The epidermis serves as a protective barrier in animals. After epidermal injury, barrier repair requires activation of many wound response genes in epidermal cells surrounding wound sites. Two such genes in *Drosophila* encode the enzymes dopa decarboxylase (*Ddc*) and tyrosine hydroxylase (*plee*). In this paper we explore the involvement of the Toll/NF-κB pathway in the localized activation of wound repair genes around epidermal breaks. Robust activation of wound-induced transcription from *plee* and *Ddc* requires Toll pathway components ranging from the extracellular ligand Spätzle to the NF-κB transcription factor. Epistasis experiments indicate a requirement for Spätzle ligand downstream of hydrogen peroxide and protease function, both of which are known activators of wound-induced transcription. The localized activation of Toll in a few cell diameters from wound edges is reminiscent of local activation of Toll in early embryonic ventral hypoderm, consistent with the hypothesis that the dorsal-ventral patterning function of Toll arose from the evolutionary cooption of a morphogen-responsive function in wound repair. Furthermore, the combinatorial activity of Toll and other signaling pathways in activating epidermal barrier repair genes can help explain why developmental activation of the Toll, ERK, or JNK pathways alone fail to activate wound repair loci.

**Significance**

After breaks in animal epidermal barriers, repair genes are activated in the cells adjacent to wound sites that help regenerate the barrier. The fruit fly *Drosophila melanogaster* is a favorable genetic system to find molecular detection systems that sense wounds and activate repair genes. In this paper, we find that the Toll signaling pathway, including the extracellular ligand Spätzle, the Toll receptor, and the NF-κB transcription factor, form a detection system to sense broken epidermis and then activate regeneration genes. The Toll pathway thus is involved not only in the activation of genes involved in fighting microbial invasion after epidermal breaks, but also in the activation of genes that regenerate epidermal barrier function.
Our previous research showed that serine protease function is required and sufficient to activate certain epidermal barrier repair genes after puncture wounding of late stage embryos (20). The activation of epidermal barrier repair genes has been shown to require different combinations of transcription factors (11, 12); however, the entire range of signaling pathways genetically required for proper barrier regeneration is still unknown. We therefore set out to explore the possibility that the protease-activated Toll/NF-κB pathway was required for the activation of epidermal barrier repair genes after wounding.

**Results**

Extracellular Regulation of Epidermal Wound Transcription by the Toll Ligand. We first asked whether the Toll ligand Spätzle (Spz) was required for the transcriptional activation of ple and Ddc wound response genes. We punctured wounded wild-type and spz null mutant embryos, fixed the embryos after an hour of recovery, and then probed for ple and Ddc RNA transcripts. At 1 h after wounding, wild-type late stage embryos showed normal activation of ple mRNA in a zone of 5- to 10-cell diameters around the sites of injury (Fig. 1A). In wounded spz mutants, the zone of ple transcript accumulation around the wounds was dramatically reduced in embryos, although there were always a few cells that showed a ple transcript signal (Fig. 1B). We also tested the function of the ple-WE1 enhancer that drives the expression of the fluorescent protein dsRed in a wound-dependent fashion (11). Note that the reporter expression is not detected until 3–5 h after wounding, because the dsRed protein has to accumulate and then oxidatively mature to the fluorescent state. In wild-type embryos, we detected ple wound reporter expression in a normal zone of 5- to 10-cell diameters from the wound in 90% of embryos (Fig. 1C), whereas 10% showed no detectable expression (n = 450 embryos; see Materials and Methods for an explanation of the variability in these assays from experiment to experiment). In contrast, the ple wound reporter in spz mutants was not activated in 43% of wounded embryos (5–10 cells), whereas the other 57% had ple reporter expression that ranged from weak and scattered (Fig. 1D) to normal levels (n = 120 embryos, Fig. S1).

When testing Ddc transcript induction in spz mutants, we did not detect a consistent difference from wild-type embryos 1 h after wounding at stage 16 (compare Fig. 1E and F). We could not reliably test wound-induced transcription by in situ hybridization at later time points because of the increasing impermeability of the developing cuticle in late embryos. However, when we tested the Ddc 0.47 wound reporter (a 0.47-kb wound enhancer from Ddc driving GFP protein expression) in living spz mutants 5 h after wounding, we did see an effect from loss of spz function. In wild type, 95% of embryos showed normal Ddc wound reporter activation 5- to 10-cell diameters from the wound site (Fig. 1G). In contrast, 71% of spz mutants showed no Ddc-GFP expression after wounding, and 29% showed expression that ranged from a narrow zone (1–3 cells) around wound sites (Fig. 1H) to expression that was indistinguishable from wild type (Fig. S1) (n = 200 embryos).

These experiments reveal a quantitative requirement for Spz in the activation of reporters driven by ple and Ddc wound-dependent enhancer elements. The above results also suggest that either the temporal requirement for Spz on the maintenance of ple and Ddc wound-induced transcription are different or that the Ddc 0.47 wound enhancer is more sensitive to the loss of Spz function than the endogenous Ddc transcription unit in its normal chromosomal location.

We next tested whether active, cleaved Spz protein was sufficient to induce the activation of the ple and Ddc fluorescent wound reporters. The Easter serine protease can cleave Spz in vitro, yielding a C-terminal fragment of 106 amino acids (Spz-C106) that induces Toll signaling (21). We filled micropipillary needles with Spz-C106 in PBS and injected embryos carrying the ple or Ddc wound reporters. Control embryos, which were injected with PBS alone, showed normal localized activation of the reporters around wound sites (Fig. 1 C and G). However, embryos

![Image](https://example.com/image.png)

Fig. 1. Spätzle is required and sufficient for wound gene activation. (A) RNA FISH with a ple coding region probe on a wild-type stage 16 embryo 1 h after puncture wounding. ple transcripts are observed in a zone of 5–10 cells around the wound site. (B) The ple probe was hybridized and visualized in a stage 16 spz mutant embryo. (C) This embryo, punctured and injected with a buffered saline solution (PBS) at stage 16, and imaged 5 h after wounding, carries the wound reporter ple-WE1, consisting of a small DNA enhancer from the ple upstream region fused to a gene for the fluorescent protein dsRed, which is fused to a nuclear localization signal (NLS) (11). (D) ple-WE1 expression in spz mutant embryos 5 h postwounding. (E) FISH using a Ddc coding region probe shows Ddc transcripts are activated in a zone of 5–10 epidermal cells surrounding the wound site at 1 h postwounding. Developmental transcription of Ddc in differentiating cells of the head skeleton (Left, anterior end of embryo in E can also be seen). (F) Wound-induced Ddc RNA transcripts are detected in a normal zone of 5–10 epidermal cells around wounds in a spz mutant embryo 1 h after wounding at stage 16. (G) The Ddc 0.47 GFP wound reporter (12) expression pattern 5 h after wounding of a stage 16 wild type embryo. (H) Ddc 0.47 expression 5 h postwounding in stage 16 spz mutant embryos. (I and J) Wounding plus injection of stage 16 embryos with the constitutively active ligand Spz-C106 results in a global activation of the ple-WE1 and the Ddc-0.47 wound reporters in epidermal cells 5 h postwounding. White arrows mark wound sites. In every panel, dashed white lines outline the embryos, and anterior is to the left. The ple-WE1 wound reporter exhibits wound-independent activation in the developing anal pad in late stage embryos, as seen in the posterior (Right) end. The individual dots of fluorescent signal in the image are nuclei of cells expressing the fluorescent protein.
injected with the Spz-C106 ligand showed a global activation of the \( Ddc \) and \( ple \) wound reporters in epidermal cells (Fig. 1 F and J). This activation of wound reporters did not require a breach of the epidermis, as injection of Spz-C106 into the perivitelline space on the apical side of epidermal cells also showed global activation of the \( Ddc \) and \( ple \) wound reporters (Fig. S2 B and D for dye entry controls). These results indicate that Spz, a signal apparently induced by tissue damage, is both quantitatively required and sufficient (at least when ectopically applied at high levels) to activate the \( ple \)-\( WE1 \) and \( Ddc \) 0.47 wound gene activation elements.

**Spätzle Acts Downstream of the Wound Gene Activators Hydrogen Peroxide, Trypsin, and Calcium Influx.** Some diffusible wound signals are evolutionarily conserved between vertebrates and *Drosophila*. For example, in both zebrafish and *Drosophila* the activation of Dual oxidase (Duox) upon wounding establishes a hydrogen peroxide (\( H_2O_2 \)) gradient that attracts blood cells to wound sites (22–24). In *Drosophila* the Duox gene is also required for activation of wound response genes in epidermal cells (25). Our previous studies have shown that Duox and hydrogen peroxide act upstream of serine protease(s) for the wound-dependent activation of the \( ple \) and \( Ddc \) genes (26).

To determine the hierarchical relationships of \( spz \), serine protease function, and hydrogen peroxide in the activation of epidermal wound response genes, we performed a series of epistasis experiments. After puncture wounding of Duox mutant embryos, \( ple \)-\( WE1 \) wound reporter expression is either barely or not detected (Fig. 24) (25). However, all Duox mutants that were punctured and injected with the Spz-C106 ligand (either into the body cavity or the perivitelline space) showed activation of the \( ple \) wound reporter in all or nearly all epidermal cells (Fig. 2B).

Wild-type embryos injected with a hydrogen peroxide solution into the body cavity or perivitelline space showed activation of the \( ple \) wound reporter in all or nearly all epidermal cells (Fig. 2C) (25). In contrast, all \( spz \) mutant embryos that were punctured and injected with a hydrogen peroxide solution into the perivitelline space showed extremely weak \( ple \) reporter expression either locally or globally in epidermal cells (Fig. 2D). These results suggest that \( spz \) functions downstream of hydrogen peroxide within the epidermal wound response pathway.

Previous developmental and innate immune studies have shown that pro-Spz has to be cleaved by extracellular proteases before it can bind and activate Toll receptor. We have not identified the endogenous protease that activates pro-Spz in the context of wound gene activation, so we tested the epistatic relationship of protease activation and \( spz \) by treating \( spz \) mutant embryos with a concentration of trypsin in the perivitelline space that is sufficient to activate wound repair genes without breaching the epidermal barrier (20).

In wild-type embryos, trypsin treatment globally activated the \( ple \) wound reporter 85% of the time (Fig. 2E), with 15% showing low or no reporter expression (\( n = 113 \)). In contrast, only 29% of the \( spz \) homozygous mutants treated with trypsin globally activated the \( ple \) wound reporter, whereas 71% of the mutants (\( n = 48 \)) showed no \( ple \) wound reporter expression (Fig. 2F and Fig. S3). For the \( Ddc \) wound reporter activation treated with trypsin, 95% of wild-type embryos showed global activation (\( n = 200 \)), whereas 70% of \( spz \) mutants showed no activation of the \( Ddc \) reporter (\( n = 60 \)) (Fig. S3). These results suggest that trypsin activates barrier repair genes either by cleaving pro-Spz directly or by cleaving another protease upstream of Spz activation. Previous studies have shown that calcium ion influx occurs rapidly around laser wound sites and triggers a Duox-dependent local inflammatory response (24). As seen in Razzell et al. (24), we also find a rapid calcium ion intracellular “flash” around puncture wound sites, and in addition observe that injection of ionomycin (a calcium ionophore) into either the body cavity or the perivitelline space triggers widespread calcium influx into epidermal cells, activating GCaMP6 driven by an *engrailed* promoter that produces striped expression in epidermal cells (Fig. 2B).
G and H). Ionomycin injection into the perivitelline space onto the apical side of epidermal cells induces ple wound reporter activation broadly in 67% of wild-type embryos (n = 40) (Fig. 2I and Fig. S3). Neither local nor extended ple wound reporter activation was observed in any of the spz mutant embryos treated with perivitelline injections of ionomycin (n = 40) (Fig. 2F). The Ddc wound reporter was also activated by perivitelline injection of ionomycin in wild-type embryos, and ionomycin did not activate the Ddc reporter in spz mutants (Figs. S2 C and D and S3). These results are consistent with calcium ion influx acting upstream of the Toll pathway in the activation of wound transcription in the epidermis.

The Toll Receptor Regulates Epidermal Wound Response Gene Activation. Having found that spz function is required to activate wound-induced gene expression, we next tested whether Toll receptor function was required to transduce the spz signal in epidermal wound gene activation. In wild-type embryos, 89% activated the ple-WE1 reporter around wound sites (n = 260) (Fig. 3A). There was no detectable reporter expression in 60% of the Toll mutant embryos, and in the remaining 40%, ple reporter expression ranged from a few cells around a wound site (Fig. 3B) to a normal amount of wound reporter expression (n = 52, Fig. S4). This result suggests that the Toll receptor is required for robust transcriptional activation of the ple gene during the barrier repair process. However, the fact that elimination of Toll function does not block all Ddc and ple reporter expression is consistent with a combinatorial and quantitative contribution in the activation of these barrier regeneration genes.

We next tested whether Toll activity was sufficient for ple and Ddc wound reporter activation. Toll receptor is normally expressed in the late embryonic epidermis at modest levels (27), and in unwounded zygotic mutants carrying the Toll106 mutation under the control of its endogenous promoter, we did not detect global activation of wound reporter expression in unwounded embryos. The Toll106 allele encodes a mutant version of Toll protein that activates the pathway without input from the Spz ligand (28). High-level expression of Toll proteins driven by the engrailed-GAL4 driver resulted in the activation of both the ple and Ddc wound reporters in epidermal stripes correlating with the localized activity of the engrailed driver (Fig. 3 C and D). This result indicates constitutively activated Toll is sufficient to activate both of these wound response genes when expressed at sufficiently high levels. Taking into account all of the above, we conclude Toll signaling has a quantitative effect on the activation of wound response genes and must require other signaling pathways to robustly activate wound response gene expression.

Intracellular Components of the Toll Signaling Pathway Regulate ple Wound Gene Transcription. To further investigate innate immune regulation of epidermal wound gene activation, we next focused on the analysis of Toll/NF-κB pathway proteins acting downstream of the Toll receptor. To determine their effect on ple-WE1 wound reporter, we wounded embryos homozygous mutant for null mutations in either tube or Myd88, both of which encode intracellular adaptor proteins (29). We also wounded embryos in mutants for dorsal (dl) and Dif, which encode NF-κB family transcription factors.

In wounded controls, 89% of wild-type embryos (n = 260) had normal ple reporter expression around the wound, and 11% had no detectable reporter activity (Fig. 4A). In wounded mutants, 78% had no ple wound reporter activation (Fig. 4C), whereas 22% activated ple reporter in a zone from a few cells to a normal range (n = 270, Fig. S4). Wounded embryos that were mutant for dorsal had ple reporter activity around wound sites that was indistinguishable from wounded controls (85% positive, n = 200) (Fig. 4D and Fig. S4). In Dif mutants, we tested the wound induction of Ddc and ple transcripts. In the wild-type controls, 85% (n = 118) had normal wound-induced transcription of ple 5- to 10-cell diameters from the wound edge (Fig. 4E). In Dif mutant embryos, 55% had no detectable ple transcripts around wound sites, whereas 45% showed expression ranging from one to two rows of ple gene activation around wound edges (Fig. 4F) to a normal zone of 5–10 cells (Fig. S4).

In wild-type embryos, 88% of wild-type embryos (n = 135) showed normal wound-induced Ddc transcription (Figs. S2E and S4). In contrast, 50% of punctured Dif mutants showed no activation of Ddc around wound sites, whereas the remaining 47% showed expression ranging from one to two rows from the wound edge (Fig. S2F) to normal Ddc induction at wound sites (Fig. S4). Cumulatively, these results indicate that intracellular Toll signaling, acting through Dif, is required for robust activation of the wound-induced ple reporter in epidermal cells.

Discussion

Both septic and clean wounding treatments result in the transcriptional activation of barrier repair and innate immunity genes in Drosophila epithelia (20, 30, 31). In this paper, we have shown that numerous signaling components in the Toll pathway, from the extracellular Spz ligand to the Dif transcription factor in the nucleus, are required to robustly induce expression of the Ddc and ple epidermal barrier repair genes after wounding late stage embryos. In addition, overexpression of constitutively activated forms of Toll receptor and injection of the activated Spz-C110 ligand are sufficient to induce wound repair reporter genes in undamaged epidermis. These results establish Spz as an epidermal wound response ligand. We found that in the hierarchy of wound-induced signals, the Spz ligand appears to act downstream of Duox and hydrogen peroxide, two previously identified activators of the epidermal wound response pathway (25).
different signals that activate Toll receptor signaling in epidermal cells near the wound edge. Dual oxidase function, which we find is required for the activation of some barrier repair genes in a Spz-dependent fashion, is also required for the attraction of hemocytes to laser-induced wounds in the late embryonic epidermis (24). How the signals activating this inflammatory response are integrated with the signals for epidermal wound closure and activation of barrier repair genes is as yet unknown, but our results that many of the signals to activate these wound-induced processes are shared, and integrated control of these processes will be a fascinating subject for future studies.

Other pathways besides Toll are crucially important for epidermal wound gene activation (Fig. 5). One pathway operates via the Stitcher Receptor Tyrosine Kinase through the downstream effectors DRK, Src42A, and ERK to the Grainty head transcription factor (12, 35, 36). DNA binding sites for Grainty head are commonly found in, and are required for the function of some wound-activated epidermal repair genes, in *Drosophila* and mammals (11, 12, 14). AP-1 sequence motifs are also required for wound-induced gene expression of *Dde* and *ple*, although it is still unclear in *Drosophila* embryos whether the JNK pathway acts upstream of the AP-1 (Jun/Fos DNA binding) sites in this context (11, 12). There are many other epidermal wound-induced genes for which the JNK pathway is unquestionably crucial (6, 7, 9, 10, 36). It seems logical that many of the scaffolding pathway interactions generate the specificity to regulate wound-induced genes, because it would be catastrophic to activate the entire suite of wound repair genes whenever the individual pathways involving ERK, JNK, or Toll are activated (Fig. 5). Relevant to the model in Fig. 5, a recent study showed that Src42A can be directly activated by a wound-induced peroxide burst, leading to the recruitment of hemocytes to a wound site (37). Our results show that peroxide injected into *spz* mutant embryos fails to activate wound reporter genes. This finding suggests to us that peroxide is not sufficient to independently activate the Src42A protein in the context of wound gene activation, although it may have a quantitative role.

Early innate immune systems evolved to recognize potential pathogens by conserved molecular signatures such as peptidoglycans of Gram-positive bacteria and lipopolysaccharides of Gram-negative bacteria. Collectively these are called pathogen-associated molecular patterns (PAMPs) (38). Concomitant with the cellular invasion or damage triggered by pathogens bearing PAMPs there are also “self” molecules that are released by damaged host cells. These signals have been called damage-associated molecular patterns (DAMPs) (39). In the danger model, proposed by Matzinger (40), optimal activation of the immune system requires both damage signals and nonself signals from pathogens (40–42). This model thus nicely aligns with the data we present here to provide an explanation as to why the epidermal wound response and innate immune signaling pathways are both activated in response to barrier damage, even in the absence of microbes.

In mammals, there is evidence that alarm or DAMP signals include heat shock proteins, extracellular matrix components (hyaluronic acid), and a chromatin-associated protein HMGB1 (43). Although the signaling pathways elicited by DAMPs are not fully defined, there is evidence that their damage signaling occurs via receptors such as the multiligand receptor for advanced glycation end products (RAGE) receptors, and Toll-like receptors (43). The common feature of these pattern recognition receptors is their multivalency, which may be achieved by having multiple receptor isoforms, or different receptor paralogs. Although not traditionally put in the DAMP category, a local increase in hydroxyperoxide can be a direct or indirect signature of pathogen-induced or wound-induced damage. Immunocompetent cells are known to use reactive oxygen species to incapacitate invaders (44), and reactive oxygen species like hydroxyperoxide can signal surrounding tissues of a nearby wound (22, 23).

In mammals, Toll-like receptors in epidermal cells can be activated by the direct binding of individual PAMPs or DAMPs (45, 46).
However, in *Drosophila*, the Toll receptor indirectly senses PAMPs by their ability to activate protease cascades that produce the Spz-106 ligand (47). It seems that signaling through the Toll receptor of *Drosophila* and probably other insects is a stripped down model of PAMP and DAMP recognition, with Toll receptor functioning as a secondary receptor hub. Acting in this way, the PAMPs recognized indirectly by Toll receptor can integrate inputs coming from different sources. In addition, and in parallel, Spz can serve as a proteolytic activity sensor that can be activated by proteases secreted by microbes (48, 49), or as shown here, by endogenous protease activities that are activated by tissue damage.

The protease(s) that activates pro-Spz in the activation of epidermal wound repair genes is as yet unknown. There are two serine proteases encoded by the genes, *persphone* (*psb*) and *ModSP* genes, that function immediately upstream of Spz activation in the context of the innate immune response, and another Spz activating protease, encoded by the *easter* (*ea*) gene, that, is required for ventral patterning in early embryos (50). We tested individual mutants in these three genes and observed no consistent changes in the wound-induced activation of the *ple* and *Ddc* genes. However, some combination of these three proteases might be involved in cleaving pro-Spz during epidermal wound gene activation.

**Materials and Methods**

*Drosophila* Stocks. The wild-type strain used was w1118. *Ddc 0.47* and *ple-WE1* were previously described (11, 12) and we refer to *Ddc 0.47* and *ple-WE1* as the *Ddc* and *ple* wound reporter lines in this paper. The following mutant lines: *ea1*, *ModSP*14511, *spz4*, *dl1*, *Duox4G07745*, *DiF*, *DiF*, *en-GAL4*, and green fluorescent balancers *CKG*, *TKG*, and *TM3 Ser GFP*, were obtained from the Bloomington Stock Center. The following lines were kindly provided as follows: *psb4* from J. M. Reichhart, Institut for Molecular and Cellular Biology, Strasbourg, France; *tube* from Steven Wasserman, University of California, San Diego, La Jolla, CA; *GCaMP6* from Chih-Ying Su, University of California, San Diego, La Jolla, CA; and the *Df(2L)4* (*Dif dl deficiency line) from Tony Ip, University of Massachusetts Medical School, Worcester, MA. Mutant flies were crossed to *Ddc 0.47* or *ple-WE1* wound reporter lines and balanced with chromosomes expressing GFP.

**Wounding and Microinjections.** *Drosophila* embryos were puncture wounded as described in Juarez et al. (51). The embryonic stage of wounding was from stages 15–16, and live imaging of wound reporters was done 5 h later. Fixed and stained images of whole mount in situ were done 1 h after wounding. All fluorescent images were collected using either a Leica SP2 or SP5 laser-scanning confocal microscope, with instrument settings at nonsaturated gain levels for both experimental and control samples, although to obtain visible photomicrographs, the images for experimental results were taken at 1.3–2.0, the gain-of-control images. Optical sections were scanned at 1-μm thicknesses, and maximum projection images are shown.

Embryonic puncture wounding, injection into the body cavity, and (especially) injection into the perivitelline space are manually demanding operations whose success varies based on the level of experience and the level of meticulous attention to detail shown by different experimenters. This variation explains most of the variation in frequency of gene or reporter activation reported in different experiments, and so the quantitation of control and experimental reporter or gene activation that we report are those obtained by the same experimenter on the same days. Although many mutants have a large proportion of embryos that show no expression, we think it best to show images of those with visible, albeit weak, wound-induced RNA or reporter expression. Even so, we usually have to boost the camera gain for mutants about 1.5- to 2-fold compared with wild type to see the weak expression we show in this paper on photomicrographs. Regarding quantitation, in the mutant backgrounds where there was a significant diminution of wound gene expression after puncture wounding, only two classes could be honestly called: class A, no expression and class B, variable expression that ranged about 1.5- to 2-fold. Optical intensity between a few cells near the wound edge to normal appearing expression. Even so, we usually have to boost the camera gain for mutants about 1.5- to 2-fold compared with wild type to see the weak expression we show in this paper on photomicrographs. Regarding quantitation, in the mutant backgrounds where there was a significant diminution of wound gene expression after puncture wounding, only two classes could be honestly called: class A, no expression and class B, variable expression that ranged about 1.5- to 2-fold. Optical intensity between a few cells near the wound edge to normal appearing expression. This is what we observed, so that is what we report in the text and graphs. In wounded wild-type embryos, we observe either normal expression of wound genes 5- to 10-cell diameters from the wound edge (the radius varies somewhat based on the size of the wound) or no expression (which we believe is due to embryonic death immediately after wounding). Almost all embryos survive puncture wounding under oil and go on to develop into first instar larvae. Trypsin and hydrogen peroxide microinjection details are reported in.
Juarez et al. (25) and Patterson et al. (20). Spz-C106 protein and the calcium ionophore, ionomycin, were injected in a similar fashion. For injections, tryptophan at 50 μM concentration, hydrogen peroxide at 2% (v/vol), ionomycin at 1 μM (from DMSO stock), and Spz-C106 protein at 50 μg/mL in 1× PBS. Embryos carrying the GCaMP6 fluorescent indicator (52), which allowed the detection of cytoplasmic influxes of intracellular calcium ion after wounding or ionophore treatment, were imaged 5 min after injection/wounding. A calcium ion-induced fluorescent signal was visible at 2 min after wounding, the earliest time we could image, and it faded to background levels by 50 min. Spz-C106 protein was kindly provided by Rob DeLotto, Rutgers University, Picataway, NJ, and is described in Delotto and DeLotto (21).

Fluorescent in Situ Hybridization. Dac and ple RNA probes were generated from partial or full-length cDNA obtained from the Drosophila Genomics Resource Center. Probe labeling, hybridization, and detection protocols were as described in Kosman et al. (53). RNA probes were labeled with digoxigenin (DIG)-conjugated ribonucleotides, and anti-DIG coupled to tyramide were used to detect transcripts in situ. Stained embryos were mounted in DTG (2.5% (v/vol) DABCO-Sigma W-2522, 50 mM Tris (pH 8.0), 90% (vol/vol) glycerol) and imaged with a Leica SPS confocal microscope as described above.

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