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/G12918 Is Required for Normal Embryonic Dorsal-Ventral Patterning

Using deficiency mapping, we mapped seele to polytene chromosome interval 4D7–4D9. Sequence analysis of genomic DNA from a seele allele-bearing stock identified a G-to-A transition affecting the 3′ splice acceptor site of the first intron of the annotated gene G12918 (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 cuticle 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 1O). 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 approximately 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/D(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
Seele Functions Upstream of Toll Activation by Spätzle
Females heterozygous for the dominant, ventralizing TollQ allele, which are also homozygous for seele04527, produce progeny with cuticles bearing rings of ventral denticles (Figure 3B), like the progeny of females carrying TollQ 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 seele04527/+ or seele04527/seele04527 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, EasterN [21] and SnakeN [22], in the germline of seele04527/+ females led to the formation of apolar, lateralized embryos (Figures 3E and 3G), seele04527/seele04527 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 Snake,N 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 seele04527/+ 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 Easter,N and Snake,N, 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

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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 seeleR283-19 and seeleR283-29 mutations.

(B) Wild-type cuticle.

(C) D0 class cuticle from snake1/snake2 mutant mother.

(D) D2 class cuticle from seele04527/seele04527 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 FltKörper.

(F) D1 class cuticle from seele04527/seele04527 mutant mother.

(G) Rescued seele04527/seele04527 embryo that was injected with in vitro-synthesized seele RNA.

(H) Gastrulating embryo from seele04527/seele04527 mutant mother.

(I) Gastrulating embryo from snake1/snake2 mutant mother.

(J) Anti-Twist staining of a gastrulating embryo from a seele04527/+ mutant mother.

(K) Anti-Twist staining of a gastrulating embryo from a seele04527/seele04527 mutant mother.

In (D)–(F), arrowheads indicate the position of ventral denticle belts and arrows indicate the position of FltKörper. 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.
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 the progeny of seele\(^{f04527}/+\) females (Figures 4A and 4D, bottom embryos). In contrast, when Easter-GFP was expressed in seele\(^{f04527}/f04527\) 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\(^{f04527}/f04527\) 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\(^{f04527}/f04527\) 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\(^{f04527}/f04527\) 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\(^{f04527}/f04527\) 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.
interacts physically with the extracellular domain of FGFR1. Factor (FGF) signaling at the midbrain/hindbrain boundary and is an ER-localized protein that influences fibroblast growth factor. The subcellular trafficking of specific target proteins. Canopy1 are several vertebrate members that appear to play a role in among the specific subgroup of SAPLIPs that includes Seele.

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+/+ (A), see+/+se+/+,Tl+/+ (B), see+/+ (C); UAS-Spätzle-GFP/nos-Gal4;Vp16 (C), seesm251,seesm253;UAS-Spätzle-GFP/nos-Gal4;Vp16 (D), seesm251;UAS-Easter-GFP/nos-Gal4;Vp16 (E), seesm251;UAS-Seele/N/nos-Gal4;Vp16 (F), seesm251;UAS-Snake/N/nos-Gal4;Vp16 (G), seesm251;UAS-Seele/N/nos-Gal4;Vp16 (H), seesm251;UAS-GD-GFP/nos-Gal4;Vp16 (I), and seesm251;UAS-GD-GFP/nos-Gal4;Vp16 (J). Arrowheads indicate the positions of ventral denticle material; arrows indicate the position of Filzkörper.

(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 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 Easterzymogens 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: see[22], snake[2], To[25], To[27], PDI-GFP, Easter-GFP, EasterN-GFP, GD-GFP, Snake-GFP, SnakeN-GFP, Splätzle-GFP, SplätzleC106-GFP, nos-Ga4:VP16, Df(2R)X1, Df(2R)X3, Df(2R)stan1.

Plasmid Constructs

pUASP-EasterN and pUASP-SnakeN carry the catalytic domains of Easter and Snake, respectively, lacking their prodomains and fused in-frame with the reporter signals described above [18], nos-Ga4: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 Antisera Directed against Seele

For Preparation of antisera, the Seele open reading frame was introduced into pET-15b (Novagen). His<sub>e</sub>-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-h-old eggs were collected on yeast-fed 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 electroluting 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 electroluting 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.

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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References


Supplemental Information

Localization and Activation of the

*Drosophila* Protease Easter Require the

ER-Resident Saposin-like Protein Seele

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Figure S1 (Related to Figure 1).
Figure S1 (Related to Figure 1). Amino Acid Sequence of Seele and Homologous SAPLIPs

The amino acid sequences of Seele, Canopy1 (from zebrafish), MSAP (human ortholog of zebrafish Canopy2), PRAT4A (mouse ortholog of zebrafish Canopy3), PRAT4B (mouse ortholog of zebrafish Canopy4) and CG11577 (putative Drosophila ortholog of Canopy3 and Canopy4). The alignment was generated using MAFFT, with modifications. The black background indicates amino acids that are conserved in all six proteins. Black triangles show the position of six cysteine residues that are conserved throughout the SAPLIPs. The dark grey background indicates strongly conserved amino acids. The light grey background denotes weak conservation. Boxes indicate the position of amino acids that are identical in Seele, Canopy1 and MSAP/Canopy2.

Figure S2 (Related to Figure 4). Perivitelline Space Localization of Easter Does Not Depend on Toll

(A-C) Confocal images of anti-Toll staining of blastoderm embryos from +/+ (A), Tl\textsuperscript{v13}/Tl\textsuperscript{v19} (B), and sel\textsuperscript{04257}/sel\textsuperscript{04257} (C) mutant mothers.

(D) Distribution of Easter-GFP in early gastrula stage embryos from Tl/+ (top) and Tl\textsuperscript{v13}/Tl\textsuperscript{v19} (bottom) mothers. Arrowheads indicate positions at which secreted Easter-GFP can be detected.
Supplemental 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. The isolation of *sel*282 is described in Luschnig et al. [4]. *sel*04527 (formerly *CG12918*04527) was isolated in a large screen for *Drosophila melanogaster* mutants carrying insertions of the lepidopteran transposon PiggyBac [5]. The isolation of *sel*04527 was described in Luschnig et al. [4]. *sel*f04527 (formerly *CG12918*f04257) was isolated in a large scale screen for *Drosophila melanogaster* mutants carrying insertions of the lepidopteran transposon PiggyBac [5]. The PDI-GFP gene trap-containing strain is described in [14]. Strains carrying transgenic insertions of the mGFP6-tagged [39] dorsal fusion proteins Easter-GFP, EasterΔN-GFP, GD-GFP, Snake-GFP, SnakeΔN-GFP, Spätzle-GFP, SpätzleC106-GFP are described in Cho et al. [23]. The germline Gal4 driver insertion nos-Gal4:VP16 is described in [18]. Stocks bearing the following deficiencies used in the mapping of *seele*, Df(2R)X1, Df(2R)X3, Df(2R)stan1, and the additional mutants *snake*1, *snake*2, *Toll*0, *Toll*Tv13, *Toll*Tv19 used in this study are described on Flybase.

Plasmid Constructs
For the construction of pUASp-EasterΔN, the two oligonucleotides 5'-CCGATTGCAGCCGGCAAAAATGCTAAAGCCATCGATTATCTG-3' and 5'-GGACATTCTCAGATCGGACTCAATAGTGTATTG-3' were used for PCR mediated amplification of a DNA fragment carrying the EasterΔN cDNA construct [36]. The resultant PCR product was digested with NotI and XbaI and ligated to NotI/XbaI-digested pUASp.

For the construction of pUASp-SnakeΔN, the two oligonucleotides 5'-CCGATTGCAGCCGGCAAAAATGCTAAAGCCATCGATTATCTG-3' and 5'-GATGCATCTAGAAACTAGTGTTGCTTGAAGGCAATC-3' were used for PCR mediated amplification of a DNA fragment carrying the SnakeΔN cDNA construct [36]. The resultant PCR product was digested with NotI and XbaI and ligated to similarly digested pUASp.

For the construction of the mCherry-*seele* expressing transgene, the oligonucleotides 5'-TAGCGTAGAGTCGACTCCCGCGAGGTCAAGTGTCAC-3' and 5'-TGACTAGGGGATCCTTACTACAGTGTCTTGGCCATCAA-3' were used to amplify a DNA fragment corresponding to codons 22 through 189 of *seele*, using embryonic cDNA as a template. The amplification product was digested with SalI and BamHI and ligated to similarly digested the pmCherry vector (Clontech, Mountain View, Ca). The resultant fused mCherry-*seele* open reading frame was then amplified using the oligonucleotides 5'-CATGATGCAGATGCAGCCGGCAATGCTAAAGCCATCGATTATCTGTCGCGCTTGCTTGCGCCCAAGGGTGTTACAGTTACCATGGTGAAGCCCGAG GAG-3' and 5'-TAGCTCAGTCTAGATTACTACAGTGTCTTGGCCATCATAAA-3' yielding an amplification product bearing the codons encoding the *seele* signal peptide, followed by mCherry and then by the remainder of *seele*. The amplification product was digested with NotI and XbaI and ligated to similarly digested pVO, a pCaSpeR-4 derivative [40] that includes a terminator region from the *K10* gene. Finally, a DNA fragment corresponding to the 2.0 kb region directly upstream of the *seele* gene was amplified from genomic DNA using the oligonucleotides 5'-GATCGTCATCGAGCCGGCAATGCTAAAGCCATCGATTATCTGTTAAATAGTTTAACCCCGG-3' and 5'-CTTGTACGTGCGATCTTGGCCATCATAAACAAAATATCGGTCA-3', digested with KpnI and NotI and ligated to similarly digested pVO-mCherry-*seele*, placing the *seele* 5' genomic sequences directly upstream of the *seele* gene was amplified from genomic DNA using the oligonucleotides 5'-GATCGTCATCGAGCCGGCAATGCTAAAGCCATCGATTATCTGTTAAATAGTTTAACCCCGG-3' and 5'-CTTGTACGTGCGATCTTGGCCATCATAAACAAAATATCGGTCA-3', digested with KpnI and NotI and ligated to similarly digested pVO-mCherry-*seele*, placing the *seele* 5' genomic sequences directly upstream of the *seele* gene was amplified from genomic DNA using the oligonucleotides.
Transgenic fly lines carrying insertions of the three constructs described above were generated by conventional P-element mediated transformation.

cDNA GH10427, which encodes the full-length Seele open reading frame, was obtained from the Drosophila Genomics Resource Center. For the construction of pBP4-seele, the two oligonucleotides 5'-GATCGACCATGGTGACGAAGGCGCTTACCTTGCTTGGC-3' and 5'-CACGATGAATTCCTACAGTTCTTTGAGGTC-3' were used for PCR-mediated amplification of the Seele open reading frame from GH10427. The resultant PCR amplification product was digested with NcoI and EcoRI and ligated to similarly cut pBP4 [36], placing the Seele open reading frame downstream of the bacteriophage SP6 promoter. pBP4-seele was used as a template for SP6 polymerase-mediated synthesis of capped mRNA using the mMessage mMachine Kit (Applied Biosystems/Ambion, Austin, Texas).

**Preparation of Antiserum Directed against Seele**
The oligonucleotides 5'-GGCAGCCTATATGTACAGCTTCACCTCCCGCGAGGTC-3' and 5'-GCTCGAGGATCCCTACAGTTCCTTTGCCATC-3' were used as primers for PCR-mediated amplification of the Seele open reading frame, using GH10427 as a template and employing high fidelity Phusion thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland). Purified amplification product was digested with NdeI and BamHI and ligated to similarly digested pET15b (Novagen). His6-tagged Seele protein was then expressed in E. coli BL21(DE3) under T7 RNA polymerase-directed transcriptional control and purified using His-select(R) Nickel Affinity Gel (P6611, Sigma Life Sciences, St. Louis, MO) under denaturing conditions. Antiserum was generated against His-tagged Seele supplied as an SDS-PAGE gel slice at Pocono Rabbit Farm and Laboratory, Inc., (Canadensis, PA).

**Western Blot Analysis**
For Western blot analysis of Seele protein, 0-4 hr old eggs were collected on yeasted apple juice/agar plates and homogenized in SDS sample buffer (2% SDS, 10% glycerol, 5% BME, 50 mM Tris pH 6.8). For each embryo extract, a volume corresponding to 30 μg of extract was subjected to SDS-PAGE, electroblotted to nitrocellulose membranes and probed with guinea pig anti-sera directed against Seele (1:2000). Blots were then washed and incubated with HRP-conjugated goat anti-guinea pig secondary antibody (cat.no. 606-103-129, Rockland, Gilbertsville, PA) at dilution of 1:2000, followed by detection using the Pierce SuperSignal Detection System (Pierce, Rockford, Il).

For Western Blot analysis of Easter-GFP and Snake-GFP, eggs from females expressing the GFP-tagged constructs were collected on yeasted apple juice/agar plates. To achieve uniformity in protein concentrations, precisely staged embryos were collected by hand for the preparation of extracts. Approximately 50 late blastoderm stage embryos were collected under hydrocarbon oil. Oil was removed with heptane and the eggs were dechorionated in 50% Chlorox bleach and homogenized with a microcentrifuge tube-compatible pestle in 50 μl of lysis buffer (25 mM Tris, pH 7.5/0.15 M NaCl/0.3% NP-40/1mM EDTA/ 1mM EGTA/0.2mM N-ethylmaleimide, containing protease inhibitors [complete Mini EDTA-free Protease Inhibitor Cocktail, Roche, Indianapolis, In]). Protein concentrations in the homogenates were determined using the Bio-Rad Protein Assay reagent (cat. no. 500-0006; Bio-Rad Laboratories, Inc., Hercules, CA). For each embryo extract, a volume corresponding to exactly 100 μg of protein was subjected to SDS polyacrylamide gel electrophoresis. Following electroblotting to nitrocellulose membranes, blots were incubated with monoclonal primary antibodies against
GFP (1/1000) (Monoclonal B-2, cat. no. sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed and incubated with goat anti-mouse IgG (1/5000) (cat. no. 31430, Thermo Scientific, Rockford, IL), followed by detection using the Pierce SuperSignal Detection System (Pierce, Rockford, IL).

**Subcellular Fractionation of Seele**
Membrane fractionation of syncytial blastoderm embryos was carried out as described in Papoulas et al. [15]. Briefly, 1-3 hr old embryos were homogenized, and then subjected to low speed centrifugation (3000 g/10 minutes) to remove debris and dense organelles. The resultant supernatant was then subjected to high-speed centrifugation (100,000g/1 hour) to pellet membrane membranes. The membrane pellet was resuspended in buffer and subsequently subjected to density gradient centrifugation in a 10-30% OptiPrep gradient (Accurate Chemical and Scientific Corporation, Westbury, NY). Following centrifugation at 340,000 g for 3 hr, 0.25 ml fractions were collected. An aliquot of each fraction corresponding to 100 µg protein was subjected to Western analysis with guinea pig anti-Seele (1/1000), rabbit anti-BiP (1/1000)[15] and rabbit anti-GM130 (1/10,000) (cat. no. ab30637, Abcam, Cambridge, MA). Secondary HRP-coupled goat antibodies directed against guinea pig (cat. no. 606-103-129, Rockland, Gilbertsville, PA) and rabbit (cat. no. 31460, Pierce, Rockford, IL) antibodies were used at a dilution of 1/5000, followed by detection using the Pierce SuperSignal Detection System (Pierce, Rockford, IL).

**Examination of Embryonic Phenotypes**
For examination of gastrulation phenotypes, embryos were dechorionated in a 50% solution of Chlorox bleach in water and placed directly into Halocarbon 27 oil (Sigma Life Sciences, St. Louis, MO). Embryos were examined and photographed under bright field illumination on a Zeiss Axioplan 2 outfitted with Axiocam digital camera.

For the examination of terminal embryo phenotypes, larval cuticles were prepared according to Van der Meer [37] and photographed under dark field illumination on a Zeiss Axioplan II with Axiocam digital camera.

Examinations of the distributions of Seele, Toll and Twist proteins were carried out by whole mount immunostaining according to the protocol of Macdonald and Struhl [38]. The Seele antiserum (pre-absorbed against 0-4 hr old fixed embryos from sel^{04527} mutant mothers) was used at a dilution of 1/1000. Alexa Fluor 568-conjugated goat anti-guinea pig antibody (Molecular Probes, Eugene, Oregon, 1/1000 dilution) was used to detect the primary antibody. The rabbit antibody against Toll [41] was used at a dilution of 1/1000 and detected with Alexa Fluor 594-conjugated goat anti rabbit antibody (Molecular Probes, Eugene, Oregon). The rabbit antibody against Twist [42] was used at a dilution of 1/5000. Alexa Fluor 594-conjugated goat anti-rabbit antibody was used to detect the primary antibody. For tests 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, imaged and photographed simultaneously. Examination and photography of embryos was carried out either by epifluorescence imaging on a Zeiss Axioplan II microscope or by confocal microscopy using a Leica SP2 Laser Scanning Confocal Microscope.
Supplemental References


