senti-

nel cells, sensing diffusive signals from tissue damage and assisting in tissue repair and in replenishing hematopoietic tissue populations upon stimulation (35–37). The Toll signaling activation in hematopoietic cells in the $dronc^{\Delta A8}$ mutant may function as an adaptive mechanism in response to extensive tissue damage caused by apoptotic deficiency.

The CLIP Domain Serine Protease Persephone Acts Upstream to Facilitate Spätzle Cleavage in $dronc^{\Delta A8}$ Hemolymph—The endogenous cytokine-like factor Spz is secreted as an inactive full-length protein and undergoes intramolecular cleavage to produce the C-terminal 106-amino acid fragment (Spz C-106) that binds to Toll and initiates signaling (28). The non-cell autonomous activation of Dorsal in the fat body prompted us to explore whether Spz C-106 is produced in $dronc^{\Delta A8}$ mutants. Hemocyte-borne Spz is reported to be both necessary and sufficient to induce the activation of NF-κB factors in fat bodies (38). We overexpressed HA-tagged pro-Spz (14) using a hemocyte-specific driver (*pxn-GAL4*) (12) in the $dronc^{\Delta A8}$ background so the tagged form of Spz would be secreted from these circulating blood cells. Indeed, Western blots of hemolymph extracted from the transgenic animals demonstrated the presence of the active form of Spz (Spz C106-HA, 19kDa) in the $dronc^{\Delta A8}$ mutants (Fig. 6*A*). This result provided additional evidence that the Dorsal activation in fat body cells was initiated by Toll receptor binding of its soluble ligand Spz, which undergoes intramolecular cleavage.

After Toll receptor ligation at the cell surface, intracellular immune signaling is initiated by recruitment of the intracellular adaptor dMyd88 (39). We next investigated whether dMyd88 is required to relay the signal for *Drs* induction in $dronc^{\Delta A8}$ mutants. In both the double-mutant lines $dronc^{\Delta A8} spz^{rm7}$ and $dronc^{\Delta A8} dMyd88^{Kra1}$, the Toll signaling activation was completely abrogated, as indicated by the absence of both *Drs* induction and Dorsal nuclear translocation (Fig. 6, *B* and *C*), the two hallmarks of autoimmune activation observed in $dronc^{\Delta A8}$ flies. Our genetic analysis clearly demonstrated that in $dronc^{\Delta A8}$, the canonical Toll signaling modules mediate Dorsal activation in the fat body.

Persephone (Psh) is an extracellular CLIP domain serine protease that activates Spz, through a proteolytic cascade that is partly independent of proteoglycan sensing (40). The proteolytic maturation of Psh can be triggered by virulent proteases secreted by fungi or Gram-positive bacteria (40, 41). To test whether Psh was involved in the DAMP-mediated Toll activation, we replaced the wild-type *psh* allele in $dronc^{\Delta A8}$ with a loss of function allele *psh¹*, which encodes a Psh protein with impaired catalytic activity (16), and assessed *Drs* expression in the double mutants. Analysis by qRT-PCR showed that, like the *spz* impairment (*spz^{rm7}*), the loss of Psh function greatly reduced *Drs* production in the $dronc^{\Delta A8}$ background (Fig. 6*D*). In conclusion, our genetic analysis suggested that in $dronc^{\Delta A8}$ mutants, endogenous, hemolymph-borne DAMPs induce systemic Toll immune signaling by activating the Psh protease, which triggers the proteolytic cleavage of the Toll ligand, Spz.

DISCUSSION

In the current study, we present evidence that the Toll signaling pathway is systemically and constitutively activated in apoptosis-deficient *Drosophila* mutant larvae. We further demonstrated that the activation of this immune pathway was independent of microbial challenge and was due to endogenous humoral factors found in the hemolymph, where the endogenous ligand Spz is processed to its active form through a proteolytic cascade. Our findings also revealed that Persephone, the *Drosophila* hemolymph serine protease, was crucial in mediating inflammatory responses to endogenous molecules, demonstrating its involvement in a danger-sensing mechanism.

In *Drosophila*, several forms of stress, including mechanical injury (42), temperature shift (43), starvation (44), and aging (45, 46) have been shown to trigger immune system activation in the absence of a pathogen. The present study using *Drosophila* third instar larvae supports the scenario that Toll signaling is a key mechanism in the host defense system that responds to endogenous inflammatory factors, consistent with the dedicated role of Toll-like receptors in vertebrate "danger" sensing (1, 47, 48). A number of previous studies that focused on nonmicrobial signal-activated host defense in *Drosophila* showed that the Imd/Relish cascade is activated by endogenous inflammatory agents, such as undigested DNA (49, 50) and the neuro-peptide bursicon (51), as well as undefined humoral factors released from the intestine after tissue damage (52). The observation of different signaling pathways that are activated in response to various forms of stress, and in the *dronc* mutant, indicate that specific detection mechanisms are used to discriminate among various forms of threat.

Is there any physiological role of Toll-mediated immune response in apoptosis-deficient organisms? In our preliminary observation, $dronc^{-/-}$ larvae lacking *spz* showed the lack of melanotic tumor. However, the mutant larvae reached the lethal point approximately at the same stage, irrespective of their inability to activate the Toll signaling. We still believe that this prominent immune pathway could benefit the animals in a way that prepares them for upcoming tissue damage, for instance, by increasing the hemocytes whose function is crucial in sequestering large debris and relaying signals that are required in tissue repair. Melanization has been shown under certain circumstances to enhance the effectiveness of defense signaling upon a life-threatening stimulus (53). Thus, we think that the activation of Toll would have some beneficial roles in apoptosis-deficient organisms.

Drosophila Toll activation involves the activation of two distinct, but parallel pathways, namely a pattern recognition receptor-dependent pathway and a Psh-dependent pathway (40), that converge on the proteolytic cleavage of Spz (54). The pattern recognition receptor-dependent pathway consists of a set of circulating receptors, including peptidoglycan recognition proteins and Gram-negative bacteria-binding proteins, capable of discriminating between bacterial and fungal determinants (55), the recognition of which triggers a subsequent proteolytic cascade leading to Spz activation. The parallel path-
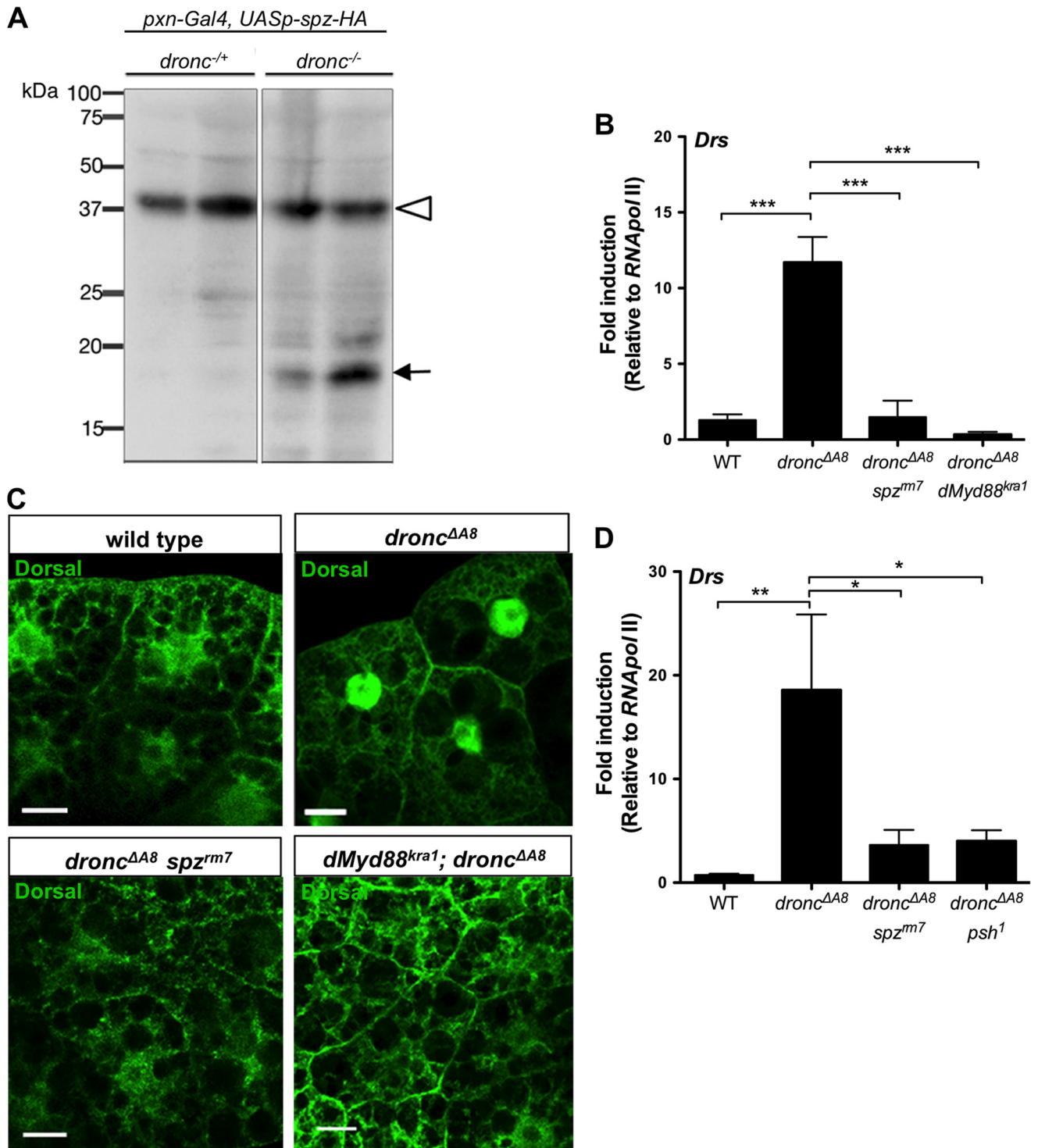
FIGURE 6. **Spz and Psh are indispensable for Toll signaling activation in the fat bodies of *dronc*ΔA8 mutants.** *A*, detection of the proteolytically processed form of Spz-HA in *dronc*$^{\Delta A8}$ hemolymph extracts by Western blot analysis. Duplicated samples of the same genotype were shown. The genotypes are *pxn-Gal4/UASp-spz-HA; dronc*$^{\Delta A8}$/+ for *dronc*$^{+/-}$ and *pxn-Gal4/UASp-spz-HA; dronc*$^{\Delta A8}$/*dronc*$^{\Delta A8}$ for *dronc*$^{-/-}$. *B* and *C*, impairment of either *spz* or *dMyd88* abolished the spontaneous Toll/Dorsal activation in *dronc*$^{\Delta A8}$, shown by quantitative RT-PCR analysis (*B*) and immunohistochemical staining (*C*). *Scale bar*, 20 μm. *D*, genetic analysis indicated that the serine protease Psh was required for Toll receptor activation. The data show means ± S.E. Statistics are results of one-way analysis of variance test. *, $p < 0.05$; **, $p < 0.01$.

way is activated when Psh senses virulence factors secreted from filamentous fungi (41) or Gram-positive bacteria (40). Although the molecular mechanism of how these enzymes are detected by Psh is currently unknown, Psh has been implicated as a key factor in the "danger signal-dependent extracellular pathway" ever since the recognition of its ability to detect abnormal protease activity (40). Our findings extend this theory by showing that Psh is responsible for endogenous DAMP-mediated systemic activation of Toll signaling. The activation of innate immunity-regulated genes including *Drs* and *Mtk* was

previously observed following serine protease activation in response to wound stimulation (56). Serine protease(s) released from necrotic cells may act to initiate the Psh-dependent Toll signaling pathway.

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