

# Spatial Regulation of Developmental Signaling by a Serpin

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## Summary

An extracellular serine protease cascade generates the ligand that activates the Toll signaling pathway to establish dorsoventral polarity in the *Drosophila* embryo. We show here that this cascade is regulated by a serpin-type serine protease inhibitor, which plays an essential role in confining Toll signaling to the ventral side of the embryo. This role is strikingly analogous to the function of the mammalian serpin antithrombin in localizing the blood-clotting cascade, suggesting that serpin inhibition of protease activity may be a general mechanism for achieving spatial control in diverse biological processes.

## Introduction

The establishment of embryonic dorsoventral polarity in *Drosophila* has been a key model system for addressing several major issues in developmental biology, including understanding the molecular mechanisms that define asymmetry and generate spatial patterning across the body axis (Morisato and Anderson, 1995). Ventral and lateral development in the *Drosophila* embryo is specified by an extracellular ligand that activates the receptor Toll. The Toll ligand is a proteolytically processed form of the Spätzle protein found within the perivitelline fluid that surrounds the early embryo (Morisato and Anderson, 1994; Schneider et al., 1994; DeLotto and DeLotto, 1998). Spätzle is processed by the serine protease Easter, which circulates as an inactive zymogen in the perivitelline space, but like Spätzle is proteolytically activated apparently only on the ventral side of the embryo (Chasan and Anderson, 1989; Chasan et al., 1992). Thus, local activation of the Easter protease appears to be crucial for the spatially asymmetric activation of Toll signaling necessary to establish embryonic dorsoventral polarity.

Easter is activated at the end of an extracellular serine protease cascade strikingly reminiscent of the mammalian blood-clotting cascade (LeMosy et al., 1999; Krem and Di Cera, 2002). The blood-clotting proteases normally circulate in the plasma as inactive zymogens but

become activated at the site of tissue damage (Furie and Furie, 1992). An additional level of control that spatially restricts the activity of these proteases is provided by serine protease inhibitors known as serpins, such as antithrombin, which inactivate proteases that diffuse away from the activation site. Serpins are suicide substrates that are cleaved by their target proteases, invariably at a reactive site near the C terminus, thereby forming a covalent complex of serpin and protease that is resistant to dissociation by the detergent SDS (Gettins, 2002).

Earlier studies suggested that negative regulation is required for spatially restricting Easter activity (Jin and Anderson, 1990; Misra et al., 1998; Chang and Morisato, 2002). Dominant mutations in the *easter* gene produce ventralized or lateralized embryos, in which the number of cells adopting a ventrolateral fate is expanded at the expense of dorsal fates. Misra et al. (1998) detected in embryonic extracts a high molecular weight form of Easter, called Ea-X, with properties suggestive of an Easter-serpin complex. In the *easter* dominant mutants, formation of Ea-X is decreased and the Easter catalytic domain remains biologically active long after zymogen activation (Chang and Morisato, 2002). The molecular nature of X has been the focus of intense interest, but has remained unidentified.

Here we report the identification of the serpin Spn27A as the inhibitor that spatially controls Easter activity in the embryo. We demonstrate that Spn27A is essential for localizing Toll signaling in order to establish embryonic dorsoventral polarity. This role for Spn27A is very analogous to the function of the mammalian serpin antithrombin in localizing the blood-clotting cascade, suggesting that serpin inhibition of protease activity is a general mechanism for achieving spatial control in biological processes.

## Results and Discussion

In order to explicitly test the hypothesis that a serpin is involved in spatially regulating the Easter protease, by analogy to the role of antithrombin in blood clotting, we searched the *Drosophila* genome for candidate serpins. A serpin that inhibits the extracellular Easter protease should have, in addition to the C-terminal reactive center loop sequence characteristically found in known inhibitory serpins (Gettins, 2002), a basic residue at the predicted reactive site to match the amino acid specificity of Easter and an N-terminal signal sequence for secretion. We identified eight serpins that fulfilled all three criteria, out of about 25 encoded in the genome (searched using “serpin” as the query term at <http://flybase.bio.indiana.edu:82/genes/fbgquery.hform>). The predicted reactive sites of two serpins, Spn1 and Spn27A, additionally showed provocative sequence similarity to the cleavage site of Spätzle, the Easter substrate (Figure 1A).

To determine whether any of the eight candidate ser-

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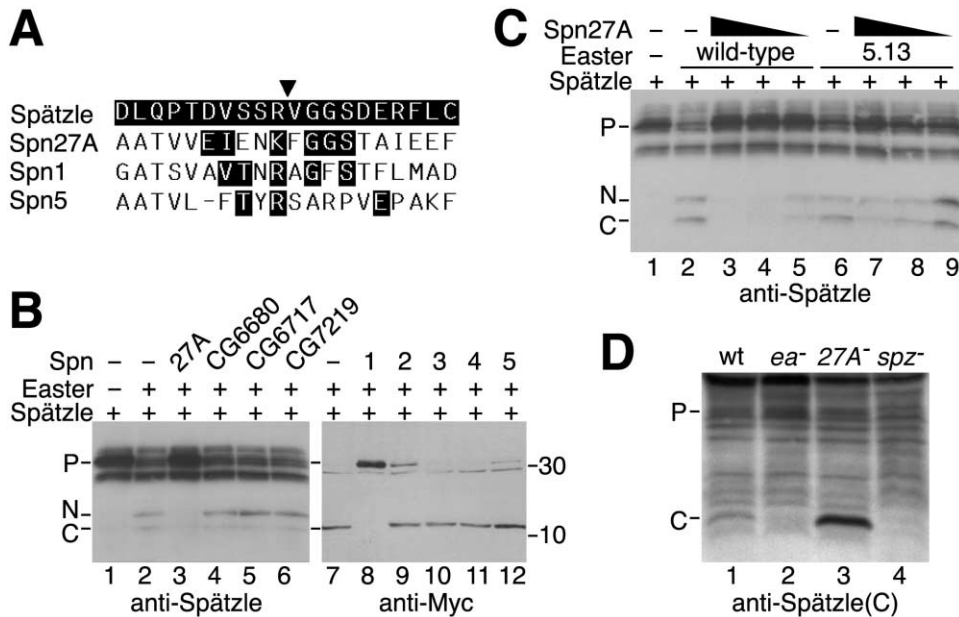


Figure 1. The Serpin Spn27A Is an Inhibitor of the Easter Protease

(A) Alignment showing sequence similarity between the cleavage site of Spätzle, Easter's substrate, and the predicted reactive sites of Spn27A and Spn1. Spn5, which lacks this similarity, is shown for comparison. The inverted triangle marks the scissile bond, and residues similar to those in Spätzle are highlighted.

(B) Of eight serpins in the *Drosophila* genome predicted to inhibit the trypsin-like Easter protease, only Spn27A (lane 3) and Spn1 (lane 8) block Easter cleavage of Spätzle in vitro. Spn2, predicted to inhibit a chymotrypsin-like enzyme, is shown for comparison (lane 9). Spätzle tagged at the C terminus with the Myc epitope was expressed with or without the Easter catalytic domain and serpin by transient transfection in *Drosophila* S2 cells, and was detected by immunoblotting (the antibodies used are indicated at the bottom). The migration of precursor (P) Spätzle and N- and C-terminal processed forms are indicated. The position of molecular weight standards (in kDa) is shown on the right.

(C) Mutant Easter protease is less efficiently inhibited by Spn27A. Decreasing amounts of Spn27A (1.5, 0.5, and 0.15  $\mu$ g plasmid DNA) were expressed in S2 cells with Easter catalytic domain (1.5  $\mu$ g plasmid DNA), either wild-type (lanes 3–5) or the *ea*<sup>5.13</sup> mutant (lanes 7–9), along with Spätzle. Two different film exposures were combined in this picture for optimal visualization of the varying levels of precursor and processed forms of Spätzle.

(D) Higher amount of processed Spätzle is detected in embryos from *spn27A* mutant. Total protein in extracts of 0–4 hr embryos from wild-type (wt; lane 1), *ea*<sup>-</sup> (lane 2), *spn27A*<sup>-</sup> (*27A*<sup>-</sup>; lane 3), and *spz*<sup>-</sup> (lane 4) females was analyzed by immunoblotting with antibodies against the Spätzle C-terminal region that react more efficiently with cleaved (C) than with precursor (P) form.

pins could inhibit Easter, we assessed their inhibitory activity in a cultured cell assay involving coexpression of the Easter catalytic domain and Spätzle (Chang and Morisato, 2002). We found that both Spn1 and Spn27A efficiently blocked Easter cleavage of Spätzle, while the other six candidates had no appreciable effect (Figure 1B). Based on these results, Spn1 and Spn27A emerged as the best candidates for a natural inhibitor of Easter.

To investigate the role of Spn1 and Spn27A in regulating Easter in vivo, we wanted to examine the genetic consequences of removing maternal serpin activity. For Spn27A, De Gregorio et al. (2002) recently generated an apparently protein null mutation to assess the zygotic role of Spn27A in regulating the melanization reaction during the immune response. Therefore, we used this mutation to remove maternal *spn27A* function and characterized the resulting phenotype by scoring embryos for the expression of dorsoventral zygotic genes (Rusch and Levine, 1996). In the wild-type embryo at the blastoderm stage, the *zen* gene is expressed in a dorsal domain, the *rho* gene in two ventrolateral stripes, and the *twi* gene in a ventral domain (Figure 2A, left panels). By contrast, embryos lacking maternal *spn27A* function showed a striking expansion of *twi* expression across

the entire dorsoventral axis, with a corresponding loss of *rho* and *zen* transcription (Figure 2A, right panels). In addition, the mutant embryos failed to differentiate a cuticle at the end of embryogenesis, consistent with the interpretation that all cells had adopted the ventral-most mesodermal fate, as dictated by uniform *twi* expression (Figure 2B, top panel). Finally, the ventralized phenotype was completely rescued by injection of embryos with in vitro synthesized *spn27A* RNA (Figure 2B, middle panel; Supplemental Table S1 at <http://www.developmentalcell.com/cgi/content/full/5/6/945/DC1>). This result demonstrates that the mutant phenotype was caused by the loss of *spn27A* function, and is consistent with a requirement for *spn27A* in germline rather than somatic tissue. The genetic characterization and rescue experiments together demonstrate that the serpin Spn27A is essential for establishing embryonic dorsoventral polarity.

The strongly ventralized phenotype produced by the loss of *spn27A* function requires wild-type *easter* activity, consistent with the interpretation that the Spn27A protein acts to regulate Toll signaling rather than another pathway important for establishing embryonic dorsoventral polarity. Females lacking *spn27A* and either *easter* or *spätzle* function produced dorsalized embryos

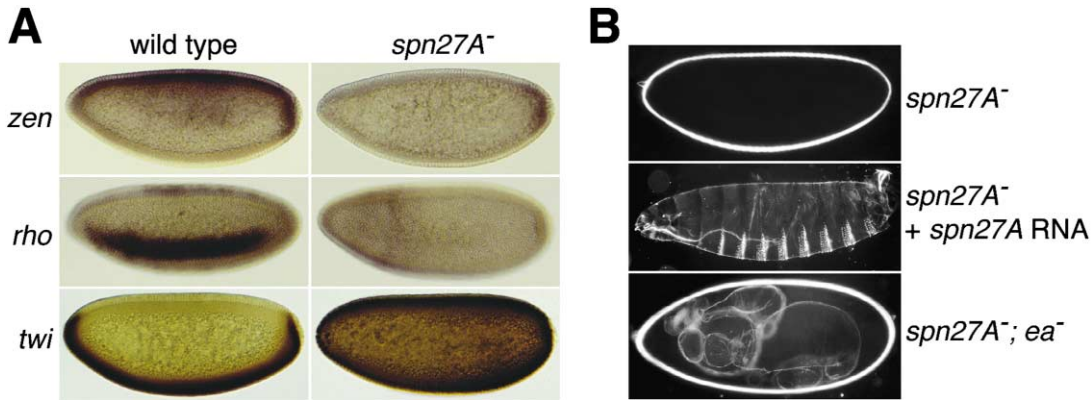


Figure 2. Spn27A Is Maternally Required for Embryonic Dorsoventral Polarity

(A) Embryos laid by *spn27A*<sup>-</sup> females are strongly ventralized. Wild-type embryos express the *zen* gene in a dorsal domain, the *rho* gene in two ventrolateral stripes, and the *twi* gene in a ventral domain (left panels). In contrast, the Spn27A-deficient embryos fail to express *zen* and *rho*, but expand *twi* expression to all dorsoventral positions (right panels). All embryos are oriented with the dorsal side up and the ventral side down.

(B) Rescue and suppression of strongly ventralized phenotype. Embryos lacking maternal Spn27A fail to differentiate cuticle, a product of dorsal and ventral ectoderm (top panel). The injection of in vitro synthesized *spn27A* RNA completely rescues the mutant phenotype to produce hatching larvae (middle panel). The ventralized phenotype resulting from a loss of *spn27A* function requires wild-type *easter* activity. Females that are *spn27A*<sup>-</sup>; *ea*<sup>-</sup> produce completely dorsalized embryos, identical to the phenotype caused by the loss of *easter* function alone (bottom panel). The bright oval structure in the top and bottom panels is the vitelline envelope.

lacking all ventral and lateral structures, indistinguishable from the phenotype produced by the *easter* or *spätzle* mutation alone (Figure 2B, bottom panel, and data not shown).

Although we have not yet been able to examine the role of Spn1 in embryonic dorsoventral patterning, the nearly complete ventralization caused by loss of Spn27A (Figure 2A) suggests that Spn1 is not functionally redundant with Spn27A. Its ability to inhibit Easter activity in vitro (Figure 1B) may therefore indicate that the natural target of Spn1 is a protease sharing substrate specificity with Easter.

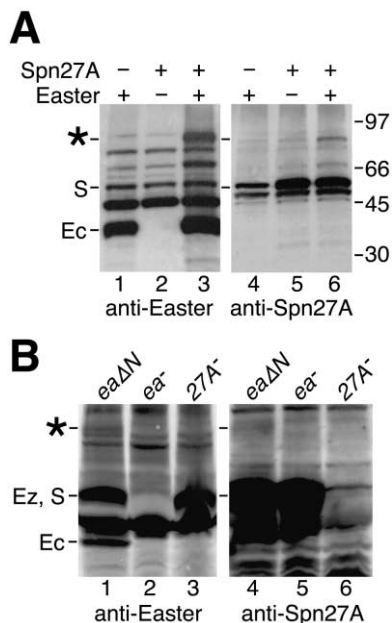
Within the Toll signaling pathway, Spn27A appears to be specifically targeting the Easter protease. In vitro, Spn27A blocked the proteolytic activity of Easter (Figure 1B), but not the upstream proteases GD and Snake (data not shown). As expected for inhibition by the classic serpin mechanism, Spn27A and Easter formed a stable 1:1 complex in vitro (Figure 3A). In embryo extracts, the Easter-Spn27A complex was present at very low levels, and thus difficult to detect consistently, presumably because only a small fraction of Easter is activated (Chasan et al., 1992). We therefore analyzed embryos containing a high level of Ea-X, the hypothetical Easter-serpin complex, produced by *ea* $\Delta$ N females bearing a transgene encoding a “preactivated” form of Easter lacking the N-terminal prodomain (Chasan et al., 1992; Misra et al., 1998). In extracts of *ea* $\Delta$ N embryos, we detected with antibodies to both Easter and Spn27A the polypeptide corresponding to Ea-X, thereby demonstrating that Easter and Spn27A form a complex in vivo (Figure 3B).

In order to obtain further evidence for the biological importance of the Easter-Spn27A complex, we carried out studies using the dominant *ea*<sup>5.13</sup> mutation, which produces lateralized embryos that lack dorsoventral polarity. Earlier studies suggested that the mutant Ea<sup>5.13</sup> protein is partially defective in its ability to form Ea-X,

resulting in continued protease activity that leads to higher levels of processed Spätzle (Chang and Morisato, 2002). Consistent with these observations in vivo, we found that the mutant protease was less efficiently inhibited by Spn27A as compared with the wild-type protease in the cell culture assay (Figure 1C).

If the role of Spn27A is to inhibit Easter and thus activation of Toll signaling, absence of Spn27A should result in increased processed Spätzle, as observed in the *ea*<sup>5.13</sup> embryonic extracts (Chang and Morisato, 2002). Indeed, we found a higher level of processed Spätzle in extracts of Spn27A-deficient embryos than of wild-type embryos (Figure 1D). Therefore, Spn27A shows all the features of the inhibitor X proposed to spatially control Easter (Misra et al., 1998).

The ventralized phenotype observed with the loss of maternal *spn27A* function implies that regulation of Easter following zymogen activation is required for maintaining embryonic polarity. If activated Easter were capable of diffusion, Spn27A may primarily act to maintain the initial asymmetry of zymogen activation, by inhibiting Easter before its diffusion to the dorsal side of the embryo. Alternatively, if Spn27A were itself localized to the dorsal side, it could be providing an opposing gradient of a signaling antagonist, as seen for the case of TGF $\beta$  signaling (Freeman and Gurdon, 2002). In fact, the following experiments support the first model. First, we found that embryos lacking Spn27A could be completely rescued by injection of cultured cell medium containing Spn27A protein into the perivitelline space surrounding the embryo, irrespective of whether injection occurred on the dorsal or the ventral side (Supplemental Table S2). This result demonstrates that Spn27A acts in the same extracellular compartment where Easter functions (Chasan et al., 1992; Stein and Nüsslein-Volhard, 1992), and suggests that there is no requirement for Spn27A to be prelocalized to a specific region along the dorsoventral axis. Second, we detected Spn27A in perivitelline



**Figure 3.** Spn27A and Easter Form an SDS-Resistant Complex In Vitro and In Vivo

(A) Easter-Spn27A complex is detected in transfected S2 cells. Easter catalytic domain and Spn27A were expressed alone (lanes 1-2 and 4-5) or together (lanes 3 and 6) in S2 cells. A polypeptide of 85–90 kDa (asterisk), matching the predicted size of a 1:1 complex of Easter catalytic domain (Ec; 35 kDa) and Spn27A (S; 50 kDa), is observed upon coexpression of the two proteins. A low level of endogenous Spn27A is detected in untransfected S2 cells.

(B) Easter-Spn27A complex is detected in the embryo. Total protein in extracts of 0–4 hr embryos from *eaΔN* (lanes 1 and 4), *ea<sup>-</sup>* (lanes 2 and 5), and *spn27A<sup>-</sup>* (*27A<sup>-</sup>*; lanes 3 and 6) females was analyzed by immunoblotting with anti-Easter (lanes 1–3) or anti-Spn27A (lanes 4–6) antibodies. The polypeptide corresponding to Ea-X (asterisk) is detected with both antibodies in the *eaΔN* extract but not the other extracts. In contrast to the Easter zymogen (Ez), the Easter catalytic domain (Ec) is only detectable in the *eaΔN* extract (Misra et al., 1998).

fluid extracted from embryos, thus providing evidence that Spn27A is a soluble and diffusible protein (Figure 4A). Finally, by immunostaining, we detected Spn27A across the entire dorsoventral axis of the embryo (Figure 4B). Thus, Spn27A appears to be a circulating component of the perivitelline space surrounding the embryo.

In conclusion, these experiments demonstrate that the serpin Spn27A is essential for spatially regulating the signal that defines embryonic dorsoventral polarity in *Drosophila*. This role for Spn27A reveals another link between development and immunity. The Toll signaling pathway was discovered for its role in *Drosophila* embryonic development, but is now also appreciated as a key defense mechanism against pathogens in the innate immune systems of both insects and mammals, for example, activating the production of antifungal peptides in *Drosophila* (Imler and Hoffmann, 2002). Spn27A was first described to have a zygotic role in regulating activation of the melanization reaction during the immune response (De Gregorio et al., 2002; Ligoxygakis et al., 2002b), and now we have discovered that it has a maternal role in regulating activation of the Toll signaling pathway during embryonic patterning. In the melanization

reaction, Spn27A presumably regulates the protease that activates phenol oxidase, a key enzyme in melanin biosynthesis. This protease may be distinct from Easter, as *easter* mutant flies do not show any gross defect in their ability to mount a melanization reaction at the site of tissue injury (our unpublished data). Interestingly, it appears that in development and in immunity the same ligand, processed Spätzle, activates the Toll signaling pathway, yet distinct serine protease cascades and serpins regulate the processing of Spätzle (Levashina et al., 1999; Ligoxygakis et al., 2002a).

Our data suggest that the role of Spn27A in establishing embryonic dorsoventral polarity is to control the spatial distribution of Toll signaling. Although its target, the Easter protease, is apparently only activated on the ventral side of the embryo, this initial asymmetry is not sufficient to establish axial polarity. As a circulating component of the extracellular fluid surrounding the embryo, Spn27A acts either by controlling the level of active Easter on the ventral side or by preventing diffusion of active Easter toward the dorsal side, thereby ensuring that the Toll ligand is ventrally restricted. In the absence of Spn27A, excess Toll ligand not bound to receptor or active Easter itself spreads toward the dorsal side, ultimately resulting in nearly uniform activation of Toll signaling that ventralizes the embryo (Figure 2A). Conversely, increased Spn27A inhibits activated Easter before it cleaves enough Spätzle precursor, leading to insufficient Toll signaling that dorsalizes the embryo (Supplemental Table S1). These studies reveal that an active mechanism for preventing Toll activation on the dorsal side of the embryo is required to establish embryonic dorsoventral polarity and depends on a critical level of Spn27A. More generally, the role of Spn27A in localizing a serine protease cascade that generates a developmental signal is very analogous to the role of the mammalian plasma serpin antithrombin in confining the blood-clotting cascade to the site of vascular damage (Roemisch et al., 2002). This striking parallel demonstrates how serine protease cascades and serpins are used to exert spatial control in two distinct biological processes that both rely on posttranslational mechanisms.

#### Experimental Procedures

##### Genetics

For a wild-type standard, we used Oregon-R or the *w<sup>1118</sup>* strain (Lindsley and Zimm, 1992). We obtained the *spn27A<sup>1</sup>* (De Gregorio et al., 2002) and the *spn27A<sup>1</sup>; spz<sup>mt7</sup>* stocks from Bruno Lemaitre (Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette), and the deficiency *Df(2L)Bsc7* from the *Drosophila* Stock Center. Because embryos from *spn27A<sup>1</sup>* homozygous females fail to develop to the syncytial blastoderm stage, apparently due to another mutation on the same chromosome, we analyzed embryos produced by *spn27A<sup>1</sup>/Df(2L)Bsc7* females. We used strongly dorsalizing alleles of *easter* (Chasan and Anderson, 1989) and *spätzle* (Morisato and Anderson, 1994) to make the *spn27A<sup>1</sup>/Df(2L)Bsc7; ea<sup>1</sup>/ea<sup>4</sup>* and *spn27A<sup>1</sup>/Df(2L)Bsc7; spz<sup>mt7</sup>/spz<sup>mt7</sup>* double mutants. Embryos lacking detectable *easter* mRNA (*ea<sup>-</sup>*) or Spätzle protein (*spz<sup>-</sup>*) were obtained from *ea<sup>4</sup>/ea<sup>5022rx1</sup>* (Chasan et al., 1992) or *spz<sup>D1-RPQ</sup>/Df(3R)T<sup>84c-RXD</sup>* (Morisato and Anderson, 1994) females. The *eaΔN* line was previously described (Misra et al., 1998).

##### Protein Analysis

For protein expression in transfected S2 cells under control of the metallothionein promoter, we constructed plasmids containing

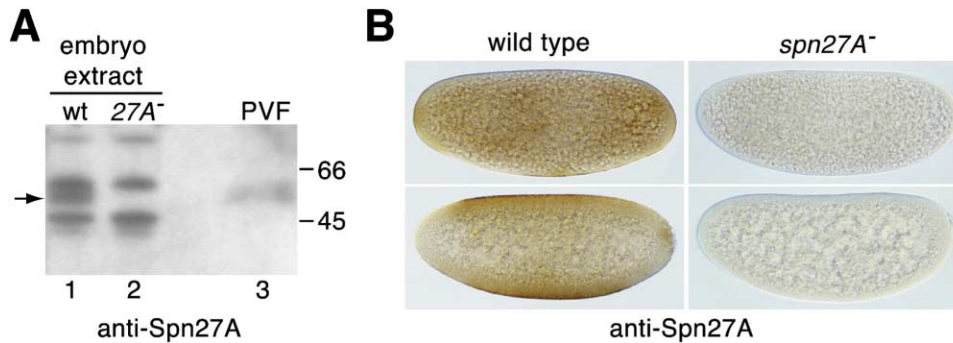


Figure 4. Spn27A Is Distributed Uniformly within the Perivitelline Compartment of the Embryo

(A) Spn27A is detected in the perivitelline fluid extracted from embryos. Extracts of 0–4 hr wild-type (wt; lane 1) and Spn27A-deficient (27A<sup>-</sup>; lane 2) embryos, along with perivitelline fluid extracted from *spz*<sup>-</sup> embryos (PVF; lane 3), were analyzed by immunoblotting with anti-Spn27A antibodies. A 50 kDa polypeptide (arrow) is detected in the wild-type extract and perivitelline fluid but not in the mutant extract. (B) Spn27A is uniformly distributed along the dorsoventral axis of the embryo. Staining by anti-Spn27A antibodies is detected along the entire dorsoventral axis of wild-type embryos at two stages when Toll signaling is known to be active (left panels). Stain is visible at the embryo periphery, but is often weakest at anterior and posterior termini of syncytial blastoderm embryo (bottom left panel). No staining is detected in Spn27A-deficient embryos at comparable stages (right panels).

cDNAs in a variant of the pRmHa-3 vector (LeMosy et al., 2001). The cDNAs for Spn1–Spn5 were isolated in an earlier study (Han et al., 2000), whereas the cDNAs for Spn27A, CG6680, and CG7219 were purchased from Invitrogen. We used PCR to construct the protein-coding region of CG6717 from genomic DNA and to attach a C-terminal Myc tag to Spätzle. All DNAs purchased or generated by PCR were sequenced to confirm the integrity of the protein-coding region. The plasmids for expressing GD, Snake, and Easter, including Easter with the *ea*<sup>5.13</sup> mutation, were described previously (LeMosy et al., 2001; Chang and Morisato, 2002). Transfection was performed as described previously (LeMosy et al., 2001). Typically, 1.5 μg of each plasmid up to 4.5 μg total was added to cells, using vector plasmid to equalize the total amount in parallel samples. Total protein in cell pellet, culture medium, or both combined was analyzed by SDS-PAGE in 12% or 15% polyacrylamide gels and Western blot with anti-Myc (Santa Cruz Biotechnology), anti-Spätzle (Chang and Morisato, 2002), anti-Easter (Chang and Morisato, 2002), or anti-Spn27A (De Gregorio et al., 2002) antibodies.

Extracts of embryos (0–4 hr at room temperature) were prepared and total protein in extracts was analyzed by Western blot essentially as previously described (Morisato and Anderson, 1994). Perivitelline fluid was extracted from *spz*<sup>-</sup> embryos (4–5 hr at 25°C) as described earlier (Stein et al., 1991). The fluid from about 180 embryos was analyzed in the blot shown in Figure 4A.

#### Embryo Analysis

We analyzed 0–4 hr embryos by RNA in situ hybridization (Lehmann and Tautz, 1994) and immunostaining (Patel, 1994) as described. Expression of *zen* and *rho* RNAs was detected with antisense RNA probes, and expression of Twist and Spn27A proteins was detected with antibodies provided by Siegfried Roth (Universität zu Köln) and Paul Brey (Institut Pasteur), respectively. The anti-Spn27A antibodies were “preadsorbed” against an excess (3- to 4-fold) of Spn27A-deficient embryos before being used for immunolocalization analysis. In all RNA in situ hybridization and immunostaining experiments, wild-type and mutant embryos were processed in parallel. Embryo cuticles were prepared as described (Wieschaus and Nüsslein-Volhard, 1986).

#### Embryo Injections

Embryos (0–1 hr at room temperature) were obtained from *spn27A*<sup>1</sup>/*Df(2L)Bsc7* females mated with Oregon-R males. T7 transcription of a cDNA template with the mMessage mMachine kit (Ambion) was used to make *spn27A* RNA, which was quantified by UV spectrophotometry after removal of free nucleotides by spin column chromatography. Culture medium of S2 cells transfected with the plasmid encoding Spn27A, and of mock transfected cells, was concentrated about 5-fold using Centricon centrifugal filters and dialyzed into

PBS. RNA was injected into the cytoplasm of embryos through the intact chorion. Culture medium was injected into the perivitelline space using the method described by Stein et al. (1991). Injected embryos were incubated at 18°C for at least 48 hr before their cuticles were prepared for examination, and the number of larvae hatching from the eggshell was counted.

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