Current Biology 22, 1013–1018, June 5, 2012 ©2012 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2012.03.065

Report

# A Ventrally Localized Protease in the *Drosophila* Egg Controls Embryo Dorsoventral Polarity

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## Summary

**_Drosophila_ embryo dorsoventral (DV) polarity is defined by serine protease activity in the perivitelline space (PVS) between the embryonic membrane and the inner layer of the eggshell [1–5]. Gastrulation Defective (GD) cleaves and activates Snake (Snk). Activated Snk cleaves and activates Easter (Ea), exclusively on the ventral side of the embryo [6–8]. Activated Ea then processes Spätzle (Spz) into the activating ligand for Toll, a transmembrane receptor that is distributed throughout the embryonic plasma membrane [9]. Ventral activation of Toll depends upon the activity of the Pipe sulfotransferase in the ventral region of the follicular epithelium that surrounds the developing oocyte [10]. Pipe transfers sulfate residues to several protein components of the inner vitelline membrane layer of the eggshell [11]. Here we show that GD protein becomes localized in the ventral PVS in a Pipe-dependent process. Moreover, ventrally concentrated GD acts to promote the cleavage of Ea by Snk through an extracatalytic mechanism that is distinct from GD's proteolytic activation of Snk. Together, these observations illuminate the mechanism through which spatially restricted sulfotransferase activity in the developing egg chamber leads to localization of serine protease activity and ultimately to spatially specific activation of the Toll receptor in the _Drosophila_ embryo.**

## Results and Discussion

Mutations affecting the serine protease Gastrulation Defective (GD) have been characterized according to their molecular lesions [12–14] (Figure 1A). Ponomareff et al. [14] additionally carried out a careful complementation analysis of available *gastrulation defective* (*gd*) mutant alleles, which demonstrated the existence of two distinct classes of alleles capable of complementing one another. Missense mutations within the protease domain constitute the $gd^{10}$ class, whereas the $gd^{2}$ class comprises missense mutations located near the amino terminal end of the protein [14]. Although females homozygous for strong $gd^{2}$ or $gd^{10}$ class alleles produce completely dorsalized embryos, embryos from $gd^{2}/gd^{10}$ class transheterozygotes exhibit normally polarized lateral and ventral pattern elements. The ability of $gd^{2}$ class mutations to complement $gd^{10}$ class mutations suggests that the $gd^{2}$ class-encoded proteins retain the ability to process Snake (Snk). To test this, we examined the processing of a GFP-tagged version of Snk (Snk-GFP) [7] in the progeny of either wild-type (WT) females or females mutant for $gd^{2}$, $gd^{10}$, or $gd^{VM90}$ (protein null, noncomplementing allele) (Figure 1B). Consistent with previous observations [7], embryos maternally mutant for $gd^{VM90}$ exhibited a low level of processed Snk-GFP whose formation does not depend upon GD protease activity. As expected, a similarly low level of processed Snk-GFP was observed in embryos from females carrying the protease-deficient $gd^{10}$ allele. In contrast, embryos maternally mutant for $gd^{2}$ exhibited a higher level of Snk-GFP processing that was comparable to the level observed in WT embryos. Thus, the $gd^{2}$-encoded protein is capable of processing Snk, raising the question of which cleavage step in the protease cascade is affected in this mutant background. We therefore examined the processing of a GFP-tagged version of Ea (Ea-GFP) [7], which failed to undergo cleavage in all three mutant backgrounds (Figure 1C). The finding that Ea is not cleaved in $gd^{2}$ maternal mutants, even though Snk is processed, suggests that in addition to processing Snk, WT GD functions to facilitate the processing of Ea by activated Snk. It is this second, extracatalytic function of GD that is disrupted by $gd^{2}$ class mutations.

We next asked whether increasing the concentration of GD molecules that retain the extracatalytic function would increase Ea processing. We moderately overexpressed in WT females transgenes encoding WT GD, or two catalytically inactive versions of GD, GD[D347N] and GD[S468A], which each carry a mutation in one of the essential amino acids in the catalytic triad that makes up the active site of the protein [5, 15]. All three led to increases in processed Ea-GFP (Figure 2B) and Spätzle (Spz)-GFP [7] (Figure 2C), indicating that GD proteins that lack protease activity can nevertheless promote Ea processing. All three transgenes were also capable of ventralizing the progeny of WT females (Figures 2D through 2F). Most of the cuticles produced by these embryos were completely encircled by ventral denticle belts. Skeletal elements of the larval head, and the dorsolaterally derived Filzkörper material, were absent from these embryos. These results indicate that the extracatalytic function does not require the presence of an active protease in the same GD molecule, provided there is another source of GD protease present in embryos to cleave and activate Snk. In contrast, overexpression of the mutant GD[2] protein, or of two preprocessed versions of GD, GDΔN211 and GDΔN253, in which a signal peptide is fused directly to amino acid 212 or 254 of the protein [5], failed to increase the levels of Ea-GFP or Spz-GFP processing (Figures 2B and 2C) or to ventralize the embryonic phenotype (Figures 2G, 2H, and 2I). Thus, the ability to increase Ea processing in a concentration-dependent manner requires the presence of sequences within the N-terminal domain. Finally, in no case did overexpression of the tested GD transgenes lead to an increase in the levels of processed Snk-GFP above that present in WT embryos (Figure 2A), indicating that the effects on Ea processing were not achieved through increased levels of active Snk protease.

Cleavage of Ea is dependent upon the function of the Pipe sulfotransferase and is the first step in the protease cascade to be localized to the ventral side of the egg [6–8]. Previous

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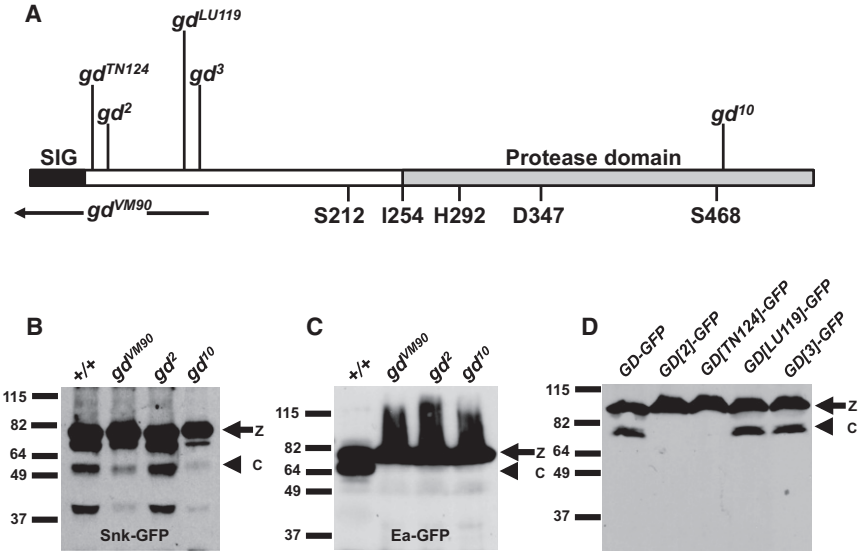


Figure 1. $gd^2$ Class Mutations Illuminate a Role for GD in Facilitating the Cleavage of Ea by Activated Snk

(A) Structure of the GD protein showing the positions of the N-terminal $gd^2$ class mutations $gd^{TN124}$, $gd^2$, $gd^{LU119}$, and $gd^3$, and the $gd^{10}$ class mutation $gd^{10}$ in the protease domain [14]. Genomic coding sequences missing from the noncomplementing null mutation $gd^{VM90}$ are indicated by the black bar. The $gd^{VM90}$ mutation results from a 717 base pair deletion that overlaps the 5′ end of the gene [14] and is likely to represent an RNA null allele (see Experimental Procedures). Also indicated are the positions of the GD signal peptide (SIG), the histidine (H292), aspartic acid (D347), and serine (S468) residues that comprise the active site of the catalytic region and the amino acid residues to which the Ea signal peptide has been fused (S212 and I254) in the GDΔN211 and GDΔN253 constructs.

(B–D) Western blot analyses of embryonic extracts. Processing of Snk-GFP (B) and Ea-GFP (C) in embryos from females bearing the *gd* allele denoted above each lane carried in *trans* to $gd^{VM90}$ is shown.

(D) Processing of GD-GFP and of mutant variants bearing the $gd^2$ class mutations shown above each lane. Z, zymogen; c, processed protease-bearing fragment.

work suggested that Pipe-sulfated, ventrally localized eggshell proteins contribute to the ventral processing of Ea by Snk [10, 11]. The observation that GD plays a role in Snk-mediated Ea processing raised the possibility that an interaction between GD and the sulfated cue in the ventral eggshell cue enables GD to bring about a productive encounter between Snk and Ea. Previous attempts to detect ventral enrichment of transgenically expressed GD-GFP in the perivitelline space (PVS) were hampered by high levels of fluorescence in the embryonic secretory pathway and throughout the PVS (Figure 3A) [16]. To eliminate this problem, we instead transplanted perivitelline fluid (PVF)

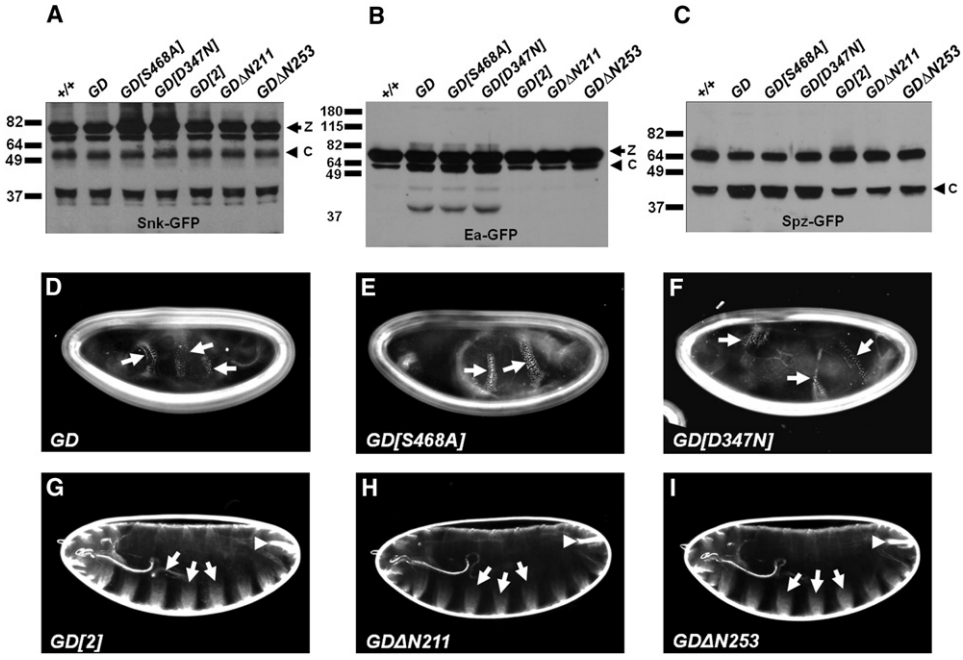


Figure 2. Proteolytically Inactive Versions of GD Illuminate a Role for the Full-Length Protein in Facilitating Processing of Ea by Snk

(A–C) Western blot analyses to examine the processing of Snk-GFP, Ea-GFP, or Spz-GFP in embryos also containing the modified versions of GD denoted above each lane. All constructs were expressed under the control of the germline-specific driver pCOG-Gal4:VP16.

(D–I) Cuticles of larval progeny of females expressing the indicated GD transgenes under the control of pCOG-Gal4:VP16. Arrows show ventral denticles or ventral denticle material; arrowheads indicate Filzkörper, a dorsolateral structure.

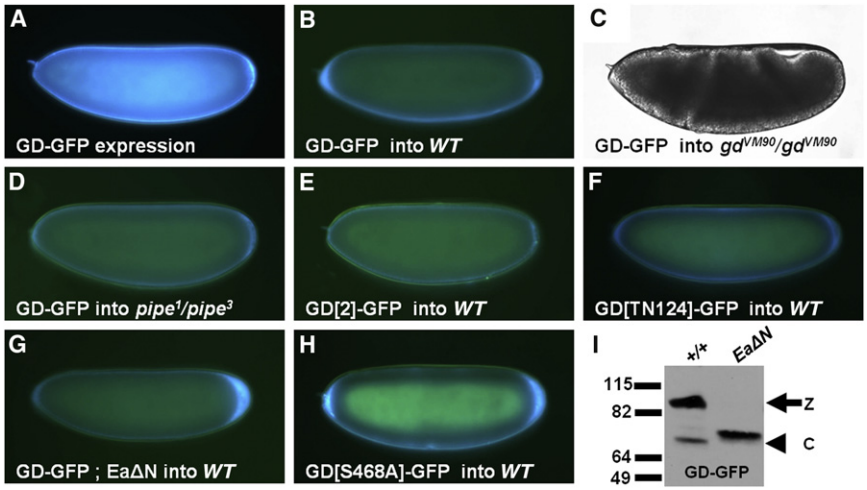


Figure 3. GD-GFP Undergoes Ventral Enrichment in the Egg PVS through a Pipe-Dependent Mechanism

(A) Embryo from a GD-GFP-expressing female. GFP signal appears blue in this and all subsequent panels displaying GFP distributions.

(B–H) Recipient embryos of the denoted maternal genotypes injected with PVF containing the indicated versions of GD-GFP.

(I) Western blot of GD-GFP processing in the embryos from WT ($^{+/+}$) and EaΔN-expressing females.

from GD-GFP-expressing embryos into the PVS of WT, non-expressing cleavage stage embryos. Regardless of the site of injection, after 1–2 hr of incubation, recipient embryos exhibited a conspicuous accumulation of fluorescence in the ventral PVS (Figure 3B). Moreover, injection of PVF containing GD-GFP into the progeny of *gd* mutant females led to phenotypic rescue of polarized gastrulation movements (Figure 3C) and the differentiation of ventral and lateral cuticular pattern elements (data not shown). Strikingly, no ventral enrichment was observed following transplantation of PVF containing GD-GFP into embryos derived from *pipe* mutant mothers. Instead, the injected protein became uniformly distributed throughout the PVS (Figure 3D). Thus, ventral enrichment of GD-GFP requires the Pipe-sulfated ventral cue. Importantly, no ventral enrichment was observed following injection into WT embryos of PVF containing GFP-tagged proteins bearing any of the four $gd^2$ class mutations ($gd^2$, $gd^{TN124}$, $gd^3$, or $gd^{LU119}$) (Figures 3E and 3F; data not shown), which lack GD's extracatalytic function. These data strongly support the hypothesis that interaction of GD with the ventral cue is essential for its ability to facilitate processing of Ea by Snk.

The proposed extracatalytic function of GD in promoting cleavage of Ea by Snk predicts that GD is present in a complex with Ea and Snk. To test this, we carried out coimmunoprecipitation (coIP) studies and observed that GFP-tagged GD coimmunoprecipitated with both Snk-hemagglutinin (HA) (Figure 4A) and Ea-HA (Figure 4B). Thus, GD is present in complexes with both downstream proteases. Interestingly, GD[2]-GFP also coimmunoprecipitated with both Snk-HA and Ea-HA (Figures 4A and 4B), suggesting that it is not the interaction of GD with Snk and Ea that requires the ventral cue but rather the productive catalytic interaction of Snk and Ea with each other.

The results described above indicate that sequences in its N-terminal domain are required for ventral localization and the extracatalytic function of GD. Like the other serine proteases in this pathway, GD undergoes processing. GD zymogen is cleaved in a process that is dependent upon the Nudel serine protease [5, 7] to generate a 44–48 kD C-terminal fragment that has been considered to be the active form of GD [4, 5]. We observed processing of GD-GFP from 90 kD to 73 kD, but no cleavage of GD[2]-GFP or GD[TN124]-GFP was detected (Figure 1D). Because our experiments previously demonstrated that Snk-GFP was cleaved normally in embryos from $gd^2$ mothers (Figure 1B), this surprising result indicates that GD does not require processing to activate its proteolytic function. This finding raised the intriguing possibility that the cleavage event that GD normally undergoes is instead required for it to undergo ventral localization and exert its extracatalytic function. To test this possibility, we examined the processing and localization of GD-GFP coexpressed with EaΔN, a constitutively active version of Ea [17] that cleaves GD at an ectopic site when they are coexpressed in tissue culture cells [4, 5]. When we coexpressed GD-GFP with EaΔN in *Drosophila* embryos, almost all of the GD-GFP zymogen was processed to a fragment that was slightly larger than that seen when GD-GFP was expressed alone (Figure 3I). When PVF from these embryos was transplanted to the PVS of WT embryos, no ventral enrichment of fluorescence was observed in the injected embryos (Figure 3G). This observation is consistent with the hypothesis that processing of GD at a specific location is a prerequisite for its ventral localization, perhaps to expose determinants that interact with the ventral cue. In contrast, GFP-tagged versions of proteins bearing the two other $gd^2$ class mutations, $gd^3$ and $gd^{LU119}$, did exhibit processed forms (Figure 1D), although they did not undergo ventral localization (data not shown). The exact site at which GD is cleaved is not known, but the size of the processed fragment is consistent with cleavage taking place between the amino acids affected by the $gd^2$ and $gd^{LU119}$ mutations (see Figure 1A). Taken together, these results suggest that the amino acids affected in $gd^2$ and $gd^{TN124}$ are required for normal processing of GD and that cleavage of GD produces a molecule that interacts with the ventral cue through determinants that are disrupted by the $gd^3$ and $gd^{LU119}$ mutations. Notably, GD's own proteolytic activity does not play a role in this process. GD[S468A]-GFP, a catalytically inactive version of GD-GFP in which the active site serine was converted to alanine, exhibited normal ventral enrichment when PVF containing this protein was introduced into the PVS of WT embryos (Figure 3H).

Our results demonstrate that GD provides two essential functions in the dorsoventral (DV) pathway: activation of the Snk protease and ventrally localized Ea cleavage. These findings support a model in which processing of GD generates a fragment with a C-terminal protease domain and N-terminal sequences that interact with the sulfated ventral cue to localize GD to the ventral region of the PVS (Figure 4C). GD may bind directly to carbohydrates associated with vitelline membrane protein that have been sulfated as a result of Pipe enzymatic action in ventral cells of the follicle layer. Consistent with this possibility, GD protein has been shown to bind to heparin

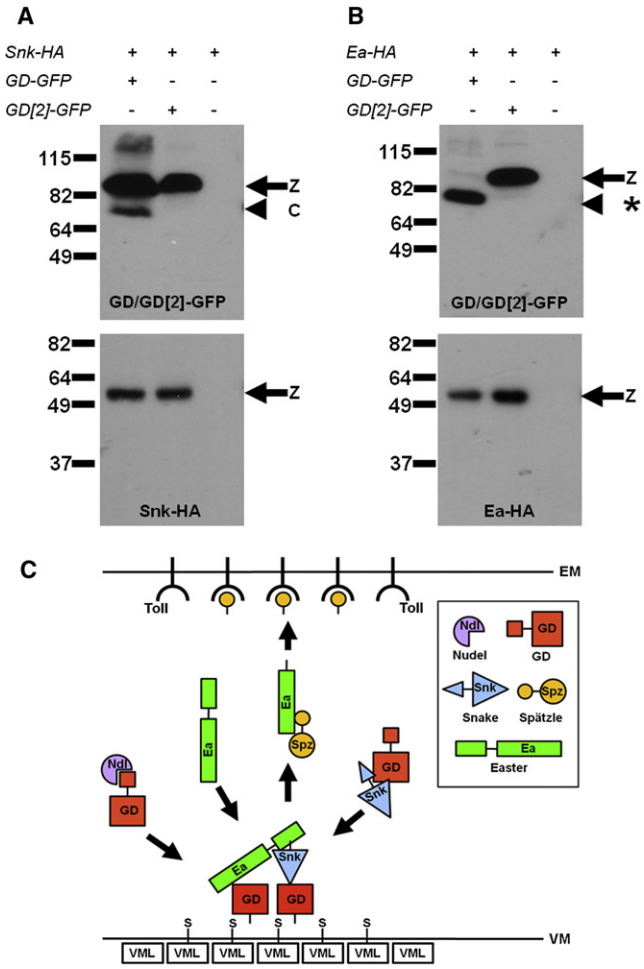


Figure 4. GD Complexes with Both Ea and Snk

(A and B) Extracts from embryos expressing Snk-HA or Ea-HA alone or together with GD-GFP or GD[2]-GFP were subjected to immunoprecipitation with GFP-Trap. Extracts were divided into two portions, and western blot analysis was carried out with anti-GFP (top panels) or anti-HA antibodies (bottom panels). When GD-GFP and Ea-HA are coexpressed, all of the GD-GFP zymogen is processed [*] due to a feedback mechanism [4, 5] in which high levels of activated Ea process GD-GFP. The same cleavage product is produced by EaΔN. Overexpressed GD[2]-GFP does not generate high enough levels of activated Ea to observe this effect.

(C) Model for ventral processing of Ea by Snk. GD (red) is processed by Nudel (purple). GD cleaves Snk (blue). Processed GD binds to Pipe-sulfated (S) proteins in the ventral VM, including vitelline membrane-like (VML) [11]. Bound GD recruits and brings together Snk and Ea zymogen (green), resulting in Ea cleavage. Processed Ea cleaves Spz (yellow) to form the active Toll ligand, which binds and activates Toll in the ventral embryonic membrane (EM).

[4, 18] and to anionic components of a highly purified *Drosophila* eggshell matrix preparation [18].

We propose that ventrally localized GD binds to both Ea and Snk and plays a direct role in promoting an interaction between them. It is possible that a single GD molecule binds either to Ea or to Snk and that ventral localization of GD acts to concentrate GD-bound Ea and Snk and bring them into proximity. Alternatively, GD bound only to Snk or Ea may undergo a conformational change when it interacts with the Pipe-sulfated ventral cue that results in an enhancement of Snk proteolytic activity or an increased susceptibility of Ea to cleavage by Snk. Lastly, GD bound simultaneously to both Ea and Snk may respond to the ventral cue by undergoing a conformational change that brings Snk and Ea into productive juxtaposition and results in Ea cleavage. A mechanism in which GD interacts with Pipe and the sulfated ventral cue to promote productive interaction between Ea and Snk can also explain the results of RNA injection studies carried out by Han et al. [15] and by DeLotto [19]. Those investigators showed that injection of very high levels of in vitro synthesized RNA encoding the GD zymogen could lateralize or reorient the polarity of embryos produced by *pipe* mutant females. They interpreted those results to indicate that Pipe is normally required for activation of GD on the ventral side of the embryo, but that at high concentrations, GD could become enzymatically active by an alternative Pipe-independent mechanism. Our current results suggest instead that high concentrations of GD can promote interactions between Easter and Snake, or conformational changes in those proteins that lead to Snake-mediated cleavage of Easter, even in the absence of Pipe and the ventral cue. In conclusion, by demonstrating that the GD serine protease is localized within the ventral PVS in a Pipe-dependent manner and that the interaction of GD with the Pipe-sulfated ventral cue enables it to bring about the ventrally restricted processing of Easter, the work reported here explains how ventrally localized sulfotransferase activity in the follicle cell layer leads to spatially localized activation of the Toll receptor and to the formation of the *Drosophila* embryonic DV axis.

## Experimental Procedures

### *Drosophila* Strains and Maintenance

All stocks were maintained employing standard conditions and procedures. The WT *Drosophila melanogaster* stock used was a *w/w* mutant derivative of Oregon R. Stocks bearing the following mutations are described in more detail on Flybase (http://flybase.org/): $gd^{2}$, $gd^{3}$, $gd^{LU119}$, $gd^{TN124}$, $gd^{VM90}$, $gd^{VO27}$ (bearing the same amino acid change as $gd^{10}$, G469E. For simplicity, we refer to this mutant as $gd^{10}$ throughout this manuscript), $pip^{1}$ (formerly $pip^{386}$), and $pip^{3}$. The strains carrying the Gal4 driver insertions nos-Gal4:VP16 and pCOG-Gal4:VP16 are described in Rorth [20]. Stocks carrying the following transgenes have been described in Cho et al. [7]: pUASp-Ea-GFP, pUASp-GD-GFP, pUASp-Snk-GFP, pUASp-Spz-GFP, pUASp-Ea-HA, and pUASp-Snk-HA.

### RT-PCR of RNA

Forty pairs of ovaries each were dissected from $gd^{VM90}/gd^{VM90}$ females and from *w/w*, Oregon R females, respectively. Total RNA was isolated from the ovaries using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). To generate cDNA, we subjected 5 μg of total RNA to reverse transcription using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). One tenth of the complementary DNA (cDNA) produced was then subjected to PCR-mediated amplification using the following two oligonucleotides: 5′-TCGA TCACCAGGGGATCGTGGCCTTGGC-3′ and 5′-TCAAATTACAAAGGCCGT GATCCAGTCCAG-3′.

An 831 base pair amplification produce corresponding to the serine protease catalytic domain was produced using cDNA from *w/w*, Oregon R-derived females. No amplification product was observed using the cDNA from $gd^{VM90}/gd^{VM90}$ females.

### Plasmid Constructs

For the construction of pUASp-GD[2]-GFP, pUASp-GD[TN124]-GFP, pUASp-GD[LU119]-GFP, and pUASp-GD[3]-GFP, the two oligonucleotides 5′-ATTCCCGCGGCCGCAAAATGAGGCTGCACCTGGCGGCGATCC-3′ and 5′-CCGTGGATACGCACCGCCGGATCCG-3′ were used for the PCR-mediated amplification of genomic DNA isolated from adult female flies homozygous for the $gd^{2}$ $gd^{TN124}$, $gd^{LU119}$, and $gd^{3}$ mutations, respectively. This resulted in the amplification of 447 bp DNA fragments bearing each of the four mutations. The DNA fragments were digested with NotI and BamHI,

and the resultant fragments were ligated to similarly digested pUASp-GD-GFP [7].

For the construction of pUASp-EaΔN, the two oligonucleotides 5′-CC GATTGCGGCCGCAAAATGCTAAAGCCATCGATTATCTG-3′ and 5′-GGACA TTCTAGATCAGGACTCAATAGTGTTTTG-3′ were used for PCR-mediated amplification of a DNA fragment carrying the EaΔN cDNA construct [5]. The resultant PCR product was digested with NotI and XbaI and ligated to similarly digested pUASp [20].

For the construction of pUASp-GD, the two oligonucleotides 5′-ATT CCCGCGGCCGCAAAATGAGGCTGCACCTGGCGGCGATCC-3′ and 5′-AC ACATTCTAGATGTGATTCAAATTACAAAGGCCG-3′ were used for PCR-mediated amplification of a DNA fragment carrying the *gd* cDNA. The resultant PCR product was digested with NotI and XbaI and ligated to similarly digested pUASp. For the construction of pUASp-GD[2], the NotI- and BamHI-digested DNA fragment bearing the $gd^2$ mutation described above was ligated to NotI- and BamHI-digested PUASp-GD. For the construction of pUASp-GD[D347N] and pUASp-GD[S468A], the same two oligonucleotides were used for PCR-mediated amplification of *gd* coding sequences from the plasmids pRMHA-3N-GDDN and pRMHA-3N-GDSA, respectively (kind gifts of Dr. Ellen LeMosy) [5]. In both cases, the resultant PCR fragment was digested with NotI and XbaI and ligated to similarly digested pUASp.

In the case of pUASp-GD[S468A]-GFP, the two oligonucleotides 5′-ATTCCCGCGGCCGCAAAATGAGGCTGCACCTGGCGGCGATCC-3′ and 5′-GATGTGAGATCTATTACAAAGGCCGTGATCCAG-3′ were used for PCR-mediated amplification of *gd* coding sequences from pRMHA-3N-GDSA [5]. The resultant PCR fragment was digested with NotI and BglII and ligated to NotI/BamHI digested pUASp-GFP [7].

For the construction of pUASp-GDΔN211 and pUASP-GDΔN253, the two oligonucleotides 5′-CCGATTGCGGCCGCAAAATGCTAAAGCCATCGATTA TCTG-3′ (encoding the amino terminus and signal peptide of Ea) and 5′-ACACATTCTAGATGTGATTCAAATTACAAAGGCCG-3′ were used for PCR-mediated amplification of *gd* coding sequences from the plasmids GD-212 and GD-254, respectively (kind gifts of Dr. Ellen LeMosy) [5]. In both cases the resultant PCR fragment was digested with NotI and XbaI and ligated to similarly digested pUASp.

For the construction of pUASp-GD-HA, the two oligonucleotides 5′-ATTCCCGCGGCCGCAAAATGAGGCTGCACCTGGCGGCGATCC-3′ and 5′-GGATGTTCTAGAAATTACAAAGGCCGTGATCCAGTCC-3′ were used for PCR-mediated amplification of a DNA fragment carrying the *gd* cDNA. The resultant PCR product was digested with NotI and XbaI and ligated to similarly digested pUASp-HA [7], a pUASp derivative that carries three tandemly arranged copies of the HA epitope of Influenza hemagglutinin inserted at the XbaI site of pUASp.

Transgenic fly lines carrying insertions of the constructs described above were generated by conventional P element mediated transformation [21].

### Perivitelline Injection

The various GFP-tagged versions of GD examined in these studies were expressed in the germline of adult females under the control of the nos-Gal4:VP16 driver line. PVF was obtained from the progeny of these females at gastrula or germband extension stages and injected into the dorsal or ventral PVS of cleavage or early syncytial blastoderm stage embryos as described in Stein et al. [22] and Stein and Nüsslein-Volhard [23]. Embryos were allowed to incubate for approximately 1.5 hr at room temperature, and were then subjected to fluorescence or bright-field microscopy on a Zeiss Axioplan2 imaging microscope.

### Embryonic Phenotypes

Larval cuticles were prepared according to van der Meer [24].

### Western Blotting and Immunoprecipitation

Collection of eggs, preparation of extracts, determination of protein concentrations in extracts, and western blot analysis of tagged versions of GD, Snk, Ea, and Spz were carried out as described in Cho et al. [7]. Following electroblotting to nitrocellulose membranes, blots were incubated with monoclonal primary antibodies against either GFP (1/1,000) (Monoclonal B-2, catalog no. sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA) or against the HA epitope (1/1,000) (Monoclonal 16B12, catalog no. MMS-101P, Covance, Emeryville, CA). Blots were washed and incubated with goat anti-mouse IgG (1/5,000) (cat. no. 31430, Thermo Scientific, Rockford, IL), followed by detection using the Pierce Super Signal Detection System (Pierce, Rockford, IL).

### Coimmunoprecipitation and Immunoblotting

For coIP studies, embryos were collected 2–4 hr after egg deposition and homogenized as for western blot analysis. Following determination of protein concentrations, a volume of extract containing 100 μg protein was incubated with GFP-Trap beads (Chromotek, GMBH, Martinsried, Germany) at 4°C for 2 hr as described by the manufacturer. Precipitates were then divided into two aliquots and subjected to SDS-PAGE followed by immunoblotting with monoclonal anti-GFP and monoclonal anti-HA, respectively, as described above.

### Acknowledgments

We are grateful to Robert DeLotto, Carl Hashimoto, Ellen LeMosy, and Trudi Schüpbach for providing *Drosophila* stocks and/or DNA clones. This work was supported by a grant from the National Institutes of Health (GM077337).

Received: February 22, 2012
Revised: March 30, 2012
Accepted: March 30, 2012
Published online: May 10, 2012

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