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Spätzle regulates the shape of the Dorsal gradient in the *Drosophila* embryo

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SUMMARY

Dorsal-ventral polarity of the *Drosophila* embryo is established by a nuclear gradient of Dorsal protein, generated by successive *gurken-Egfr* and *spätzle-Toll* signaling. Overexpression of extracellular Spätzle dramatically reshapes the Dorsal gradient: the normal single peak is broadened and then refined to two distinct peaks of nuclear Dorsal, to produce two ventral furrows. This partial axis duplication, which mimics the ventralized phenotype caused by reduced *gurken-Egfr* signaling, arises from events in the perivitelline fluid of the embryo and occurs at the level of Spätzle processing or Toll activation. The production of two Dorsal peaks is addressed by a model that invokes action of a diffusible inhibitor, which is proposed to normally regulate the slope of the Dorsal gradient.

Key words: Dorsal-ventral polarity, Oogenesis, Embryogenesis, Axis duplication, *gurken*, *pipe*, *spätzle*

INTRODUCTION

An enduring problem in developmental biology is understanding how spatial asymmetry is initiated and gradually modified to produce pattern across a body axis. In amphibians, early development involves establishment of the organizer, which was defined by its ability to induce a second axis in the classic Spemann-Mangold transplantation experiment (reviewed by Hamburger, 1988). Secreted growth factors are required not only for the induction of the organizer, but account for its subsequent instructive properties (reviewed by Harland and Gerhart, 1997). Yet, the relative roles of cellular signaling sources and secreted morphogen gradients in embryonic patterning remain controversial. In the *Drosophila* embryo, genetic mutations and transplantation experiments can cause partial duplications of the dorsal-ventral axis (Schüpbach, 1987; Stein and Nüsslein-Volhard, 1992). Analysis of these embryos has helped define the symmetry-breaking events in axis formation, while raising questions regarding the respective contributions of spatial cues determined by groups of cells surrounding the oocyte in the egg chamber, and positional information generated by the diffusion of extracellular morphogens in the embryo.

The shape of the signal that generates the dorsal-ventral pattern of the *Drosophila* embryo undergoes progressive refinement during development. The initial cue that coordinates embryo and eggshell polarity is generated in the ovarian egg chamber, where the developing oocyte is surrounded by an epithelium of somatically derived follicle cells (reviewed by Ray and Schüpbach, 1996; Nilson and Schüpbach, 1999). Dorsal-ventral asymmetry arises during mid-oogenesis, when the *gurken* (*grk*) transcript, which encodes a transforming growth factor α-like molecule, becomes localized to the dorsal anterior corner of the oocyte (Neuman-Silberberg and Schüpbach, 1993). Gurken protein apparently serves as a secreted ligand for the epidermal growth factor receptor (Egfr) in neighboring follicle cells (Price et al., 1989; Schejter and Shilo, 1989; Sapir, et al., 1998), activating the canonical Ras signal transduction cassette in a group of cells on the dorsal side (Wasserman and Freeman, 1998; Peri et al., 1999).

As a consequence of repression by Egfr signaling on the dorsal side of the follicular epithelium, *pipe* RNA is expressed as a broad stripe in ventral follicle cells (Sen et al., 1998). Pipe protein is predicted to act as a glycosaminoglycan-modifying enzyme in the Golgi on an undetermined substrate (Sen et al., 2000).

The signal originating in ventral follicle cells is transmitted to the embryo through the perivitelline space, which lies between the vitelline layer of the eggshell and the embryo plasma membrane (reviewed by Morisato and Anderson, 1995). The establishment of embryonic polarity is dependent on the ventrally restricted activation of the uniformly distributed receptor Toll (Hashimoto et al., 1988; Hashimoto et al., 1991; Stein et al., 1991). The ligand for Toll is apparently encoded by the *spätzle* (*spz*) gene, which produces a protein containing a C-terminal cystine knot motif found in many vertebrate growth factors (Morisato and Anderson, 1994). The Spätzle protein is secreted into the perivitelline space as an inactive precursor, and is cleaved into the active ligand (Morisato and Anderson, 1994; Schneider et al., 1994) through the activity of a serine protease cascade that includes the products of the genes *nudel* (Hong and Hashimoto, 1995), *gastrulation defective* (Konrad et al., 1998), *snake* (DeLotto and Spierer, 1986) and *easter* (*ea*) (Chasan and Anderson, 1989). Regrettably, there have been two technical obstacles to

visualizing the shape of the ventral signal within the perivitelline space. First, all of the biochemical reactions involve the conversion of a precursor protein to an activated form, and no reagent that specifically recognizes the active species has been reported. Second, the vitelline membrane acts as an impermeable barrier to larger molecules; embryo fixation and detachment of the vitelline membrane also removes many perivitelline proteins.

Activation of Toll initiates an intracellular signaling pathway that results in the nuclear translocation of the transcription factor encoded by *dorsal*, a member of the NF-κB/*rel* family (reviewed by Drier and Steward, 1997). Dorsal is initially present throughout the embryonic cytoplasm, where it is retained by the inhibitory IκB protein encoded by *cactus*. Signaling on the ventral side leads to the proteolysis of Cactus, thereby releasing Dorsal (Belvin et al., 1995; Bergmann et al., 1996; Reach et al., 1996). Along the dorsal-ventral axis, high levels of Dorsal protein are present in ventral nuclei, progressively lower levels in lateral nuclei, and no detectable protein in dorsal nuclei (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The shape of the Dorsal gradient is characterized by the size of the ventral domain (assessed by the number of nuclei expressing peak Dorsal), and a distinct slope (functionally defined by the number of nuclei that lie between highest and lowest nuclear Dorsal). Changing the shape of the Dorsal gradient causes patterning defects that lead to embryonic lethality.

The Dorsal gradient subdivides the axis into distinct domains by setting the expression limits of key zygotic regulatory genes, which are responsible for initiating the differentiation of various tissues. High levels of nuclear Dorsal lead to the expression of Twist protein in mesodermal precursor cells (Thisse et al., 1988; Jiang et al., 1991; Pan et al., 1991). The Twist protein is itself expressed in a graded fashion in the most ventral 16-18 cells (20% embryo circumference), and this domain can be subdivided into smaller threshold responses (reviewed by Rusch and Levine, 1996). Intermediate levels of nuclear Dorsal activate the expression of *short gastrulation* (*sog*) in two lateral stripes that flank the ventral Twist domain, each 12-14 cells wide (combined 32% embryo circumference; François et al., 1994). In many of the experiments described below, Twist expression has been used as a marker for high nuclear Dorsal concentrations, rather than identifying cells that exceed a particular ratio of nuclear to cytoplasmic Dorsal staining. Likewise, changes in the number of cells that express *sog* have been correlated with changes in the slope of nuclear Dorsal.

In trying to understand how the shape of the Dorsal gradient is regulated, one must grapple with the provocative phenotype exhibited by embryos reduced in *grk* and *Egfr* activity. As first described by Schüpbach (Schüpbach, 1987), these ventralized embryos gastrulate with two ventral furrows instead of the single wild-type ventral furrow. The partial axis duplication is dictated by a change in the shape of the Dorsal gradient. In contrast to wild type, the gradient contains two peaks separated by a shallow ventral minimum (Roth and Schüpbach, 1994). This patterning could be occurring in the follicular epithelium, in which an initially broad ventral domain is refined into two smaller groups of cells, perhaps involving lateral inhibition, which then establish the two peaks of nuclear Dorsal (Roth and Schüpbach, 1994). Alternatively, patterning might be occurring within the perivitelline fluid of the embryo, involving the extracellular signaling pathway that leads to the ventral activation of Toll. We reasoned that addressing this issue would provide insights into the regulation of the wild-type Dorsal gradient.

In this paper, we describe experiments designed to analyze the respective contributions of the *gurken-Egfr* and *spätzle-Toll* signaling pathways to the shape of the Dorsal gradient. We confirm earlier observations that the *gurken-Egfr* pathway regulates the region of *pipe* expression (Sen et al., 1998). Although the domain of peak nuclear Dorsal in the embryo can vary over a wide range, and depends on the level of Spätzle production, it never exceeds the boundary defined by *pipe* expression. In embryos that express the highest levels of Spätzle, the shape of the ventral domain is changed from a single broadened peak to two distinct peaks of nuclear Dorsal, and thus resembles the ventralized phenotype caused by a reduction in the *grk* signal. These observations suggest that the patterning process leading to the reshaping of the Dorsal gradient occurs in the embryo, rather than in the follicular epithelium. Additional experiments suggest that refinement takes place at the level of the Spätzle processing reaction. We present a model that accounts for the production of two Dorsal peaks by invoking the action of a diffusible inhibitor, possibly the N-terminal domain of Spätzle, which is proposed to normally regulate the slope of the gradient.

MATERIALS AND METHODS

Fly stocks

The maternal effect mutations used in these studies have been described previously as follows: grk^{WG}, grk^{HG}, grk^{DC} and $Egfr^{1}$ (top^{1}) (Schüpbach, 1987); ea^{83l} (Jin and Anderson, 1990); and spz^{D1}, spz^{rm7} and $Df(3R)Ser^{+82f}$ (Morisato and Anderson, 1994).

DNA and RNA methods

The translational efficiency of the *spz* transcript was increased by replacing the 0.3 kb *Xba*I-*Not*I *spz* 3′UTR fragment with a 0.7 kb *Xba*I-*Not*I *bicoid* 3′UTR fragment. In the 1.9 kb *spz* cDNA (Morisato and Anderson, 1994), an *Xba*I site was introduced by oligonucleotide-directed mutagenesis at bp 1628 immediately 3′ to the coding region, thereby flanking the 0.3 kb *spz* 3′ UTR sequences with an *Xba*I site and a *Not*I site in the polylinker. In the *bicoid* cDNA c53.46.6 (Berleth et al., 1988), an *Xba*I site was introduced at the *Eco*RV site (bp 1725) and a *Not*I site at the *Eco*RI site (bp 2456). The 0.7 kb *bicoid* 3′ UTR contains the sequences responsible for localizing the *bicoid* transcript to the anterior pole. However, RNA localization is not germane to these experiments, as RNA transport normally occurs during oogenesis, while *spz-(bcd 3′UTR)* transcripts were injected posteriorly into 1.5-2.0 hour embryos. The relative level of Spätzle protein made from the *spz-(bcd 3′UTR)* RNA was compared with wild-type *spz* RNA in an injection assay. The *spz-(bcd 3′ UTR)* RNA rescued spz^{-} embryos to hatching at concentrations five- to tenfold lower than wild-type *spz* RNA.

The *N-spz* construct was generated by introducing a termination codon and *Xba*I site at bp 1305 by PCR. The truncated *spz*-coding region, lacking sequences that encode the C-terminal 106 amino acids, was subcloned into the *spz-(bcd 3′ UTR)* cDNA backbone.

Transcripts from the 1.9 kb *spz* cDNA, the *spz-(bcd 3′UTR)* cDNA, the spz^{D1}*-(bcd 3′UTR)* cDNA, and the *N-Spz* templates were synthesized by SP6 polymerase in vitro and injected into strongly dorsalized embryos. Recipient embryos were laid by females with the following genotypes: spz^{-} $(spz^{rm7}/Df(3R)\ Ser^{+R82f})$; $Egfr^{1}$; spz^{-}

(Egfr[1]; spz[rm7]/Df(3R) Ser[+R82f]); ea[83l] spz[−] (ea[83l] spz[rm7]/Df(3R) Ser[+R82f]).

In situ hybridization and antibody staining

Expression of *pipe* RNA in ovarian follicle cells was detected by in situ hybridization using digoxigenin-labeled antisense RNA probe synthesized from *pipe* cDNA, kindly provided by David Stein (University of Texas, Austin, TX). Hybridization and histochemical detection using alkaline phosphatase were carried out as described by Hong and Hashimoto (Hong and Hashimoto, 1995). Stage 10 egg chambers were embedded in Spurr (Polysciences), and cross-sections containing the oocyte nucleus were identified.

Expression of *sog* RNA in embryos was detected by an antisense RNA probe synthesized from *sog* cDNA, kindly provided by Ethan Bier (University of California, San Diego, CA). Hybridization and detection were carried out as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989). Young cellularized embryos were embedded in Spurr resin and sectioned every 10 μm.

Expression of Dorsal protein was detected with rabbit anti-Dorsal antibodies, kindly provided by Steve Wasserman (University of California, San Diego, CA). Expression of Twist protein was detected with rabbit anti-Twist antibodies, kindly provided by Siegfried Roth (Universität zu Köln). Primary antibodies were visualized with biotin-conjugated anti-rabbit antibodies and streptavidin-horseradish peroxidase (HRP) using Vectastain ABC (Vector Laboratories) as described by Patel (Patel, 1994). In the figures, embryos show brown staining (DAB precipitation) or dark-blue staining (DAB precipitation enhanced with NiCl). Embryos were embedded in Spurr resin and sectioned every 10 μm.

Injected embryos were fixed and individually dissected out of their vitelline membrane case before proceeding to in situ hybridization or antibody staining.

Preparation and analysis of *Drosophila* embryo extracts

Standard embryonic extracts were prepared as previously described (Morisato and Anderson, 1994) from embryos laid by females with the following genotypes: *spz[−] (spz[RPQ]/Df(3R) Ser[+R82f]); ea[−] (ea[4]/ea[5022rx1]); Toll[−] (Df(3R) Tl[5BREQ]/ Df(3R) Tl[9QRE])*. For sorted embryonic extracts, approximately 500 stage 4-5 embryos laid by wild-type (Oregon R) or *Egfr[1]* females were individually examined under Series 27 oil. Embryos were rinsed in heptane and approximately 30 μl of extracts (4 μg/μl) were prepared. Protein samples were separated on a 12.5% polyacrylamide gel and blotted to PVDF.

Rabbit antibodies were generated against bacterially produced Spätzle protein and affinity-purified against the N-terminal or C-terminal domain of Spätzle protein using previously described methods (Morisato and Anderson, 1994).

RESULTS

gurken-Egfr signaling determines the extent of the single domain of *pipe* expression

Establishment of the Dorsal gradient in the embryo is initiated by the *gurken-Egfr* pathway in the ovary. A reduction in *Egfr* signaling results in more embryonic nuclei that express high levels of Dorsal, leading to an expanded domain of Twist expression. With moderate and strong *grk* alleles, this enlarged ventral region exhibits two maxima in the Dorsal gradient, giving rise to two peaks of Twist expression (Fig. 1D-F; Roth and Schüpbach, 1994). We asked whether this change in the shape of the Dorsal gradient is determined during oogenesis (e.g. by lateral inhibition between groups of cells within the follicular epithelium), or at a subsequent step during embryogenesis.

To address this question, we first analyzed *pipe* expression, which is regulated by *Egfr* signaling. In wild-type stage 10 egg chambers, *pipe* RNA was expressed in the ventral 40% of the follicle cells that surround the oocyte (Fig. 1A; Sen et al., 1998). When the strength of the *grk* dorsalizing signal was reduced by mutations in the *Egfr* signaling pathway, more

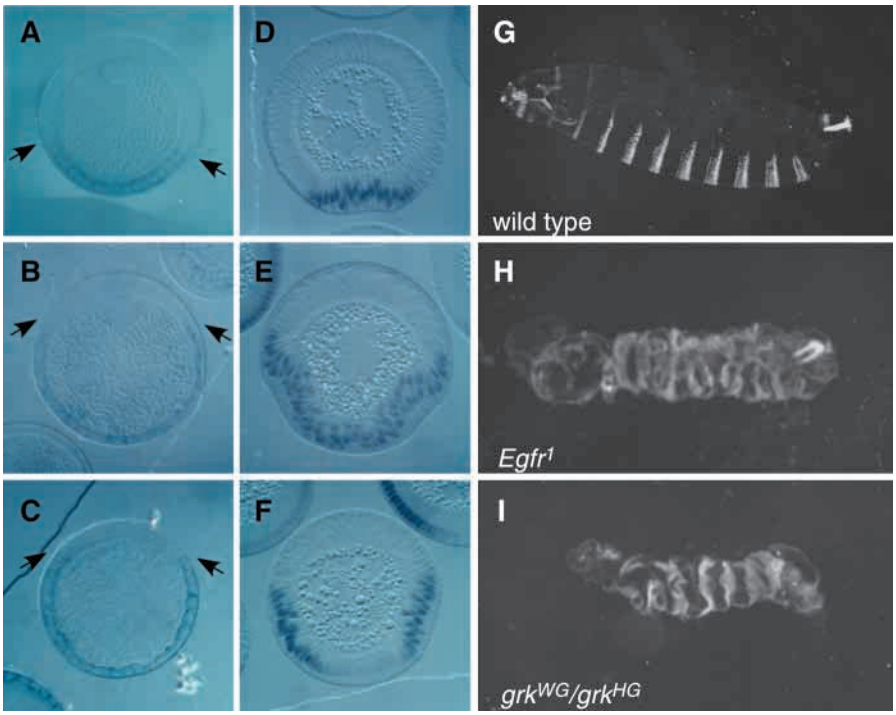

Fig. 1. Reduction in Egfr signaling expands the domain of *pipe* expression in the follicular epithelium. (A-C) Ovaries dissected from wild-type (A), *Egfr[1]/Egfr[1]* (B), and *grk[WG]/grk[HG]* (C) females were visualized for the expression of *pipe* RNA. Panels show cross-sections of an egg chamber in mid-oogenesis at the position of the oocyte nucleus. Arrows indicate the dorsal borders of staining. In B,C, the domain of *pipe* expression appears uniformly expanded, with no distinct peaks apparent. The dorsal side is upwards in all figures. (D-F) Gastrulating embryos were stained for Twist protein. Panels show cross-sections of embryos at 50% egg length. Wild-type embryos (D) gastrulate with a single ventral furrow. Embryos produced by *Egfr[1]* females (E) broaden the domain of cells expressing Twist, and a fraction gastrulate with two ventral furrows, as shown here. Embryos produced by *grk[WG]/grk[HG]* females (F) exhibit two distinct domains of cells that express Twist, and each domain invaginates as a distinct ventral furrow. (G-I) Cuticles of embryos produced by wild-type (G), *Egfr[1]/Egfr[1]* (H) and *grk[WG]/grk[HG]* (I) females were dissected out of their vitelline membrane cases and are shown in dark-field illumination. The images in H,I are magnified 1.5× relative to G.

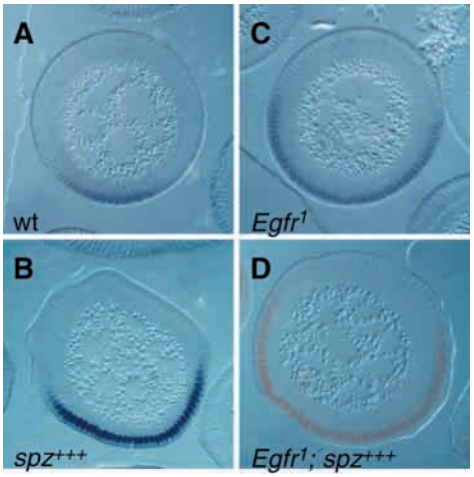

Fig. 2. Increasing *spätzle* dose expands the Twist domain. Young cellularized embryos were stained for Twist protein. Panels show cross-sections of embryos at 50% egg length. (A,B) In wild-type embryos (A), Twist is expressed in the ventral 20% of the embryo. In embryos laid by spz^{-} females that were injected with high levels of *spz* RNA (B), Twist expression is expanded to the ventral 40% of the embryo. (C,D) In embryos produced by $Egfr^{1}$ females (C), Twist is expressed in the ventral 50% of the embryo. In embryos laid by $Egfr^{1}; spz^{-}$ females that were injected with high levels of *spz* RNA (D), Twist expression is expanded to the ventral 70% of the embryo.

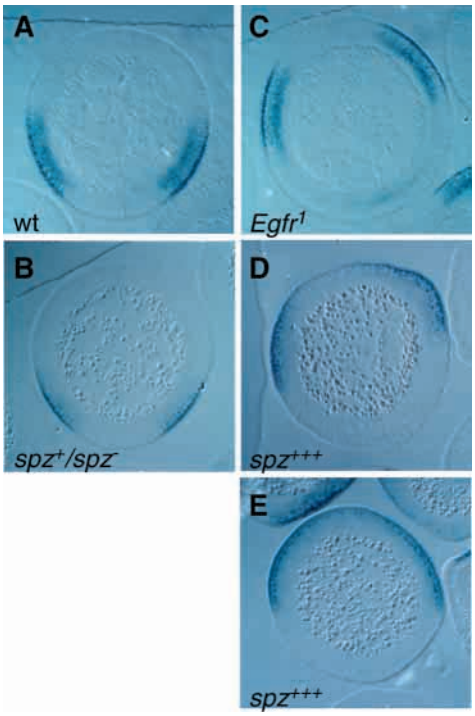

Fig. 3. Changing *spätzle* dose does not affect the size of the *sog* domain. Young cellularized embryos were hybridized to detect expression of *sog* RNA. Panels show cross-sections of embryos at 50% egg length. In wild-type embryos (A), *sog* RNA is expressed in two stripes that together occupy 31.5% of the embryo circumference. In embryos produced by spz^{+}/spz^{-} females (B), *sog* is expressed in 30% of the embryo. In embryos produced by $Egfr^{1}$ females (C), *sog* is expressed in 31% of the embryo. In most embryos laid by spz^{-} females that were injected with high levels of *spz* RNA (D), *sog* is expressed in two stripes occupying 33% of the embryo. In a few embryos injected with high levels of *spz* RNA (E), *sog* is expressed in a single domain, although the RNA level appears lower at the fusion border.

follicle cells adopted the ventral fate. Mutant ovaries ($Egfr^{1}$, grk^{WG}/grk^{HG}, and grk^{DC}/grk^{HG}) showed an increase in the number of *pipe*-expressing follicle cells (Table 1), with the extent of *pipe* RNA expansion corresponding to the strength of the allele (Fig. 1A-C), as characterized by the expansion of mesoderm and ventral structures in cuticles (Fig. 1G-I). The extent of *pipe* expression appeared as a single expanded domain and was never observed to form two distinct domains (Fig. 1B,C). Thus, pattern refinement does not appear to occur during oogenesis, but rather at a step after the establishment of *pipe* transcription.

pipe expression during oogenesis spatially restricts Spätzle action in embryogenesis

The ovarian follicle cells degenerate shortly after depositing the vitelline membrane and chorion around the oocyte. We asked how spatial information defined by the region of *pipe*-expressing ventral follicle cells was related to the domain of high nuclear Dorsal in the embryo determined hours later, by quantitating the domains of *pipe* and Twist expression. In the wild-type ovary, follicle cells occupying the ventral 40% of the egg chamber expressed *pipe* RNA (Fig. 1A). Later in development in the young wild-type embryo, 16-18 cells occupying 20% of the embryo circumference expressed high

Table 1. Spatial relationship between the ventral signal in the ovary and the embryo

Maternal genotype	Ovarian *pipe* expression (% oocyte circumference)*	*spz* dose‡	Embryonic Twist expression (% embryo circumference)§
Wild type	40±3 (*n*=12)	+/+	20±1 (*n*=18)
		+++	43±3 (*n*=13)
		+/–	15±2 (*n*=21)
$Egfr^{1}/Egfr^{1}$	71±3 (*n*=15)	+/+	49±3 (*n*=16)
		+++	70±3 (*n*=12)
grk^{WG}/grk^{HG}	82±2 (*n*=21)	+/+	54±3 (*n*=14)
grk^{DC}/grk^{HG}	85±3 (*n*=11)		

*The % oocyte circumference is the number of follicle cells expressing *pipe* RNA divided by the total number of follicle cells around the egg chamber circumference (38-44 cells).

‡High (+++) *spz* dose was achieved by injecting *spz* RNA synthesized in vitro into embryos laid by spz^{-} or $Egfr^{1}; spz^{-}$ females (see Materials and Methods).

§The % embryo circumference is the number of cells expressing nuclear Twist divided by the total number of cells in the embryo circumference (78-85 cells) at 50% egg length.

nuclear Dorsal, as assessed by the activation of Twist (Fig. 2A). In other words, there was a substantial narrowing of the ventral domain defined during oogenesis and the corresponding domain of high nuclear Dorsal in the embryo. With mutations in the *Egfr* signaling pathway (*Egfr*^{1} and *grk*^{WG}/*grk*^{HG}), a similar narrowing of the ventral signal was observed (Table 1). For example, in *Egfr*^{1} ovaries, *pipe* RNA was expressed in the ventral 70% of the egg chamber (Fig. 1B); in embryos resulting from these mutant ovaries, Twist was expressed in the ventral 50% of the embryo circumference (Fig. 2C).

In order to further investigate how spatial information generated in the ovary is used to pattern the embryo, we assessed the effect of changing *spz* dose on the shape of the Dorsal gradient. An increase in *spz* dose was achieved by injecting *spz* RNA synthesized in vitro into pre-cellular embryos laid by *spz*$^{-}$ females. The timing of injection defined the period within embryonic development when *spz* must be acting (between injection at 1.5 hours and cellularization at 3 hours), and prevented a possible effect of *spz* overexpression on oogenesis. In all the injection experiments described here, we used embryos laid by *spz*$^{-}$ rather than wild-type females to avoid possible complications arising from the timing of translation of endogenous and injected *spz* transcripts.

The dorsalized phenotype produced by the absence of maternal *spz* activity can be rescued by the injection of *spz* RNA into the embryo. When moderate levels of *spz* RNA (0.5 mg/ml) are injected into embryos laid by *spz*$^{-}$ females, these embryos express Twist in a wild-type domain and proceed to hatch (Morisato and Anderson, 1994; data not shown). In comparison, injection of higher concentrations of *spz* RNA (2-5 mg/ml) expanded the Twist domain to about 40% of the embryo circumference (Fig. 2B; Table 1). Injection of *spz* RNA into embryos laid by *Egfr*^{1}*; spz*$^{-}$ females expanded the Twist domain to about 70% of the embryo circumference (Fig. 2D; Table 1). Thus, in each case, the Twist domain expanded to, but did not exceed, the boundary defined by *pipe* expression in the ovarian follicle cells. We interpret these observations to mean that the domain of *pipe* expression establishes the spatial limits of the Spätzle processing machinery or binding of processed Spätzle to Toll.

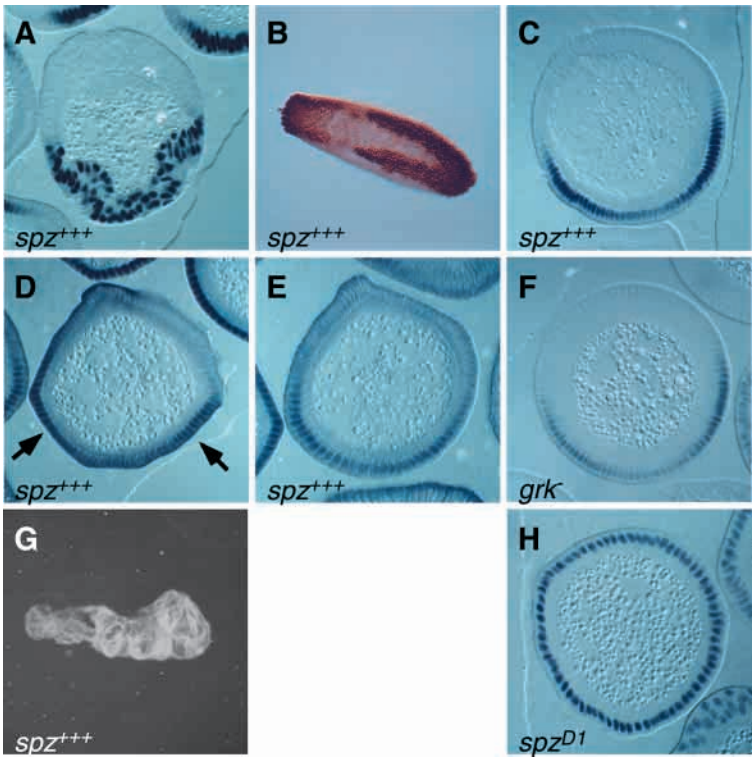

Fig. 4. High levels of Spätzle can generate two peaks of nuclear Dorsal and Twist expression. (A) Embryos laid by *spz*$^{-}$ females were injected with *spz* RNA and stained for Twist protein. Note the invagination of two ventral furrows. (B,C) Embryos laid by *spz*$^{-}$ females were injected with *spz-(bcd 3′UTR)* RNA and stained for Twist protein. (B) Two stripes of Twist are visible in this embryo, viewed from the ventral side. In cross-section (C), this embryo with two peaks of Twist expression is virtually indistinguishable from the embryo shown in F. (D,E) Embryos laid by *spz*$^{-}$ females were injected with *spz-(bcd 3′UTR)* RNA and stained for Dorsal protein. In a young embryo (D), the two peaks of nuclear Dorsal (arrows) are separated by several cells in which Dorsal protein is present in both the nucleus and the cytoplasm. (The region of non-staining (upper right) was caused by the incomplete removal of vitelline membrane before incubation with antibodies.) In an older embryo (E), the two peaks of nuclear Dorsal are clearly visible, as cytoplasmic Dorsal protein has been degraded. (F) Young cellularized embryos produced by *grk*^{WG}/*grk*^{HG} females were stained for Twist protein. Two peaks of Twist expression are apparent in this cross-section. (G) Cuticle of embryo laid by *spz*$^{-}$ female and injected with *spz-(bcd 3′UTR)* RNA is strongly ventralized. (H) Embryos laid by *spz*$^{-}$ females were injected with *spz*^{D1}*-(bcd 3′UTR)* RNA and stained for Twist protein. Twist is expressed uniformly around the embryo circumference.

At subsaturating concentrations of *spz*, the initial region of *pipe* expression in the ovary is transformed into a narrower domain of peak Dorsal nuclear translocation in the embryo, as indicated by the characterization of embryos laid by wild-type females. Further, when the *spz* dose was halved, the Twist domain was reduced from 20% to 15% (Table 1; ventral domain bounded by *sog* in Fig. 3B). In other words, embryos derived from *spz*$^{+}$/*spz*$^{-}$ females were capable of generating a narrower peak of the Dorsal gradient than that found in wild-type embryos, starting with the same broad stripe of *pipe* RNA. These embryos proceeded to develop and hatch, indicating that there are corrective mechanisms downstream of the Dorsal gradient capable of generating a final 'wild-type' pattern.

These experiments demonstrated that the size of the ventral Twist domain could vary quite dramatically as a function of the amount of *spz* expressed in the embryo. In contrast, the slope of the Dorsal gradient remained relatively constant. Slope was measured by counting the number of cells expressing *sog* RNA to determine the number of nuclei lying between high nuclear Dorsal and no nuclear Dorsal within the gradient (François et al., 1994). In young wild-type embryos, *sog* RNA was expressed in two stripes of 12-14 cells flanking the Twist domain, occupying 31.5% of the embryo circumference (Fig. 3A). A change in *spz* dose shifted the relative positions of the two *sog* stripes, but did not significantly affect the domain of expression (Fig. 3B-D; Table 2). For example, 90% of the embryos injected with high levels of *spz* RNA showed two stripes of *sog*-expressing cells occupying 33% of the embryo

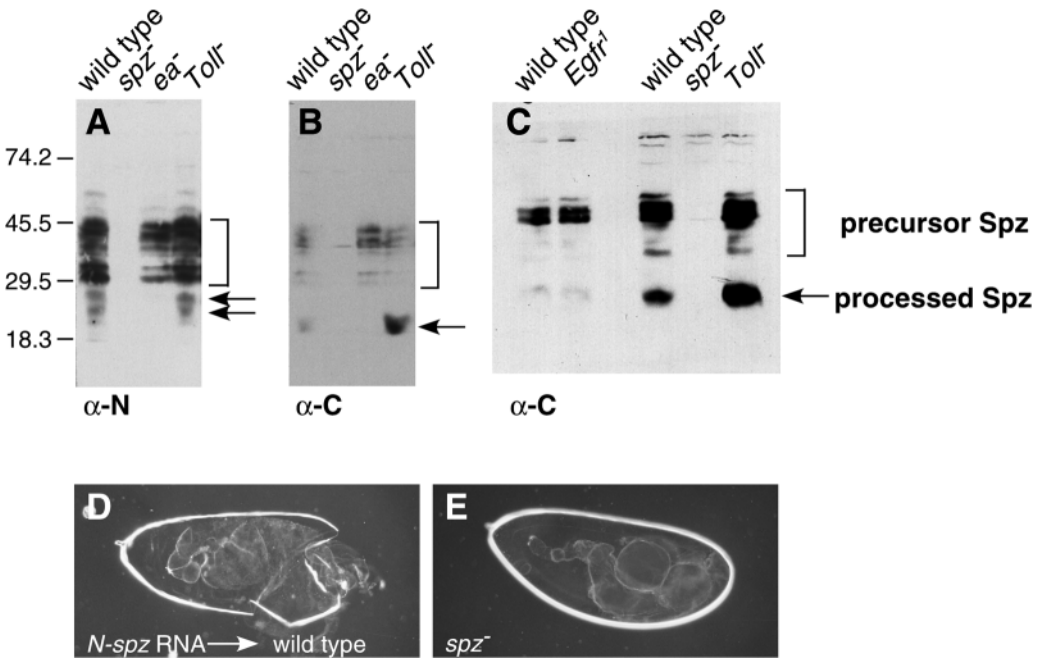

Fig. 5. The Spätzle processing reaction in wild-type and *Egfr*^{1} embryos. (A) Extracts were prepared from 0-4 hour embryos laid by wild-type, *spz*$^{-}$, *ea*$^{-}$, and *Toll*$^{-}$ females. Proteins were separated on a 12.5% polyacrylamide gel and transferred to PVDF. The mobility of molecular weight markers is shown on left. The immunoblot was probed with antibodies directed against the N-terminal half of Spätzle protein. Full-length forms of Spätzle are marked by the bracket. Prominent proteolytically processed forms of N-terminal Spätzle, present in wild-type embryos and absent in embryos laid by *ea*$^{-}$ females, are indicated by arrows (migrating as 23 and 27 kDa proteins). (B) An immunoblot with the same samples as in A was probed with antibodies directed against the C-terminal half of Spätzle protein. The arrow marks the proteolytically processed form of C-terminal Spätzle (migrating as a 21 kDa protein). (C) On the left, extracts were prepared from sorted stage 4-5 embryos laid by wild-type or *Egfr*^{1} females. On the right, conventional extracts were prepared from 0-4 hour embryos laid by wild-type, *spz*$^{-}$, and *Toll*$^{-}$ females. The immunoblot was probed with antibodies directed against the C-terminal half of Spätzle protein. (D) Cuticle of embryo laid by wild-type female and injected with *N-spz* RNA is strongly dorsalized. The bright outline is the vitelline membrane. (E) Cuticle of dorsalized embryo laid by *spz*$^{-}$ female.

circumference (Fig. 3D). The observation that the size of the *sog* domain remains relatively constant over such a broad range of *spz* concentrations suggests that the slope of the Dorsal gradient is normally regulated (see Discussion).

Pattern duplication occurs downstream of Spätzle expression

Embryos injected with high concentrations of *spz* RNA expressed Twist in a single broad domain (Fig. 2B), as described in the previous section. A small portion of these injected embryos invaginated two ventral furrows (Fig. 4A), and were remarkably similar to the phenotype observed in embryos produced by *Egfr*^{1} females.

In order to further increase the level of Spätzle protein production, the endogenous *spz* 3′ untranslated region (UTR) was replaced with the *bicoid* 3′ UTR to increase translational efficiency. Injection of RNA synthesized from this *spz-(bcd 3′ UTR)* template produced higher levels of Spätzle protein (see Materials and Methods), resulting in embryos that showed two distinct peaks within the Twist domain (Fig. 4B,C; Table 3). There was no detectable difference in the overall size of the Twist domain (40% embryo circumference) in embryos exhibiting two peaks, compared with embryos expressing one expanded domain. These peaks of Twist staining indeed reflected two maxima in the Dorsal gradient (Fig. 4D,E). Injected embryos differentiated cuticles with expanded mesoderm and ventral structures (Fig. 4G) that resembled embryos produced by *grk* females (Fig. 1I).

Table 2. Effect of *spätzle* dose on the size of the *sog* domain

Egfr allele	*spz* dose*	*sog* expression in embryo (% embryo circumference)‡
Wild type	+/+	31.5±2 (*n*=10)
	+/–	30±2 (*n*=21)
	+++	33±3 (*n*=18)
Egfr^{1}/*Egfr*^{1}	+/+	31±1 (*n*=11)

*High (+++) *spz* dose was achieved by injecting *spz* RNA synthesized in vitro into embryos laid by *spz*$^{-}$ females (see Materials and Methods).

‡The % embryo circumference is the number of cells expressing *sog* RNA divided by the total number of cells in the embryo circumference (78-85 cells) at 50% egg length.

The ventralized embryos produced by Spätzle overexpression bore a striking resemblance to the ventralized embryos produced by *grk*^{WG}/*grk*^{HG} females (compare Fig. 4C with 4F). In particular, the ventral minimum lying between the two peaks was relatively shallow in young cellular blastoderm embryos; the minimum became deeper in older cellularized and gastrulating embryos (Fig. 4D,E). These observations suggest that formation and maintenance of the Dorsal gradient is dynamic, capable of change over a period of time (see Discussion).

In summary, these experiments demonstrate that the pattern refinement that generates two maxima in the Dorsal gradient acts in the embryo, rather than in the ovarian follicle cells. Furthermore, they argue that the patterning process occurs downstream of the production of Spätzle protein.

Pattern refinement appears to act upstream of Toll

Embryos that are constitutively activated for *Toll* signaling (by carrying the *Toll*^{10b} mutation) express Twist around the embryo circumference (Leptin and Grunewald, 1990). The failure to

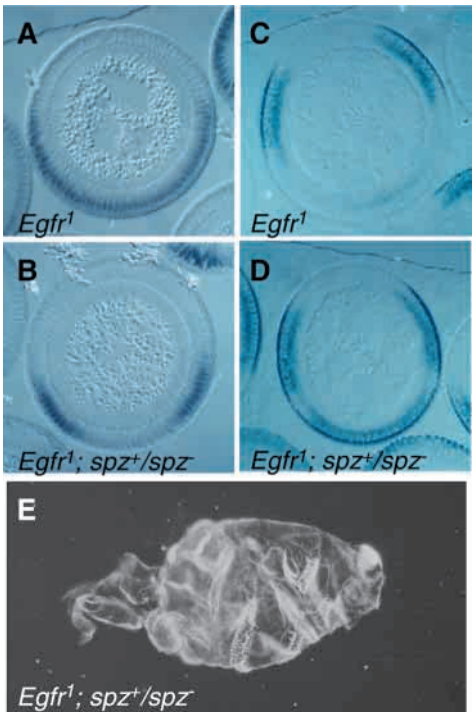

Fig. 6. Reduction of *spätzle* dose in *Egfr*^{1} background can generate two Twist peaks. (A,B) Cellularized embryos were stained for Twist protein. In embryos produced by *Egfr*^{1} females (A), Twist is expressed in the ventral 50% of the embryo. In embryos produced by *Egfr*^{1}*; spz*$^{+}$*/spz*$^{-}$ females (B), Twist is expressed in two stripes. (C,D) Young cellularized embryos were hybridized to detect expression of *sog* RNA. In embryos produced by *Egfr*^{1} females (C), *sog* is expressed in two stripes flanking the ventral Twist domain. In embryos produced by *Egfr*^{1}*; spz*$^{+}$*/spz*$^{-}$ females (D), *sog* expression is expanded ventrally, but is absent in two domains where Twist is expressed. (E) Cuticle produced by embryos laid by *Egfr*^{1}*; spz*$^{+}$*/spz*$^{-}$ females.

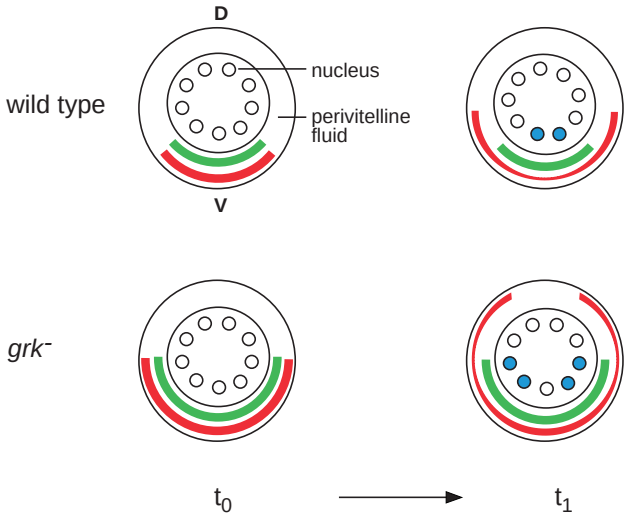

Fig. 7. Model for generating two peaks of nuclear Dorsal. This cartoon depicts events within the perivitelline space at two different time points for embryos laid by wild-type and *grk*$^{-}$ females. The Spätzle cleavage reaction is hypothesized to produce an inhibitor (red), in addition to activated Spätzle (green). If processed Spätzle and the inhibitor possess different diffusion rates, the relative distribution of the two molecules would change from t_0 to t_1, giving rise to the final shape of the gradient characterized by one or two peaks of high nuclear Dorsal (blue).

observe two Twist peaks in these embryos suggests that refinement cannot be carried out by the cytoplasmic signaling pathway downstream of *Toll*. We investigated the effect of enlarging the region of activated wild-type Toll by taking advantage of two mutations that broaden the distribution of processed Spätzle. We reasoned that if pattern refinement were occurring by communication between receptor molecules, we should be able to observe refinement of an expanded domain into two or more peaks.

In the first case, we used a dominant ventralizing *easter* mutation, *ea*^{83l}. In embryos laid by *ea*^{83l}*/ea*$^{+}$ females, Easter protease activity is spatially misregulated, leading to an expansion of ventrolateral fates (Jin and Anderson, 1990; A. J. Chang and D. M., unpublished). Injection of high levels of *spz-(bcd 3′UTR)* RNA into a recipient embryo derived from *ea*^{83l} *spz*$^{-}$ females resulted in significant expansion of the Twist domain compared with wild type (70-75% of embryo circumference). However, embryos that refined this broadened domain into two peaks were never detected (Table 3).

In the second case, we analyzed the consequence of overexpressing the protein encoded by a dominant lateralizing *spz* mutation, *spz*^{D1}. Embryos produced by *spz*^{D1}*/spz*$^{-}$ females lack detectable dorsal-ventral polarity (Morisato and Anderson, 1994). The *spz*^{D1} mutation maps to the C-terminal cystine knot motif, and is predicted to affect the interaction between processed Spätzle and Toll (L.-K. Chiang and D. M., unpublished). Injection of high levels of *spz*^{D1}*-(bcd 3′UTR)* RNA into a *spz*$^{-}$ recipient resulted in uniform Twist staining around the embryo circumference (Fig. 4H), demonstrating that the mutant protein produced contains sufficient activity to generate high levels of nuclear Dorsal (manifested by Twist expression). However, embryos that exhibited refinement to more than one peak were never detected (Table 3).

The failure to observe two peaks within a broadened Twist domain suggested that simply expanding the region of high nuclear Dorsal is not sufficient to induce pattern refinement. Furthermore, pattern refinement requires *ea* and *spz*, the same activities that are involved in generating a wild-type slope of the Dorsal gradient. These experiments argue that patterning occurs upstream of *Toll*, either at the level of the Spätzle processing reaction or binding of processed Spätzle to Toll. This interpretation is in agreement with earlier studies involving cytoplasmic transplantations into *Toll*$^{-}$ embryos, which argued against a *Toll*-dependent local self-enhancement or lateral inhibition process (Roth, 1993).

The Spätzle processing reaction generates both an activator and inhibitor of signaling

The transition from a single broadened domain to two distinct peaks observed during pattern refinement could be explained by invoking the activity of a hypothetical inhibitor. In the wild-type embryo, such an inhibitor would be responsible for narrowing the relatively broad ventral domain established during oogenesis to the stripe of high nuclear Dorsal, and for regulating the gradient slope (see Discussion). In the Spätzle

Table 3. Characterization of embryos showing an expanded Twist domain

Maternal genotype		% embryos with one Twist domain	% embryos with two Twist peaks
Wild type (*n*=50)		100	0
Egfr^{1}/Egfr^{1} (*n*=50)		100	0
grk^{WG}/grk^{HG} (*n*=60)		52	48
Maternal genotype of injected embryos	RNA injected	% embryos with one Twist domain	% embryos with two Twist peaks
spz^{rm7}/Df(3R)			
(*n*=119)	*spz-(bcd 3′UTR)* (1 mg/ml)	80	20
(*n*=117)	*spz-(bcd 3′UTR)* (10 mg/ml)	79	21
ea^{83l} spz^{rm7}/Df(3R)			
(*n*=92)	*spz-(bcd 3′UTR)* (1 mg/ml)	100	0
(*n*=62)	*spz-(bcd 3′UTR)* (10 mg/ml)	100	0
spz^{rm7}/Df(3R)			
(*n*=106)	*spz^{D1}-(bcd 3′UTR)* (1 mg/ml)	100	0
(*n*=72)	*spz^{D1}-(bcd 3′UTR)* (10 mg/ml)	100	0

processing reaction, Easter cleavage generates N-terminal and C-terminal processed Spätzle forms. Although C-terminal processed Spätzle has been shown to be directly required for activating Toll (Morisato and Anderson, 1994; Schneider et al., 1994; DeLotto and DeLotto, 1998; G. Denton and D. M., unpublished), no activity has been ascribed to N-terminal Spätzle. We tested whether N-terminal Spätzle could act as an inhibitor of signaling.

In the embryo, alternative splicing of the *spz* gene results in the production of a number of proteins (indicated by the bracket in Fig. 5A) that diverge in the N terminus, while sharing a common C terminus containing the cystine knot motif (Morisato and Anderson, 1994; L.-K. Chiang and D. M., unpublished). When embryonic extracts were probed with anti-N-Spätzle antibodies (Fig. 5A), several proteins corresponding to the N-terminal products of the Spätzle processing reaction were identified. When embryonic extracts were probed with anti-C-Spätzle antibodies (Fig. 5B), a single processed form was observed. This C-terminal processed Spätzle was present at much higher levels in embryos lacking Toll, as reported earlier and interpreted to result from the absence of receptor-mediated uptake and degradation (Morisato and Anderson, 1994). The lack of an effect on the level of N-terminal Spätzle by Toll suggests that the products of the cleavage reaction are not physically associated and undergo different fates. Consistent with this interpretation, when N-terminal Spätzle was affinity purified from embryonic extracts, cleaved C-terminal Spätzle was not co-purified (M. Byrne and D. M., unpublished). These observations raise the possibility that the N- and C-terminal forms have different functions in signaling.

To ask whether N-terminal Spätzle could exhibit dorsalizing activity, we constructed a template for generating truncated Spätzle protein *(N-Spz)*, based on the site of Easter cleavage (DeLotto and DeLotto, 1998; Materials and Methods). When *N-Spz* RNA was injected into wild-type embryos (*n*=154 with 1 mg/ml *N-Spz* RNA; *n*=214 with 5 mg/ml *N-Spz* RNA), nearly all of the developing embryos showed evidence of dorsalization. Embryos that were injected at the earliest pre-cellular stages showed the most significant effects, gastrulating as strongly dorsalized embryos, and developing dorsalized cuticle (Fig. 5D) virtually indistinguishable from embryos lacking maternal *spz* (Fig. 5E). Not surprisingly, the dorsalizing activity of N-terminal Spätzle appeared to be weaker than the ventralizing activity of C-terminal Spätzle, as assessed by experiments co-injecting varying concentrations of full-length *spz* and *N-Spz* RNA into embryos laid by *spz*$^{-}$ females (data not shown). These experiments suggest a possible role for N-terminal processed Spätzle in modulating the shape of the Dorsal gradient.

Pattern refinement does not require increased Spätzle processing

The partial axis duplication caused by Spätzle overexpression raises the possibility that the *grk* phenotype also arises from an increase in the amount of activated Spätzle. Such elevation might occur at the level of the proteolytic processing reaction (e.g. by controlling the initiation of the protease cascade) or, more indirectly, at the level of translational regulation of the Spätzle precursor. In either case, pattern refinement to two peaks in this scenario would be a consequence of excessively high levels of Spätzle processing. In fact, results from two experiments show that pattern refinement does not require increased Spätzle processing, and imply that partial axis duplication involves mechanisms that generate the wild-type Dorsal gradient.

First, we directly compared the level of precursor and processed Spätzle proteins in embryos laid by wild-type and *Egfr^{1}* females. As *Egfr* signaling is also required for properly patterning the eggshell, a large fraction of eggs laid by strong *grk* alleles are unfertilized because they lack a properly formed micropyle. To address this problem, we identified individual precellular blastoderm stage embryos laid by wild-type and *Egfr^{1}* females. We prepared embryonic extracts from these embryos and probed immunoblots with anti-C-Spätzle antibodies (Fig. 5C). The level of both precursor and processed Spätzle proteins in *Egfr^{1}* embryos appeared comparable with the amount detected in wild-type embryos. This result suggests that the ventralized phenotype caused by the *Egfr^{1}* mutation, and in stronger *grk* alleles by implication, arises from a broadened spatial distribution of the ventral signal, rather than from an increase in the overall level of processed Spätzle.

Second, we determined the effect of changing *spz* dose in an *Egfr^{1}* background. A fraction of the embryos laid by *Egfr^{1}* females clearly have two peaks in the Dorsal gradient, as they

invaginate two ventral furrows (Fig. 1E). Such embryos show two peaks of *folded gastrulation* expression, which is restricted to a subdomain of the presumptive mesoderm, and is required for ventral furrow invagination (Costa et al., 1994). Yet these embryos do not show two peaks of Twist expression, apparently because the Dorsal concentration in the ventral minimum is sufficiently high to activate Twist. When the level of *spz* expression was increased by injecting *spz-(bcd 3′ UTR)* RNA into embryos laid by $Egfr^1$; spz^- females, these embryos showed a greatly expanded Twist domain, as described earlier (Fig. 2D).

In contrast, a reduction in *spz* dose caused a refinement of the broadened Twist domain to two distinct peaks (Fig. 6A,B). The majority of embryos laid by $Egfr^1/Egfr^1$; spz^+/spz^- females showed two weakly staining Twist stripes, demonstrating that pattern refinement could occur even at low levels of *spz* expression. In these embryos, *sog* was newly expressed in the domain lying between the two Twist stripes (Fig. 6C,D), demonstrating the creation of two maxima in the Dorsal gradient. With a reduction in *spz* dose, these embryos failed to invaginate ventral furrows, and the cuticles they differentiated showed a reduction in ventral fates as compared to embryos laid by $Egfr^1$ females (Fig. 6E).

Pattern refinement caused either by a reduction in *Egfr* signaling or by the overproduction of precursor Spätzle is observed only when the domain of peak nuclear Dorsal is expanded beyond the wild-type size. This condition could be primarily spatial, in that refinement requires a minimum distance between the two peaks. Alternatively, this condition could be an indirect effect of the requirement for high levels of Spätzle processing. The results described here show that refinement can occur even when Spätzle production is at or below wild-type levels, if the distribution of the ventral signal is broadened beyond its normal restricted domain. These observations suggest that pattern refinement is determined by a delicate balance between the domain of the Spätzle processing machinery (as determined by *pipe* expression) and the level of Spätzle proteolytic cleavage.

DISCUSSION

The Dorsal gradient in the wild-type embryo possesses a characteristic shape. We show here that the domain of peak nuclear Dorsal in the embryo can vary over a wide range, depending on the level of Spätzle production, but never exceeds the limits presaged by the expression of *pipe* in the ovary. In contrast, the slope of the Dorsal gradient, as measured by the extent of *sog* expression, is relatively constant under these conditions.

The shape of the Dorsal gradient is dramatically changed in embryos laid by females carrying mutations in the *gurken-Egfr* signaling pathway. Not only do these embryos expand Twist expression, as a consequence of a reduction in the dorsalizing signal that establishes egg chamber asymmetry, but they exhibit two distinct peaks within the Twist domain that give rise to two ventral furrows. In the experiments described here, this partial axis duplication is not evident during oogenesis, as *pipe* RNA was found to be expressed in a single broad domain in follicle cells. The production of two Dorsal peaks could be mimicked by injecting high levels of *spz* RNA into the pre-cellular embryo cytoplasm, suggesting that pattern refinement occurs during embryogenesis. We suggest that while the size of the ventral domain is expanded in *grk* and *Egfr* ovarian egg chambers, the partial axis duplication observed in mutant embryos is caused by reactions occurring later in the embryo.

It may have been easier to imagine how the selection of one or two gradient peaks would involve signaling within the follicular epithelium, because spatial information could then be stably maintained and transmitted by cells. The elaboration of the two dorsal appendages in the *Drosophila* eggshell results from a series of such intercellular signaling events. Activation of Egfr by Gurken stimulates transcriptional induction of Argos, a secreted Egfr inhibitor, which then downregulates Egfr activity in the initial central domain, leaving two lateral domains of signaling (Wasserman and Freeman, 1998).

In fact, the findings described in this paper argue that events involving the diffusion of an extracellular morphogen not only regulate the gradient slope, but perhaps unexpectedly, determine the position and number of maxima within the axis in response to the broad cues generated during oogenesis. Reaction-diffusion models have been applied to analyze the respective contributions of the *gurken-Egfr* and *spätzle-Toll* pathways in generating embryonic pattern (Meinhardt, 1989). The current studies provide experimental support for this theoretical work, and present opportunities for understanding the underlying mechanisms.

Formation and maintenance of the Dorsal gradient appear dynamic. The shape of the Dorsal gradient in the wild-type embryo does not change markedly after nuclear translocation is first detected. In embryos laid by *grk* females or embryos expressing high levels of Spätzle, however, the shape of the Dorsal gradient is subtly modified. In particular, the minimum lying between the two Dorsal peaks becomes deeper in older embryos (Fig. 4). This observation suggests that signaling takes place over a period of time, and explains how an initial asymmetry, in the form of the broad stripe of *pipe*, might be gradually refined into a gradient of positional information.

We present the following model, which accounts for many of the observations described above. The initial shape of the gradient (at t_0) is established by the proteolytic activation of Spätzle in a relatively broad domain, reflecting the ventral region of the egg chamber that expresses *pipe* RNA (Fig. 7). We propose that the Spätzle processing reaction generates an inhibitor that negatively regulates the production of the ventral signal, possibly at the level of Easter protease activity or the interaction between processed Spätzle and Toll. Whereas processed C-terminal Spätzle is believed to bind to Toll quickly and show limited movement after cleavage, we postulate that the hypothetical inhibitor undergoes broader diffusion. (The term diffusion in this discussion indicates relative movement within the aqueous perivitelline space, and does not imply usage in a chemically more rigorous sense.) In the wild-type embryo, inhibitor action is responsible for establishing the region of high nuclear Dorsal, corresponding to the Twist domain, to be narrower than the ventral region of the egg chamber expressing *pipe* RNA. The final shape of the Dorsal gradient (at t_1) is generated over time by the opposing effects of processed Spätzle and the inhibitor.

In embryos produced by *grk* females, we infer that Spätzle processing is occurring at wild-type levels, but the reaction is distributed over a broader domain. The ventral region becomes

sufficiently expanded such that the difference between the diffusion rates of processed Spätzle and the inhibitor can reshape the ventral domain itself. In particular, rapid diffusion of the inhibitor results in a lower concentration at each border, compared with the center of the domain. This change in the ratio of processed Spätzle to inhibitor eventually produces a peak at each border of the expanded domain. By this reasoning, an expanded ventral domain never generates more than two peaks because there are never more than two borders.

Embryos synthesizing high levels of precursor Spätzle increase the amount of processed Spätzle, thereby expanding the domain of high nuclear Dorsal. In contrast to embryos produced by *grk* females, where a wild-type level of processed Spätzle is distributed over a broader area, an increased level of processed Spätzle appears to generate a broader domain in these injected embryos. Pattern refinement is observed only at the highest levels of Spätzle production, perhaps because only in this situation can the minimum domain size be created.

The complexity of the patterning process is underscored by the observation that partial axis duplication can be induced by both an increase and decrease in *spz* dosage, depending on the extent of *pipe* expression dictated by *gurken-Egfr* signaling. A deeper understanding of this dynamic behavior will probably require the application of mathematical approaches.

In order to explain the production of two Dorsal peaks, the inhibitor must be generated in a spatially asymmetric manner. In the model outlined here, inhibitor production has been linked to the proteolytic processing of Spätzle to satisfy this condition. The results described above raise the possibility that N-terminal processed Spätzle is acting as an inhibitor to shape the Dorsal gradient, although the model does not exclude action of other negative regulators. For example, pattern refinement may involve maternal Dpp signaling, acting parallel or downstream of Toll, which has recently been shown to reduce the magnitude of Dorsal nuclear translocation (Araujo and Bier, 2000). At the mechanistic level, the N-Spätzle inhibitor could be recruiting molecule X, found in a stable complex with Easter and suggested to be a serpin (Misra et al., 1998), or it could be acting on one of the proteases that act upstream of Easter. Alternatively, the inhibitor could be negatively regulating the binding of processed Spätzle to Toll.

The strongest genetic support for diffusion playing a role in the formation of the Dorsal gradient comes from the mosaic analysis of Nilson and Schüpbach (Nilson and Schüpbach, 1998). Ventral *pipe*$^{-}$ clones not only resulted in the absence of Twist expression, but also produced a corresponding loss of *sog* expression in lateral regions of the embryo. These results rule out the presence of a pre-existing gradient in the follicular epithelium. The requirement for a ventral source of the Dorsal gradient suggested that the slope is generated by the diffusion of a component in the embryonic signaling pathway, although a sequential induction mechanism within the follicular epithelium was not formally excluded.

An output dependent on the ratio of the respective diffusion rates of activator and inhibitor, rather than diffusion of the activator alone, may allow the embryo to generate a more stable gradient shape in response to the broad spatial signals defined during oogenesis. Moreover, such a mechanism may help the embryo cope with changes in perivitelline fluid viscosity, caused by fluctuations in temperature and humidity after egg deposition, that would otherwise result in developmental defects. Coupling diffusion of an activator and inhibitor may represent a general strategy for regulating extracellular signaling in other patterning reactions.

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