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Uncoupling Dorsal-mediated activation from Dorsal-mediated repression in the *Drosophila* embryo

Girish S. Ratnaparkhi, Songtao Jia* and Albert J. Courey†

The Rel family transcription factor Dorsal patterns the dorsoventral axis of the *Drosophila* embryo by activating genes such as *twist* and *snail* and repressing genes such as *decapentaplegic* and *zerknüllt*. Dorsal represses transcription by recruiting the co-repressor Groucho. However, repression occurs only when Dorsal-binding sites are close to binding sites for other factors that also bind Groucho. The need for additional factors to assist Dorsal in repression may result from the intrinsically weak interaction between Dorsal and Groucho. To test this idea, we generated a Dorsal variant containing a high-affinity Groucho recruitment motif at its C terminus. As predicted, this variant functions as a dedicated repressor, silencing *decapentaplegic* and *zerknüllt* while failing to activate *twist* and *snail*. We also converted Dorsal into a dedicated activator by replacing its weak Groucho-recruitment motif with heterologous activation domains. Although the dedicated activator alleles fail to repress *decapentaplegic* and *zerknüllt* in the syncytial blastoderm embryo, they are able to pattern the dorsoventral axis. This indicates that dorsoventral patterning is not dependent upon Dorsal-mediated repression, reflecting the existence of redundant mechanisms to block Decapentaplegic signaling.

KEY WORDS: Dorsal, Twist, Decapentaplegic, Groucho, Short gastrulation, Dorsoventral pattern formation

INTRODUCTION

Dorsal (DL) is a *Drosophila* Rel homology domain (RHD) protein that patterns the embryonic dorsoventral (DV) axis. Selective nuclear import of maternally encoded DL in the syncytial blastoderm embryo leads to the formation of a DL nuclear concentration gradient, with highest concentrations in ventral nuclei and lowest concentrations in dorsal nuclei (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). As many as 50 zygotically active genes may be directly regulated by DL, which functions as both an activator and a repressor of transcription (Ray et al., 1991; Rusch and Levine, 1996; Stathopoulos and Levine, 2002; Stathopoulos et al., 2002). Experimentally verified activation targets include *snail* (*sna*), *twist* (*twi*), *short gastrulation* (*sog*) and *brinker* (*brk*), whereas experimentally verified repression targets include *decapentaplegic* (*dpp*), *zerknült* (*zen*) and *tolloid* (*tld*).

Repression by DL requires the co-repressor Groucho (GRO) (Dubnicoff et al., 1997). Well-characterized GRO-binding motifs include the engrailed homology 1 (eh1) and WRPW motifs (Goldstein et al., 2005; Paroush et al., 1994; Smith and Jaynes, 1996). DL lacks these high-affinity GRO-recruitment motifs, but contains a weak eh1-like sequence in its C-terminal domain (CTD) that is required for DL-mediated repression (Flores-Saaib et al., 2001). We have hypothesized that the low affinity of this motif for GRO renders DL bifunctional, allowing it to activate or repress transcription depending on the binding-site context (Courey and Jia, 2001).

Several zygotically active DL target genes, including *dpp*, *sog* and *brk*, encode components of a second pattern-forming system that continues the task of patterning the DV axis initiated by DL (Ashe, 2002; Ashe, 2005; O'Connor et al., 2006). DL-mediated repression of *dpp*, which encodes a secreted signaling protein, restricts its expression to the dorsal-most 40% of the embryo, where it interacts with other factors to subdivide this region into multiple developmental domains. Diffusion of DPP through the pervitelline space could potentially result in the ventral spread of DPP signaling. As this would upset the DV developmental program, redundant mechanisms employing SOG and BRK block the ventral spread of DPP signaling (Ferguson and Anderson, 1992b; Hasson et al., 2001; Holley et al., 1995; Jazwinska et al., 1999a; Zhang et al., 2001). Given these redundancies, it is possible that ventral repression of *dpp* by DL is partially or wholly dispensable for pattern formation.

To test ideas about the mechanisms of DL-mediated activation and repression, and about the role of repression in patterning, we have altered DL in ways predicted to selectively abolish activation or repression. Our findings provide support for the idea that low-affinity GRO binding is required for Dorsal bifunctionality. In addition, they show that DL-mediated repression is largely redundant with other mechanisms for blocking the ventral spread of DPP signaling. We speculate that DL-mediated repression has evolved to ensure that the DPP pattern-forming system will behave in a robust manner in the face of environmental fluctuations that alter the rates at which DPP pathway components diffuse through the extraembryonic space.

MATERIALS AND METHODS

DNA constructs and transfection assays

pPac-FLAG (Bhaskar et al., 2000) was used for expression of DL and DL variants in S2 cells. pPac-FLAG-*dl-p65* encodes the DL RHD (amino acid residues 1-345) fused to the mouse p65 activation domain (residues 305-550). pPac-FLAG-*dl-VP16* encodes the DL RHD (residues 1-380) fused to the VP16 activation domain (residues 411-490). pPac-FLAG-*dl-WRPW* encodes full-length DL fused to sequences encoding a C-terminal WRPW motif, with a single proline residue before the WRPW motif. pPac-FLAG-DL, pPac-DL380, pPac-*gro* and pPac-Gal4-*gro* have been described previously (Chen et al., 1999; Jia et al., 2002). The P-element expression vectors are based on UASp (Rorth, 1998) and encode fusion proteins identical to those encoded by the pPac-FLAG vectors, but without the FLAG tag.

Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, CA 90095-1569, USA.

*Present address: Department of Biological Sciences, Columbia University, 1114 Fairchild Center, 1212 Amsterdam Avenue, New York, NY 10027, USA
†Author for correspondence (e-mail: courey@chem.ucla.edu)

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Transient transfections in S2 cells and RNAi were carried out as described previously (Bhaskar and Courey, 2002; Jia et al., 2002).

Transgenic flies

UASp transgenes were introduced into females homozygous for a null allele of *dl* (*dl*^{1}) and containing the MatGal4 driver, which encodes Gal4-VP16 under the control of the maternally active *α4-Tubulin* promoter (gift of Daniel St Johnston, University of Cambridge, UK). Genotypes of the transgenic flies used in this paper are: (1) *dl*^{1}*/dl*^{1}*,P[dl];MatGal4/+*; (2) *dl*^{1}*/dl*^{1}*,P[dl-VP16]; MatGal4/+*; (3) *dl*^{1}*;P[dl-p65]/ MatGal*; and (4) *P[dl-WRPW]/+; dl*^{1}*; MatGal4/+*.

Embryos produced by these females were subjected to antibody staining and in situ hybridization according to previously described procedures (Hauptmann, 1999; Kosman et al., 2004; Tautz and Pfeifle, 1989).

RESULTS AND DISCUSSION

Construction of dedicated activator and repressor forms of DL

As repression by DL is largely dependent upon an eh1-like motif in the CTD that weakly binds GRO (Flores-Saaib et al., 2001), we attempted to create dedicated activator forms of DL by replacing the CTD with heterologous activation domains. We therefore generated plasmids encoding fusions between the DL RHD, which contains the DNA-binding domain, and either the p65 or VP16 activation domains, creating DL-p65 and DL-VP16 (Fig. 1A). Although wild-type DL binds GRO, this interaction is too weak to allow DL to recruit GRO on its own in vivo (Flores-Saaib et al., 2001; Valentine et al., 1998). Dedicated GRO-dependent repressors contain high-affinity GRO-recruitment motifs such as the WRPW motif found at the C-terminal end of Hairy family proteins (Fisher et al., 1996). Therefore, we attempted to convert DL into a dedicated repressor by fusing the WRPW motif to its C-terminal end, creating DL-WRPW (Fig. 1A).

In vivo, DL works on its own to mediate simple activation and in conjunction with TWI to mediate synergistic activation (Shirokawa and Courey, 1997). We examined the ability of the DL chimeras to mediate both types of activation using the D4-luc reporter (Fig. 1B), which contains four DL-binding sites and was used to test simple activation, as well as the DE5-luc reporter (Fig. 1C), which contains five DL-binding sites alternating with five TWI-binding sites and was used to test synergistic activation (Chen et al., 1999; Jia et al., 2002; Pan and Courey, 1992; Shirokawa and Courey, 1997). The two reporters yielded similar results. Wild-type DL activated each of these reporters by about 10- to 20-fold, whereas DL-p65 and DL-VP16 activated them by 150- to 350-fold. Appending the WRPW motif to wild-type DL dramatically attenuated its ability to activate both reporters. Wild-type DL and the three DL chimeras are expressed at similar levels (Fig. 1B, inset).

The effect of increasing GRO levels was tested by co-transfecting an expression vector encoding GRO (pPac-*gro*) with the DL constructs and the DE5-luc reporter (Fig. 1C). Increasing GRO levels leads to a small but reproducible decrease in wild-type DL-mediated activation. This is probably due to the weak interaction between DL and GRO, as a similar decrease is not observed for DL-p65 and DL-VP16, which lack the DL CTD containing the GRO-recruitment motif. A more dramatic (10-fold) attenuation of reporter activity was observed when GRO was co-transfected with DL-WRPW. This repression results in reporter gene expression that is lower than the basal level observed in the absence of transfected DL.

We also examined the GRO requirement for the function of some of the chimeras by using double-stranded RNA interference (RNAi) to remove GRO from transfected cells (Fig. 1D). An immunoblot with anti-GRO antibodies (inset) shows that RNAi significantly

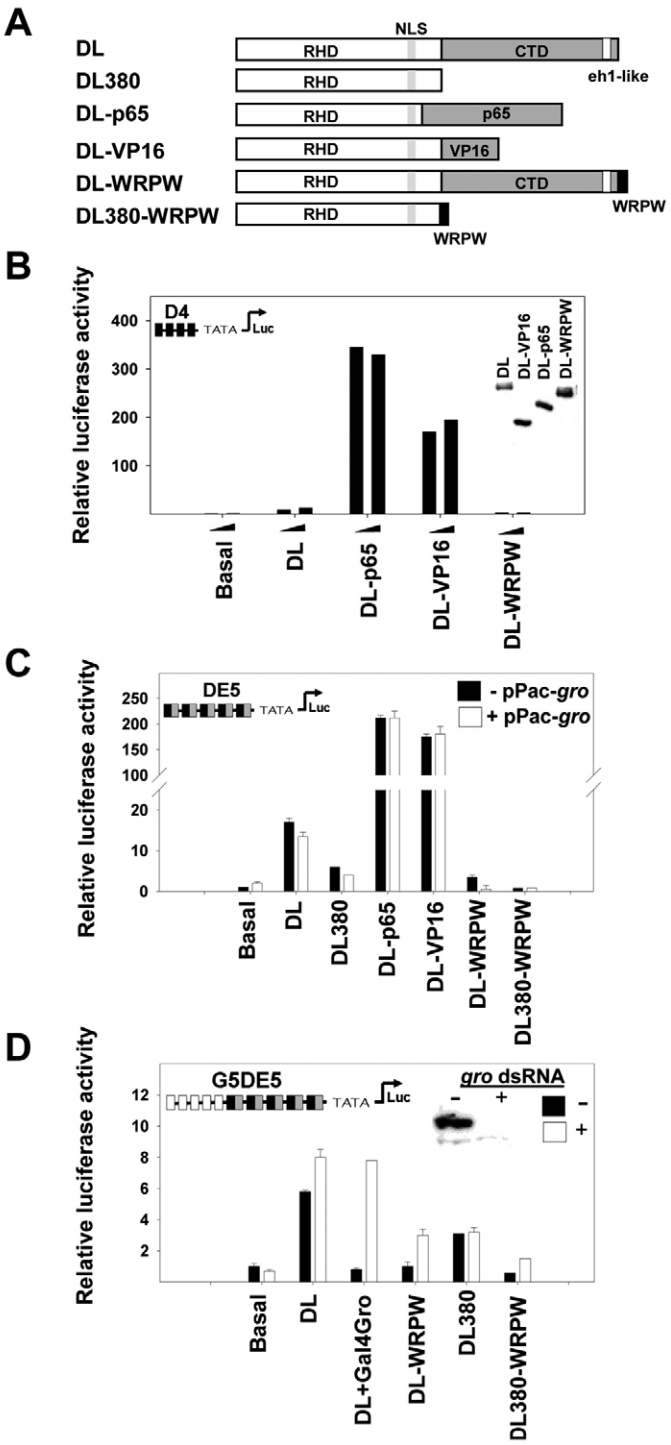

Fig. 1. Activation and repression by DL variants in S2 cells. (**A**) Structure of DL variants. (**B**) Activation of the D4-luciferase reporter by addition of increasing amounts (50 ng, 150 ng) of vectors encoding DL variants. (Inset) Anti-DL immunoblot of lysates of S2 cells transiently expressing the FLAG-DL constructs. (**C**) Synergistic activation of the DE5-luciferase reporter by vectors encoding DL variants (60 ng) and TWI (20 ng). An expression vector encoding GRO (500 ng) was also included in the indicated transfection mixtures. (**D**) Synergistic activation of the G5DE5-luciferase reporter by vectors encoding DL variants (60 ng) and TWI (20 ng). *gro* dsRNA (1 μg) was also included in the indicated transfection mixtures. (Inset) Anti-GRO immunoblot of lysates of S2 cells treated or not treated with *gro* dsRNA.

reduces the level of endogenous GRO. The reporter used in these experiments (G5DE5-luc) is identical to DE5-luc except for the presence of five Gal4-binding sites upstream of the DL and TWI sites. The presence of these sites allowed us to test the effect of a Gal4-GRO fusion protein. As was expected from previous studies (Song et al., 2004), this reporter is repressed by Gal4-GRO, and this repression is eliminated by GRO RNAi, providing further evidence for the effectiveness of the RNAi.

Co-transfection of G5DE5-luc with DL results in about a 6-fold increase in reporter expression. GRO RNAi results in a small but reproducible increase in the DL-activated level of reporter expression, in accord with the idea that GRO binds weakly to DL and attenuates activation by DL. A truncated form of DL (DL380) containing just the RHD activates transcription weakly and this activation is not influenced by GRO, as there is no effect of GRO knockdown. By contrast, transcription in the presence of DL-WRPW or DL380-WRPW is strongly stimulated by GRO knockdown. In conclusion, these tissue culture findings are consistent with the idea that DL-VP16 and DL-p65 function as GRO-insensitive dedicated activators, whereas DL-WRPW functions as a GRO-dependent dedicated repressor.

Regulation of DL target genes by dedicated activator and repressor DL alleles

To determine whether these DL variants also function as dedicated activators and repressors in vivo, they were maternally expressed in a *dl* null background using the Gal4/UAS system. Western blots (data not shown) indicated that the transgenes were expressed at levels similar to that of the endogenous *dl* gene. Like wild-type DL, all the DL variants were localized in ventral to dorsal nuclear concentration gradients (see Fig. S1 in the supplementary material). However, variants lacking the DL CTD (DL-VP16 or DL-p65) exhibited somewhat expanded gradients, consistent with the ability of the CTD to enhance cytoplasmic retention of DL (Isoda et al., 1992).

As expected, expression of the activation targets *twi*, *sna*, *sog* and *brk*, as determined by in situ hybridization, was nearly normal in embryos produced by females bearing the wild-type *dl* transgene under the control of a maternal Gal4 driver (Fig. 2, compare A with C). Expression of DL-VP16 (Fig. 2D) and DL-p65 (Fig. 2E) also resulted in ventral activation of *sna*, *twi*, *sog* and *brk*. Consistent with the expanded nuclear localization of these variants, the domain of ventral activation was also expanded relative to wild type. By contrast, DL-WRPW failed to activate *twi*, *sna*, *sog* or *brk* (Fig. 2F).

The repression targets *zen* and *dpp* are both expressed in the dorsal 40% of the precellular wild-type embryo (Fig. 2A), and show uniform expression along the DV axis in *dl* null embryos (Fig. 2B). Expression of transgenic DL restores a near wild-type pattern of *zen* and *dpp* expression (Fig. 2C). By contrast, early *zen* and *dpp* expression are greatly expanded in DL-VP16 and DL-p65 embryos – expression was frequently almost uniform along the DV axis (Fig. 2D,E). This is consistent with the function of DL-VP16 and DL-p65 as dedicated activators. By contrast, like wild-type DL, DL-WRPW repressed *zen* and *dpp* (Fig. 2F).

To further confirm that DL-VP16 is unable to repress, while DL-WRPW is unable to activate, we carried out double-labeling experiments to examine TWI protein and *zen* mRNA expression, simultaneously. In agreement with the single-labeling studies, DL-VP16 directed ventral activation of *twi*, but failed to repress *zen* (Fig. 3C), whereas DL-WRPW repressed *zen* but failed to activate *twi* (Fig. 3D).

Thus, the addition of a high-affinity GRO-binding motif to DL converts it to a dedicated repressor. This is consistent with the idea that that low-affinity binding of factors such as DL to GRO is crucial for their bifunctionality. DL-dependent silencers in genes such as *dpp* and *zen* contain sites adjacent to the DL-binding sites that are crucial for repression (Huang et al., 1995; Jiang et al., 1993; Kirov et al., 1993). Our findings fit a model in which factors that bind these adjacent sites assist DL in recruiting GRO. Evidence in favor of this model comes from studies of the *zen* silencer, in which the adjacent

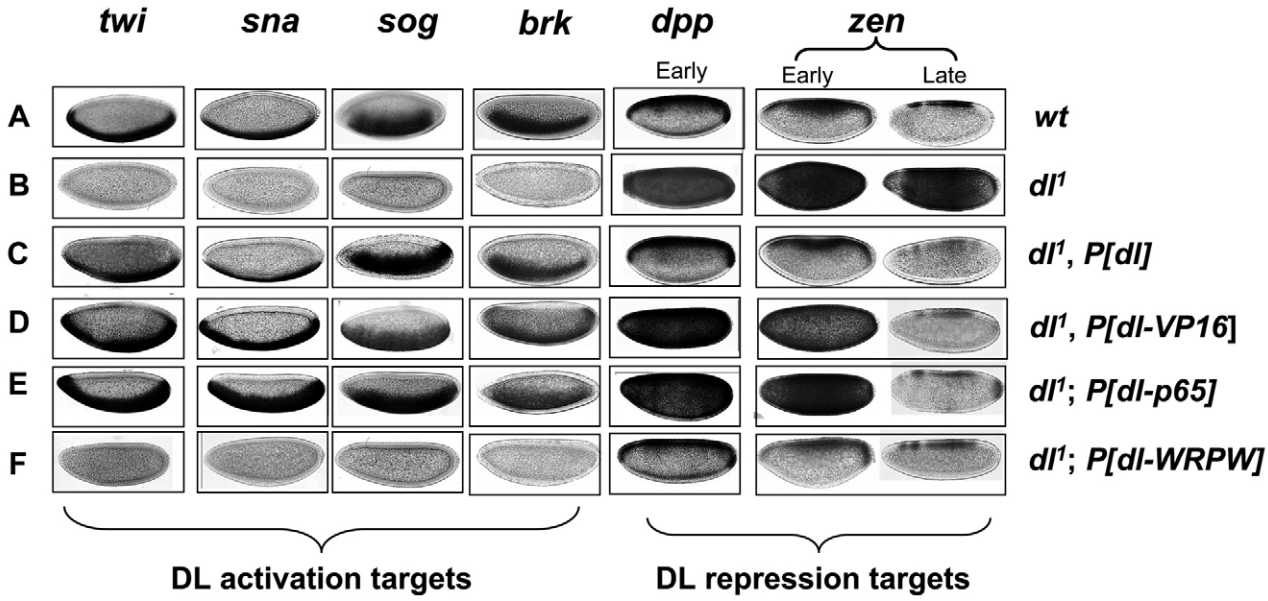

Fig. 2. Activation and repression of DL target genes by maternally expressed DL variants. (**A-F**) The expression of target genes was monitored by in situ hybridization using digoxigenin-labeled probes against *twi*, *sna*, *sog* or *brk* (genes activated by DL), and *dpp* or *zen* (genes repressed by DL). Sagittal views of the embryos are oriented with anterior on the left and the dorsal side at the top. Maternal genotypes are described in the Materials and methods.

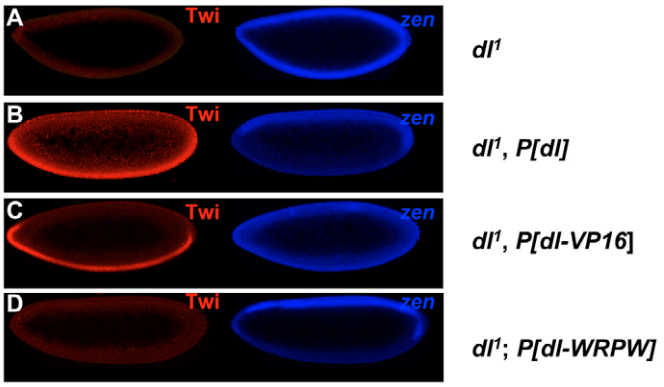

Fig. 3. Activation and repression by DL variants visualized by double labeling. (**A-D**) TWI protein expression was monitored by antibody staining (red, left), while *zen* mRNA expression was monitored by in situ hybridization (blue, right). Sagittal, confocal sections of the embryos are oriented with anterior on the left and the dorsal side at the top. Maternal genotypes are described in the Materials and methods.

sites were found to bind Dead ringer (DRI; Retained – FlyBase). Like DL, DRI binds GRO weakly, and it is likely that the combination of DL and DRI bound to adjacent sites results in the formation of a high-affinity platform for GRO recruitment (Hader et al., 2000; Valentine et al., 1998).

DV patterning in the absence of DL-mediated repression

Although *zen* is negatively regulated by DL in the syncytial blastoderm embryo, it comes under the positive control of the DPP signal during cellularization. At this stage, *zen* expression is lost from the dorsolateral region and is only maintained around the dorsal midline where the DPP signal is maximal (Fig. 2A,C) (Ferguson and Anderson, 1992a; Irish and Gelbart, 1987; Rushlow and Levine, 1990). In embryos carrying the dedicated activator alleles of DL, in which *zen* and *dpp* expression are nearly uniform in the syncytial embryo, *zen* expression is nonetheless restricted to a narrow dorsal stripe during cellularization (Fig. 2D,E). This indicates that although early *dpp* expression is almost uniform in these embryos, DPP signaling is still graded in the normal manner as a result of the action of SOG and BRK.

To assess further the ability of the DL variants to pattern the DV axis, we examined cuticle preparations. Embryos produced by *dl* null females were completely dorsalized developing into hollow tubes of dorsal epidermis (Fig. 4B). The wild-type *dl* transgene restored DV patterning, although the embryos were slightly ventralized when compared with wild-type embryos, as indicated by an incomplete head skeleton and missing or partially deleted Filzkörper (Fig. 4C). This ventralization may reflect a slight overexpression of the transgenic wild-type DL. DL-VP16 and DL-p65 substantially restored DV patterning, as demonstrated by the appearance of ventral denticle belts, Filzkörper and head cuticle (Fig. 4E,F). These embryos exhibited ventralization that was more severe than that observed in embryos expressing transgenic wild-type DL, as indicated by the greater widening of the ventral denticle belts and the greater severity of the head and Filzkörper defects. This greater ventralization is consistent with the expanded nuclear localization of the dedicated activators relative to wild-type DL.

In contrast to embryos expressing dedicated activator variants, embryos expressing DL-WRPW were almost as strongly dorsalized as the *dl* null embryos (Fig. 4G). The only sign of rescue of the

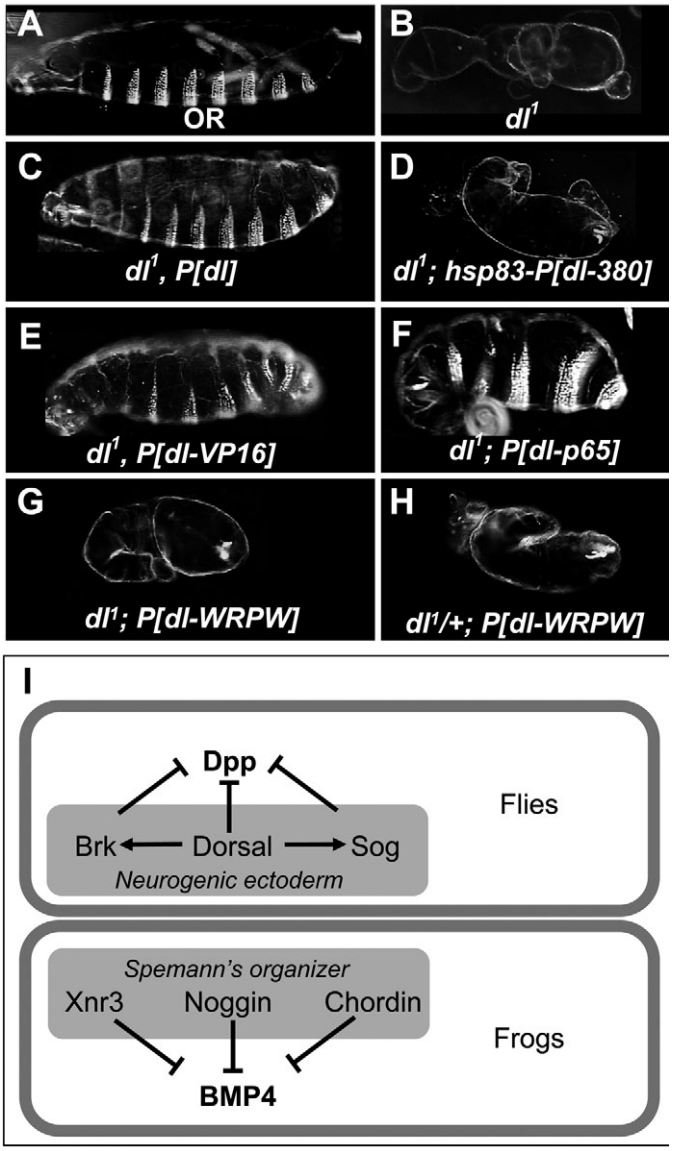

Fig. 4. DV patterning of the cuticle is rescued by dedicated activator DL variants. (**A-H**) Cuticle preparations of fully developed embryos expressing DL variants produced by females of the indicated genotypes (see Materials and methods). Anterior is to the left. (**I**) DPP/BMP signaling is redundantly blocked during DV patterning in invertebrate and vertebrate embryos.

dorsalized cuticles by DL-WRPW function was the frequent appearance of Filzkörper, structures that form at very low levels of DL activity (Fig. 4D) (Anderson et al., 1985; Roth et al., 1991). Consistent with the idea that the WRPW motif confers upon DL the ability to recruit GRO to targets that it would normally activate, DL-WRPW also leads to dorsalization in the presence of wild-type DL (Fig. 4H).

Redundant pathways to prevent DPP signaling in ventral regions

The above studies show that DL-mediated repression is dispensable, whereas DL-mediated activation is necessary and sufficient for patterning of the DV axis. This likely reflects the existence of multiple pathways to block DPP activity ventrally. These partially

redundant pathways include transcriptional repression by DL (Huang et al., 1993; Ray et al., 1991), interference with the binding of DPP to its transmembrane receptor by SOG (Biehs et al., 1996; Ferguson and Anderson, 1992b; Francois et al., 1994; Holley et al., 1995), and interference with the activation of downstream targets by BRK (Hasson et al., 2001; Jazwinska et al., 1999a; Saller and Bienz, 2001; Zhang et al., 2001) (Fig. 4I).

A number of previous findings suggest that these mechanisms for blocking DPP activity are partially redundant with one another. For example, *sog* or *brk* single mutants exhibit only modest defects in the neurogenic ectoderm, whereas *sog brk* double mutants are much more severely affected (Jazwinska et al., 1999b). Furthermore, the DL protein in the ancestral insect *Tribolium* probably does not function as a repressor, only as an activator, suggesting that the repression function of DL is a relatively recent acquisition (Chen et al., 2000).

The analogous vertebrate DV patterning forming system also exhibits redundancy. In the vertebrate embryo, the Spemann organizer opposes the signaling activity of BMP4 (a DPP homolog). This dorsal domain secretes a cocktail of partially redundant BMP4 inhibitors, including Chordin (a SOG homolog), Noggin and XNR3 (Fig. 4I). As a result of this redundancy, Chordin knockdown results in only minor patterning defects (Khokha et al., 2005). The widespread use of redundant mechanisms to block DPP/BMP4 activity (Niehrs, 2005) suggests that redundancy may be required to fine-tune developmental axes. It may also help to ensure the robustness of pattern formation in the face of environmental fluctuations that could adversely perturb gradients of signaling activity.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/22/4409/DC1

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