DWnt-2, a Drosophila Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate

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The sexually dimorphic characteristics of the reproductive tract in Drosophila require that cells of the gonad and the genital disc be assigned sex-specific fates. We report here that DWnt-2, a secreted glycoprotein related to wingless, is a signal required for cell fate determination and morphogenesis in the developing male reproductive tract. Testes from DWnt-2 null mutant flies lack the male-specific pigment cells of the reproductive tract sheath and the muscle precursors of the sheath fail to migrate normally. However, other cell types of the testis are unaffected. DWnt-2 is expressed in somatic cells of the gonad throughout development, implicating it as a signal that can influence pigment cell fate directly. Indeed, the ectopic expression of DWnt-2 in females results in the appearance of male-specific pigment cells in otherwise morphologically normal ovaries. Thus, the presence of pigment cells is a sexually dimorphic trait that is controlled by DWnt-2 expression. DWnt-2 is also expressed in regions of the male genital disc and gonad, which we have identified as sites of contact with muscle precursor cells, suggesting that secreted DWnt-2 protein is a signal for the migration or attachment of these cells.

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tors of male development in the reproductive tract, were left unpursued.

In a screen for mutations in DWnt-2, a member of the Wnt family of genes (Russel et al. 1992), we have found it to be required for the development of the sheath of the male reproductive tract and testis morphogenesis. We have also found that DWnt-2, when expressed ectopically in females, results in the appearance of male-specific pigment cells. Pigment cells, the outer cells of the testis sheath, are absent in the mutants, indicating that DWnt-2 is required for their specification in males. The inner muscle layer of the testis sheath fails to develop in the male mutants and the testis does not undergo its normal morphogenesis. The muscle defect in the DWnt-2 mutant has led us to investigate the origin of the precursor cells of the muscle layer. From our observations we propose a model for the formation of the male reproductive tract and discuss the role of DWnt-2 in this process. We also discuss the relation of DWnt-2 to the signals proposed half a century ago to explain testis morphogenesis and the induction in females of male-specific pigment cells.

Results

Generation of mutations in DWnt-2

To determine the developmental requirement of DWnt-2 we created mutations in this gene. We first generated a deficiency chromosome by X-irradiating male flies of the 5R13A line, a homozygous viable P-element insertion at 45E (Kassis et al. 1992), the cytological location of DWnt-2 (Russell et al. 1992). We screened progeny of the irradiated flies for the loss of the P-element eye color marker ry" and obtained a homozygous lethal deficiency Df(2R)11. The cytological band to which DWnt-2 maps is deleted in this deficiency and DWnt-2 expression is absent in embryos homozygous for the deficiency (data not shown), indicating that this deficiency lacks DWnt-2 sequences. We then performed a chemical mutagenesis and screened for mutations that failed to complement Df(2R)11 (see Materials and Methods). Four lethal complementation groups were recovered, as well as one adult viable complementation group that is male sterile. To determine the cause, testes were dissected from DWnt-2 mutant males and examined. With variable penetrance, testes from null mutant males are much smaller than wild-type, with the most severe examples having an abnormal oblong shape compared to the wild-type spiral. In addition, the null mutant testes do not possess the yellow pigmentation of the testis sheath (Fig. 2A,B). As noted above, males with the hypomorphic allele DWnt-2RJ have a less severe phenotype in which regions of the sheath produce yellow pigment (Fig. 2C).

Analysis of the adult testis phenotype in DWnt-2 mutants

Flies with null mutations in DWnt-2 are male sterile. To determine the cause, testes were dissected from DWnt-2 mutant males and examined. With variable penetrance, testes from null mutant males are much smaller than wild-type, with the most severe examples having an abnormal oblong shape compared to the wild-type spiral. In addition, the null mutant testes do not possess the yellow pigmentation of the testis sheath (Fig. 2A,B). As noted above, males with the hypomorphic allele DWnt-2RJ have a less severe phenotype in which regions of the sheath produce yellow pigment (Fig. 2C).

Figure 1. Mutations detected in DWnt-2. Schematic representation of the primary amino acid sequence of DWnt-2; vertical bars indicate cysteine residues.

Table 1. Alleles, mutagenic agents, and mutations detected in DWnt-2 mutants.

| Allele | Mutagen      | Mutation |
|--------|--------------|----------|
| O      | EMS          | Q40Z     |
| I      | EMS          | G57D     |
| 66     | EMS          | W74R     |
| 4      | DEB          | W74R     |
| 99     | EMS          | V220D    |
| E      | EMS          | K232Z    |
| 80     | EMS          | W265Z    |
| K      | EMS          | C281Y    |
| L      | EMS          | C281Y    |
| P      | EMS          | R295W    |
| RJ     | EMS          | C342Y    |

The molecular lesions of the DWnt-2 alleles are summarized in Figure 1. The pair of alleles DWnt-2^56 and DWnt-2^4 contain the same nucleotide change, although they were isolated independently. The alleles DWnt-2^K and DWnt-2^L also contain the same mutation, however, as they were generated in the same screen, may not represent independent events. Five alleles are missense mutations that cause either the gain or loss of a charged residue. Substitutions at conserved cysteine residues occur in three of the alleles. Stop codons are present in three of the alleles. DWnt-2^2L appears to be a null allele, as it is predicted to be truncated shortly after the signal sequence by a stop codon introduced at Q40.

All of the alleles exhibit the same phenotype, whether in a homozygous state or in heteroallelic combinations. The only exception is DWnt-2^66, which has a less severe phenotype, as discussed below. The phenotype seen for all the other alleles cannot be exacerbated when in trans to Df(2R)11, defining these as amorphic alleles.
To determine whether the cell types necessary for spermatogenesis are present in the mutants, we assayed the expression of β-galactosidase from P-element enhancer trap lines specific for cells of the testis (Gönczy et al. 1992; Gönczy 1995). Expression from the germ-line-specific enhancer trap S346 (Gönczy 1995) clearly shows that a germ cell lineage is maintained in the mutants (Fig. 3A,B). All of the specialized somatic cells of the testis required for spermatogenesis (Fuller 1993) are also present in the mutants. These include the hub cells, cyst cells (Fig. 3C,D), and terminal epithelial cells (Fig. 3E,F), as assayed by expression from the markers I72 (Gönczy 1995) and 34 (Gönczy et al. 1992), respectively. The presence of these cell types, along with the observation that mutant testes occasionally possess motile sperm (data not shown), suggests that the male sterility of the mutants is not attributable to a failure in the process of spermatogenesis.

The somatic cells necessary for the structure of the testis are affected in the mutants. These cells form a sheath composed of an outer pigment cell layer, an inner muscle cell layer, and their respective basal laminae (Bairati 1967). Assaying for expression from the pigment cell marker 365 (Gönczy 1995) shows that the testes from null mutant adults completely lack the outermost pigment cell layer, which normally covers both the testis and the seminal vesicles (Fig. 4A,B). Expression from the enhancer trap L44a (Fasano and Kerridge 1988), which marks muscle cells of the testis sheath (Gönczy 1995), reveals that the muscle cell layer is malformed or incomplete in these mutants (Fig. 4D). Cells in this morphologically aberrant tissue do have a muscle cell identity, as they express muscle myosin (Fig. 4F). The phenotype of the mutant testis muscle layer varies from a partially complete muscle layer to a tangle of muscle cells at the base of the testis.

The testis muscle layer derives from the genital disc. To understand how DWnt-2 affects the formation of a complete muscle layer, we have studied the development of this tissue in wild-type animals during pupation. An early study of testis morphogenesis had concluded that the inner cell layer of the testis sheath derived from somatic cells at the base of the gonad and that these either grew or stretched after the genital disc-derived

Figure 2. Morphology of the testes from DWnt-2 mutants. (A) Wild-type male reproductive tract. Testes are elongated and assume a helical spiral shape. (t) Testis; (sv) seminal vesicle; (ag) accessory gland; (ad) anterior ejaculatory duct. (B) DWnt-2 mutant male reproductive tract of the genotype DWnt-2^{209}/Df(2R)11. The internal genital disc-derived structures (sv, ag, ad) have a normal morphology, but the testes are small, abnormally shaped, and do not have the yellow pigmentation of wild type. (C) DWnt-2 mutant male reproductive tract of the genotype DWnt-2^{299}/Df(2R)11. The male reproductive tract has some patches of cells that produce yellow pigment (arrowheads).

Figure 3. Specialized cells of the testis required for spermatogenesis are present in DWnt-2 mutants. All mutants were X-gal stained, showing β-galactosidase activity from P-element enhancer traps that mark specific cell types of the testis. Here and in Fig. 4, specific genotypes are noted for completeness, but no differences were seen when any of the following alleles were examined in trans to Df(2R)11: DWnt-2^{66}, DWnt-2^{80}, DWnt-2^{99}, and DWnt-2^{299}. (A) Wild-type testis; the marker line S346 is expressed in germ cells. (B) DWnt-2^{66}/Df(2R)11 mutant testis; S346 expression shows that germ cells are present. (C) Wild-type testis; the marker line I72 is expressed in cells of the hub (arrowhead) and cyst cells (arrow). (D) DWnt-2^{299}/Df(2R)11 mutant testis; I72 expression is normal, indicating the presence of a hub (arrowhead) and cyst cells (arrow). (E) Wild-type testis; the marker line 34 labels cells of the terminal epithelium. (F) DWnt-2^{299}/Df(2R)11 testis; expression from the marker 34 shows that the mutant possesses a terminal epithelium.
Seminal vesicles had contacted the gonad (Stern 1941a). At that time, the inner cell layer was not recognized as being muscle tissue.

To determine the origin of the muscle layer we have used Twist expression as a marker for myoblasts. Expression of Twist is initiated in the mesoderm during embryogenesis (Thisse et al. 1988), persists in adult muscle precursors (Bate et al. 1991), and is required at high levels for myogenesis (Baylies and Bate 1996). To determine the location of the testis muscle precursors we dissected gonads and genital discs before the formation of a connection between them and stained them with an anti-Twist antibody. Twist is expressed in the adephelial cells that are closely associated with the male genital disc, but not in any cells of the gonad (Fig. 5). The lack of Twist expression in cells of the pupal gonad strongly argues against the possibility that they contribute to the testis muscle layer and supports the conclusion that precursors of the testis muscle sheath reside in the genital disc.

To determine the fate of the adephelial cells of the male genital disc, we have made use of the testis muscle marker L44a, from which we have also seen expression earlier in development. The gonads and genital disc were dissected from individual pupae of the L44a marker line at various stages after puparium formation and were stained for β-galactosidase activity to visualize the muscle precursor cells (Fig. 6A–C). During development, muscle precursor cells are present first on the genital disc as adephelial cells (Fig. 6A,A'), then contact the base of the gonad (Fig. 6B,B') and migrate to ensheathe it (Fig. 6C,C'). Pseudopodial extensions from cell bodies are seen during this process (Fig. 6D), which are indicative of cell migration.

Having observed the appearance of muscle precursors on the testis after its connection to the genital disc, we asked how a failure of the genital disc to contact the testis would affect muscle development. For this purpose we have used the male sterile mutant sexcombless (sx). In sx/Y males, internal structures of the reproductive tract are missing or partially formed and consequently, one or both testes remain unattached to the seminal vesicles (Stern 1941a). If the hypothesis that the testis muscle derives from the genital disc is correct, then one would predict that the failure of the gonad to contact the genital disc would abolish testis muscle formation. Expression from the L44a enhancer trap in testes dissected from adult sx/Y males shows that the muscle layer forms when a rudimentary genital disc has contacted the testis but does not form in a free unattached testis (Fig. 6E,F). Together these findings support the conclusion

Figure 4. Abnormalities in the sheath of DWnt-2 mutant testes. (A–D) Mutants are X-gal stained for β-galactosidase activity from marker lines; (E,F) mutants are immunostained for muscle myosin. (A) Wild-type testis; β-galactosidase expression from the pigment cell marker 365. β-Galactosidase activity is targeted to the nuclei of the pigment cells that form the outer layer of the testis. (B) DWnt-2^{97}/Df(2R)11 mutant testis; a lack of β-galactosidase activity from the marker 365 shows that no pigment cells are present. The testes in A and B may appear slightly yellow, but this is caused by the fixation procedure, not the presence of yellow pigment. The 365 marker is present in a white genotype background, which eliminates the yellow pigment in both the wild-type and the mutant. (C) Wild-type testis; β-galactosidase is expressed from marker line L44a in the cells of the muscle layer. (D) DWnt-2^{7}/Df(2R)11 mutant testis; β-galactosidase expression from the marker line L44a shows muscle tissue that does not ensheathe the testis. (E) Wild-type testis; immunostaining with an antibody to muscle myosin illustrates the continuity of the testis muscle layer. (F) DWnt-2^{7}/Df(2R)11 mutant testis; immunostaining for muscle myosin shows that myosin-expressing tissue is present but does not form a continuous layer over the testis.

Figure 5. Expression of Twist in the male pupal gonad and genital disc. Wild-type male gonad (A) and genital disc (B) were dissected 24 hr after pupal formation and were immunostained with anti-Twist antibody. There are many Twist-expressing adephelial cells associated with the genital disc, but none are in the gonad or are associated with it.
that the testis muscle layer derives from muscle precur-
sor cells of the genital disc.

DWnt-2 expression in the developing reproductive
tract

To determine how DWnt-2 might affect male repro-
ductive tract development, we have identified sites of
DWnt-2 expression by in situ hybridization. DWnt-2 is ex-
pressed in all mesodermal cells of the gonad, before
the mesoderm and germ cells have condensed to form a
compact gonad (Fig. 8A,B). As previously reported,
DWnt-2 expression is limited to the posterior mesoder-
mal cells of the gonad late in embryogenesis (Fig. 8C;
Russell et al. 1992). This late pattern of expression is
apparently maintained in the male, as DWnt-2 is ex-
pressed at the posterior of the pupal gonad in the cells
that will become the terminal epithelia (Fig. 8D). In the
male pupal genital disc, DWnt-2 is expressed in the ep-
ithelial cells at the apical tip of each developing seminal

Figure 6. Testis muscle cells originate in the genital disc. (A–
D). Male larvae of the muscle-specific marker line L44a were
allowed to pupate and the genital disc and gonads from indi-
vidual pupae were dissected and stained with X-gal at increasing
times after pupal formation (APF). (A) gonad ∼24 hr APF (A')
genital disc from same pupa as A; (B) gonad ∼30 hr APF (B')
genital disc from same pupa as B; (C) gonad ∼40 hr APF (C')
genital disc from same pupa as C; the connection between the
two broke during dissection. (D) Muscle precursors show cellu-
lar extensions as they migrate over testis (arrows) (higher mag-
nification than in other panels). (E) A rudimentary genital disc
and attached testis from an In(1)sex male was stained for β-
galactosidase activity from the L44a enhancer trap. Expression
from this marker shows that the testis has acquired a muscle
layer. (F) An unattached gonad from an adult In(1)sex male was
stained for β-galactosidase expression from the L44a muscle
marker. No muscle cells are associated with it.

Figure 7. DWnt-2 and the specification of pigment cells. Go-
nads were dissected from male pupae at 24 hr APF and X-gal
stained for β-galactosidase activity from the 365 marker. (A)
Wild-type male gonad. The bulging nuclei of the pigment cells
(arrowheads) are distinct from other cell types. (B) Male gonad
from a DWnt-2⁴/Df(2R)¹¹ mutant. The mutant completely
lacks cells with the distinctive pigment cell morphology.
Expression of DWnt-2 in primordia of the male reproductive tract. (A–C) Gonads were hybridized with a DWnt-2 DNA probe. (D–H) Gonads were hybridized with an antisense DWnt-2 RNA probe. (A) Stage 13 embryo; DWnt-2 is expressed in mesodermal cells that will coalesce to form the somatic mesoderm of the gonad. (B) Stage 15 embryo; DWnt-2 is expressed in all mesodermal cells of the newly formed gonad. (C) Stage 16 embryo; DWnt-2 expression is limited to the posterior mesodermal cells of the gonad. (D) Testis dissected at 24 hr APF shows DWnt-2 expression in the terminal epithelia. (E) Male genital disc dissected at 24 hr APF shows expression in the primordia of the seminal vesicles. (F) Male genital disc from a late third instar larva shows DWnt-2 expression in the male genital primordium (mgp) but not in the repressed female primordium (rfp). (G) A pupal female genital disc dissected at 24 hr APF shows no DWnt-2 expression in the oviducts. (H) Female genital disc from a late third instar larva shows no DWnt-2 expression in the repressed male primordium (rmp), nor in the female genital primordium (fgp).

This is a refinement of expression that was present earlier in the third instar larval disc (Fig. 8F) in the male genital primordium (Epper and Notthiger 1982). DWnt-2 is not expressed in the genital disc of the female third instar larva (Fig. 8H), nor is it expressed during pupation in the developing oviducts, the female structures analogous to the developing seminal vesicles (Fig. 8G). Thus, DWnt-2 expression occurs in sexually dimorphic patterns.

Although the pigment cells are a sexually dimorphic cell type found only in the male, it has been shown that the ovary possesses some cells that can differentiate into pigment cells if they have been exposed to a male genital disc during development (Hadorn and Bertani 1948). Our finding that DWnt-2 is expressed only in the male third instar larval genital disc suggested that DWnt-2 is the signal provided by a transplanted male genital disc to induce pigment cell fate in females. To test this hypothesis, we expressed DWnt-2 ectopically in clones during female development. This was accomplished by using a heatshock-driven Flp recombinase to remove an interruption cassette from an actin>cd2>gal4 transgene. The ensuing clonal Gal4 activity allows for expression from a UAS transgene (Pignoni and Zipursky 1997). We induced clones expressing Gal4 in a genetic background containing either UAS:DWnt-2 and the enhancer trap 365, or UAS:DWnt-2 and a wild type copy of the white gene, which is necessary for the yellow pigment of the testis to be produced (Lindsley and Zimm 1992). When clones were induced in third instar female larvae, all of the resulting adults have ectopic pigment cells, as assessed by either their production of yellow pigment (Fig. 9B,D), or the expression of β-galactosidase from the 365 pigment cell enhancer trap (Fig. 9F).

Discussion

Mutations in the DWnt-2 gene of Drosophila result in a male sterile phenotype. Mutants exhibit structural abnormalities in the muscle layer of the testis sheath and lack the pigment cells of the testis. From these defects we conclude that DWnt-2 functions to determine the fate of somatic cells of the male gonad and aid the migration or attachment of muscle precursors, by either a direct or an indirect mechanism. Ectopic expression of DWnt-2 in females results in the formation of ectopic pigment cells. This misexpression of a male cell fate implicates DWnt-2 as a signal that controls a sexually dimorphic trait.

DWnt-2 is required for the formation of the male reproductive tract

Males mutant for DWnt-2 are sterile. Testes from DWnt-2 null mutants have an abnormal morphology and are moderately to severely reduced in size. There is also variation in the extent to which testis muscle cell migration has proceeded. One invariant aspect of the null phenotype is the lack of an outer pigment cell layer. DWnt-2 is required for the differentiation or survival of this sex-specific cell type in the male. The germ cells and the somatic cell types that support spermatogenesis are present in the mutant testes, indicating that sterility is caused by structural and mechanical defects of the reproductive tract.
Role of DWnt-2 in the male reproductive tract

Figure 9. Ectopic expression of DWnt-2 in females induces pigment cells. (A) Wild-type male reproductive tract; testes and seminal vesicles possess yellow pigmentation. (B) Ovaries dissected from adults of the genotype white/yw, actin<cd2-gal4; UAS-DWnt-2+/--; hsFLP, Sb/+. Some cells in close association with the ovary are producing yellow pigment (arrowheads). As third instar larvae, these animals had been heatshocked for 20 min to initiate clonal expression of DWnt-2. The boxed area of the whole mount ovary in B is the same subject as the slide preparation in D, which is shown at an enlargement of 4× relative to B and rotated 90° counterclockwise. C-F are all presented at the same magnification. (C) Cell bodies of a wild-type testis. (D) Wild-type testis; β-galactosidase expression from the 365 marker. The pigment cells shown are those that cover the seminal vesicle. (E) Ovaries dissected from adults of the genotype: yw, actin<cd2-gal4/UAS-DWnt-2; 365/++; hsFLP, Sb/+. That had been heat-shocked as third instar larvae, as in B; cells associated with the ovaries are expressing β-galactosidase from the pigment cell marker 365.

Figure 10. A model for the migration of pigment and muscle precursors cells. The first cells of the genital disc to contact the gonad are the muscle precursor cells, which must gain access to the basal surface of the pigment cells. Migration of muscle and pigment cells then proceeds in opposite directions until the gonad and the seminal vesicle have each acquired an inner layer of muscle tissue and an outer layer of pigment cells. (te) Terminal epithelial cells of the testis; (p) pigment cell; (m) muscle precursor cell; (sv) epithelial cells of the seminal vesicle.
and testis muscle precursors are influenced by independent factors.

**DWnt-2 affects muscle migration**

The role of DWnt-2 in testis muscle cell development, as revealed by the mutant phenotype, is to ensure the proper morphogenesis of the muscle precursor cells. This is different from the role of wingless (wg) in determining muscle cell fate in the embryonic mesoderm (Baylies et al. 1995; Ranganayakulu et al. 1996), and from the conserved function of Wnt-1 and Wnt-3, which induce myogenesis in the somites of chick explants (Munsterberg et al. 1995; Stern et al. 1995). In the case of the testis muscle layer, DWnt-2 appears not to be required to specify muscle cell fate, as myosin-expressing testis muscle layer, served function of Wnt-1 and Wnt-3, which induce myogenesis in the somites of chick explants (Munsterberg et al. 1995; Stern et al. 1995). In the case of the testis muscle layer, DWnt-2 appears not to be required to specify muscle cell fate, as myosin-expressing testis muscle tissue is present (Fig. 4F) and covers mutant testes to variable degrees.

Instead of initiating cell differentiation, DWnt-2 contributes to either the migration of the muscle precursors away from the genital disc or to their adherence and migration over the testis. This contribution may be a direct one, in which DWnt-2 regulates motility or adherence, or an indirect one, in which the role of DWnt-2 is to specify the pigment cell substrate. The variation in penetrance of the muscle phenotype, seen for all alleles, indicates that DWnt-2 is not essential for muscle precursor migration and may argue that the loss of pigment cells is the primary reason why the muscle fails to develop.

However, DWnt-2 is expressed in the seminal vesicle and the terminal epithelium of the gonad before the contact of the genital disc and the gonad (Fig. 8D,E). This expression pattern strongly suggests that DWnt-2 may have a function in the recognition or migration events that occur when the myoblasts of the seminal vesicle contact the pigment cells of the gonad. At this stage, DWnt-2 may be a signal required by either or both of the migrating cell types. Because of the absence of the pigment cells in the mutants we have not been able to determine whether this late expression of DWnt-2 affects pigment cell migration. Likewise, we have not yet separated effects attributable to a lack of pigment cells from any direct requirement of DWnt-2 for muscle cell migration.

DWnt-2 is necessary and sufficient to specify pigment cell fate

DWnt-2 activity is required in males for the presence of pigment cells. Although the pigment cells are a male specific cell type, this cell fate can be induced in females into which has been transplanted a male genital disc. By expressing DWnt-2 ectopically, we have also induced this pigment cell fate in females (Fig. 9). This result and our observation that DWnt-2 is expressed only in the male genital disc (Fig. 8E,F) suggest that DWnt-2 is the activity responsible for the ectopic pigment cells seen in transplant experiments (Hadorn and Bertani 1948).

Possible mechanisms of DWnt-2 signal transduction

Our results indicate that DWnt-2 has the ability to direct the differentiation of a particular cell fate. We presume that as an instructive signal for the differentiation of pigment cells DWnt-2 affects changes in gene transcription. It is known that wg affects transcription by stabilizing Armadillo (van Leeuwen et al. 1994), a β-catenin homolog (Peifer and Wieschaus 1990; McCrea et al. 1991). When Armadillo is bound to pangolin, a Tcf DNA-binding protein, the complex acts as a transcriptional activator (Brunner et al. 1977; van de Wetering et al. 1991). It will be interesting to determine whether DWnt-2 also uses the same signaling components. We might predict that this will be true; however, there are examples of Wnt functions that are not mediated by transcriptional regulation through β-catenin and Tcf. This is the case for the Caenorhabditis elegans Wnt gene mom-2, which controls orientation of the mitotic spindle, a cytoskeletal effect that occurs in the absence of transcription (Rocheleau et al. 1997; Thorpe et al. 1997).
precursor cell migration could be mediated in several ways. DWnt-2 might affect motility through the regulation of cytoskeletal elements. Alternatively, the migration defects seen in DWnt-2 mutants might not be attributable to a loss of motility, but rather a loss of the ability of cells to determine where their final position should be. This specific function is implicated for the C. elegens gene lin-17, a Wnt receptor homolog required for the migration of certain neuronal cells to their proper position (Harris et al. 1996). A third possible explanation for the affect of DWnt-2 on myoblast migration is through alterations in adhesion. Wingless has a positive effect on E-cadherin transcription in vitro (Yanagawa et al. 1997), suggesting that DWnt-2 may also affect adhesiveness at the transcriptional level. The phenotype of the DWnt-2 mutant raises many questions about how this signal functions to specify cell fate and promote the morphogenetic process of migration. Further studies of the DWnt-2 signaling mechanism should greatly advance our understanding of how Wnt genes control development.

Materials and methods
Fly stocks
The 5R13A stock was obtained from Judith Kassis (Food and Drug Administration, Washington, D.C.). The deficiency stock w; In(2LR)w45-32n, cn, Df(2R)Np5/CyO was obtained from the University of New York, NY). The sexcomless stock of the genotype In(1)sx, sc, ec, cv, ct, v/sc, ec, cv, ct, v, g, f was obtained from the Bloomington stock center. The fly stock with the actin<cd2->gal4 and hsFLP transgenes was obtained from Larry Zampursky (University of California, Los Angeles) and the hsFLP transgene on the M/KRS chromosome (Chou and Perrimon 1992) was obtained from the Bloomington stock center. To create the UAS:DWnt-2 transgenic stock, the DWnt-2 cDNA was cloned into the pUAST plasmid (Brand and Perrimon 1993) and this was injected into white1Ga embryos. Wild-type flies used for in situ and immunohistochemistry were of the Canton-S stock. Mutant larvae and pupae were identified through the use of an attached SM5-TM6,Tb balancer.

X-ray mutagenesis
Males of the line 5R13A (P[y]+, cn) were irradiated with 3500 rads in a cabinet X-ray machine and crossed to females of the genotype Sco/CyO, cn; ry. Approximately 22,700 progeny were screened for the loss of the P[y] marker. Flies that were cn/ CyO; ry were crossed to flies of the genotype Sco/CyO; ry. Progeny of the genotype cn/CyO; ry were crossed among one another to establish a stock and to determine whether the loss of the P-element had created a lethal deficiency. In this manner, deficiency Df(2R)11 was created. By examining polytene chromosomes from salivary glands of third instar larvae of the genotype Df(2R)11/+ we have mapped the approximate breakpoints of this deficiency as 45C6; 45E1.

Chemical mutagenesis
Wild-type males with an isogenized second chromosome (iso1) were mutagenized with ethylmethanesulfonate (EMS) or diethoxybutane (DEB) according to standard methods (Grigliatti 1986) and were crossed with Sco/CyO, cn females. Single F1 males with the mutagenized iso1/CyO, cn were crossed at 29°C to females of the genotype Df(2R)11, cn/CyO, cn. The F2 generation was screened for lethality of iso1/Df(2R)11, cn by the absence of straight-winged flies. Flies with iso1 carrying a lethal mutation over CyO were crossed among one another to establish a stock. Of the chromosomes screened, 3186 were mutagenized with 24 mM EMS, 1678 were mutagenized with 30 mM EMS, and 1102 were mutagenized with 5 mM DEB. Sixteen independent mutations were recovered, representing five complementation groups.

The DWnt-2 mutations in trans to Df(2R)11 are adult viable and have the visible phenotype of held-out wings, which allowed them to be identified in a screen for lethals. However, all chromosomes bearing the original alleles (4, 66, 80, 99) of the DWnt-2 complementation group are lethal in any heteroallelic combination. This is apparently a case of synthetic lethality caused by a genetic locus or loci present on the isogenized chromosome used for the mutagenesis. This was revealed when the DWnt-2 alleles were allowed to recombine with a recessively marked chromosome. Recombination events that exchanged genetic material distal to the gene curved and proximal to the gene plexus, resulted in chromosomes carrying the DWnt-2 alleles that were no longer lethal in trans to each other.

Once the DWnt-2 complementation group was identified, more alleles were generated by the following procedure. Males of the genotype yw; iso1a (a more recently isogenized second chromosome) were mutagenized with EMS and crossed to yw; Sco/CyO females. Single males of the genotype yw; iso1/CyO were crossed at 29°C to females carrying a P[w+] marker on the same chromosome arm as the DWnt-2 allele (Df(2R)11, P[w+]A7a/CyO). Crosses in which all the straight-winged flies had held-out wings were screened for defects in the male reproductive tract. Flies of the genotype iso1/CyO were mated to create stocks of new alleles. Of the chromosomes screened, 931 were mutagenized with 30 mM EMS and 1727 were mutagenized with 24 mM EMS. Seven alleles were isolated independently (E, I, K, L, O, P, Rj) and when sequenced all had mutations in DWnt-2.

SSCP analysis
Primers to intronic sequences of DWnt-2 were used in PCR with genomic DNA from flies of the following genotypes: mutant/Df(2R)11, mutant/CyO, iso1/iso1, and Df(2R)11/CyO, the latter two serving as controls. Reactions were labeled by including the following in a 25-µl reaction: 2.5 µl 10× Hot Tub buffer (Amersham), 400 ng of genomic DNA template, 40 pmoles of each primer, 200 nmoles each of dATP, dCTP, dGTP, and dTTP, 0.25–0.375 µl [32P]dCTP (3000 Ci/nmole, 10 µCi/µl), and 1.25 units of Taq DNA polymerase. Amplification was carried out for 30 cycles at temperatures optimized for each primer set. One microliter of each PCR reaction was diluted with 5 µl of stop solution (95% formamide, 20 µm EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were denatured at 95°C for 3 min, placed on ice, and loaded on a 0.5× Hydrolink MDE (mutation detection enhancement) gel (AT Biochem) in 0.6×TBE. Electrophoresis was carried out at a constant 8 W for 12–16 hr at room temperature. Gels were dried on filter paper and exposed to film for 1–112 hr. For some PCR reactions the sizes of the DNA fragments were reduced by restriction enzyme digestion to enhance the ability to detect polymorphisms. In these cases, PCR samples were phenol/chloroform extracted, ethanol precipitated, and then resuspended in 15 µl of 1× restriction digestion buffer with enzyme. One microliter of this
reaction was diluted with 5 µl of stop solution and electrophoresed as above. Exons of alleles in which a polymorphism was detected were either subcloned for sequencing or were sequenced directly from PCR template. Each of the original Dwnt-2 alleles gave rise to either the gain or loss of a restriction enzyme site, which was used to confirm each mutation by checking for the corresponding restriction fragment polymorphism on Southern blots.

PCR amplification of mutant alleles

For the alleles generated by failure to complement Dwnt-2, the coding sequences of Dwnt-2 were cloned and sequenced. This was accomplished either by amplifying genomic exons of Dwnt-2, as mentioned above for SSCP analysis, or by reverse transcription PCR (RT–PCR) from RNA isolated from homozygous mutant adults. RT–PCR was carried out with the SuperScript Preamplification System (GIBCO-BRL) according to the manufacturer’s directions. PCR fragments were then cloned in Bluescript plasmid and sequenced.

X-gal staining of tissues

Testes from adults, and gonads and genital discs from larval and pupal stages were dissected in PBS with 0.1% Tween (PBTT). Tissues were fixed in PBTT with 1% glutaraldehyde for 30 min then washed three times with PBTT. Tissues were placed in staining buffer [7.2 mM Na2HPO4, 2.8 mM NaH2PO4, 1 mM MgCl2, 0.15 mM NaCl, 5 mM KFe(CN)6, 5 mM K3Fe(CN)6 and 0.2% X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside)] and incubated at 37°C for 12-24 hr. Tissues were washed with PBTT and mounted in 50% glycerol.

In situ hybridization

Hybridization with DNA probes was as described previously (Russell et al. 1992). Hybridization with RNA probes was as follows. RNA probe was labeled with digoxigenin (DIG; Boehringer Mannheim) using linearized DNA template according to the manufacturer’s directions. Reactions were phenol/chloroform extracted, then ethanol precipitated. Probe was then suspended in 20 µl of hybridization buffer [40 mM NaHCO3, 60 mM Na2CO3 (pH 10)] and incubated at 60°C for 60 min. To this, 10 µl of 1 M Tris (pH 7.5) and 300 µl hybridization solution (50% formamide, 5× SSC, 100 µg/ml salmon sperm DNA, 100 µg/ml tRNA, 50 µg/ml heparin, 0.1% Tween) were added. Testes and genital discs from larval and pupal stages were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 15 min on ice. Deoxycytolate and Triton X-100 were each added to a concentration of 0.1% and fixation was continued at room temperature for 15 min. The fixative was then removed and the tissues washed twice in 0.3 M NH4OAc. An equal volume of ethanol was then added dropwise. Tissues were washed in ethanol and stored at −20°C, if necessary. Tissues to be hybridized were then washed in 50% ethanol/50% xylene for 10 min. The tissues were then washed three times in ethanol, twice in methanol, once in 50% methanol/50% PBTT +4% formaldehyde (PBTF). All washes were for 2 min. The tissues were then fixed for 20 min in PBTF. The tissues were washed five times for 5 min in PBTF and then once in 50% PBTT/50% hybridization solution. Tissues were incubated in hybridization solution at 56°C for 1–4 hr. DIG-labeled RNA probe prepared as described above was added at a 1:1000 dilution and incubated at 56°C for 12–16 hr. Tissues were washed at 56°C for 30 min each in the following washes: once in hybridization solution, once in 50% hybridization solution/50% PBTT, then five times in PBTT. The hybridized tissues were then incubated overnight at 4°C with anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) at a final dilution of 1:2000. The tissues were then washed four times for 30 min in PBTT, washed twice in staining buffer [0.1 M NaCl, 50 mM MgCl2, 0.1 M Tris-HCl (pH 9.5), and 0.1% Tween 20], and put into 1 ml of staining buffer plus 4.5 µl of nitroblue tetrazolium and 3.5 µl of X-phosphate (Boehringer Mannheim). The staining reaction was allowed to proceed in the dark for 30 min to 2 hr. Tissues were postfixed in PBTF for 30 min, washed in PBTT, and mounted in 50% glycerol.

Immunohistochemistry

Tissues were dissected in PBS with 0.1% Triton X-100 (PBX), fixed in PBX plus 4% formaldehyde for 30 min at room temperature, and washed in 2% H2O2 in methanol for 20 min to block endogenous peroxidase activity. The tissues were incubated in PBXT with 1% bovine serum albumin and 0.01% azide (BBT) with a primary antibody at a 1:1000 dilution overnight at 4°C. Tissues were washed in BBT three times for 30 min, then blocked with 2% normal serum in BBT for 1 hr, washed in BBT for 30 min, and incubated with 1:500 biotin-conjugated secondary antibody (Vector Laboratories) in BBT overnight at 4°C. Tissues were washed five times in PBX, then incubated with a biotinylated horseradish peroxidase-avidin complex (Vector Laboratories) and washed three times for 20 min with PBX. Samples were then incubated in PBX with 0.5 mg/ml diamobenzidine and 0.03% nickel chloride. Staining was stopped by washing tissues in BBT. Tissues were mounted in 50% glycerol.

The antibodies used were rabbit anti-muscle myosin (Kiehart and Feghali 1986) at a 1:500 dilution and rabbit anti-Twist (Roth et al. 1989) at a 1:1000 dilution.

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