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Report

Localization and Activation of the *Drosophila* Protease Easter Require the ER-Resident Saposin-like Protein Seele

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Summary

Drosophila **embryonic dorsal-ventral polarity is generated by a series of serine protease processing events in the egg perivitelline space. Gastrulation Defective processes Snake, which then cleaves Easter, which then processes Spätzle into the activating ligand for the Toll receptor [1–3].** ***seele*** **was identified in a screen for mutations that, when homozygous in ovarian germline clones, lead to the formation of progeny embryos with altered embryonic patterning; maternal loss of** ***seele*** **function leads to the production of moderately dorsalized embryos [4]. By combining constitutively active versions of Gastrulation Defective, Snake, Easter, and Spätzle with loss-of-function alleles of** ***seele*****, we find that Seele activity is dispensable for Spätzle-mediated activation of Toll but is required for Easter, Snake, and Gastrulation Defective to exert their effects on dorsal-ventral patterning. Moreover, Seele function is required specifically for secretion of Easter from the developing embryo into the perivitelline space and for Easter processing. Seele protein resides in the endoplasmic reticulum of blastoderm embryos, suggesting a role in the trafficking of Easter to the perivitelline space, prerequisite to its processing and function. Easter transport to the perivitelline space represents a previously unappreciated control point in the signal transduction pathway that controls** ***Drosophila*** **embryonic dorsal-ventral polarity.**

Results and Discussion

Seele/CG12918 Is Required for Normal Embryonic Dorsal-Ventral Patterning

Using deficiency mapping, we mapped *seele* to polytene chromosome interval 46D7–46D9. Sequence analysis of genomic DNA from a $seele^{282}$ allele-bearing stock identified a G-to-A transition affecting the 3′ splice acceptor site of the first intron of the annotated gene CG12918 (Figure 1A). A second allele, $seele^{f04527}$, carries a PiggyBac transposon insertion in the second intron of CG12918 [5]. Sixty percent (680 of 1124) of the embryonic cuticles produced by females homozygous for $seele^{f04527}$ exhibited ventral denticles of narrower than normal width (Figure 1D) and the dorsolaterally derived tracheal structures referred to as Filzkörper (Figure 1E), a phenotype characterized as moderately severe (the D2 phenotype) [6]. Thirty-nine percent (438 of 1124) of cuticles lacked denticles but produced Filzkörper, the strongly dorsalized (D1) phenotype (Figure 1F), and fewer than 1% (2 of 1124) of embryos were completely dorsalized, lacking both ventral denticles and Filzkörper, like embryos produced by dorsal group null mutant females (Figure 1C). Finally, fewer than 1% (4 of 1124) of the progeny displayed the weakest phenotype (D3), in which the embryos had ventral denticle bands of normal width and Filzkörper but exhibited a tail-up or twisted phenotype. Consistent with their moderate and strongly dorsalized phenotypes, embryos produced by *seele* mutant females exhibited appropriately polarized gastrulation movements (Figure 1H). Also consistent with these phenotypes, embryos from $seele^{f04527}$ mutant females failed to stain for the ventral mesodermal marker Twist (Figure 1K) [7]. The ability of injected in vitro-synthesized RNA encoding the CG12918 open reading frame to rescue the progeny of *seele* mutant females confirmed that CG12918 corresponds to the *seele* locus. Following injection of 48 cleavage/blastoderm-stage embryos produced by $seele^{f04527}/Df(2R)X3$ mutant females with *seele* RNA at a concentration of 0.5 mg/ml, 13 embryos hatched (Figure 1G), and 5 embryos exhibited the weak D3 phenotype. None of 68 cleavage/blastoderm-stage embryos injected with water hatched or exhibited the D3 phenotype.

seele Encodes a Member of the Saposin-like Class of Proteins

CG12918 encodes a putative protein product of 189 amino acids with a predicted molecular weight of 21.3 kDa that exhibits significant amino acid sequence similarity to the saposin-like proteins (SAPLIPs), a group of proteins found in a diverse range of organisms [8] (see Figure S1 available online). Notably, Seele carries six conserved cysteine residues characteristic of all SAPLIPS (see Figure S1). Seventeen amino acids at the amino terminus of the protein are likely to act as a secretory signal peptide, whereas the carboxyl terminus of the protein bears four amino acids, KEEL, which are known to act as an endoplasmic reticulum (ER) retention signal in *Drosophila* [9]. Among the known SAPLIPs, Seele is most similar to two vertebrate proteins, the putative zebrafish orthologs of which are Canopy1 and Canopy2 (MSAP in mammals) [10, 11]. Seele is more distantly related to two additional zebrafish/vertebrate SAPLIPs, Canopy 3 and Canopy 4 (PRAT4A and PRAT4B in mammals) [12, 13]. The product of the *Drosophila* gene CG11577 appears to be the bona fide fly ortholog of both Canopy3 and Canopy4.

Seele Protein Is Present in the Endoplasmic Reticulum of Blastoderm Embryos

Western blot analysis of extracts of embryos from wild-type females using an antibody against Seele detected a protein of about 28 kDa that was not seen in *seele* mutant-derived

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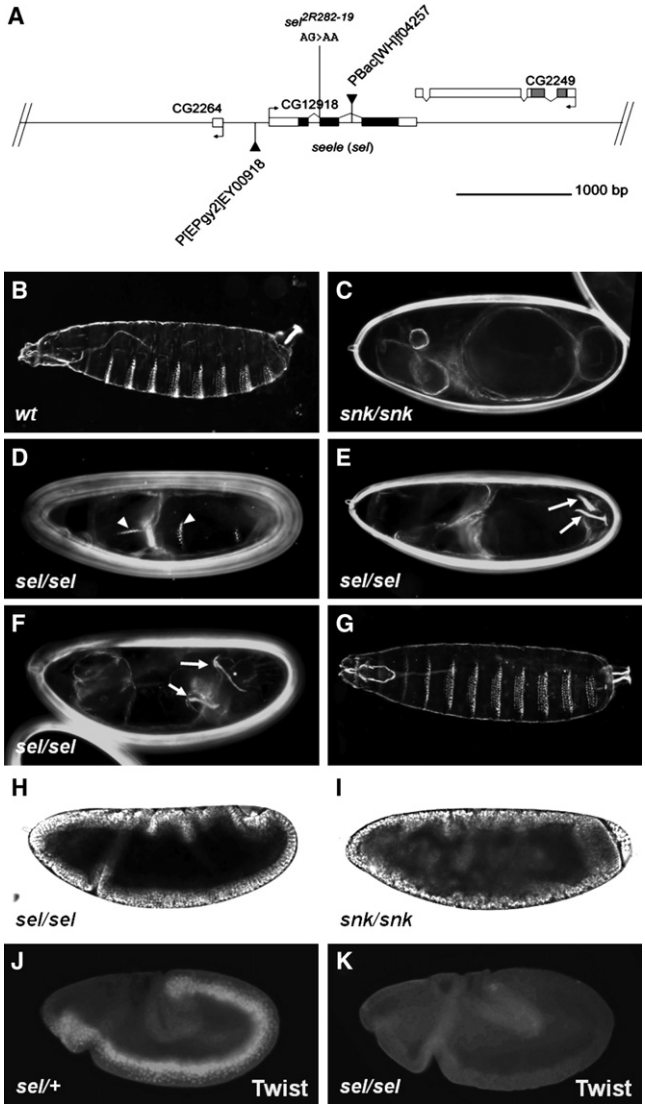

Figure 1. *seele*/CG12918 Is Required Maternally for the Establishment of *Drosophila* Embryo Dorsal-Ventral Polarity

(A) Diagram of the exon/intron structures of *seele*/CG12918 and the two nearby genes CG2264 and CG2249, and the position of the $sel^{2R282-19}$ (sel^{282}) and sel^{f04257} mutations.

(B) Wild-type cuticle.

(C) D0 class cuticle from $snake^{1}/snake^{2}$ mutant mother.

(D) D2 class cuticle from $sel^{f04257}/sel^{f04257}$ mutant mother showing ventral denticle bands of narrow width.

(E) The same D2 class cuticle as in (D) photographed at a different focal depth and showing the position of Filzkörper.

(F) D1 class cuticle from $sel^{f04257}/sel^{f0425\ 7}$mutant mother.

(G) Rescued $sel^{f04257}/sel^{f04257}$-derived embryo that was injected with in vitro-synthesized *seele* RNA.

(H) Gastrulating embryo from $sel^{f04257}/sel^{f04257}$mutant mother.

(I) Gastrulating embryo from $snake^{1}/snake^{2}$ mutant mother.

(J) Anti-Twist staining of a gastrulating embryo from a sel^{f04257}/+ mutant mother.

(K) Anti-Twist staining of a gastrulating embryo from a $sel^{f04257}/sel^{f04257}$ mutant mother.

In (D)–(F), arrowheads indicate the position of ventral denticle belts and arrows indicate the position of Filzkörper. Maternal genotypes are shown at bottom left. The cuticles in (B) and (G) were photographed at half the magnification of (C)–(F). See also Figure S1.

embryos (Figure 2A). Similarly, blastoderm-stage wild-type embryos stained with anti-Seele displayed a pattern of expression (Figure 2B) that was absent from blastoderm embryos produced by *seele* mutant females (Figure 2C). In late-stage embryos, a more complex pattern of zygotic expression was seen, which included abundant expression in the developing salivary glands (Figure 2D); late-stage embryos produced by $seele^{f04527}/seele^{f04527}$ mothers that had been fertilized by wild-type males also expressed Seele in various tissues, including structures that correspond to developing salivary glands (Figure 2E). No Seele was detected in embryos from $seele^{f04527}/seele^{f04527}$ mothers that had been fertilized by $seele^{f04527}/seele^{f04527}$ males (data not shown).

When expressed in syncytial blastoderm embryos, the stage at which dorsal-ventral patterning is occurring, a functional, transgenic version of Seele fused to mCherry exhibited considerable colocalization with a GFP-tagged version of protein disulfide isomerase (PDI-GFP), an ER-resident protein (Figures 2F–2H) [14]. Moreover, following fractionation of extracts from syncytial blastoderm embryos by density-gradient centrifugation [15], Seele was observed to cofractionate with the ER protein BiP [16], but not with the Golgi protein GM130 [17] (Figure 2I).

Seele Functions Upstream of Toll Activation by Spätzle

Females heterozygous for the dominant, ventralizing $Toll^{9Q}$ allele, which are also homozygous for $seele^{f0452}$, produce progeny with cuticles bearing rings of ventral denticles (Figure 3B), like the progeny of females carrying $Toll^{9Q}$ alone (Figure 3A). These results indicate that the ventralizing signal transmitted by activated Toll receptor does not require Seele function and that Seele acts upstream of Toll. To extend these findings and to determine the step in the dorsal-ventral pathway at which Seele acts, we generated transgenic, ventralizing versions of Spätzle, Easter, Snake, and Gastrulation Defective (GD) and examined the phenotypes of embryos produced by *seele* mutant females expressing these constructs.

Nanos-Gal4VP16-mediated germline expression [18] of the ventralizing SpätzleC106 derivative of Spätzle [19, 20] fused in-frame to GFP, in either $seele^{f04527}$/+ or $seele^{f04527}/seele^{f04527}$ females, led to the formation of lateralized progeny embryos (Figures 3C and 3D) and hence to constitutive Toll activation. This indicates that Seele functions upstream of Spätzle-mediated activation of Toll. In contrast, whereas expression of the two "preactivated" versions of Easter and Snake, EasterΔN [21] and SnakeΔN [22], in the germline of $seele^{f04527}$/+ females led to the formation of apolar, lateralized embryos (Figures 3E and 3G), $seele^{f04527}/seele^{f04527}$ females expressing either of these transgenes produced strongly dorsalized (D1) progeny (Figures 3F and 3H). The likely explanation for these observations is that Seele is required for Easter function, with the epistasis of *seele* over SnakeΔN resulting from the inability of preactivated Snake to transmit its lateralizing signal in the absence of downstream functional Easter. Finally, whereas transgenic overexpression of GD-GFP protein in the germline of $seele^{f04527}$/+ females led to the formation of lateralized and ventralized progeny (Figure 3I), *seele* mutant females expressing this transgene produced dorsalized embryos (Figure 3J). Thus, like EasterΔN and SnakeΔN, *seele* acts downstream of GD.

Seele Is Required for Easter-GFP Localization and Processing

The ER localization of Seele and the epistasis analysis described above led us to examine the distributions, within

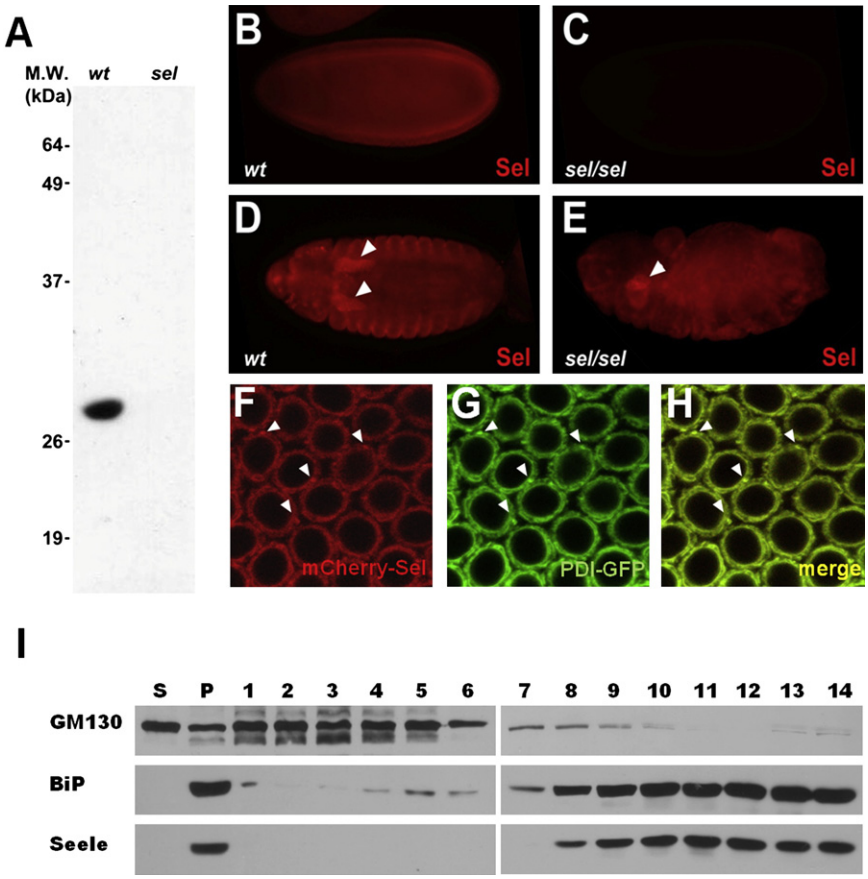

Figure 2. Seele Protein Localizes to the Endoplasmic Reticulum

(A) Western blot analysis of 0- to 4-hr-old wild-type (left lane) and *sel*^{f04257}/*sel*^{f04257} mutant-derived (right lane) embryo extracts probed with anti-Seele antibody.

(B–E) Whole-mount immunohistochemical staining of a wild-type blastoderm (B), *sel*^{f04257}/*sel*^{f04257} mother-derived blastoderm (C), germband-retracted-stage embryo from a wild-type mother (D), and germband-retracted-stage embryo from a *sel*^{f04257}/*sel*^{f04257} mother fertilized by a wild-type male (E). Arrowheads in (D) and (E) indicate the position of embryonic salivary glands.

(F–H) Confocal images of the cortical cytoplasm at the surface of a wild-type syncytial blastoderm embryo showing mCherry-Seele (F), PDI-GFP (G), and a merged image of the two fluorescent proteins (H). Arrowheads in (F)–(H) indicate positions of conspicuous overlap.

(I) Western blot analysis of membrane fractions collected from a 10%–30% OptiPrep density-gradient separation of membranes prepared from syncytial blastoderm-stage wild-type embryos. Blots were probed with antibodies against the Golgi protein GM130, the ER protein BiP, and Seele. The S and P lanes contain aliquots of the supernatant and pellet, respectively, obtained following the 100,000 × g spin. The pellet fraction was subsequently resuspended and fractionated on the OptiPrep gradient.

the egg, of previously generated GFP-tagged transgenic versions of Easter, Spätzle, Snake, and GD [23] in the progeny of *seele* mutant females. Following expression of GD-GFP, Easter-GFP, and Spätzle-GFP in the germline of *seele*^{f04527}/+ females, green fluorescence was detected in the perivitelline space of progeny embryos (Figures 4A, 4C, and 4D, top embryos). This fluorescence was most conspicuous in the spaces generated between the eggshell and embryo produced by folds in the embryonic membrane that form during gastrulation. There was no change in the perivitelline localization of GD-GFP and Spätzle-GFP in *seele*^{f04527}/*seele*^{f04527} mutant females (Figures 4A and 4D, bottom embryos). In contrast, when Easter-GFP was expressed in *seele*^{f04527}/*seele*^{f04527} mutant females, a dramatic decrease in green fluorescence in the perivitelline space was observed (Figure 4C, bottom embryo). Moreover, western blot analysis of embryonic extracts obtained from females expressing Easter-GFP in either a wild-type or a *seele* mutant background demonstrated that the abundance of processed Easter-GFP was dramatically decreased in extracts of *seele*^{f04527}/*seele*^{f04527} mutant-derived embryos (Figure 4E, left panel). This is consistent with a situation in which Easter needs to be secreted into the perivitelline space in order to undergo Pipe-dependent processing by Snake.

In contrast to GD-GFP, Easter-GFP, and Spätzle-GFP, most of the green fluorescence associated with transgenic Snake-GFP was detected in the cytoplasm of embryos produced by both *seele*^{f04527}/+ and *seele*^{f04527}/*seele*^{f04527} mothers (Figure 4B). The low levels of Snake-GFP present in the perivitelline space of wild-type-derived embryos precluded the determination of whether perturbation of Seele activity affects the perivitelline levels of Snake-GFP. However, western blot analysis of Snake-GFP showed no difference in processing of the protein in embryos from wild-type versus *seele* mutant embryos (Figure 4E, middle panel), suggesting that Snake-GFP localization and function are insensitive to the presence or absence of Seele activity. Similarly, no alteration in the pattern of processing of GD-GFP was observed in extracts from wild-type-derived versus *seele*^{f04527}/*seele*^{f04527}-derived embryos (Figure 4E, right panel).

Easter-GFP Localization and Processing Do Not Depend on Toll

As noted above, Seele exhibits some structural similarity to the zebrafish proteins Canopy3 and Canopy4, the mammalian homologs of which, PRAT4A and PRAT4B, have been shown to interact physically with and regulate the subcellular trafficking of members of the Toll-related group of receptors that operate during the innate immune response [12, 13, 24, 25]. This suggested the possibility that the effect of Seele upon Easter-GFP secretion might be an indirect consequence of a primary role for Seele in the trafficking of Toll to the membrane, for example if Easter and Toll were to interact physically during the secretion of Toll.

Wild-type embryos stained with an antibody against Toll display a characteristic honeycomb-like staining pattern [26] (Figure S2A) that is absent from the progeny of *Toll* mutant females (Figure S2B). Embryos from *seele*^{f04527}/*seele*^{f04527} mutant females exhibited a staining pattern that was indistinguishable from that of wild-type embryos (Figure S2C). Moreover, abundant Easter-GFP was present in the perivitelline space of the progeny of females lacking Toll protein

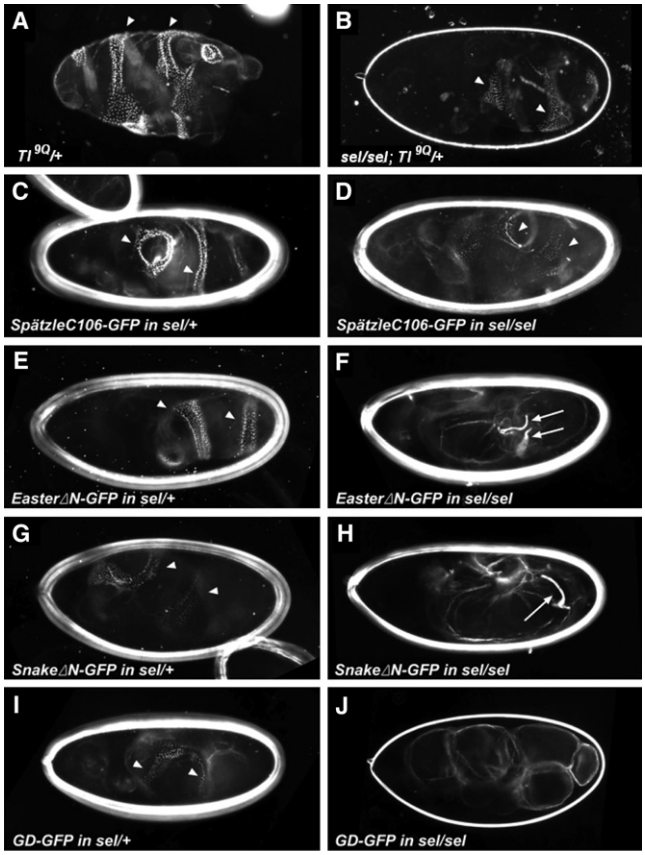

Figure 3. *seele* Is Epistatic over *easter*, *snake*, and *gastrulation defective* but Not *Toll* and *spätzle*

Maternal genotypes of mothers producing the progeny embryo cuticles shown are as follows: Tl^{9Q}/+ (A), sel^{278}/sel^{278};Tl^{9Q}/+ (B), sel^{f04257}/+; *UAS-Spätzle-GFP/nos-Gal4:VP16* (C), $sel^{f04257}/sel^{f04257}$;*UAS-Spätzle-GFP/nos-Gal4:VP16* (D), sel^{f04257}/+;*UAS-EasterΔN/nos-Gal4:VP16* (E), $sel^{f04257}/sel^{f04257}$;*UAS-EasterΔN/nos-Gal4:VP16* (F), sel^{f04257}/+;*UAS-SnakeΔN/nos-Gal4:VP16* (G), $sel^{f04257}/sel^{f04257}$;*UAS-SnakeΔN/nos-Gal4:VP16* (H), sel^{f04257}/+;*UAS-GD-GFP/nos-Gal4:VP16* (I), and $sel^{f04257}/sel^{f04257}$;*UAS-GD-GFP/nos-Gal4:VP16* (J). Arrowheads indicate the positions of ventral denticle material; arrows indicate the position of Filzkörper.

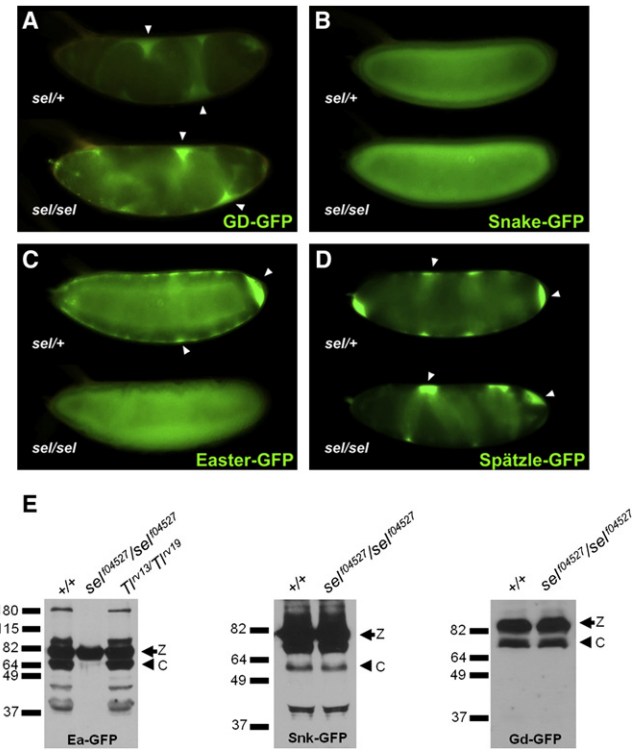

Figure 4. Seele Is Required for Perivitelline Space Localization and Processing of Easter

(A) GD-GFP in late gastrula embryos from sel^{f04257}/+ (top) and $sel^{f04257}/sel^{f04257}$ (bottom) mothers.

(B) Snake-GFP in blastoderm embryos from sel^{f04257}/+ (top) and $sel^{f04257}/sel^{f04257}$ (bottom) mothers. Abundant secretion of Snake-GFP is not detected at blastoderm or later stages of development.

(C) Easter-GFP in early gastrula embryos from sel^{f04257}/+ (top) and $sel^{f04257}/sel^{f04257}$ (bottom) mothers.

(D) Spätzle-GFP in late gastrula embryos from sel^{f04257}/+ (top) and $sel^{f04257}/sel^{f04257}$ (bottom) mothers.

In (A)–(D), pairs of embryos were oriented adjacent to one another and imaged and photographed simultaneously. For consistency of presentation, in (A) and (C), the digital photographs were divided horizontally between the embryos, and the images of the embryos were reversed so that the sel^{f04257}/+-derived embryo was above the $sel^{f04257}/sel^{f04257}$-derived embryo. Arrowheads indicate positions at which secreted GFP-tagged protein can be observed.

(E) Western blot analysis of Easter-GFP (Ea-GFP, left), Snake-GFP (Snk-GFP, middle), and GD-GFP (right) processing in embryonic extracts from wild-type and *seele* mutant mothers. "Z" and "C" indicate the zymogen and cleaved forms of the proteins, respectively. Maternal genotypes are shown above each lane. The processing of Easter-GFP is also shown in extracts of progeny from *Tl* mutant mothers. The higher-molecular-weight bands observed in the wild-type and Tl-derived extracts correspond to cleaved, activated Easter-GFP species complexed to Spn27A. Identification of the zymogen and cleaved forms of Ea-GFP, Snk-GFP, and GD-GFP is described in [23]. See also Figure S2.

(Figure S2D). Finally, Easter-GFP is processed normally in the progeny of *Toll* null mutant females, as shown by western blot analysis (Figure 4E). Together, these results indicate that the trafficking of Toll to the embryonic plasma membrane does not depend upon Seele and that neither the presence of Easter-GFP in the perivitelline space nor its processing by Snake depends on the trafficking of Toll to the embryonic membrane.

Conclusions

Members of the SAPLIP class of proteins participate in a variety of processes, including lipid metabolism, membrane fusion, antimicrobial and cytolytic activity, apoptosis, neurite outgrowth, and receptor signaling [8]. A common feature of many of these proteins is their interaction with lipids [27–30]. Among the specific subgroup of SAPLIPs that includes Seele are several vertebrate members that appear to play a role in the subcellular trafficking of specific target proteins. Canopy1 is an ER-localized protein that influences fibroblast growth factor (FGF) signaling at the midbrain/hindbrain boundary and interacts physically with the extracellular domain of FGFR1 [10]. It may act as a molecule-specific molecular chaperone, either in the maturation or the modification of FGFR1 or by facilitating the localization of FGFR1 to membrane microdomains with specific lipid compositions. Similarly, available evidence suggests that the mammalian PRAT4A and PRAT4B proteins act in the ER, either to facilitate the folding, maturation, or assembly of their cognate TLR proteins or more directly to regulate transit through the secretory pathway [12, 13, 24, 25]. These data, together with our observations, strongly suggest a role for Seele, acting in the lumen of the

ER to control the localization and activity of Easter. Seele could participate in the folding or maturation of Easter or alternatively could play a more direct role in Easter trafficking, by accompanying Easter from the ER to the Golgi apparatus, acting to mediate the selective uptake of Easter protein into transport vesicles, or modifying the properties of transport vesicles in which Easter resides.

Easter represents a key nexus of regulation of the dorsal group signal transduction pathway. The ventrally restricted step in the protease cascade is the Pipe-dependent activation of Easter by Snake [23, 31]. An additional layer of regulatory control of Easter is its interaction, following activation, with the serine protease inhibitor Spn27A [32–34]. The presence of inhibitory proenzyme domains in the Snake and Easter zymogens provides a means of preventing inappropriate activation of the two proteins during transit through the secretory pathway of the embryo. Localization of Easter to a specific class of secretory vesicles with a unique lipid composition could provide an additional means of ensuring that Easter is not precociously processed by Snake. Alternatively, Seele-dependent folding, glycosylation, or maturation of Easter could represent a way of preventing its precocious processing by Snake. Elucidating the step during secretory transit of Easter that is influenced by Seele and the extent to which Seele physically interacts with Easter or with membrane lipids should allow the determination of which of these mechanisms Seele employs to regulate Easter function.

Experimental Procedures

Stocks and Maintenance

All stocks were maintained employing standard conditions and procedures. The wild-type *Drosophila melanogaster* stock used was a *w/w* mutant derivative of Oregon R. Stocks bearing the following mutations, transgenes, and chromosomal deficiencies are described in more detail in the Supplemental Experimental Procedures: sel^{282}, $snake^{1}$, $snake^{2}$, $Toll^{9Q}$, $Toll^{rv13}$, $Toll^{rv19}$, PDI-GFP, Easter-GFP, EasterΔN-GFP, GD-GFP, Snake-GFP, SnakeΔN-GFP, Spätzle-GFP, SpätzleC106-GFP, *nos-Gal4:VP16*, *Df(2R)X1*, *Df(2R)X3*, *Df(2R)stan1*.

Plasmid Constructs

pUASp-EasterΔN and pUASp-SnakeΔN carry the catalytic domains of Easter and Snake, respectively, lacking their prodomains and fused in-frame to the Easter secretory signal peptide [35]. *nos-Gal4:VP16*-mediated expression of these transgenes in the female germline [18] results in secretion of active versions of the proteases. Details of the construction of these transgenes, the transgene encoding the mCherry-Seele fusion protein, and the pBP4-*seele* plasmid [36], which facilitates SP6 polymerase-mediated in vitro synthesis of *seele* mRNA, are described in the Supplemental Experimental Procedures.

Preparation of Antiserum Directed against Seele

For preparation of antiserum, the Seele open reading frame was introduced into pET-15b (Novagen). His_6-tagged Seele protein was then expressed in *E. coli* BL21(DE3) under T7 RNA polymerase-directed transcriptional control, purified by affinity chromatography under denaturing conditions, and sent to Pocono Rabbit Farm and Laboratory Inc. (Canadensis, PA) for the production of antibodies in guinea pigs.

Western Blot Analysis

For western blot analysis of Seele protein, 0- to 4-hr-old eggs were collected on yeasted apple juice/agar plates, homogenized in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel lanes contained 30 μg of embryo extract. Following electroblotting onto nitrocellulose membrane, blots were incubated with HRP-conjugated secondary antibody, followed by detection with the Pierce SuperSignal detection system. For the preparation of embryo extracts used in western blot analysis of Easter-GFP and Snake-GFP, in order to achieve uniformity in protein concentrations, approximately 50 late-blastoderm-stage embryos were collected by hand. For each embryo extract, a volume corresponding to exactly 100 μg of protein was subjected to SDS-PAGE, followed by electroblotting and detection as described above.

Subcellular Fractionation of Seele

Membrane fractionation of syncytial blastoderm embryos was carried out as described in [15]. Following low-speed centrifugation (3,000 × g for 10 min) to remove debris and dense organelles, the resultant supernatant was then subjected to high-speed centrifugation (100,000 × g for 1 hr) to pellet membranes. The membrane pellet was resuspended and subjected to density-gradient centrifugation in a 10%–30% OptiPrep gradient (Accurate Chemical and Scientific Corporation). Following centrifugation at 340,000 × g for 3 hr, 0.25 ml fractions were collected. Aliquots of these fractions were then examined by western blot analysis with antibodies directed against Seele, the ER protein BiP, and the Golgi protein GM130, respectively.

Examination of Embryonic Phenotypes

Gastrulating embryos were examined under Halocarbon oil 27 (Sigma Life Sciences). Larval cuticles were prepared according to [37]. Examinations of the distributions of Seele, Toll, and Twist proteins were carried out by whole-mount immunostaining according to the protocol of [38]. For tests of the influence of *seele* and *Toll* mutations on the distribution of GFP-tagged fusions proteins, similar-stage embryos from wild-type and mutant females were oriented adjacent to one another, and GFP-associated fluorescence of the two embryos was imaged simultaneously.

Supplemental Information

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.09.069.

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