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Summary

The maternal Toll signaling pathway sets up a nuclear gradient of the transcription factor Dorsal in the early *Drosophila* embryo. Dorsal activates *twist* and *snail*, and the Dorsal/Twist/Snail network activates and represses other zygotic genes to form the correct expression patterns along the dorsoventral axis. An essential function of this patterning is to promote ventral cell invagination during mesoderm formation, but how the downstream genes regulate ventral invagination is not known. We show here that *wntD* is a novel member of the Wnt family. The expression of *wntD* is activated by Dorsal and Twist, but the expression is much reduced in the ventral cells through repression by Snail. Overexpression of WntD in the early embryo inhibits ventral invagination, suggesting that the de-repressed WntD in *snail* mutant embryos may contribute to inhibiting ventral invagination. The overexpressed WntD inhibits invagination by antagonizing Dorsal nuclear localization, as well as *twist* and *snail* expression. Consistent with the early expression of WntD at the poles in wild-type embryos, loss of WntD leads to posterior expansion of nuclear Dorsal and *snail* expression, demonstrating that physiological levels of WntD can also attenuate Dorsal nuclear localization. We also show that the de-repressed WntD in *snail* mutant embryos contributes to the premature loss of *snail* expression, probably by inhibiting Dorsal. Thus, these results together demonstrate that WntD is regulated by the Dorsal/Twist/Snail network, and is an inhibitor of Dorsal nuclear localization and function.

Key words: Dorsal, *Drosophila*, Gastrulation, Snail, Toll, WntD

Introduction

Mesoderm is the middle germ layer formed during gastrulation. In *Drosophila*, the mesoderm arises from the invagination of the ventral cells of the blastoderm. The mesoderm provides the precursor cells for muscles, hemocytes, lymph glands, the somatic gonad and the heart. The maternal Toll signaling pathway has a crucial role in establishing the ventral cell fate and thus mesoderm formation (Anderson, 1998; Roth, 2003; Stathopoulos and Levine, 2002; Wasserman, 2000).

Toll is a single-pass transmembrane receptor and is activated by a series of upstream serine proteases that processes the ligand Spätzle (Hashimoto et al., 1988; Hu et al., 2004; LeMosy et al., 1999; Morisato, 2001; Weber et al., 2003). The activated Toll recruits the cytoplasmic components MyD88, Tube and Pelle to regulate the nuclear transport of the transcription factor Dorsal (Charatsi et al., 2003; Kambris et al., 2003; Sun et al., 2004). Dorsal, a NF-κB homolog, is normally retained in the cytoplasm by Cactus, an IκB homolog. Toll signaling causes the phosphorylation and degradation of Cactus, thereby allowing Dorsal to enter the nucleus and regulate gene expression (Belvin et al., 1995; Bergmann et al., 1996; Fernandez et al., 2001; Reach et al., 1996). These signaling components are ubiquitously distributed, but the pathway is activated only in the ventral side of the embryo (LeMosy et al., 1999; Roth, 2003). Thus, activation of Toll by the diffusible Spätzle leads to the formation of a nuclear gradient of Dorsal, with the highest concentration in ventral nuclei (Anderson, 1998; Roth, 2003; Roth et al., 1989; Rushlow et al., 1989; Stathopoulos and Levine, 2002; Steward, 1989; Wasserman, 2000).

A single gradient of nuclear Dorsal can generate multiple patterns of zygotic gene expression along the dorsoventral axis (Jiang and Levine, 1993; Stathopoulos et al., 2002). Dorsal acts as both a transcriptional repressor and activator. For example, *zerknüllt* and *decapentaplegic* are repressed by Dorsal and therefore can be expressed only in the dorsal side of the embryo where the dorsal ectoderm is formed (Huang et al., 1993; Ip et al., 1991; Jiang et al., 1992; Pan and Courey, 1992). Meanwhile, Dorsal activates other zygotic genes, such as *twist*, *snail*, *rhomboid*, *short gastrulation*, *lethal of scute* and *single-minded* (*sim*). Depending on the affinity of the Dorsal-binding sites and on the presence of co-activator sites on their promoters, these target genes are activated by different thresholds of the Dorsal gradient, and thus have ventral expression with variable lateral limits (Stathopoulos and Levine, 2002).

High levels of nuclear Dorsal activate the expression of *twist* and *snail*, and the Dorsal/Twist/Snail network regulates ventral cell invagination to form the mesoderm (Ip and Gridley, 2002; Leptin, 1999; Stathopoulos and Levine, 2002). In *dorsal*, *twist* or *snail* mutants, no ventral invagination occurs and no mesodermal tissues are formed. Twist is a basic helix-loop-helix transcription factor and acts as a co-activator for Dorsal to optimally activate other zygotic target genes, including *snail*. Snail contains five zinc fingers and functions as a transcriptional repressor (Hemavathy et al., 2000; Nieto, 2002). A model for this gene regulatory network in promoting mesoderm formation is that Dorsal/Twist activates multiple zygotic genes that are expressed in the ventral region with different lateral limits. These target genes may promote the ventral (mesodermal) cell fate or the lateral (neuroectodermal) cell fate. Snail specifically represses those genes that are not compatible with mesoderm formation. Consistent with this model, many genes, including *rhomboid*, *sim*, *lethal of scute*, *short gastrulation*, *crumbs*, *Delta* and *Enhancer of split*, are repressed by Snail in the ventral region and their expression is, therefore, restricted to the lateral regions. In *snail* mutant embryos, these genes are de-repressed into the ventral region. However, it has not been demonstrated that any of these Snail target genes can directly inhibit ventral invagination and mesoderm formation (Hemavathy et al., 1997).

To identify novel components in the dorsoventral pathway, we carried out a microarray assay using embryos derived from gain-of-function and loss-of-function mutants of the Toll pathway. Among the novel genes identified, we analyzed the expression and function of *wntD* because the Wnt family of secreted proteins regulates patterning, cell polarity and cell movements (Nelson and Nusse, 2004; Veeman et al., 2003). Our results show that *wntD* is activated by Dorsal and Twist but repressed by Snail. Increased expression of WntD in wild-type early embryos inhibits ventral invagination. Thus, *wntD* is the first Snail target gene shown to have an interfering function in mesoderm invagination. We also demonstrate that the overexpressed WntD blocks invagination by inhibiting Dorsal nuclear localization. Loss-of-function analyses also show that physiological levels of WntD can attenuate Dorsal nuclear localization and function. Therefore, *wntD* is a novel downstream gene of the Dorsal/Twist/Snail network and can feed back to inhibit Dorsal.

Materials and methods

Drosophila stocks and genetics

Control strains used were OregonR or *y w*. Transgenic lines were generated by microinjection of the *P* element plasmid together with the Δ2-3 transposase helper plasmid into *y w* embryos. The *UAS-wntD* flies used for most of the experiments were homozygous for the transgenes on both second and third chromosomes. The p*CaSpeR-wntD* transgenic flies and the *Df(3R)l26c* flies were mated with a double balancer chromosome strain, and then mated together to establish a stable line containing the homozygous rescue construct on the second chromosome and *Df(3R)l26c* on the third chromosome, with a balancer. The $snail^{HG31}$, $twist^{ID96}$, $dorsal^{H}$, *Df(2L)TW119*, $Toll^{10b}$, $twist^{IIH}$ $snail^{IIG}$ double mutant, $Delta^{B2}$ and *nanos-Gal4* stocks were used.

Plasmids and cloning

OregonR genomic DNA was used as the template for PCR amplification of the *wntD* ORF. To generate the *pUAST-wntD* for embryonic expression experiments, the primers GATCGCGGCC-GCTCAGTCGATCTAACGACATCGCAG and GATCGGTACCG-TTGTGGTAATAAATTAGAGGTGG were used to amplify the *wntD* ORF together with 58 bp 5′ and 117 bp 3′ of the ORF. This fragment was subcloned into the *Not*I and *Asp*718 sites of pBluescript KS(+). This entire fragment was then excised with *Not*I and *Asp*718, and subcloned into the *Not*I and *Asp*718 sites of pUAST. To generate the *pCaSpeR-wntD* genomic rescue construct, a 5 kb genomic DNA region was amplified in two fragments using PCR. The 5′ fragment of 3184 bp was amplified using the primers GATCGGTACC-GATCTGGTCGGTGGCCTCTTCAAC and GATCGGTACCGTT-GTGGTAATAAATTAGAGGTGG, and then digested with *Asp*718 and *Nco*I . The 3′ fragment of 2842 bp was amplified using the primers GATCGCGGCCGCTCAGTCGATCTAACGACATCGCAG and GA-TCGCGGCCGCCAGACATCGACTTGTGCGACTGGC, and then digested with *Nco*I and *Not*I. The fragments were then ligated into the *Asp*718 and *Not*I sites of pBluescript KS(+). The 5 kb genomic clone was then digested with *Apa*I and *Age*I and blunted with Klenow polymerase. This yielded a 2721 bp fragment that included the *wntD* ORF plus 1558 bp of 5′ flanking sequence and 233 bp 3′ flanking sequence. This region does not contain any other annotated ORF. This 2721 bp fragment was blunt-end ligated into the *Xba*I site of the pCaSpeR vector.

Embryo in situ and antibody staining

Embryo in situ hybridization using digoxigenin-labeled probes was carried out as previously described (Hemavathy et al., 2004; Hemavathy et al., 1997). Double in situ hybridization for the simultaneous detection of *snail* and *wntD* transcripts was performed using digoxigenin-labeled *snail* (diluted 1:200) and biotin-labeled *wntD* (diluted 1:100) probes together during hybridization. After washing with buffers, embryos were incubated overnight at 4°C with sheep anti-digoxigenin Fab fragments conjugated to peroxidase (Roche, 1:1200 dilution). The peroxidase stain was developed using 0.5 mg/ml DAB in 1×PBS and 0.006% H_2O_2. Embryos were then washed five times in 1×PBT to remove the DAB/H_2O_2. They were incubated overnight with anti-Biotin Fab fragments conjugated to alkaline phosphatase (Roche, 1:2000 dilution) at 4°C. The alkaline phosphatase staining was developed with NBT/BCIP. Embryos were mounted in Permount and visualized under Nomarski optics. The monoclonal antibody 7A4 was used to stain for Dorsal, using the procedure as described (Hemavathy et al., 2004; Hemavathy et al., 1997). Goat anti-mouse IgG (Fab fragments) conjugated with the Alexa 488 fluorochrome (Molecular Probes) was used and the samples were mounted in Vectashield with DAPI (Vector Laboratories).

Results

Drosophila wntD is expressed in a complex pattern during early embryogenesis

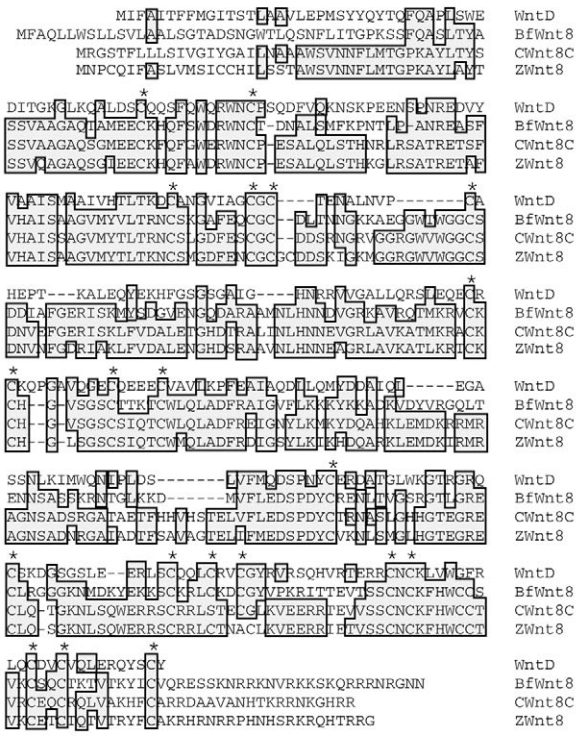
We used microarray chips (Affymetrix) to study the gene expression profiles of gastrulating embryos derived from wild-type, $dorsal^{-/-}$ and $Toll^{10b}$ flies. $Toll^{10b}$ codes for a gain-of-function Toll receptor (Schneider et al., 1991). Many known target genes, such as *twist*, *snail*, *short gastrulation*, *tinman* and *mef2*, showed lower expression in the $dorsal^{-/-}$ sample and increased expression in the $Toll^{10b}$ sample, as predicted (data not shown). Among the novel targets, we selected the annotated gene CG8458 for further study because it encodes a member of the Wnt family, and Wnt proteins have been implicated in controlling cell polarity and cell movement in many organisms (Nelson and Nusse, 2004; Veeman et al., 2003).

The predicted amino acid sequence of CG8458 has the

closest homology to cephalochordate Wnt8, chicken Wnt8C, and zebrafish Wnt8. Sequence alignment and pair-wise comparison show that CG8458 is a distal member of this subfamily (Fig. 1A,B). The average identity between CG8458 and other Wnt8 molecules is approximately 27%, while the identity among other members is higher than 50%. Nonetheless, 20 out of the 22 characteristic cysteine residues of Wnt proteins are conserved in CG8458 (Fig. 1A, asterisks). FlyBase (http://flybase.bio.indiana.edu/) has named this gene *Drosophila Wnt8*. However, a recent report suggests that this gene may not be an ortholog of vertebrate *Wnt8* but instead an orphan Wnt gene (Kusserow et al., 2005). Based on our functional analysis, we elected to use the name *Drosophila wntD* for the annotated gene CG8458, and the encoded protein WntD (Wnt inhibitor of Dorsal). A similar microarray analysis was reported, but *Drosophila wntD* was not included probably because of the different criteria used for selection (Stathopoulos et al., 2002).

In situ analysis reveals that *wntD* mRNA is expressed in a dynamic pattern in the early embryo. There is no detectable maternally deposited RNA and the earliest zygotic expression is present at the anterior and posterior poles of early stage 4 embryos (Fig. 1C). Soon after, *wntD* is expressed in a few patches of ventral cells (Fig. 1D). This low level of expression remains in the ventral cells throughout the blastoderm stage. Meanwhile, expression arises in two lines of cells abutting the mesoderm (Fig. 1E,F). These two lines of staining coincide with the mesectoderm, the precursor of ventral midline cells (see Fig. 2). The expression of *wntD* in the mesectoderm persists during germ band extension and gradually disappears (Fig. 1G,H). De novo expression appears around stage 8 in the ventral neuroectodermal cells adjacent to the midline (Fig. 1G,H). This expression continues in the neuroectoderm through stages 9 and 10 (Fig. 1I,J), and is reduced to an undetectable level by stage 11. No expression was detected in other stages of embryonic development. The expression pattern

A



B

	BfWnt8	CWnt8C	ZWnt8
WntD	28%/45%	27%/45%	26%/43%
BfWnt8		55%/69%	50%/68%
CWnt8C			74%/85%

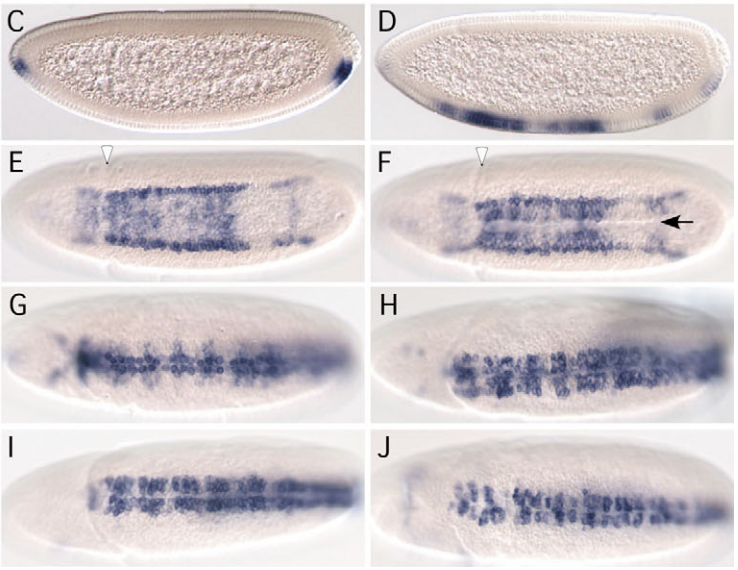


Fig. 1. (A) Alignment of Wnt8/WntD protein sequences. Sequences of Wnt proteins from *Drosophila melanogaster* (WntD), *Branchiostoma floridae* (cephalochordate, BfWnt8), *Gallus gallus* (chicken, CWnt8), and *Danio rerio* (zebrafish, ZWnt8) are shown. The lightly shaded boxes highlight the conserved amino acid residues. WntD has fewer conserved residues when compared with other members of this subfamily. However, 20/22 of the characteristic cysteine residues are conserved (asterisks). (B) Degree of conservation among WntD and Wnt8 family members. The percent identity/percent similarity is shown in the table. WntD is more distally related to other members in the Wnt8 subfamily. (C-J) Expression pattern of *Drosophila wntD* in wild-type embryos. In situ hybridization was performed using an antisense probe generated from a *wntD* cDNA clone. The embryos are oriented with the anterior to the left. For sagittal views, the dorsal side is up (C,D); for other embryos, the ventral views are shown (E-J). The embryo in C is a pre-cellular blastoderm (stage 4), D is a cellular blastoderm (stage 5), E is an early gastrula-stage embryo (stage 6), and F is a gastrula-stage embryo with a ventral furrow, indicated by the arrow (stage 6). The embryos in G-J are at various stages of germ-band extension (stages 7, 8, 9 and 10). During gastrulation, the cephalic furrow (arrowhead in panels E,F) is formed at approximately the same time as the ventral furrow. The expression of *wntD* appears first in the anterior and posterior regions of the pre-cellular blastoderm (C), and then in the ventral cells and mesectoderm (D-F). Expression continues in the ventral mesectoderm (G), and de novo expression appears in the ventral neuroectoderm (G-J).

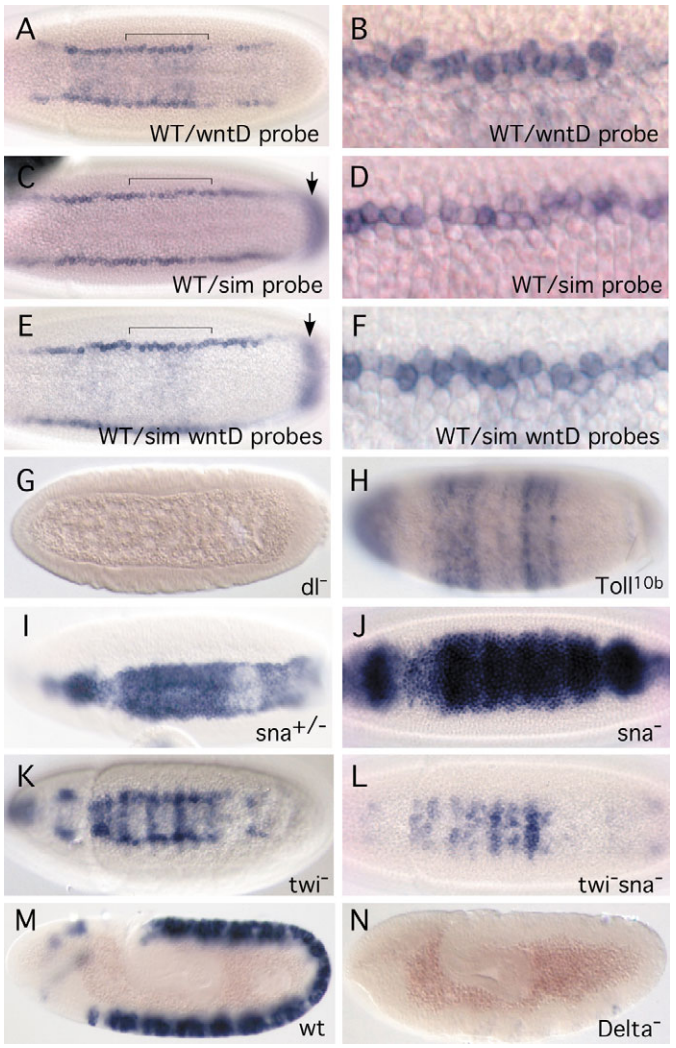


Fig. 2. Genetic regulation of *wntD* expression. (A-F) The expression of *wntD* and *sim* in wild-type (WT) embryos. Both genes have lateral stripes of expression. Only *wntD* shows a lower level of expression in ventral cells, and only *sim* has a characteristic stripe in the posterior region (arrows in C and E). The embryo in E and F showed both characters, indicating that it contained both *wntD* and *sim* probes. Panels B, D and F are higher magnifications of the regions indicated by the brackets in A, C and E, respectively. The double in situ hybridization (E,F) shows that the *wntD* and *sim* patterns overlap in the mesectoderm. (G) In embryos laid by *dorsal*$^{-/-}$ mothers, no *wntD* staining was observed at any stage of embryogenesis. (H) In embryos laid by *Toll*^{10b} mothers, an expansion of *wntD* expression into the dorsal side was observed. (I) Heterozygous *snail* embryos had increased expression in ventral cells but the mesectodermal expression was unchanged. (J) In the *snail* homozygous background, the mesectodermal expression of *wntD* disappeared, and the ventral staining became stronger. (K) In *twist* mutant embryos, the *wntD* pattern was narrower but overall was similar to that observed in wild-type embryos. (L) In *twist snail* double-mutant embryos, the mesectodermal staining disappeared, while a weaker ventral staining remained. (M) Sagittal view of a wild-type embryo during germ-band extension, showing *wntD* expression in the neuroectoderm. (N) In embryos that were zygotically mutant for *Delta*, the *wntD* expression in the neuroectoderm disappeared.

of *wntD* is largely different from other *Drosophila* Wnt genes (Eisenberg et al., 1992; Graba et al., 1995; Janson et al., 2001; Russell et al., 1992).

Genetic control of *wntD* expression by the dorsoventral pathway

We first performed double in situ hybridization to determine the location of the two lateral lines of *wntD* expression (Fig. 2A-F). Previous reports demonstrate that *sim* is expressed in the mesectoderm (Fig. 2C,D) and is strongly repressed by Snail in ventral cells (Kasai et al., 1992; Kosman et al., 1991; Leptin, 1991). Embryos that contained both *wntD* and *sim* in situ probes (Fig. 2E,F) showed essentially the same lateral pattern as embryos that contained either probe (Fig. 2A-D), demonstrating that the two patterns overlap. Double in situ hybridization also showed that, similar to *sim*, the lateral expression of *wntD* is at the border of the *snail* pattern (see Fig. 6A). Thus, we conclude that the two lateral lines of *wntD* expression are in the mesectoderm.

To understand the regulation of *wntD*, we analyzed its expression in various genetic mutants. No signal was observed in embryos derived from *dorsal*$^{-/-}$ mothers (Fig. 2G), demonstrating that the expression in both the trunk and the poles is absolutely dependent on Dorsal. In embryos derived from *Toll*^{10b} mothers, the expression of *wntD* was expanded into the dorsal side but the overall staining was not stronger than wild type (Fig. 2H), probably as a result of both activation by Dorsal and repression by Snail (see below). In conclusion, the mRNA staining in *dorsal*$^{-/-}$ and *Toll*^{10b} embryos corroborates the results of the microarray analysis.

In *snail* homozygous mutant embryos, a higher level of *wntD* expression was present throughout the ventral region but mesectodermal expression was not obvious (Fig. 2J). We also observed that in some heterozygous embryos there was normal mesectodermal staining but higher ventral expression of *wntD* (Fig. 2I). Gene expression in the mesectoderm is regulated by a complex interaction between the Notch pathway and Snail, such that the mesectodermal expression of *sim* also requires the positive input of Snail (Cowden and Levine, 2002; Morel et al., 2003; Morel and Schweisguth, 2000). The mesectodermal expression of *wntD* in both wild-type and *snail* mutant embryos is similar to that of *sim*, suggesting that *wntD* and *sim* are regulated by a similar mechanism. More importantly, the results demonstrate that Snail also represses *wntD* expression in the ventral cells.

In *twist* mutant embryos, *wntD* showed a narrower version of the wild-type pattern, centered on the ventral midline (Fig. 2K). The Snail pattern is significantly reduced in *twist* mutant embryos (Kosman et al., 1991; Leptin, 1991; Ray et al., 1991). Therefore, the narrower *Wnt8* pattern in *twist* mutants can be explained by the reduced expression of the repressor Snail. In *twist snail* double-mutant embryos, the expression of *wntD* was weak and only present in the ventral-most cells (Fig. 2L). We speculate that high levels of Dorsal are sufficient to activate this weak expression of *wntD* in the ventral nuclei. However, the overall ventral staining of *wntD* in the double mutant was much weaker than that in *snail* mutants (compare with Fig. 2J), suggesting that *wntD* is weakly activated by Dorsal and strongly activated by Dorsal/Twist cooperation, as has been

shown for other target genes of the dorsoventral pathway (Ip et al., 1992b; Jiang and Levine, 1993; Shirokawa and Courey, 1997). A stronger activation by the Dorsal/Twist combination may also explain the detectable expression of *wntD* in the ventral cells of wild-type embryos despite the repression by Snail. Within 1.6 kb of the 5′ flanking sequence of *wntD*, there are seven sites that are similar to the Snail-binding consensus and five sites that are similar to Dorsal-binding consensus (data not shown). However, the demonstration of whether *wntD* is a direct target requires further evidence.

wntD expression in the neuroectoderm depends on Delta. In zygotic *Delta* mutant embryos, the early *wntD* pattern was largely unaffected but the late pattern during germ-band extension was reduced and subsequently lost (Fig. 2M,N). Early embryos contain a significant maternal load of *Delta* gene products. As a result, the expression of target genes such as *sim* remains unaffected until later stages (Martin-Bermudo et al., 1995). The regulation of *wntD* by Delta in the neuroectoderm may depend on a similar mechanism.

Increased expression of WntD blocks presumptive mesoderm invagination

An essential biological function of the Dorsal/Twist/Snail network is to promote invagination of the ventral cells to form the mesoderm (Ip and Gridley, 2002; Leptin, 1999; Stathopoulos and Levine, 2002). Although the repressor function of Snail is required for ventral invagination, none of the known target genes normally repressed by Snail has been directly implicated in disrupting ventral invagination (Hemavathy et al., 2004; Hemavathy et al., 1997). Because *wntD* is repressed by Snail, we increased *wntD* expression in wild-type embryos in an attempt to phenocopy the defects in *snail* mutant embryos. The maternal *nanos-Gal4* line was used to direct the ubiquitous expression of *UAS-wntD* in early embryos. We found that approximately 50% of these embryos at the gastrulation stage had observable defects in ventral invagination. Approximately one quarter of these defective embryos had completely lost the ventral furrow (Fig. 3B), and the others showed varying degrees of invagination with the anterior regions always being worse than the posterior regions. The ventral invagination defect is not a result of general problems in cell shape changes or cell movements because cephalic furrow formation and germ-band extension occurred normally in these embryos. Tissue sectioning confirmed the phenotype that the mesoderm was largely missing in gastrulating embryos (Fig. 3D).

The *nanos-Gal4* female flies deposit maternally the *Gal4* gene products, which direct the UAS-dependent WntD expression ubiquitously in pre-blastoderm stage embryos. We also tested the *rhomboid-Gal4* driver; this *rhomboid* promoter contains mutations in its Snail-binding sites and directs zygotic *Gal4* expression in the ventral half of the blastoderm (Ip et al., 1992a). In these experiments, approximately 5% of embryos at gastrulation stage showed slightly defective invagination (data not shown). The *rhomboid* promoter, as well as other ventral zygotic promoters, is activated by Dorsal. Thus, the expression of WntD by zygotic promoters may be too late to induce a substantial phenotype. This speculation is consistent with the mechanism of feedback inhibition of Dorsal by WntD as shown below.

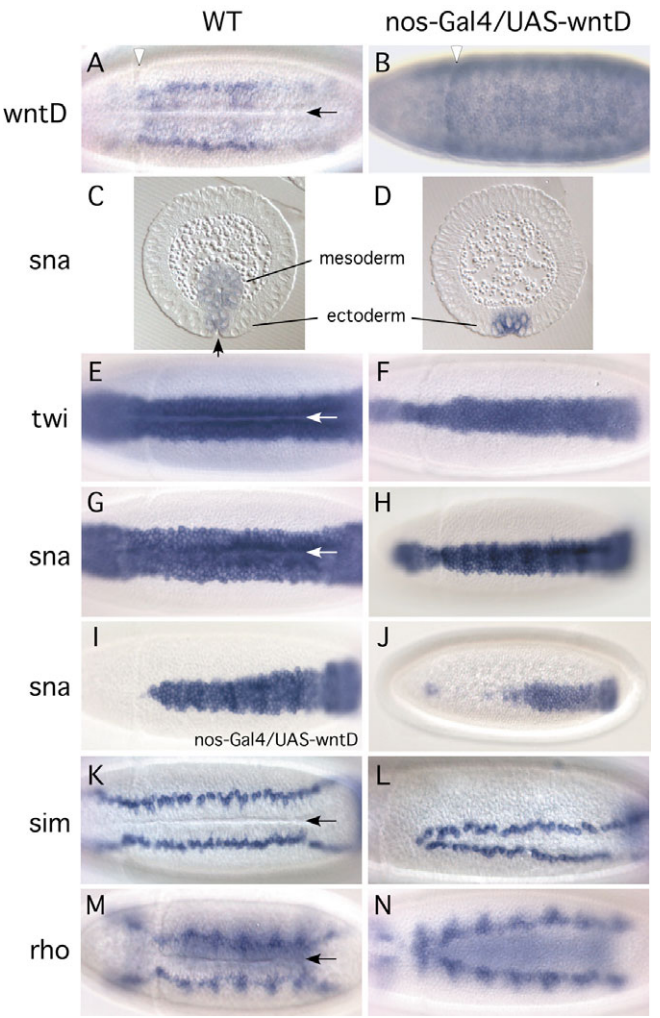


Fig. 3. Increased WntD expression blocks ventral invagination by interfering with *twist* and *snail* expression. The panels in the left column, except panel I, are wild-type embryos. The panels in the right column are embryos expressing WntD by the *nanos-Gal4-UAS* system. The in situ hybridization probes used are indicated on the left. Panels C and D are cross-sections with dorsal side up, and all other panels are ventral views with anterior to the left. Arrow indicates ventral furrow; arrowhead indicates cephalic furrow. (A,B) The overall expression level of *wntD* was higher in *nanos-Gal4-UAS-wntD* embryos than in wild-type embryos and the expression was ubiquitous. The pictures were underexposed to show the cell morphology. The embryo in panel B had no ventral furrow, whereas the cephalic furrow appeared normal. (C,D) Cross-sections of gastrulating embryos showing that no mesoderm was formed during gastrulation in embryos overexpressing WntD. (E,F) The *twist* expression pattern was much reduced in embryos overexpressing WntD. In wild-type embryos, *twist* expression is approximately 22 cells wide along the dorsoventral axis at the onset of gastrulation. The embryo shown in E already had some of the cells invaginated. (G) A wild-type embryo showing the normal *snail* pattern. (H-J) The panels show the reduced *snail* pattern with increasing severity in embryos overexpressing WntD. Some embryos showed narrower patterns of expression whereas others showed no expression in the anterior regions. (K-N) WntD overexpression also causes *sim* and *rhomboid* to show abnormal expression patterns. In wild-type embryos, the expression of *sim* and *rhomboid* in the ventral cells is repressed by Snail. Moreover, the positioning of *sim* also requires Snail. Thus, the abnormal patterns of *sim* and *rhomboid* in panels L and N correlate well with the reduced *snail* pattern.

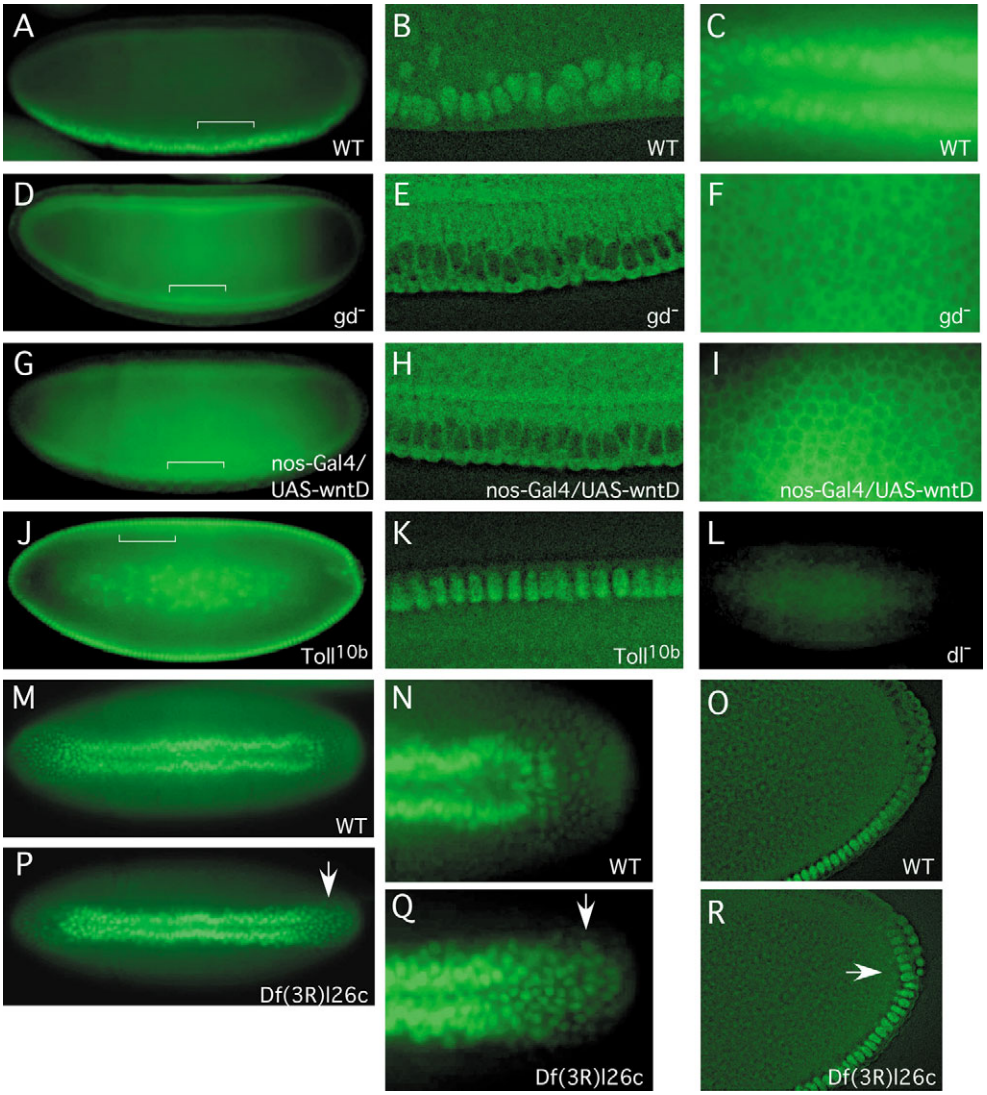


Fig. 4. WntD regulates Dorsal nuclear localization. All the panels show immunofluorescence staining using an anti-Dorsal antibody. A,D,G,J and L are side views; M and P are ventral views of whole embryos. B,E,H and K are sagittal views, after 2D deconvolution, of the regions indicated by the brackets in A,D,G and J, respectively. C,F and I are ventral views of gastrulae. N and Q are higher magnification views of the posterior regions of the embryos shown in M and P, respectively. O and R are sagittal views, after 2D deconvolution, of cellular blastoderms at the posterior region, including the pole cells. The genotype of each embryo is shown at the bottom right-hand corner. *gd*, *gastrulation-defective*; *Toll*^{10b} is a gain of function *Toll*. (A-C) In wild-type blastoderm and gastrula, Dorsal protein is localized in the ventral nuclei. (D-F) In *gastrulation-defective* mutant embryos, the Dorsal protein remains cytoplasmic. (G-I) In many WntD-overexpression embryos, Dorsal is also cytoplasmic. (J,K) In *Toll*^{10b} embryos, the nuclear Dorsal staining extended into the dorsal side of the embryo. (L) Essentially no signal was detected in a Dorsal protein null embryo. (M,N) Ventral view of a wild-type gastrula showing high levels of nuclear Dorsal around the ventral furrow. The higher magnification in N shows that the posterior region (right side) had less staining. (O) Sagittal view of a wild-type cellular blastoderm at the posterior end, showing the staining of Dorsal changing from nuclear to cytoplasmic in cells ventral to the pole cells. (P-R) Embryos derived from the *Df(3R)l26c* strain, which has many genes, including *wntD*, uncovered, showed increased Dorsal nuclear staining in the posterior region, as indicated by the arrow. (R) In a cellular blastoderm before germ-band extension, the nuclear staining of Dorsal already extents further to the dorsal side, using pole cells as a reference.

WntD blocks invagination by disrupting the expression of mesoderm determinants

The Wnt family of secreted proteins regulates cell fate, cell polarity, cytoskeleton and cell movement (Nelson and Nusse, 2004; Veeman et al., 2003). To elucidate the mechanism that underlies the invagination defect induced by WntD, we stained for various markers of cell fate and cell shape. We were surprised to find that *twist* and *snail* expression became highly abnormal in the *nanos-Gal4*-driven WntD-expressing embryos. The *twist* pattern was narrower than 12-cell widths along the circumference, compared with 20-cell widths in wild-type embryos (Fig. 3E,F) (Kosman et al., 1991). The *snail* expression pattern was even more severely affected. A total of 93% (n=147) of WntD-expressing embryos at the blastoderm and gastrulation stages showed abnormality in the *snail* expression pattern. The abnormality is variable and ranges from a few cells narrower to an almost complete disappearance of the pattern (Fig. 3G-J). The anterior expression was always more severely affected, and the posterior expression was affected to various extents in different embryos. We quantitated the phenotype by counting the width of the *snail* expression domains at 50% egg-length. In WntD-overexpression embryos that we assigned to have a phenotype, the *snail* pattern varied from zero to 11 cells, with an average width of seven cells. For wild-type embryos, the width of the *snail* domain is 13 to 17, with an average of 15 cells. Thus, all the embryos that we assigned to have a phenotype showed quantitative defects.

sim and *rhomboid* are normally repressed by Snail in the presumptive mesoderm (Fig. 3K,M) (Kasai et al., 1992; Kosman et al., 1991; Leptin, 1991). In WntD-overexpression embryos the *sim* pattern disappeared in the anterior region and the lateral rows of staining came closer in the posterior region (Fig. 3L). As described above, Snail represses *sim* expression in the ventral cells but the expression and positioning of *sim* in the presumptive mesectoderm also requires Snail (Cowden and Levine, 2002; Morel et al., 2003; Morel and Schweisguth,

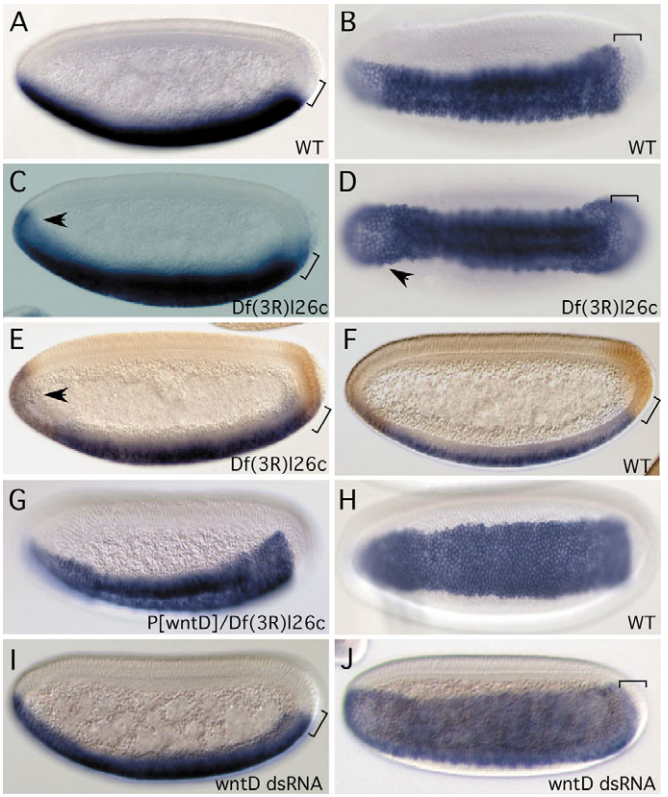


Fig. 5. Loss of WntD function leads to expansion of a Dorsal target gene. The blue staining in all the panels is RNA in situ staining using an antisense *snail* probe. The brown staining in E and F is in situ staining using an antisense *huckebein* probe. (A) Sagittal view of a wild-type blastoderm. The bracket at the posterior end indicates the retracted expression from the pole. (B) Ventral view of a wild-type gastrula, showing the sharp pattern of *snail* in the lateral and posterior regions. (C) Sagittal view of a *Df(3R)l26c* blastoderm. The bracket and the arrow indicate the expanded staining in the posterior and anterior regions, respectively. (D) Ventral view of a *Df(3R)l26c* gastrula; the expanded staining is similarly indicated by the bracket and the arrow. (E,F) Double staining of *snail* and *huckebein*, showing their complementary patterns in the posterior region of a wild-type embryo but overlapping pattern in a *Df(3R)l26c* embryo. (G) Ventrolateral view of an embryo derived from *Df(3R)l26c* strain that also contained a transgenic *wntD* genomic construct. All embryos from this rescued strain showed *snail* expression identical to that observed in wild-type embryos. (H) Ventral view of another wild-type blastoderm, with a retracted posterior pattern. (I) Sagittal view of a wild-type blastoderm previously injected with *wntD* dsRNA, showing a slightly expanded posterior expression. (J) Ventral view of a wild-type blastoderm previously injected with *wntD* dsRNA. The posterior sharpening is not as obvious as in wild-type embryos injected with buffer alone.

2000). Therefore, the abnormal *sim* pattern follows exactly the reduced *snail* expression. *rhomboid* showed similar narrowing of the pattern (Fig. 3N), consistent with the model that Snail is a simple repressor of *rhomboid*. In conclusion, increased WntD expression causes highly reduced *twist* and *snail* expression, leading to abnormal expression of other genes in ventral cells. Even though increased WntD expression may also cause other defects, the reduced *twist* and *snail* expression is probably sufficient to account for the loss of invagination.

Negative regulation of Dorsal nuclear localization by WntD

The direct activator of *twist* and *snail* expression in the blastoderm is Dorsal (Ip et al., 1992b; Jiang et al., 1991). Therefore, we examined the distribution of the Dorsal protein. In wild-type blastoderm and gastrulating embryos, Dorsal shows the characteristic ventral nuclear pattern (Fig. 4A-C). By contrast, WntD overexpression caused low-level staining around the periphery of the whole embryo (Fig. 4G), and high-resolution imaging showed that the ventral cells had Dorsal proteins predominantly in the cytoplasm (Fig. 4H,I). This phenotype was similar to that of embryos derived from a *gastrulation-defective* mutant (Fig. 4D-F), which causes no activation of the Toll pathway. Embryos derived from the opposite $Toll^{10b}$ gain-of-function mutant showed nuclear staining all around the embryo (Fig. 4J,K). The phenotype induced by WntD overexpression was different from that in the Dorsal protein null mutant, which essentially showed no staining (Fig. 4L). These results together suggest that the overexpressed WntD inhibits Dorsal nuclear localization.

Specific mutants of *wntD* are not yet available. Therefore, we examined a few deficiency strains by staining for *wntD* mRNA expression in the embryo and confirmed that *Df(3R)l26c*, which has the 87E1-87F11 region deleted, has uncovered *wntD*. We used this deficiency to assess whether endogenous WntD regulates Dorsal. In wild-type blastoderm, the Dorsal nuclear gradient extends into the neuroectoderm and the posterior end (Stathopoulos and Levine, 2002). Before the onset of gastrulation, the posterior Dorsal staining is normally retracted (Fig. 4M-O). However, in embryos derived from the *Df(3R)l26c* strain, Dorsal nuclear staining expanded in the posterior region (Fig. 4P-R). Because the earliest *wntD* expression is at the anterior and posterior regions (Fig. 1C), the loss of *wntD* in the deficiency could be the underlying reason for the posterior expansion of Dorsal in these embryos.

WntD attenuates the function of Dorsal

The posterior expression of *snail* in wild-type embryos is retracted and shows a sharp pattern before the onset of gastrulation (Fig. 5A,B). *snail* expression in *Df(3R)l26c* mutant embryos, however, expanded into the posterior region (Fig. 5C,D). Double staining shows that, in wild-type embryos, the posterior gene *huckebein* is complementary to the *snail* pattern (Fig. 5F). Moreover, we did not detect a change in the *huckebein* pattern in the *Df(3R)l26c* mutant embryos (data not shown). Using *huckebein* expression as a position marker, we found that the *snail* pattern expanded into the posterior region so that it overlapped with that of *huckebein* in the deficiency mutant embryo (Fig. 5E). Quantitation by using the *snail* pattern revealed that 24% (n=55) of all gastrulating embryos from *Df(3R)l26c* heterozygous parents showed the posterior expansion. Based on Mendelian ratios, this result represents an almost full penetrance. Thus, there is a posterior expansion of *snail* expression that correlates with the posterior expansion of nuclear Dorsal shown in Fig. 4. Subtle broadening of the *snail* pattern was also observed in the anterior region (Fig. 5C,D), suggesting that there is an increase of nuclear Dorsal in the anterior region, but the increase was not detectable by immunofluorescence staining.

Because *Df(3R)l26c* removes a number of genes in addition to *wntD*, we performed a genetic rescue experiment to confirm

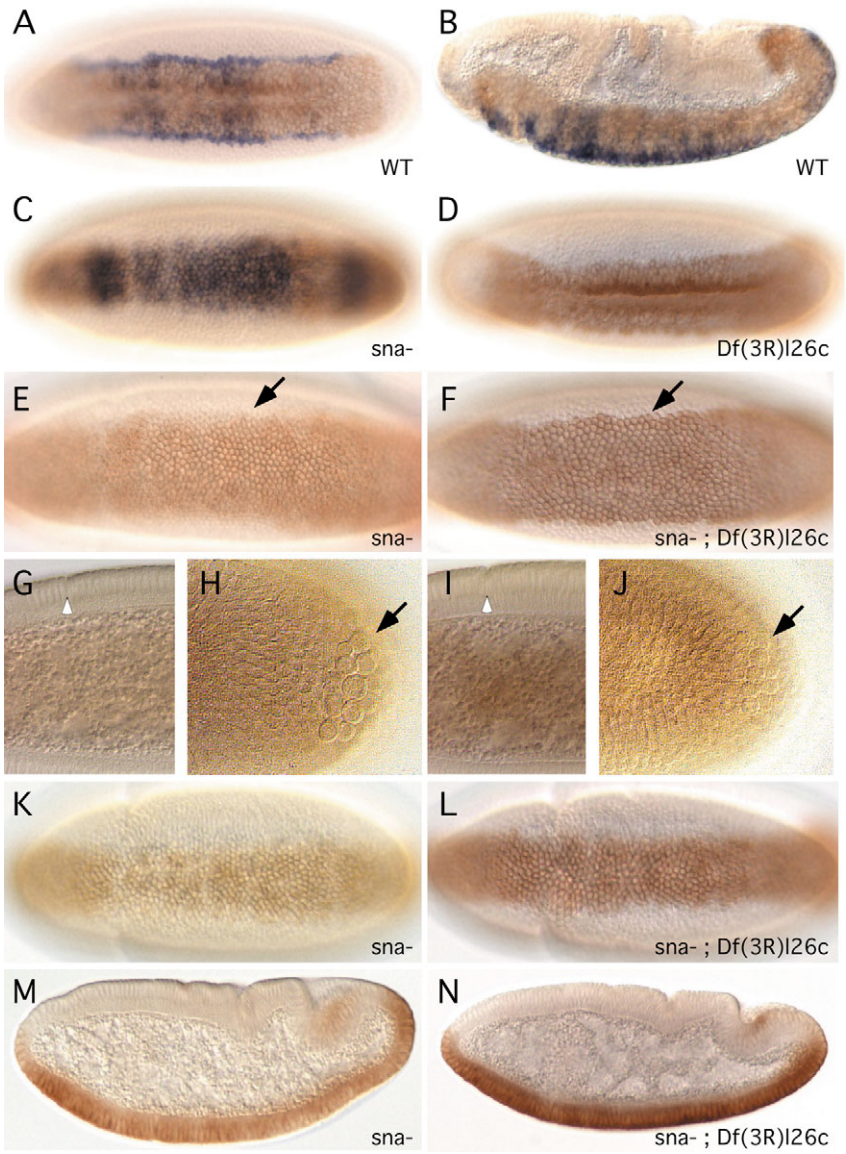


Fig. 6. Feedback regulation of *snail* expression by de-repressed WntD expression. *snail* in situ probe, brown; *wntD* probe, blue. The embryos shown in E,K and M were from an experiment using the *snail* probe only; other embryos were from experiments using the *snail* and *wntD* probes together. (A,B) Wild-type embryos showing the patterns of *snail* and *wntD* expression. (A) Ventral view of an early gastrula-stage embryo; (B) Sagittal view of a mid-gastrula-stage embryo. (C) A *snail* mutant at germ-band extension stage showing the de-repressed *wntD* and the reduced *snail* mRNA expression. (D) A *Df(3R)l26c* embryo double stained for *wntD* and *snail*. The *snail* pattern expanded into the anterior and posterior regions, and the staining of *wntD* was absent, demonstrating that it was a homozygous deficiency embryo. (E) A gastrulating *snail* mutant embryo stained for *snail* mRNA alone. The mutant embryo did not have a ventral furrow, although the cephalic furrow had already formed. The lateral border of the *snail* expression (arrow) was fuzzy in contrast to the sharp pattern observed in wild-type embryos. (F) A double-mutant embryo stained for both *snail* and *wntD*. The lateral borders of the *snail* pattern were sharp (arrow). (G,H) Higher magnifications of the embryo shown in E, showing the cephalic furrow (arrowhead) and the cellularization in the sagittal view (G), and the slightly dorsally moved pole cells (arrow, H). (I,J) Higher magnification of the embryo shown in F, showing that it was at a similar stage to the embryo shown in E. (K) Ventral view of a *snail* mutant during early germ-band extension showing the disappearing *snail* mRNA. (L) Ventral view of a similar stage double-mutant embryo showing a higher level of the *snail* mRNA staining. (M,N) Sagittal views of gastrulating embryos of the genotype indicated. The *snail* staining disappeared more slowly in the double-mutant background.

the involvement of *wntD*. We generated a transgenic line that contained a *wntD* genomic fragment, which showed all the normal expression patterns of *wntD* in the early embryo (data not shown). When we crossed this genomic construct into the *Df(3R)l26c* mutant, the posterior and anterior expansion phenotype of *snail* was completely rescued (Fig. 5G). We also did not observe posterior Dorsal expansion in any of the embryos derived from the *wntD*-rescued *Df(3R)l26c* strain (data not shown). The rescue experiment demonstrates that the deletion of *wntD* in the deficiency strain is responsible for the observed Dorsal and *snail* expression phenotypes. We also performed RNA interference of *wntD* by injecting double-stranded RNA into wild-type pre-blastoderm stage embryos. Approximately 10% of these injected embryos at late blastoderm stage had a mild posterior expansion of *snail* (Fig. 5I,J), and none of the embryos injected with buffer alone showed such a phenotype. This result further supports the idea that loss of WntD causes posterior expansion of *snail* expression.

We then examined whether the de-repressed WntD expression in the ventral cells of *snail* mutant embryos can inhibit Dorsal function. A previous report demonstrated that in mutant embryos that produced non-functional Snail proteins, the expression of *snail* mRNA disappeared prematurely (Ray et al., 1991). This premature loss of *snail* mRNA expression could be due to the inhibition of Dorsal function by the de-repressed WntD. We surmised that the removal of *wntD* in a *snail* mutant should lead to enhanced Dorsal function. Thus, we established a *snail;Df(3R)l26c* double-mutant strain. The double homozygous embryos were identified by the lack of *wntD* mRNA staining (blue color in Fig. 6) and the lack of a ventral furrow. The *snail* mRNA pattern (brown color) in *snail* mutant embryos exhibited a fuzzy border around the onset of gastrulation (Fig. 6E). By contrast, double-mutant embryos showed a *snail* pattern with sharp borders (Fig. 6F), similar to that observed in wild-type or *Df(3R)l26c* embryos (Fig. 5 and Fig. 6D). The developmental stages of these embryos were very similar based on the position of the pole cells, the degree of cellularization, and the cephalic furrow formation (Fig. 6G-J), supporting our argument that the genetic defect is the cause

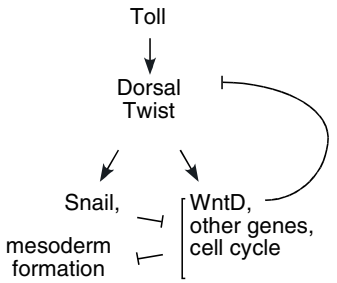


Fig. 7. A model of WntD and Dorsal/Twist/Snail interaction in the *Drosophila* embryo. Dorsal and Twist cooperate to activate the expression of *snail*, *wntD*, and other genes in the ventral and lateral regions. Snail represses *wntD* and other neuroectodermal genes in the ventral region, thereby restricting their expression to the lateral regions and allowing ventral invagination to proceed normally. WntD in turn can negatively regulate Dorsal, probably at a step upstream of Dorsal nuclear localization.

for the change of *snail* pattern. By mid-germ band extension, the *snail* mRNA staining became very weak in *snail* mutant embryos (Fig. 6K,M), but in double-mutant embryos the *snail* mRNA level was better sustained (Fig. 6L,N). The establishment and maintenance of the sharp *snail* pattern requires Dorsal (Ip et al., 1992b; Kosman et al., 1991; Ray et al., 1991). The *Df(3R)l26c* deficiency strain has many genes deleted and the effect cannot be attributed directly to the loss of *wntD*, but the result is consistent with our speculation that deleting *wntD* in the *snail* mutant embryo allows Dorsal to function more efficiently in activating target genes.

Discussion

We have shown that *Drosophila wntD* is a novel downstream gene, and a negative regulator, of the Dorsal/Twist/Snail network. The dynamic pattern of *wntD* expression in the early embryo is a combined result of activation by Dorsal/Twist and repression by Snail. Overexpressed WntD negatively regulates Dorsal nuclear localization, leading to an inhibition of ventral cell invagination. Physiological levels of WntD can also negatively regulate Dorsal, as loss of WntD leads to detectable expansion of both Dorsal nuclear localization and *snail* expression in the posterior regions. Furthermore, de-repressed WntD expression in the ventral region of *snail* mutant embryos can also attenuate Dorsal function. However, the loss of WntD could not rescue the invagination defect of the *snail* mutant embryo, suggesting that in the *snail* mutant embryo there are other de-repressed genes that can interfere with ventral invagination.

The *wntD* loss-of-function phenotype correlates with the expression of *wntD* at the poles of pre-cellular blastoderms (Fig. 1C). *wntD* is also expressed a bit later in the mesectoderm, and weakly in the mesoderm. Because WntD can inhibit Dorsal, one speculation is that WntD in the early mesectoderm may help to establish the sharp *snail* expression at the mesectoderm-neuroectoderm boundary (Kosman et al., 1991). However, we did not detect any changes in the Dorsal protein gradient or *snail* pattern in the trunk regions of the *Df(3R)l26c* embryos. We speculate that the timing of early expression of *wntD*, which may have additional input from the Torso pathway at the poles, is important for the feedback inhibition of Dorsal. By the time of cellularization, the Dorsal protein gradient is well established. This well-established Dorsal gradient activates the *wntD* gene in the trunk regions, but the subsequently translated WntD protein may not be capable of exerting a strong negative-feedback effect on the already formed Dorsal gradient. This timing argument is supported by the results of WntD-overexpression experiments. The use of maternal *nanos*-Gal4 caused a strong inhibition of Dorsal nuclear localization and of ventral invagination, whereas the use of zygotic promoters did not result in a significant phenotype.

Snail acts as a transcriptional repressor for at least 10 genes in the ventral region where mesoderm arises (Hemavathy et al., 2000; Kosman et al., 1991; Leptin, 1991; Ray et al., 1991). In *snail* mutant embryos, all of these target genes are de-repressed in the ventral cells, concomitant with severe ventral invagination defects. However, no direct evidence has been reported on whether these de-repressed genes interfere with invagination (Hemavathy et al., 2004; Hemavathy et al., 1997). We show here for the first time that a target gene of Snail, namely *wntD*, can block ventral invagination when overexpressed. If de-repressed WntD is solely responsible for inhibiting ventral invagination, we would expect that, in the *snail;Df(3R)l26c* double-mutant embryos, ventral invagination will appear again. We did not observe a rescue of ventral invagination in the double-mutant embryos (Fig. 6), suggesting that *wntD* is not the only de-repressed target gene that inhibits invagination. Nonetheless, the de-repressed WntD can attenuate Dorsal function (Fig. 6), and may contribute to the ventral invagination defect.

Previous reports have shown that overexpression of String/Cdc25 leads to early mitosis in the ventral cells and a block in ventral invagination (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). The zygotic transcription of *string* in the ventral region is activated by the Dorsal/Twist/Snail network. Meanwhile, the String protein is kept at a low level in the ventral cells by Tribbles through protein degradation, and this process requires the positive input of Snail (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Therefore, in the *snail;Df(3R)l26c* double-mutant embryos, the ventral cells should have increased String protein, as well as many other de-repressed gene products (Fig. 7). Perhaps the cumulative effect contributed by many of these *snail* target genes causes the severe invagination defect observed in the *snail* mutant embryo (Hemavathy et al., 2004); the simultaneous deletion of *wntD* and other interfering genes may be required to suppress the ventral invagination phenotype in *snail* mutants.

WntD may inhibit a component in the Toll pathway, or a component in the nuclear import/export pathway, leading to the cytoplasmic localization of Dorsal. However, the downstream mediators of *Drosophila* WntD signaling are not known. Being the closest homologs of *Drosophila* WntD, vertebrate Wnt8 proteins regulate mesoderm patterning, neural crest cell induction, neuroectoderm patterning, and axis formation (Hoppler and Moon, 1998; Lekven et al., 2001; Lewis et al., 2004; Popperl et al., 1997). These vertebrate Wnt8 proteins may transmit the signal through the canonical pathway, but the exact mechanism remains unclear (Lekven et al., 2001; Lewis et al., 2004; Momoi et al., 2003). We examined *Drosophila*

embryos that lacked maternal and zygotic functions of both Frizzled 1 and Frizzled 2 but did not observe any obvious defects in Dorsal or *snail* expression. A similar experiment using a *dishevelled* null mutant also did not reveal any such defects (data not shown). Furthermore, overexpression of Dishevelled or dominant-negative Gsk3 did not cause a detectable change of dorsoventral patterning (data not shown). These results suggest that *Drosophila* WntD may use other components for signaling. Wnt molecules employ multiple receptors and pathways to regulate various processes (Nelson and Nusse, 2004; Veeman et al., 2003). For example, *Drosophila* Wnt5 interacts with the receptor tyrosine kinase Derailed to regulate axon guidance (Yoshikawa et al., 2003). There are seven Wnt proteins and five Frizzled receptors in *Drosophila*, and WntD showed detectable affinity towards Frizzled 4 in cell culture assays (Wu and Nusse, 2002), but the in vivo relevance of this interaction is not clear. It is important to elucidate how *Drosophila* WntD transmits its signal. Equally important is to find out whether WntD interacts with the Toll pathway, and whether the interaction also occurs in processes such as the immune response and cancer progression in other organisms.

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