EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the Drosophila embryonic ventral ectoderm

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SUMMARY

The induction of different cell fates along the dorsoventral axis of the Drosophila embryo requires a graded activity of the EGF receptor tyrosine kinase (DER). Here we have identified primary and secondary target genes of DER, which mediate the determination of discrete ventral cell fates. High levels of DER activation in the ventralmost cells trigger expression of the transcription factors encoded by ventral nervous system defective (vnd) and pointed P1 (pntP1). Concomitant with the induction of pntP1, high levels of DER activity lead to inactivation of the Yan protein, a transcriptional repressor of Pointed-target genes. These two antagonizing transcription factors subsequently control the expression of secondary target genes such as otd, argos and tartan. The simultaneous effects of the DER pathway on pntP1 induction and Yan inactivation may contribute to the definition of the border of the ventralmost cell fates.

Key words: Drosophila, EGF receptor, Pointed, Yan, spitz group, Cell fate, ventral nervous system defective

INTRODUCTION

The Drosophila EGF receptor (DER), a receptor tyrosine kinase, is required in multiple sites during development. In some contexts DER was shown to mediate binary decisions, e.g. the generation of posterior follicle cell fates (González-Reyes et al., 1995; Roth et al., 1995). In other cases however, different levels of DER activity are correlated with the induction of multiple cell fates in a given tissue. High levels of DER activity are required for the formation of wing veins, while intermediate levels are responsible for determining cell morphology and size in the intervein regions (Diaz-Benjumea and Hafen, 1994). During embryonic development the DER cascade represents the zygotic pathway responsible for patterning the embryonic ventral ectoderm. High levels of DER activity induce the ventralmost cell fates, while lower levels are required to induce ventrolateral cell fates (Schweitzer et al., 1995b).

In view of the ability of the DER pathway to induce several cell fates within a given tissue, it is important to elucidate the mechanisms responsible for the formation and interpretation of graded DER activity. Analysis of DER signaling has been aided by the fact that the activity of RTKs is mediated via the common, well conserved Ras signaling cascade. Within the cell, activation of the Sos/Ras/MAP kinase pathway by DER has been demonstrated (Diaz-Benjumea and Hafen, 1994; Schweitzer et al., 1995b).

Further understanding of DER regulation was facilitated by the identification of a class of mutants (termed the spitz group) that display similar phenotypes to DER and genetically interact with it (Mayer and Nüsslein-Volhard, 1988; Sturtevant et al., 1993; Raz and Shilo, 1993). Spitz is a DER ligand, which is produced as a transmembrane precursor (Rutledge et al., 1992). Its processing to generate the active, secreted form is the limiting event in several instances of DER function (Schweitzer et al., 1995b). Rhomboid and Star, two novel transmembrane proteins (Bier et al., 1990; Kolodkin et al., 1994), modulate the level of DER activity, possibly by facilitating Spitz processing (Schweitzer et al., 1995b).

Finally, based on the analysis of the mutant cuticle phenotype, a requirement for pointed (pnt) in patterning the embryonic ventral ectoderm, as a member of the spitz group, has been suggested (Mayer and Nüsslein-Volhard, 1988). The pnt gene harbors two promoters separated by 50 kb, which generate two alternative transcripts encoding transcription factors of the ETS family: Pointed P1 acts as a constitutive transcriptional activator, while Pointed P2 requires phosphorylation by MAP kinase to become a potent transcriptional activator (Klämbt, 1993; Klaes et al., 1994; O’Neill et al., 1994; Brunner et al., 1994). pntP1 is normally expressed in the ventral ectoderm and tracheal cells. pntP2 is expressed in the mesoderm and midline glial cells, and was shown to be a crucial downstream target for DER in the midline glial cells (Scholz, H., Sadlowski, E., Klaes, A. and Klämbt, C., unpublished data). An inhibitory ETS type transcription factor, which competes with Pointed for binding of common sites on the DNA, has been identified and termed Yan (Lai and Rubin, 1992; O’Neill et al., 1994; Brunner et al., 1994; Tei et al., 1992). Phosphorylation of Yan by MAP kinase induces its translocation from the nucleus to the cytoplasm (Rebay and Rubin, 1995).

A novel feature of the DER signaling cascade is an...
inhibitory secreted polypeptide, Argos, which is likely to serve as an antagonistic ligand of DER (Freeman et al., 1992; Schweitzer et al., 1995a). *argos* transcription is induced by the DER pathway (Golenbo et al., 1996a). It thus serves as an inhibitory feedback loop: the cells exhibiting the highest level of DER activation express Argos, which is secreted and could serve to terminate or reduce DER signaling in the neighboring cells. Argos therefore appears to be required for defining a restricted time window of DER signaling and for preserving the graded effects of DER activation.

This work examines the transcriptional responses to DER activation during development of the embryonic ventral ectoderm. Two cohorts of DER-target genes were identified. Primary targets (*pntP1, vnd* or *fasIII*) are induced in different ectodermal domains. Secondary target genes of DER (*otd, argos and tartan*) are activated by Pointed P1 in response to DER signaling. The proper induction of these genes requires the concomitant inactivation of Yan, mediated by DER signaling.

**MATERIALS AND METHODS**

**DNA probes and antibodies**

To analyze pointed expression a general *pnt* probe as well as *pntP1- and *pntP2*-specific cDNA fragments were used (Klämbt, 1993). *otd* cDNA (Finkelstein et al., 1990), *argos* cDNA (Freeman et al., 1992), *tartan* cDNA (Chang et al., 1993) and *yan* cDNA clones (Lai and Rubin, 1992) were used to monitor the respective gene expression. Monoclonal anti-FasIII antibodies (obtained from T. Volk) were used and polyclonal anti-βGal rabbit antibodies (Cappel) were utilized to detect the balancer chromosomes.

**Staining**

In situ hybridization experiments have been carried out with digoxigenin-labeled cDNA probes as described (Tautz and Pfeifle, 1989), with minor modifications. A detailed protocol is available on request. Antibody stainings have been performed according to Klämbt et al. (1991).

**Fly lines**

The following mutant lines have been used: *pnt*8874 (Jürgens et al., 1984), *pnt*888 and *pnt*H234 (Klämbt, 1993; Scholz et al., 1993). The mutation *pnt*16.1 is an additional EMS-induced allele (Scholz, unpublished data) which like *pnt*8874 and *pnt*888 represents an amorphic pointed mutation. Amorphic *yan* alleles aoP15 and aoP17 (Nüsslein-Volhard et al., 1984; Rogge et al., 1995) gave essentially the same results in our experiments. The allele *yan*2d (Rogge et al., 1995) behaved as a semi cold-sensitive, and showed an amorphic *yan* phenotype only at 18°C. The *fly*F26 allele was used at 29°C in which it is amorphic (Raz and Shilo, 1992). The double mutant *yan*2d, *fly*F26 chromosome was generated by recombination and the embryos maintained at 29°C. A fly strain directing Gal4 expression in the Krüppel domain (Kr-Gal4) was provided by M. Leptin, the rho-Gal4 strain was provided by M. Levine, UAS-*pnt*P1.0 was isolated in a local jump experiment originating from the insertion UAS-P1.3 on the third chromosome (Klaes et al., 1994), a UAS-activated Yan line was provided by I. Rebay (Rebay and Rubin, 1993) and a UAS-sSpok4a integrated on the second chromosome was used (Schweitzer et al., 1995b).

**RESULTS**

**Pattern formation in the ventral ectoderm**

Pattern formation in the ventral ectoderm has been reported to depend on the action of the *spitz* group genes and the *Drosophila* EGF receptor homologue (DER). In *spitz* group mutants as well as in *fly*DER embryos, loss of ventral cell fates is observed (Mayer and Nüsslein-Volhard, 1988; Raz and Shilo, 1993). By mechanisms discussed below, the activity of these proteins is converted into several discrete cell fates, which can be identified at stage 10/11 by the expression of various markers. The ventralmost 1-2 cell rows on each side of the midline express orthodenticle (*otd*) (Wieschaus et al., 1992), *argos* (aos) (Freeman et al., 1992), tartan (*trn*) (Chang et al., 1993), ventral nervous system defective (*vnd*) (Jimenez et al., 1995) and *fasIII* (Patel et al., 1987). The ventrolateral 2-3 cell rows express only *fasIII* (schematized in Figs 1, 3A-C).

![Fig. 1. A scheme for expression of markers in the ventral ectoderm. Schematic summary depicting the expression domains of the following genes: yan, pointed P1, ventral nervous system defective (vnd), fasciclinIII (fasIII), orthodenticle (otd), argos (aos) and tartan (trn). Only expression in the neuroectoderm is shown. Gray shading and yellow nuclei indicate the neurogenic region or neuroectoderm. Nuclei of delaminating neuroblasts are in bright yellow. Dark gray shading and green nuclei indicate the mesectoderm. The mesoderm is indicated by red nuclei. (A) During the cellular blastoderm (stage 5/6), yan and pointed are expressed in the neuroectoderm. Expression at the ventral margin of the neuroectoderm is lowest. vnd is expressed in the ventral region of the neuroectoderm. (B) At the extended germband stage (stage 10), the neuroblasts segregate. yan expression is now found in a broad ventral region, whereas pointed, otd, argos, trn and vnd are expressed in a two-cell-wide row of cells. FasIII is expressed by 4-5 cell rows (in the medial and mediolateral cells). The hierarchy of the different genes is discussed in the text.](image-url)
Generating a perpendicular gradient of DER activation

Ectopic expression of secreted Spitz resulted in expression of otd within the entire ventral ectoderm, suggesting that ventral expression of otd is normally induced by higher levels of DER activity (Schweitzer et al., 1995b). otd expression does not extend to the dorsal ectoderm under these conditions, presumably because dorsal fates have already been specified by the Dpp pathway. Since it was not possible to directly visualize the putative graded distribution of DER activity, we have generated a gradient of DER activation perpendicular to the dorsoventral axis, by expressing secreted Spitz in the Krüppel (Kr) domain (see experimental procedures and Fig. 2A). Kr is expressed in parasegments T2-A4. In the T1/T2 boundary, where Kr expression terminates, an anterior-posterior gradient of DER activation should be generated by graded reduction in Kr levels and the diffusion of secreted Spitz. Induction of lacZ expression in the Kr domain was followed. At stage 9 a patchy expression was detected in the ventral ectoderm. However, Kr expression in the T1/T2 boundary terminates in the same row of cells along the anterior-posterior axis (Fig. 2B).

The cells in the intersection between the two gradients are likely to monitor the cumulative effects of secreted Spitz. Their response should thus reveal the nature of the normal gradient of DER activation in the dorsoventral axis. On each side of the midline, expression of otd in this region appears to form a "wedge-like" shape, with 8-10 cells on each axis (Fig. 2C). This symmetrical response of the cells to the endogenous DER activity, and the artificially induced gradient of DER activation, suggests that, in wild-type embryos, a dorsoventral gradient of DER activation is operating. This gradient is likely to reflect the availability of secreted Spitz, which may be generated in the ectoderm in a restricted pattern or alternatively could emanate from the ventral midline (Golembo et al., 1996b). This paper will examine how the graded DER signal is converted into distinct domains of ventral cell fates.

Pointed is required for ventral ectoderm formation

Previous work has shown that ventral cell fates, as monitored by the expression of otd and argos, depend on the DER pathway (Schweitzer et al., 1995b; Golembo et al., 1996a). Ventral pattern formation also depends on the spitz group gene pointed (Mayer and Nüsslein-Volhard, 1988). Of the two pointed transcripts, only pntP1 is expressed in the ventral ectoderm. It first appears prior to gastrulation in the entire neuroectoderm region. As gastrulation commences, pntP1 expression is confined to the 2-3 ventralmost cell rows. By stage 10, the transcript is restricted to 1-2 cell rows in the ventral ectoderm, and disappears by stage 11/12 (Klämbt, 1993; Fig. 4A).

To further analyze the function of Pointed in ventral patterning, we assayed the expression of several markers in pnt mutant embryos. The genes otd, argos and trn are expressed at stage 10/11 in the ventralmost 1-2 cell rows (see Figs 1, 3A-C). In pnt null mutant embryos, the expression of otd, argos and trn in these cells is abolished or significantly reduced (Fig. 3D-F).

To determine whether Pointed is sufficient for the induction of these genes, ectopic expression of pntP1 was induced by Kr-Gal4. For all three markers, induction of expression in the Kr domain was observed (Fig. 3G-I), suggesting a direct transcriptional activation of these three genes by Pointed P1. Other markers normally expressed in a similar pattern in the ventralmost cells (e.g. vnd) are not affected by ectopic Pointed P1 expression. This strengthens the notion that the expanded expression of otd, argos and trn does not represent a secondary response to a general cell fate alteration induced by Pointed. Expression of Pointed P2, the non-constitutive form of

![Fig. 2. Perpendicular gradients of DER activation generate 'wedge'-like responses.](image)

- **A** A scheme for the effects of the endogenous gradient of secreted Spitz and the perpendicular gradient induced by Kr-Gal4/UAS-secreted spitz. Expression of otd reflecting the ventralmost cell fate is monitored. Arbitrary units of DER activation, contributed by each of the two gradients, are shown. When the sum equals, or is greater than 3, expression of otd is induced (shaded circles). The resulting pattern is a "wedge" of otd expression in the intersection between the gradients, on each side of the midline.
- **B** Expression of Kr-Gal4 as monitored by induction of UAS-lacZ in a stage 9 embryo, viewed from the ventral side.
- **C** Expression of otd in a Kr-Gal4/UAS-sspi embryo at stage 11 (ventral view). Note the formation of the 'wedge' shape, representing the domain of highest DER activity (arrow).
- **D** Expression of pnt in a Kr-Gal4/UAS-sspi embryo stage 9 (ventral view, arrow marks the wedge). Note: in all figures anterior is to the left.
Pointed, in the Kr domain did not give rise to an ectopic induction of target genes such as otd (not shown).

DER signaling induces pointedP1 transcription

In view of the above results, we wanted to ask whether the previously observed effects of DER on otd and argos expression are mediated by Pointed. Since Pointed P1 is thought to be a constitutively active transcription factor, with no requirement for modulation of its activity by the DER/MAP kinase signaling pathway, a direct induction of pointedP1 transcription appeared possible. The early onset of pointedP1 expression takes place prior to the time in which the DER pathway is required to pattern the ectoderm (Raz and Shilo, 1993). Since early pointedP1 expression in the ectoderm is maintained in flb/DER mutant embryos (not shown), it is clearly not DER dependent. However, at stage 9/10, prior to the appearance of pointedP1 in the tracheal pits, expression of pointedP1 is not observed in the ventral ectoderm of flb/DER mutant embryos (Fig. 4B), indicating the transition of pointedP1 expression to a DER-dependent mode.

In a complementary experiment, activation of DER by expression of secreted Spitz in the Kr domain, resulted in the induction of pointedP1 expression in the same region (Fig. 4C). A ‘wedge’ of pointedP1 expression was observed in the T1/T2 boundary, similar to the one observed for otd (Fig. 2D). Utilization of pointed-specific probes demonstrated that only the pointedP1 transcript is induced by secreted Spitz (Fig. 4D).

Yan is required for patterning the ventral ectoderm

In view of the essential role of Pointed in patterning the ventral ectoderm, it was important to determine the function of yan which encodes a negative regulator of ETS transcriptional activators. yan expression in the embryo is first detected at stage 5/6 where it is found in the dorsal ectoderm (Fig. 5A). yan expression declines in a dorsal-ventral gradient and is not found in the mesoderm (Fig. 5B). To define the ventral borders of yan expression, we performed in situ hybridization experiments using yan and single minded (sim) probes. sim expression demarcates the developing midline cells directly flanking the mesoderm; yan expression is not observed in the presumptive midline or in the mesodermal cells up to stage 10. At stage 8, yan expression is found in the head and marks a broad region in the thoracic and abdominal segments, again excluding the mesoderm and midline cells. This broad ectodermal expression is maintained until stage 10/11 (Fig. 5C), when it is reduced and appears more pronounced in the tracheal pits. From stage 11 onwards, yan transcripts are also detected in the midline cells (Scholz, H., Sadlowski, E., Klaes, A. and Klämbt, C., unpublished data). Expression of yan does not depend upon DER, as it is unaltered in flb/DER embryos, or in
embryos in which the expression of secreted Spitz is induced by Kr-Gal4 (not shown).

The function of yan in patterning the ectoderm was investigated by examining the expression of otd, argos and trn in yan mutant embryos. A clear expansion of the ventralmost markers otd, argos and trn is observed, such that 5-8 cell rows on each side of the midline are expressing these genes (Fig. 5D-F). This expanded region is much broader than the zone in which the DER-dependent induction of pntP1 transcription takes place at stage 9/10. In addition, all three analyzed genes showed increased levels of expression in yan mutant embryos. Absence of the Yan protein may thus allow the Pointed P1 protein, which is expressed earlier in a broader domain, to efficiently induce ventralization. This suggestion was confirmed by analysis of embryos that were mutant for both yan and flb. In these embryos, expression of otd in a pattern that is broader than the wild-type one is observed (not shown). Therefore, in the absence of DER signaling and Yan, the early DER-independent expression of pntP1, is capable of triggering otd expression.

In a complementary experiment, the activated form of Yan, which is unable to undergo phosphorylation by MAP kinase (Rebay and Rubin, 1995), was expressed in wild-type embryos. Indeed, the expression of otd and argos was significantly reduced or abolished, in the region where activated Yan was expressed (Fig. 5G-I).

pnt transcription does not appear to be regulated by Yan, since no alteration in the expression of pntP1 was observed in yan mutant embryos (not shown). Furthermore, pnt expression appeared unchanged in two EMS-induced amorphic pnt alleles (pnt16.1 and pnt16.1), suggesting that no autoregulatory feedback loop is controlling pnt expression.

**Additional candidate primary genes triggered by DER**

The response of the ventralmost cells to DER activation described above, occurs in two phases. First, DER triggers the expression of pntP1. Subsequently, Pointed P1 in conjunction with reduced Yan levels, triggers the expression of the second wave of genes. The initial response to DER activation may include other genes in addition to pnt. One candidate is vnd, which was shown to be expressed in the ventralmost cells (Jimenez et al., 1995; Mellerick and Nirenberg, 1995). The level of vnd expression is not reduced in pnt mutant embryos (not shown). To test if vnd is triggered by DER, expression of the gene was monitored in flb/DER mutant embryos. Indeed, while the early phases of expression are not affected, no expression of vnd is detected from stage 11 in the mutants (not shown). Following induction of secreted Spitz by Kr-Gal4, ectopic expression of vnd in the ventral ectoderm is observed (Fig. 4F).

Another class of genes that are likely to be triggered directly by the DER pathway are the ones expressed in a broader domain of the ventral ectoderm, which includes ventrolateral cells in which the DER-dependent expression of pntP1 does not take place. fasIII is a candidate for this category of genes. Expression of FasIII in 4-5 cell rows of the ventral ectoderm on each side of the midline requires the activity of DER (Raz and Shilo, 1993) (see Fig. 1). The FasIII domain is also expanded following hyperactivation of DER (Schweitzer et al., 1995b). The expression of FasIII was examined in different backgrounds of pnt and yan. No alterations in the pattern of fasIII expression were detected in pnt16.1 or yan16.1 mutant embryos and embryos in which the expression of pntP1 was induced by Kr-Gal4 (not shown). The results indicate that expression of fasIII, a representative DER-dependent ventrolateral marker, does not appear to be regulated by Pointed or Yan. Previous experiments have also documented in pnt mutant embryos only weak effects on the expression of other ventrolateral markers (Kim and Crews, 1993).

**DISCUSSION**

**DER activation gradient**

In the developing embryonic ventral ectoderm, the DER RTK is expressed in a uniform pattern (Zak et al., 1990). By the generation of a perpendicular gradient of DER activation, we have shown that DER is triggered in a graded manner along the dorsoventral axis. The source of this gradient may lie in graded Spitz processing in the ectoderm. Alternatively, specific signals may emanate from the midline and generate a graded response of DER (Kim and Crews, 1993; Golembo et al., 1996b). The DER activation gradient is inherently different from other gradients that were previously described. In the case of the Bicoid or Dorsal gradients, for example, cooperative responses

![Fig. 4. pointed P1 transcription is induced by DER. (A) In a wild-type embryo (stage 10), expression of pnt in the ventralmost 1-2 cell rows (arrowhead). (B) In a homozygous flb1F26 embryo (stage 10), pnt staining in the ventral cells is diminished (arrowhead). (C) In a Kr-Gal4/UAS-sspi embryo (stage 9), expanded expression of pnt is observed in the Kr domain (marked by arrows). (D) Staining of a similar embryo with a pntP1-specific probe shows the same result. (E) Staining with a pntP2-specific probe shows only the normal expression in the mesoderm, without ectopic expression in the Kr domain. (F) Staining of a similar embryo (stage 10/11) with a vnd probe shows expanded expression within the Kr domain, in the ventral region. A and F are ventral views, B is dorsal and C-E are lateral views.](image-url)
to their nuclear concentration account for the generation of borders (St. Johnston and Nüsslein-Volhard, 1992; Jiang and Levine, 1993). These responses are stable over time, as long as the initial concentration of the morphogen is maintained.

In contrast, DER possesses a catalytic tyrosine kinase domain and activates a cytoplasmic cascade of kinases. Thus, graded DER activation generates a gradient of enzymatic activities. The resulting responses to these activities are not fixed over time. Cells in which a lower level of DER activation takes place, may eventually reach the same ‘state’ as the cells in which the highest level of DER activation has commenced. This temporal constraint highlights the requirement for Argos as a negative element, which is induced by the DER pathway to terminate signaling and maintain the graded response to DER activation (Golembo et al., 1996a). The downstream consequences of DER activation, which display distinct borders of gene expression, should therefore reflect the response to the enzymatic activity of the DER pathway.

**DER induces pointedP1 expression**

This work has identified pntP1 as a central response element of high DER activity in the developing ectoderm. Although pntP1 has an early embryonic DER-dependent expression phase, it becomes transcriptionally dependent upon DER at stage 9/10. Subsequently, Pointed P1 mediates (directly or indirectly) the induction of several transcriptional responses to high DER activity. Induction of argos by Pointed P1 in the ventralmost cells followed by Argos diffusion, results in a shutdown of DER signaling. Thus, the simultaneous transcriptional induction of positive and negative elements, generates a time window of DER activity, which can last until the Argos protein is produced and secreted at effective concentrations.

**DER inactivates Yan**

The cellular consequences of DER signaling in the ectoderm are further modulated by the post-translational modification of the Yan protein. Yan, which like Pointed is an ETS domain protein, was shown to counteract the positive effects of Pointed, presumably by competing for the Pointed-binding site(s) on target genes (O’Neill et al., 1994). In regions of high DER activity, Yan is likely to be inactivated by MAP kinase phosphorylation. In C. elegans, lin-1 encodes an inhibitory ETS-domain protein (Beitel et al., 1996) and was placed in the Let-23 EGF receptor pathway downstream to MAP kinase (sur-1) (Wu and Han, 1994).

The ventralization of yan mutant embryos is dramatic. Up to eight cell rows express the ventralmost markers on each side of the midline. This ventralization is wider than the domain in which pointed is induced by DER, and may reflect the response to the initial, DER-independent and broader domain of pntP1 expression. Thus, Yan may normally serve to restrict the early activity of Pointed. Only upon triggering of the DER pathway is pntP1 transcription induced in the ventralmost cells, and its activity manifested in the region where Yan is efficiently inactivated.

Yan appears to have an antagonistic interaction with the DER pathway in other instances as well. Expression of Yan in the embryonic dorsal head ectoderm blocks cell divisions, and allows the neuronal and ectodermal differentiation of these cells to take place. In the eye imaginal disc, Yan blocks cell proliferation, which is also likely to be driven by DER, and permits neuronal differentiation to commence (Rogge et al., 1995). Inactivation of transcriptional repressors by an RTK, subsequently leading to transcriptional induction, was also demonstrated recently for Torso, which triggers phosphorylation of NTF-1/Grainyhead (Liaw et al., 1995).

**Conversion of the DER gradient to distinct domains – further refinement**

Several features responsible for the restricted induction of ventral-most markers are conserved throughout embryonic and larval development (Fig. 5). Yan antagonizes the induction of ventralmost cell fates by Pointed. (A) A wild-type stage 5 embryo simultaneously hybridized with digoxigenin-labeled yan and single minded DNA probes. single minded expression demarcates the presumptive midline cells (arrowheads). yan is first expressed in the cellular blastoderm in the neurogenic region and the dorsal ectoderm. Very low levels of yan expression were found in the dorsalmost blastoderm cells. yan expression is below the detection limit after 2-3 cell rows dorsal to the presumptive midline. (B) During gastrulation, yan expression is increased in the ventralmost ectodermal cells. (C) Expression in about 6-8 ventralmost cell rows flanking the ventral midline. This ventralization is wider than the domain in which yan expression is elevated compared to an otherwise more general expression pattern (stage 9/10). No yan expression is found in the midline cells (arrowhead). (D) Expression of otd in a stage 12 yan<sup>pos/pos</sup> embryo can be observed in a broad domain of up to eight cells rows flanking the ventral midline. In addition, the level of otd expression appears enhanced when compared to wild type (see Fig. 3A). (E) aos expression in a stage 11 yan<sup>pos/pos</sup> embryo. The domain of aos expression, as well as the level is increased. (F) trn expression in a stage 11 yan<sup>pos/pos</sup> embryo. Strong trn expression is found in up to eight cell rows flanking the ventral midline. (G) Induction of activated Yan by Kr-Gal4 (anterior border of Kr domain is shown by arrow); or rho-Gal4 (H) results in a dramatic reduction in the level of otd expression in the domain in which Yan was induced. (I) Similarly, expression of aos in the ventral domain (arrowhead) is abolished in rho-Gal4/UAS-activated Yan embryos. Note: rho-Gal4 drives the expression of target genes in the neuroectoderm.
response to the Ras/MAP kinase pathway (Rebay and Rubin, 1995). However, phosphorylation of the other sites may be required to modulate or amplify the response and could provide an additional factor in restricting the region where Yan inhibitory effects are efficiently eliminated by DER. The simultaneous requirement for the induction of pntP1 transcription by DER should provide another spatial constraint. Concomitant activation of Pointed and inactivation of Yan was also demonstrated in the eye imaginal disc, where a combined response is achieved by MAP kinase phosphorylation of Pointed P2 and Yan (O’Neill et al., 1994; Brunner et al., 1994). It is interesting to note that, although the two forms of Pointed are affected in different ways by RTKs (transcriptional induction for pointed P1 versus post-translational activation for Pointed P2), the end result is similar, in terms of activation of Pointed-target genes. However, how different target genes are activated in each tissue in response to Pointed activation, needs to be further analyzed.

**Primary and secondary DER-target genes**

The ventralmost markers otd, args and trn respond to the nuclear levels of Pointed P1 and Yan. Since pntP1 is induced by DER, they represent the secondary-target genes responding to DER activation. In contrast, the vnd and fasIII genes, on the contrary, may represent additional primary DER-target genes. Their expression is altered in DER mutant embryos or following hyperactivation of DER, but they do not respond to modifications in Pointed P1 levels. Whereas induction of pntP1 is required for the generation of ventral ectodermal structures, induction of vnd by DER may influence the specification of ventral neuroblasts.

Among the candidate primary-target genes, pntP1 and vnd are induced in the ventralmost cells, while fasIII is expressed also in the ventrolateral cell rows. It is not known which molecules mediate the transcriptional activation of the primary DER-target genes. A possible candidate is the Jun protein, which is ubiquitously expressed in the embryo (Zhang et al., 1996), and can be phosphorylated and activated by receptor tyrosine kinase pathways. An attractive possibility is that the same molecules are involved in triggering the ventralmost and the ventrolateral target genes, and differences in affinities to the promoter sites would dictate the expression borders of each gene. A model for the transcriptional responses to DER activation in the ventral ectoderm is presented in Fig. 6.

*pntP1* and *yan* are expressed in additional tissues in which DER activation takes place. In the ovary, *yan* is expressed in both anterior and posterior follicle cells. In the posterior follicle cells, DER-dependent induction of *pntP1* transcription takes place (Gabay and Shilo, unpublished data). The accumulation of Pointed in the follicle cells, in conjunction with the DER-dependent inactivation of Yan, could provide the binary switch, triggering differentiation to the posterior fate and subsequent signaling into the oocyte by these cells. The identification of *pntP1* and *yan* as downstream targets of the DER pathway may thus represent a general paradigm for different developmental decisions in which the DER pathway is involved.

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