Regulation of the *dorsal* morphogen by the *Toll* and *torso* signaling pathways: a receptor tyrosine kinase selectively masks transcriptional repression

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The *dorsal* (*dl*) nuclear gradient initiates the differentiation of the mesoderm, neuroectoderm, and dorsal ectoderm by activating and repressing gene expression in the early *Drosophila* embryo. This gradient is organized by a *Toll* signaling pathway that shares many common features with the mammalian IL-1 cytokine pathway. Here we present evidence that a second signaling pathway, controlled by the *torso* (*tor*) receptor tyrosine kinase, also modulates *dl* activity. Evidence is presented that the *tor* pathway selectively masks the ability of *dl* to repress gene expression but has only a slight effect on activation. Intracellular kinases that are thought to function downstream of *tor*, such as *D-raf* and the *rolled* MAP kinase, mediate this selective block in repression. Normally, the *Toll* and *tor* pathways are both active only at the embryonic poles, and consequently, target genes (*zen* and *dpp*) that are repressed in middle body regions are expressed at these sites. Constitutive activation of the *tor* pathway causes severe embryonic defects, including disruptions in gastrulation and mesoderm differentiation, as a result of misregulation of *dl* target genes. These results suggest that RTK signaling pathways can control gene expression by antirepression, and that multiple pathways can fine-tune the activities of a single transcription factor.

[Key Words: *dorsal* morphogen; *Toll*; *torso*; receptor tyrosine kinase; transcriptional repression]

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The *dorsal* (*dl*) morphogen initiates the differentiation of the embryonic mesoderm, neuroectoderm, and dorsal ectoderm (for review, see Govind and Steward 1991; Ip and Levine 1992; St Johnston and Nüsslein-Volhard 1992). *dl* is a member of the REL family of transcription factors (Steward 1987), which are all regulated at the level of nuclear transport (Liou and Baltimore 1993). In the case of *dl*, selective nuclear transport results in the formation of a broad protein gradient, with peak levels present in ventral regions of early embryos and progressively lower levels in lateral and dorsal regions (Roth et al. 1989; Rushlow et al. 1989; Steward 1989).

Peak levels of *dl* activate the expression of regulatory genes such as *twist* (*twi*) and *snail* (*sna*) in ventral regions; these are required for initiating the differentiation of the mesoderm (Boulay et al. 1987; Thisse et al. 1987). Low levels of *dl* trigger the expression of neuroectodermal regulatory genes in lateral regions, which define the neurogenic territory (Ip et al. 1992a). *dl* may be unique among the maternal gradients of transcription factors by functioning as both a transcriptional activator and repressor (Jiang et al. 1992; Pan and Courey 1992). *dl* not only activates mesodermal and neuroectodermal target genes in a concentration-dependent manner but also represses the expression of certain genes, such as *zerknüllt*

(*zen*) and *decapentaplegic* (*dpp*), thereby restricting them to the dorsal ectoderm (Doyle et al. 1989; Ip et al. 1991; Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993), where they are important for the differentiation of the amnioserosa and dorsal epidermis, respectively (Wakimoto et al. 1984; St. Johnston and Gelbart 1987). In *dl*[−] embryos there is a failure to activate mesoderm and neuroectoderm determinants, so derivatives of these embryonic tissues do not form (Simpson 1983). Moreover, *zen* and *dpp* are not restricted to dorsal regions but are instead expressed throughout *dl*[−] mutants (see Fig. 1C; Rushlow et al. 1987). This derepression causes all cells of *dl* mutants to differentiate into derivatives of the dorsal ectoderm.

The formation of the *dl* gradient depends on a cell signaling pathway, which includes a transmembrane receptor, *Toll*, as well as several intracellular components, including a putative kinase, *pelle* (for review, see Govind and Steward 1991; Wasserman 1993). Several lines of evidence suggest that a cascade of extracellular serine proteases locally activate the *spätzle* ligand in ventral and polar regions of the perivitelline fluid that surrounds the egg and early embryo (Morisato and Anderson 1994). Activated *spätzle* locally interacts with *Toll* receptor in ventral regions and the poles. This leads to the dissoci-

Figure 1. Regulation of *zen* by *dl* and *tor*. All embryos are at the 14th nuclear cleavage cycle and are oriented with anterior to the left and dorsal up. Expression patterns were visualized by in situ hybridization using digoxigenin-UTP-labeled RNA probes. (A) *zen* expression pattern in a wild-type embryo. Staining covers the dorsal half of the embryo and includes both poles (arrowheads). (B) *zen* expression in an embryo derived from a female homozygous for the strong *tor*^{PM51} mutation. Staining is lost from the poles (arrowheads) but is normal in middle body regions. (C) *zen* expression in an embryo derived from a female homozygous for the *dl*^{l5} mutation. Staining is completely derepressed in ventral regions and the poles. Only the pole cells are unstained. (D) *zen* expression in an embryo derived from a *dl*^{l5}, *tor*^{PM51} double mutant. The staining pattern is identical to that observed in C.

ation of an inactive cytoplasmic complex containing *dl* and its inhibitor, *cactus* (*cact*), so that *dl* is released to the nucleus where it functions as a transcriptional activator and repressor (Geisler et al. 1992; Kidd 1992).

There is considerable information regarding the way in which *dl* regulates gene expression. Target promoters containing low-affinity *dl*-binding sites are activated only in ventral regions, the presumptive mesoderm, where there are peak levels of *dl* protein (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991; Ip et al. 1992b; Jiang and Levine 1993). In contrast, promoters containing high-affinity *dl*-binding sites and E boxes (recognition sequences for helix-loop-helix proteins) are activated in both ventral (presumptive mesoderm) and lateral (presumptive neuroectoderm) regions (Ip et al. 1992a; Jiang and Levine 1993). Restricted expression in the neuroectoderm depends on the *sna* repressor, which is localized to the mesoderm (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991; Ip et al. 1992b).

dl is intrinsically a weak transcriptional activator in the early embryo. The efficient activation of target promoters such as *twi* and *sna* depends on multiplicative interactions among numerous *dl*-binding sites (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991; Ip et al. 1992b). Moreover, *dl*-HLH interactions also augment the levels of target gene expression (Kosman et al. 1991; Ip et al. 1992b; González-Crespo and Levine 1993; Jiang and Levine 1993). Recent studies suggest that the *dl* activator is converted into a potent repressor when it interacts with neighboring "corepressors" bound to the *zen* and *dpp* promoters (Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993). For example, a minimal silencer element has been identified in distal regions of the *zen* promoter that can repress the ventral expression of heterologous promoters (Doyle et al. 1989; Ip et al. 1991; Jiang et al. 1993; Kirov et al. 1993). It contains several *dl*-binding sites as well as closely linked binding sites for

one or more corepressors. These corepressors have not yet been characterized; however, there is evidence that they interact with *dl* and foster cooperative binding to DNA (Jiang et al. 1992). Mutations in the corepressor sites convert the *zen* silencer element into a weak enhancer (Jiang et al. 1993; Kirov et al. 1993). Because the *dl*-binding sites are intact, they can mediate weak activation in response to high levels of *dl* in ventral regions of early embryos. Only when both the *dl* sites and the nearby corepressor sites are intact can *dl* function as a repressor.

Although *dl* represses *zen* and *dpp* in ventral and lateral regions of the embryo, both genes are expressed at high levels at the poles (Doyle et al. 1986; St Johnston and Gelbart 1987). However, there are equally high levels of *dl* in the nuclei of ventral and polar regions, and both sites of expression are lost in *Toll*⁻ mutants (Roth et al. 1989; Steward, 1989). Nonetheless, *dl* fails to repress *zen* at the poles. Previous studies suggest that the *torso* (*tor*) receptor tyrosine kinase (RTK) plays a role in this process. *zen* normally is expressed in dorsal regions of *tor* mutants but is lost at the poles (Rushlow et al. 1987). This observation suggests that *tor* might regulate a transcriptional activator at the poles that overrides *dl*-mediated repression. Alternatively, it is possible that the *tor* signaling pathway somehow masks the ability of *dl* to function as a repressor.

Here, we present evidence favoring the latter model, whereby *tor* functions as an antirepressor that blocks *dl*-mediated repression. Constitutive activation of the *tor* RTK causes *zen* to be completely derepressed in ventral regions of early embryos. However, target genes that are normally activated by *dl* are only slightly affected, suggesting that *tor* selectively blocks the ability of *dl* to function as a repressor but has little or no effect on *dl*-mediated activation. Intracellular kinases that are thought to function downstream of *tor* (for review, see

Perrimon 1993) appear to mediate this selective block in repression. In particular, the removal of maternal *D-raf* products results in a loss of *zen* expression at the poles, identical to that observed in *tor*[−] mutants. Similarly, a hyperactivated form of the *Drosophila* MAP kinase homolog, *rolled* (Brunner et al. 1994; Biggs et al. 1994), attenuates *dl*-mediated repression. These results suggest that two independently acting signaling pathways, *Toll* and *tor*, fine-tune the activities of the *dl* morphogen. In middle body regions *dl* functions as both an activator and repressor because it is only regulated by *Toll*, but at the poles *dl* functions only as an activator because of the combined action of both the *Toll* and *tor* pathways.

Results

In situ hybridization was used to monitor the effects of the *tor* signaling pathway on *dl* activity. Embryos were collected from mutant females carrying various maternal-effect mutations and hybridized with digoxigenin-labeled RNA probes (Tautz and Pfeifle 1989; Jiang et al. 1991). This allowed the visualization of a number of different expression patterns, including endogenous genes that normally are activated or repressed by *dl*, as well as various defined synthetic promoters (see Jiang and Levine 1993).

tor exerts an antirepression effect on *dl*

zen is an early target gene that is directly repressed by *dl* (Jiang et al. 1992). Both high levels of *dl* in ventral regions and low levels in lateral regions repress *zen*, so that its expression is restricted to the dorsal half of the embryo (Fig. 1A). In addition, the *zen* pattern extends into both poles, despite the high levels of nuclearly localized *dl* protein present at these sites (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). In embryos derived from *dl*[−] females, the early *zen* pattern is derepressed throughout the embryo and includes all somatic cells (Fig. 1C). As shown previously, *zen* expression is lost

from the poles in embryos derived from *tor*[−] females, although there is no effect on the normal dorsoventral limits of the pattern in middle body regions (Fig. 1B; Rushlow et al. 1987). This loss in polar expression could reflect a failure to induce a transcriptional activator or might result from an “antirepression” effect, whereby *tor* masks the ability of *dl* to function as a transcriptional repressor.

In an effort to distinguish between these possibilities, we examined *zen* expression in embryos derived from *dl*[−], *tor*[−] double mutants (Fig. 1D). Staining is detected throughout the embryo, similar to the pattern observed in *dl*[−] mutants (Fig. 1, cf. D with C). The simplest interpretation of this result is that *tor*⁺ gene activity normally blocks *dl* repression at the poles, but it is not required for polar expression in the absence of the *dl* repressor. A similar result was obtained for the early pattern of *dpp* expression, which is another target gene that is directly repressed by *dl* (Casanova 1991).

Ectopic activation of *tor* blocks *dl* repression throughout the embryo

There are several dominant *tor* mutations that cause constitutive, ligand-independent activation of *tor* RTK activity (Casanova and Struhl 1989; Sprenger et al. 1989). Normally, *tor* is ubiquitously expressed on the cell surface of early embryos but is activated only at the poles by a localized ligand encoded by the *torso-like* (*tsl*) gene (Savant-Bhonsale and Montell 1993; Martin et al. 1994). Localized activation of the *tor* RTK results in the restricted expression of the gap gene *tailless* (*tll*) at the poles (Fig. 2C). The *tor*⁴⁰²¹ mutation causes the most severe constitutive activation of *tor* RTK activity among the known dominant *tor* alleles (Klingler et al. 1988; Casanova and Struhl 1989). Embryos derived from *tor*⁴⁰²¹/+ females display a strongly abnormal *tll* expression pattern, such that staining is nearly ubiquitous and spans almost the entire length of the embryo (Fig. 2D). This ectopic expression of *tll* causes severe disruptions in the embryonic segmentation pattern (Steingrimsen

Figure 2. Constitutive activation of *tor* disrupts *zen* and *tll* expression. Embryos are staged and presented as in Fig. 1. (A) Wild-type pattern of *zen* expression. (B) *zen* expression pattern in an embryo derived from a *tor*⁴⁰²¹/+ female. Staining is strongly derepressed in ventral regions, similar to that observed in *dl*[−] mutants (cf. with Fig. 1C). (C) *tll* expression pattern in a wild-type embryo. Staining is restricted to the anterior and posterior poles, although the anterior pattern is repressed from ventral regions (arrowhead). (D) *tll* expression pattern in an embryo derived from a *tor*⁴⁰²¹/+ female. Staining nearly encompasses the entire length of the embryo, and there is a derepression of the anterior pattern so that it now includes ventral regions (arrowheads; cf. with C).

et al. 1991). The absence of expression in the anterior-most regions might reflect a slight expansion of the normal mechanisms that establish the anterior limit of the wild-type pattern (Fig. 2D; cf. D with C), including the attenuation of the *bicoid* (*bcd*) activator by the *tor* pathway (Ronchi et al. 1993).

Constitutive activation of *tor* also results in a dramatic derepression of the *zen* pattern in early embryos (Fig. 2B; cf. B with A). In some embryos, staining extends almost uniformly along the entire length of the embryo while in others (such as the one shown in Fig. 2B), there is a slight repression of the pattern in anterior, ventral regions. This site of residual repression activity corresponds roughly to the location of the ventral limit of the normal anterior *tll* stripe (see Fig. 2C). The derepression of the *zen* pattern observed in *tor*⁴⁰²¹ mutants is not the result of a block in *dl* nuclear transport, because these mutants contain normal levels of nuclearly localized *dl* repressor in ventral and ventrolateral regions (data not shown). This observation suggests that *tor* activity can mask the ability of *dl* to function as a transcriptional repressor whenever the *tor* and *Toll* pathways coincide. In wild-type embryos, *tor* activity is restricted to the poles and normally blocks *dl*-mediated repression only in these regions. However, in embryos where *tor* is constitutively activated, *dl* repression is blocked both at the poles and in middle body regions.

Previous studies suggest that *dl* either directly or indirectly represses the anterior domain of the *tll* pattern in ventral regions (arrowhead in Fig. 2C). In *dl*⁻ embryos this anterior pattern expands so that expression is uniformly intense in both dorsal and ventral regions (Pignoni et al. 1992). Constitutive activation of *tor* results in a similar ventral derepression of the *tll* pattern (arrowheads in Fig. 2D). This result suggests that genetic interactions between *dl* and *tor* lead to a general block in the

ability of *dl* to function as a transcriptional repressor. Consistent with this view is the finding that repression of the *dpp* pattern is also impaired in *tor* gain-of-function mutants (data not shown). Another implication of this result is that *dl* might directly repress *tll* expression. In principle, it is possible that ventral repression of the anterior *tll* pattern (arrowhead in Fig. 2C) could be mediated indirectly by a *dl* target gene such as *twi* or *sna*. However, as shown below, constitutive activation of *tor* does not significantly disrupt the initiation of these genes.

tor exerts only a slight effect on *dl* activation

The *twi* and *sna* expression patterns were examined to determine whether dominant *tor* mutations influence the ability of *dl* to function as a transcriptional activator (Fig. 3). Embryos were collected from *tor*⁴⁰²¹/+ females and stained to reveal the patterns of endogenous *twi* and *sna* RNAs. The initiation of both patterns is essentially normal (Fig. 3A,C), although there is a severe premature loss in *sna* expression by the onset of gastrulation. This is associated with defects in ventral furrow formation and mesoderm invagination (data not shown). *sna* is normally repressed from the posterior pole of wild-type embryos (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991), and it seems likely that the expanded limits of *sna* repression observed in *tor* gain-of-function mutants result from expanded expression of one or more *tor* target genes, such as *huckebein* (*hkb*) or *tll* (Brönner and Jäckle 1991; see Discussion).

Further evidence that *tor* has little or no effect on the ability of *dl* to work as an activator was obtained by analyzing the expression of synthetic promoters. Previous studies have shown that a truncated *sna* promoter containing the first 1.6 kb of *sna* 5'-flanking sequence is

Figure 3. Constitutive activation of *tor* causes only mild defects in the initiation of target genes. All embryos were derived from *tor*⁴⁰²¹/+ females. (A) *twi* expression pattern. Staining extends along the length of the ventral surface and encompasses the poles. This pattern is quite similar to that observed in wild-type embryos (not shown). (B) Expression of a *lacZ* fusion gene containing the first 1.6 kb of the *sna* promoter. Staining encompasses ventral regions but does not extend to the poles. This pattern is quite similar to that observed in wild-type embryos (Ip et al. 1992b), although there is about a two-fold reduction in the levels of staining. (C) *sna* expression pattern. Staining includes ventral regions and the poles. This early pattern is quite similar to that observed in wild-type embryos (not shown), although there is a progressive weakening in expression particularly in posterior regions (arrowheads). (D) Expression pattern of the synthetic PE-Et promoter, which contains low-affinity *dl*-binding sites and closely linked *twi* sites (Et E boxes). Staining extends to the poles and encompasses the entire presumptive mesoderm (20 cells in ventral and ventrolateral regions). This pattern is nearly identical to that observed in wild-type embryos.

activated only by peak levels of *dl* protein present in the ventral-most 12 cells of early embryos (Ip et al. 1992b). A *lacZ* fusion gene containing this promoter sequence can be thought of as a "sensitized" element because just a two-fold reduction in the levels of *dl* (embryos derived from *dl*^{-/+} mothers) causes nearly a complete loss in expression. Consequently, it provides a sensitive assay for investigating the effects of *tor* on *dl*-mediated activation. The *sna-lacZ* fusion gene was crossed into an embryo derived from *tor*^{4021/+} females, and its expression was monitored by *in situ* hybridization using a digoxigenin-labeled *lacZ* antisense RNA probe. An essentially normal pattern is observed, with expression spanning the entire ventral surface of the embryo (Fig. 3B). However, there is a mild reduction in the levels of expression and a slight narrowing of the pattern (10 cells rather than 12). Nonetheless, this result suggests that the constitutive activation of *tor* RTK activity causes less than a two-fold reduction in the effective levels of *dl* activator.

The *dl* protein is distributed in a steep concentration gradient so that there are substantially lower levels of protein in ventrolateral versus ventral regions (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). To determine whether *tor* might disrupt the ability of intermediate levels of *dl* to activate gene expression, we analyzed the expression of a synthetic *twi* promoter fragment, PE-Et. This 440-bp regulatory sequence directs expression in the entire presumptive mesoderm (spanning ~18 cells in width), including both ventral and ventrolateral regions of early embryos (Jiang and Levine 1993). The normal PE sequence directs expression in the ventral-most 10–12 cells, similar to the pattern generated by the 1.6-kb *sna-lacZ* fusion gene (Jiang et al. 1991; Ip et al. 1992b). A modified PE containing two recognition sequences for the *twi* protein ("Et") directs

an expanded pattern that includes ventrolateral regions where there are intermediate levels of *dl* (Jiang and Levine 1993). A PE-Et-*lacZ* fusion gene shows a normal pattern of expression in *tor*⁴⁰²¹ mutants (Fig. 3D), with expanded dorsoventral limits as seen in wild-type embryos. These results suggest that *tor-dl* interactions do not impair *dl*-mediated activation in either ventral or ventrolateral regions.

Uncoupling of *dl*-mediated activation versus repression is observed most dramatically with fusion genes containing the *zen* silencer element. Previous studies have shown that *dl* represses *zen* in ventral regions by interacting with a 180-bp ventral repression element (VRE) located ~1.4 kb upstream of the transcription start site (Doyle et al. 1989; Ip et al. 1991; Jiang et al. 1992, 1993; Kirov et al. 1993). This element contains three high-affinity *dl*-binding sites, as well as closely linked binding sites for one or more corepressors. The *dl* protein is intrinsically a weak transcriptional activator; however, interactions between *dl* and neighboring corepressors in the *zen* VRE convert *dl* into a repressor that silences the ventral expression of heterologous promoters. For example, ventral expression of *even-skipped* (*eve*) stripe 2 is silenced when the *zen* VRE is placed upstream of the 480-bp *eve* minimal stripe 2 enhancer ("MSE"; Fig. 4A). Mutations in the corepressor binding sites abolish the ability of the *zen* VRE to function as a silencer (Fig. 4C). In addition, because the *dl*-binding sites are intact, the VRE-MSE fusion gene directs weak expression along the entire ventral surface in response to peak levels of *dl* (Jiang et al. 1993; Kirov et al. 1993).

A wild-type VRE-MSE fusion promoter containing an intact VRE directs an abnormal pattern of expression in embryos derived from *tor*^{4021/+} females (Fig. 4B). There is no ventral repression of the *eve* stripe 2 pattern; in

Figure 4. Constitutive activation of *tor* selectively disrupts *dl*-mediated repression. (A,B) Expression of the 2×180 VRE-MSE fusion promoter in a wild-type (A) and *tor*⁴⁰²¹ embryo (B). This promoter contains two tandem copies of a minimal *zen* silencer element (VRE) placed upstream of the *eve* stripe 2 enhancer (MSE). In wild-type embryos (A), expression of the stripe 2 enhancer is restricted to dorsal regions and is repressed in ventral regions by the *zen* silencer (arrowhead). (B) There is no ventral repression in embryos derived from *tor*^{4021/+} females. In addition, weak staining extends along the ventral surface where there are peak levels of *dl* (indicated by brackets). The overall reduction in the levels of stripe 2 expression (see dorsal surface) is probably the result of the down-regulation of *bcd* by *tor*

(see text). (C,D) Expression of a mutagenized *zen-eve* fusion promoter, mVRE-MSE, in a wild-type embryo (C) and in a mutant embryo derived from a *tor*^{4021/+} female (D). This promoter contains one copy of the 180-bp VRE placed upstream of the *eve* MSE. Corepressor sites were eliminated in the VRE by site-directed mutagenesis. As a result, there is no ventral repression of the expression pattern in wild-type embryos (C; arrowhead). In addition, there is weak ventral activation by *dl* (brackets). A similar expression pattern is observed in *tor*⁴⁰²¹ mutants (D), except that there is an overall reduction in the levels of stripe 2 expression.

addition, there is weak staining along the ventral surface. This expression pattern is quite similar to that observed for the mutagenized VRE–MSE fusion promoter containing a defective VRE lacking corepressor binding sites (Fig. 4C). These results suggest that the constitutive activation of the *tor* RTK selectively blocks *dl*–corepressor interactions that are essential for *dl*-mediated repression. However, *tor* does not interfere with the ability of *dl* to bind the VRE and mediate weak activation. The reduced levels of staining directed by the *eve* MSE (see dorsal surfaces in Fig. 4A,B) might result from *tor*–*bcd* interactions. *bcd* is the primary activator of *eve* stripe 2, and previous studies have shown that *tor* attenuates *bcd* activation at the anterior pole (Ronchi et al. 1993); it is reasonable to suppose that this *bcd*–*tor* interaction occurs in a more expanded domain in *tor*⁴⁰²¹ mutants (see Discussion). A similar reduction in stripe 2 expression is observed when the mutagenized VRE–MSE fusion promoter lacking corepressor sites is expressed in a *tor*⁴⁰²¹ background (Fig. 4D).

tor–*dl* interactions are mediated by downstream signaling components

Several intracellular kinases have been implicated in the *tor* signaling pathway, including the *Drosophila* homolog of the mammalian Raf serine/threonine kinase (*D-raf*; for review, see Perrimon 1993). To determine whether this intracellular pathway is also important for mediating the *tor*–*dl* interaction, particularly the masking of *dl*-mediated transcriptional repression, we examined the expression of *dl* target genes in embryos derived from eggs lacking *D-raf*⁺ products. Because *D-raf* is an essential gene that is active throughout the *Drosophila* life cycle, germ-line mosaics were induced in *D-raf*⁺/*D-raf*[−] heterozygotes. For this purpose, a heterozygous strain was employed that carries the yeast flip recombinase binding site (FRT) near the base of the chromosome arm. The strain also contains the flip recombinase under the control of the heat-inducible *hsp70* promoter and a dominant female sterile mutation (Chou and Perrimon

1992; Melnick et al. 1993; Ronchi et al. 1993). When larvae are heat-shocked there is efficient recombination of the FRT-marked chromosomes, so that large *D-raf*[−] patches of tissue are generated in the developing germ line. Nearly all of the eggs laid by these females lack *D-raf*⁺ products, as judged by the loss of *tll* expression at the posterior pole (data not shown). Embryos derived from these females display an abnormal *zen* pattern, whereby expression is lost at the poles (Fig. 5B cf. B with A). This pattern is indistinguishable from that observed in embryos derived from *tor*[−] females (Fig. 1B), suggesting that the ability of *tor* to mask *dl* repression is mediated by the intracellular *D-raf* kinase.

Previous studies have shown that the repression of *sna* from the anterior and posterior poles is also controlled by *tor*. In *tor*[−] mutants, *sna* expression is sustained at the posterior pole (Casanova 1991), whereas in wild-type embryos, this expression is extinguished during cellularization (Fig. 5C). This posterior repression probably is mediated by the *tor* target gene *hkb* (M. Leptin, pers. comm.). Embryos derived from *D-raf*[−] eggs show a persistence of *sna* expression at the posterior pole (Fig. 5D), identical to the situation observed in *tor*[−] mutants. This observation suggests that intracellular signaling components downstream of the *tor* RTK and *ras* mediate *sna* repression at the posterior pole.

Recent studies on the *sevenless* (*sev*) RTK have identified a MAP kinase, *rolled*, that is essential for signal transduction in this pathway (Brunner et al. 1994; Biggs et al. 1994). A dominant, hyperactivated form of the *rolled* kinase, *Sevenmaker* (*Sem*), results in the formation of supernumerary R7 photoreceptor cells, similar to the situation observed for dominant gain-of-function mutations in the *sev* RTK (Brunner et al. 1994). Embryos derived from *Sem*⁺ females show segmentation defects that are consistent with an expanded pattern of *tll* expression (data not shown). However, disruptions in the segmentation pattern are erratic and generally not as severe as those observed in embryos carrying strong *tor* dominant mutations. To determine whether the *rolled* MAP kinase mediates aspects of the *tor*–*dl* interaction,

Figure 5. *D-raf* mediates the *dl*–*tor* interaction. Embryos in B and D were obtained from *l(1)ph^{EA75}/+* females that were induced to deposit eggs completely lacking *D-raf* activity (see Materials and methods). (A) *zen* expression pattern in a wild-type embryo. (B) *zen* expression in an embryo lacking maternal *D-raf* products. Staining is lost from both poles, similar to the situation observed in *tor*[−] embryos (cf. Fig. 1B). (C) *sna* expression pattern in a wild-type embryo. Staining is lost from the poles (arrowheads) by this stage in development (late nuclear cycle 14). (D) *sna* expression pattern in embryos derived from *D-raf*[−] eggs. There is no repression from the poles, similar to the situation observed in *tor*[−].

the *zen* expression pattern was examined in embryos derived from *Sem*/+ females (Fig. 6A). The *zen* pattern shows expanded limits of expression, such that staining extends into ventrolateral regions of the embryo where the gene is normally off (i.e., cf. Fig. 6A with Fig. 5A). This observation suggests that hyperactivation of the *rolled* MAP kinase causes *dl* to be a less effective repressor.

Further evidence that *rolled* attenuates *dl* repression was obtained by examining the expression of the *zen* VRE–*eve* MSE fusion promoter in embryos derived from *Sem*/+ females (Fig. 6B). The MSE drives strong expression in both dorsal and ventral regions, with only a slight reduction in ventral regions. In wild-type embryos, staining is restricted to dorsal regions because the VRE silences ventral expression (cf. Fig. 6B with Fig. 4A). This result suggests that the hyperactivated MAP kinase impairs, but does not abolish, *dl*-mediated repression from the *zen* VRE. However, the effect is not as dramatic as that observed in *tor*^D mutants (i.e., Fig. 4B), suggesting that the *rolled* MAP kinase might play a relatively dedicated role in transmitting the *sev* signal (see Discussion).

Discussion

dl functions as both a sequence-specific transcriptional activator and repressor (for review, see Ip and Levine 1992). Here, we present evidence that the *tor* RTK signaling pathway fine-tunes *dl* activity by selectively blocking its ability to repress target genes such as *zen*. This block in repression helps “dorsalize” the embryonic poles and delimit the anteroposterior boundaries of the presumptive mesoderm. Evidence is presented that the *D-raf* serine–threonine kinase transduces the signal generated by the activation of the *tor* receptor. The newly identified *rolled* MAP kinase appears to function downstream of *D-raf* to suppress *dl* repression, but it is possible that additional, as yet unidentified, intracellular signaling components also participate in this process. These findings, together with previous studies on the *Toll* signaling pathway (for review, see Govind and Steward 1991; Wasserman 1993), suggest that the detailed activities of a single transcription factor can be modu-

lated by multiple pathways. Thus, the *dl* morphogen is subject to exquisite regulation: Its activity is influenced by divergent signaling pathways and the context of its target promoters (Jiang et al. 1992; Pan and Courey 1992).

Convergence of multiple signaling pathways

The most important finding of this study is that the *tor* RTK pathway exerts a relatively specific effect on *dl*-mediated repression and that multiple signaling pathways can act on a single transcription factor, as summarized in Figure 7. However, it should be emphasized that the *tor* pathway might not lead to the direct modification of *dl*, but, instead, could function by modulating one or more *dl* corepressors (see Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993, 1994). The *Toll* pathway is activated in both ventral regions and the poles by the *spätzle* ligand (Anderson et al. 1992; Morisato and Anderson 1994). This leads to the dissociation of the inactive *dl*–*cact* complex so that *dl* can enter the nucleus and function as both an activator of *twi* and *sna* and a repressor of *zen* and *dpp* (Govind and Steward 1991; Ip and Levine 1992; Wasserman 1993). Both the *Toll* and *tor* pathways are active at the poles. As a result, *dl* activates *twi* and *sna* but fails to repress *zen* and *dpp*, so all of these genes are expressed at the poles.

Role of *tor* in dorsoventral patterning

Previous studies on the *tor* pathway have focused primarily on segmentation. The follicular epithelium surrounding the developing oocyte is thought to secrete a *tor* ligand, possibly encoded by *tsl*, only at the polar regions of the egg chamber (Stevens et al. 1990; Savant-Bhonsale and Montell 1993; Martin et al. 1994). This leads to the selective activation of the *tor* RTK at the poles and localized expression of two target genes, *tll* and *hkb* (Brönner and Jäckle 1991; Liaw and Lengyel 1993). Embryos derived from *tor*[−] females lack the posterior domain of *tll* expression and display an abnormal pattern of expression at the anterior pole (Pignoni et al. 1990). Most of the segmentation defects observed in *tor* mutants appear to result from altered expression of *tll* (Ste-

Figure 6. Hyperactivation of the *rolled* MAP kinase disrupts *dl*-mediated repression. Embryos were collected from *Sem*/+ females. (A) *zen* expression pattern. There is a slight ventral derepression of the pattern so that staining extends into ventrolateral regions where the gene is normally off (cf. with Figs. 1A, 2A, and 5A). However, this derepression is not as severe as that observed

in the *tor*⁴⁰²¹ background (cf. Fig. 2B). (B) Expression of the 2×180VRE–MSE fusion promoter. Stripe 2 is expressed at nearly uniform levels in both dorsal and ventral regions, indicating that *dl* fails to mediate efficient repression in this mutant. In wild-type embryos, stripe 2 expression is restricted to the dorsal surface and there is complete ventral repression (see Fig. 4A). However, unlike the situation observed in *tor*⁴⁰²¹ mutants, there is no staining detected along the ventral surface nor attenuation of the stripe 2 pattern (see Fig. 4B).

Figure 7. Coordinate regulation of *dl* by the *tor* and *Toll* signaling pathways. The *Toll* receptor is selectively activated in ventral regions, and the poles, by the *spätzle* ligand. This induces two cytoplasmic proteins, *tube* and *pelle*, to modify the *dl-cact* complex so that *dl* is released from the cytoplasm and enters nuclei. Once in the nucleus, *dl* acts as both activator of zygotic target genes such as *twi* and *sna* and as repressor of *zen* and *dpp*. In middle body regions, the *tor* receptor tyrosine kinase is expressed but not activated because of the lack of *tsl* ligand. At the poles, the ligand activates *tor* and induces an intracellular signaling pathway that includes the products of *l(1)ph* (*D-raf*) and *rolled* (MAP kinase). This leads to a specific impairment in the ability of *dl* to repress targets like *zen* and *dpp* but has only slight effects on its ability to activate genes like *twi* and *sna*. The mechanism might involve modification of the *dl* protein itself, e.g., by phosphorylation, or of other components interacting with *dl* in the repression of target genes. For example, the *tor* pathway might modify one or more *dl* corepressors and thereby block the formation of a *dl* repression complex on the *zen* promoter.

ingrímsson et al. 1991). For example, in strong *tor^D* mutants there is ectopic expression of *tll*, which in some cases encompasses nearly the entire length of the embryo (i.e., Fig. 2D). There is a tight correlation between the extent to which *tll* is ectopically expressed and the suppression of middle body segments. In severe cases there is a virtual loss of all segments, whereas slight expansion of the *tll* pattern results in the loss of just one or two abdominal segments (Warrior and Levine 1990). Most of the segmentation defects observed in *tor^D* mutants can be suppressed by removing *tll⁺* gene activity (Strecker et al. 1991).

There is evidence that *tor* also plays an important role in dorsoventral patterning. Some of the head defects observed in *tor⁻* mutants could result from the loss of *zen* at the anterior pole, because *zen⁺* gene activity is required for the differentiation of head structures and is also important for head involution (Wakimoto et al. 1984). In addition, there are a number of dorsoventral patterning defects observed in *tor^D* mutants, including disruptions in ventral furrow formation, mesoderm differentiation, and germ-band elongation (J. Rusch and M. Levine, unpubl.). Finally, *tor⁺* gene activity is required for repressing *sna* at the posterior pole; in *tor⁻* mutants, *sna* expression is sustained in these regions and there is a corresponding expansion of the ventral furrow and embryonic mesoderm (Casanova 1991). Consequently, it would appear that *tor* is important for defining the anteroposterior boundaries of the ventral furrow.

Mechanism of *tor*-*dl* interaction

dl functions as a repressor that restricts the expression of

tissue determinants such as *zen* and *dpp* to dorsal regions where they are important for initiating the differentiation of the dorsal ectoderm (Rushlow et al. 1987; St Johnston and Gelbart 1987). Repression involves local protein-protein interactions between *dl* and neighboring corepressors bound to *zen* and *dpp* silencer elements (Jiang et al. 1992, 1993; Pan and Courey 1992; Huang et al. 1993; Kirov et al. 1993). These interactions convert the weak *dl* activator into a long-range repressor that can silence the ventral expression of various heterologous promoters, including *hunchback*, *Krüppel*, and *eve* (Doyle et al. 1989; Ip et al. 1991). It would appear that the *tor* pathway selectively blocks *dl*-corepressor interactions, thereby permitting *zen* and *dpp* expression at the poles where there are high levels of *dl* protein. In principle, *dl*-corepressor interactions could be disrupted by either modification of *dl* or the corepressor. Constitutive activation of the *tor* RTK results in ubiquitous expression of *zen* and *dpp*, presumably because of a breakdown in *dl*-corepressor interactions in both middle body regions and the poles. This constitutive activity does not significantly alter the expression of target genes that are activated by *dl*, such as *twi* and *sna*.

The uncoupling in the ability of *dl* to function as an activator versus repressor is illustrated most dramatically by the expression pattern of a *zen* VRE-*eve* MSE fusion promoter in embryos derived from *tor⁴⁰²¹/+* females (Fig. 4). The VRE not only fails to silence the ventral expression of *eve* stripe 2 but actually mediates weak activation in response to peak levels of *dl* present along the ventral surface. The block in *dl*-corepressor interactions does not result from a general failure of *dl* to con-

tact cooperating factors in a target promoter element. For example, activation of the synthetic PE-Et promoter throughout the presumptive mesoderm in response to both high and intermediate levels of *dl* depends on cooperative DNA-binding interactions between *dl* and HLH proteins bound to neighboring E-box sequences (Jiang and Levine 1993). The dorsoventral limits of the PE-Et pattern are not altered in embryos derived from *tor*⁴⁰²¹/+ females, suggesting that *dl*–HLH interactions are unaffected (Fig. 3; data not shown).

RTK pathways and antirepression

A number of intracellular signaling molecules mediate the *tor* RTK signal. These include *corkscrew*, a tyrosine phosphatase; *Son of sevenless*; *ras1*; *D-raf*; *Dsor1*, a homolog of MEK kinase (MAP kinase kinase); and most recently, *rolled*/*Sem*, a putative MAP kinase (for review, see Perrimon 1993; Brunner et al. 1994; Biggs et al. 1994). Several of these signaling components have been implicated in other RTK pathways, including the epidermal growth factor (*EGF*) and *sev* RTKs (Dickson et al. 1992). However, it is not clear whether all of these components are equally important for the different RTKs.

For example, *Sem*/+ heterozygotes display a severe eye phenotype, extra R7 cells, which is quite similar to that observed upon constitutive activation of the *sev* RTK or *ras1* (Brunner et al. 1994). This observation suggests that the dominant *Sem* mutation represents a hyperactivated form of the *rolled* MAP kinase. However, the kinase might play a variable role in the *EGF* RTK pathway because *Sem*/+ females lay normal eggs but display extra wing veins; both of these processes are regulated by the *EGF* receptor (Clifford and Schüpbach 1992). The observation that the *tll* and *zen* expression patterns are only mildly disrupted in embryos derived from *Sem*/+ eggs suggests that the *rolled* kinase might also play a limited role in transducing the *tor* signal. Perhaps the efficient derepression of *zen* depends on the activation of additional, as yet unidentified, signaling components that function downstream of *ras1* and *D-raf*.

RTKs have been implicated in the activation of gene expression in a variety of systems, including the *Caenorhabditis elegans* vulva (Sternberg and Horvitz 1991) and the fly ovary (Shilo 1992), embryo (Sprenger et al. 1989; Casanova and Struhl 1989), and eye (Dickson et al. 1992) and numerous mammalian tissues including T cells (e.g., Margolis et al. 1992). It is conceivable that one or more of these processes depend on the same type of antirepression mechanism underlying the *dl*–*tor* interaction. *tor* does not regulate *zen* and *dpp* expression by inducing a transcriptional activator. Instead, it masks the action of a transcriptional repressor. Previous studies suggest that *zen* is activated by one or more ubiquitously distributed factors, which interact with multiple elements distributed throughout the *zen* promoter (Doyle et al. 1989; Jiang et al. 1993). The distal VRE silences this activation whenever *dl* and its corepressors are bound to the VRE. *dl*-mediated repression is relieved at the poles

by the *tor* RTK pathway. Perhaps *tll* expression is restricted to the poles by the masking of a ubiquitous repressor, and it is conceivable that the activation of R7-specific genes also involves antirepression by the *sev* RTK (Lai and Rubin 1992).

Materials and methods

Fly stocks

Fly stocks used in this study were *yw*^{67c23}, *dl*^{l5} (Steward and Nüsslein-Volhard 1986), *tor*^{PM51} (Schüpbach and Wieschaus 1986), and *tor*⁴⁰²¹ (Klingler et al. 1988). The *tor*^{PM51}; *dl*^{l5} double mutant was obtained by recombination. Germ-line *l(1)ph*^{EA75} (*D-raf*) mutant females were obtained by inducing site-specific mitotic recombination using the FLP–DFS technique (Chou and Perrimon 1992). X chromosomes have been constructed containing an FRT sequence near the centromere and either the dominant female sterile *ovo*^{D1} or the *l(1)ph*^{EA75} mutation. Males carrying the FRT *ovo*^{D1} chromosome and the *flp* recombinase under heat shock control on the second chromosome (F38) were crossed to females carrying the FRT *l(1)ph*^{EA75} chromosome and F38. These parental strains were kindly provided by Norbert Perrimon (Harvard Medical School, Boston, MA) and Claude Desplan (Rockefeller University, NY). The progeny of this cross were heat-shocked during the first-instar larval stage for 2 hr at 37°C and allowed to develop at 25°C. The fertile female progeny laid eggs that developed with a strong terminal phenotype as shown by *eve* antibody stainings (data not shown). *Sem* mutant females were obtained by outcrossing males from a *l(1)ph*^{HM7}; *Sem*/+ stock to *yw* females and selecting for the *Sem* phenotype (rough eyes and extra wing veins) among the female offspring. The *Sem* strain was kindly provided by Ernst Hafen (Zoologisches Institut Universität Zürich, Switzerland).

Transgenic lines containing various *lacZ* fusion genes have been described previously: 1.6-kb *sna*–*lacZ* (Ip et al. 1992b); PE-Et (Jiang and Levine 1993); 2×180 VRE–MSE (Jiang et al. 1993). To examine the expression of the various *lacZ* fusion genes in mutant backgrounds, males carrying a given fusion gene were crossed with females of the appropriate genotype. For the wt background, *yw* females were used. All embryo collections were done at 25°C.

Whole mount in situ hybridization

Embryonic expression patterns were visualized by in situ hybridization using antisense RNA probes labeled with digoxigenin–UTP and antidigoxigenin antibodies conjugated to alkaline phosphatase (Tautz and Pfeifle 1989; Jiang et al. 1991; Kosman et al. 1991). The stained embryos were photographed using Nomarski optics.

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