Interaction Between Wingless and Notch Signaling Pathways Mediated by Dishevelled

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In *Drosophila*, the Wingless and Notch signaling pathways function in many of the same developmental patterning events. Genetic analysis demonstrates that the dishevelled gene, which encodes a molecule previously implicated in implementation of the Wingless signal, interacts antagonistically with Notch and one of its known ligands, Delta. A direct physical interaction between Dishevelled and the Notch carboxyl terminus, distal to the cdc10/ankyrin repeats, suggests a mechanism for this interaction. It is proposed that Dishevelled, in addition to transducing the Wingless signal, blocks Notch signaling directly, thus providing a molecular mechanism for the inhibitory cross talk observed between these pathways.

**Drosophila** Wingless (Wg), the homolog of the mouse oncogene protein Wnt-1, is a secreted glycoprotein signaling molecule required for a variety of inductive signaling events during both embryonic and imaginal development (1, 2). Genetic analysis has identified several genes, dishevelled (dsh), zeste-white 3 (zw3, also known as shaggy), and armadillo (arm), whose products are required for transduction of the Wg signal. dsh encodes a conserved protein (3) of unknown function (4, 5), zw3 encodes multiple serine-threonine kinases homologous to mammalian glycogen synthase kinase–3 (6), and arm codes for a homolog of β-catenin (7). To date, molecules directly involved in reception of the Wg signal on the cell surface have remained elusive. The following model has been proposed for Wg signal transduction: Upon signaling, Dsh, the most proximal known component of the response pathway, antagonizes Zw3 activity, thereby derepressing a Wg-specific function of Arm, resulting in activation of target gene expression (4, 8, 9; reviewed in (2)).

During wing imaginal disc development, Wg is required in spatially and temporarily separable steps. Between 48 to 96 hours after egg laying (AEL), Wg expression in the ventral compartment is required for correct dorsal/ventral (DV) patterning (10, 11). Establishment of the DV boundary precedes the later pattern of Wg expression—a stripe at the presumptive margin (11–16). Beginning shortly after 96 hours AEL (mid-third instar), signals emanating from the margin determine the position of margin-specific structures, including the innervated sensory bristles anteriorly and noninnervated bristles posteriorly. Four observations indicate that Wg determines the position of bristle development: (i) The stripe of Wg expression coincides with the presumptive wing margin during this period (11–14). (ii) Reduction of Wg activity during late third instar results in wings devoid of margin bristles (13, 14). (iii) Wg controls expression of the achaete-scute complex (AS-C) proneural genes at the margin, whose activation is required to achieve the neural fate (14, 15). (iv) Ecotopic Wg expression produces ectopic margin structures (16).

Several observations have suggested a role for the Notch (N) gene in a variety of Wg-mediated signaling events. These include related phenotypes in loss-of-function mutants (13, 18, 19), isolation of wou mutations in screens for genetic modifiers of N (20) and vice versa (21), and genetic interactions between N and wou (22–20).

N encodes a receptor in an evolutionarily conserved signaling mechanism that, through local cell interactions, controls the fate of a broad spectrum of cells (23). The best-characterized function of N is in mediating lateral signaling (also called lateral inhibition) between immobile neighboring precursor cells, thus regulating their responses to specific developmental signals. N is a 2703-amino acid protein with a large extracellular domain containing 36 tandem epidermal growth factor (EGF)–like repeats and 935–amino acid intracellular domain bearing 5 tandem cdc10/ankyrin repeats (24). Genetic and molecular studies have identified several genes that are believed to encode elements of the N signaling pathway. These include the membrane-bound ligands Delta (Dl) and Serrate (Serr), the cytoplasmic protein DeltaExon 1 (DEx), and the nuclear proteins encoded by numb (numb), Hairless (H), the Enhancer of split complex (E(spl)), and Suppressor of Hairless (Su(H)) (25). Dl and Serr bind to specific SOF repeats in the N extracellular domain (26), whereas the intracellular ankyrin repeats are necessary for N signaling activity (27–28). The cytoplasmic proteins Dl (29, 30) and Su(H) interact with N at or adjacent to the ankyrin repeats (27, 32). Su(H) appears to translocate to the nucleus, where it activates transcription, perhaps in a complex with a proteolytic fragment of N (31, 33).

Genetic analyses have implicated both N and Dsh in establishment of DV pattern in the wing as well as in induction of bristles at the margin (13, 34–36, and references cited above). However, the precise relation between N and Wg signaling pathways remains unclear. We have therefore attempted to gain insight into the relation between N and Wg by studying specifically the role of N in the Wg-dependent induction of bristles. Our results indicate that Dsh mediates Wg signaling to specify sensory mother cell (SMC) development. N signaling, in contrast, represses SMC development. We find that in addition to transducing the Wg signal, Dsh also inhibits N activity to facilitate implementation of the Wg signal. We have identified a physical interaction between Dsh and N and show that this interaction maps to the COOH-terminal cytoplasmic tail of N, a domain with no previously assigned function. The molecular interaction between Dsh and N provides a direct molecular link that can account for the cross talk between the two signaling pathways.

An ectopic bristle-induction assay. At least two distinct roles for Dsh in Wg-dependent signaling at the wing margin can be discerned. Disruption of Wg signaling by induction of cell clones mutant for dsh gives rise to two distinct wing phenotypes. When large clones intersect the margin (induced before 72 hours AEL), the margin is frequently lost, resulting in nicks (Fig. 1B) (4, 37). In contrast, smaller clones (induced after 72 hours AEL) often leave the margin intact, but bristles fail to develop in mutant cells (Fig. 1C). These results are reminiscent of the phenotypic consequences of loss of wou activity in the wing, which also results in nicks or bristle loss (13, 14, 19). Specifically, earlier loss of Wg activity results in failure to establish DV compartmentalization (10, 14, 15), whereas late loss can result in either loss of growth-organizing activity (16) or the absence of bristles (13, 34–36).
We propose that establishment of dsh mutant cell clones before determination of the D/V boundary can result in loss of the margin. However, if dsh mutant cell clones are established after growth-organizing activity is completed or if they abut but do not span the margin, clones are recovered that result only in loss of bristles. Therefore, distinct events dependent on wg and dsh can be identified in wing patterning. The remainder of our analysis will focus solely on the later role of Dsh and Wg in bristle development.

Because studying bristle development at the margin is complicated by the earlier D/V patterning and growth-organizing events, we devised an ectopic bristle-induction assay. Ubiquitous overexpression of dsh during the time of endogenous bristle induction results in induction of ectopic bristles. Pulsed dsh overexpression, driven from the usp70 promoter (Hs:dsh) during late third instar (108 to 120 hours AEL) (38), results in the development of supernumerary bristles in the interior of the wing (Fig. 1, D to F). The bristle types (scout, slender, and recurved) are appropriate for the dorsal or ventral surface on which they appear, and are biased toward the margin. Because endogenous dsh mRNAs are uniformly expressed in imaginal discs (39), it appears that Dsh overexpression rather than misexpression is responsible for the occurrence of ectopic bristles. The ectopic bristle phenotype is not restricted to the wing. Overexpression of dsh also results in duplication of the Wg-dependent scutellar bristles (Fig. 1C) (13, 38). However, we will argue that the wing provides a semi-quantitative readout of the strength of the inductive signal, and have therefore focused our attention on the wing bristles.

Formation of the ectopic bristles can be visualized in imaginal discs by use of the enhancer trap line A101, which labels the SMCs (40). In Hs:dsh animals, ectopic SMCs first appear in late third instar and are evident at succeeding stages in a pattern suggestive of the ensuing pupal phenotype (Fig. 1, H and I). The ectopic bristles clearly develop well beyond the domain in which wild-type AS-C expression is seen (17) and thus represent de novo proneural induction. Thus, dsh overexpression induces extra bristle formation by recruiting extra epithelial cells to the neural developmental pathway.

At the wing margin, wg has been suggested to encode the inductive signal controlling bristle development (13, 14). To demonstrate this hypothesis, we ectopically expressed wg in the interior of the wing blade. The GAL4 system (41) was used to drive wg expression in the patched (ptc) expression domain, resulting in ectopic bristle development in the corresponding region (Fig. 2A) (38). Therefore, Wg can act as a bristle-inducing signal. We also tested whether ectopic bristles in Hs:dsh animals are Wg-dependent by inducing Hs:dsh in a w^118 background (38). Loss of Wg activity beginning shortly before Dsh is overexpressed completely abolished the ectopic bristle response (Fig. 2, B and C). Conversely, overexpression of Wg enhanced the ectopic bristle response. Whereas misexpression of wg from the usp70 promoter (Hs:wg) is too weak to induce a phenotype alone, induction of Hs:wg together with Hs:dsh potentiates the response to dsh, causing bristles to form well into the interior of the wing in the vast majority of wings, as compared with induction of Hs:dsh alone (~90% versus ~10%) (38). This finding is consistent with the model that dsh overexpression activates bristle induction by potentiating Wg signaling.

Several observations indicate that the maximum distance of ectopic bristles from the margin depends on the levels of both Wg and Dsh proteins and therefore reflects the strength of the inductive signal. (1) The extent of ectopic bristle induction depends on the amount of Dsh overexpression. The territory in which ectopic bristles appear stretches to progressively greater distances.
from the margin with increasing levels of \( Hs \)-\( dsh \) (compare Fig. 1, D and F). (ii) The distance of ectopic bristles from the margin is increased by superimposing ectopic Wg expression on the endogenous pattern, thereby increasing Wg levels at all positions in the wing. (iii) The ectopic bristles are more numerous near the margin, where endogenous Wg is most highly expressed. This graded, morphogenetic pattern suggests that dsh overexpression acts to potentiate the response to the endogenous stripe of Wg expression at the margin. Thus, the maximum distance of ectopic bristles from the margin induced by dsh overexpression serves as a semiquantitative indicator of the strength of the inductive signal.

We conclude that the ectopic bristles developing in response to overexpression of dsh are generated by a mechanism similar to that governing formation of bristles at the wing margin. Their induction is Wg-dependent, and the timing of the requirement for dsh overexpression coincides with that observed for Wg and Dsh in induction of bristles at the margin. Because the formation of ectopic bristles is temporally and spatially distinct from the establishment of the DV boundary, we can specifically assay the effects of altering expression of other genes on the bristle-induction process. The mechanism underlying formation of ectopic bristles can thus serve as a model system for understanding the development of bristles at the margin.

**Antagonism between N and D.** To elucidate the function of N during bristle induction on the wing, we tested the effect of altering the level of N activity during the induction of ectopic bristles by \( Hs \)-dsh (38). Ectopic bristles generated by strong pulses of dsh overexpression were found well into the interior of the wing at a much higher frequency in \( N \) heterozygotes than in a wild-type background (>90% versus ~10%, Fig. 3A). Reduction of N activity thus potentiates bristle formation in the interior of the wing blade, indicating that N and Dsh have opposing effects on bristle induction. This relation is clearly observed at the level of SMC induction, where a reduced expansion of the SMC domain is seen (Figs. 3B and I). A similar, though less robust, result was obtained when a dominant-negative N construct, \( N^{\alpha \text{dn}} \) (26), was expressed together with \( Hs \)-dsh (39). Conversely, duplication of the N locus, or expression of an activated N (\( Hs \)-N\(^{\alpha \text{act}} \)) (42), suppresses the ectopic bristle phenotype (39). We conclude that in the interior of the wing blade, N and the Wg pathway exert opposite, dosage-dependent effects on bristle induction, with Wg activating and N inhibiting induction. It follows that, in a wild-type background, induction of ectopic bristles by \( Hs \)-dsh must overcome the antagonism mediated by wild-type levels of N.

Because DI is a ligand for N, and both act together in regulation of bristle development on the notum, we tested whether DI inhibits bristle induction on the wing. Accordingly, ectopic bristles were induced with \( Hs \)-dsh in a DI heterozygous background. As with N, decreasing the dosage of DI potentiated the \( Hs \)-dsh-dependent ectopic bristle response, thereby enhancing the severity of the ectopic bristle phenotype (Fig. 3D). Conversely, if ectopic bristles were induced in the presence of an extra copy of DI, the severity of the phenotype was reduced (39). Thus, DI has an activity similar to N in suppressing bristle development. In summary, both N and DI mediate a signal that is antagonistic to that of Wg and Dsh in ectopic bristle induction. We propose that a similar antagonism exists between the two pathways during wild-type development.

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**Fig. 2.** Wg dependence of ectopic bristles. (A) Ectopic bristles induced by expression of Wg in the ptc expression domain. ptc is expressed along the anterior-posterior boundary of the wing, and the occurrence of bristles in this pattern indicates that ectopic Wg is able to induce ectopic bristles. (B and C) Pupal wing phenotypes resulting from loss of Wg activity in third instar, either without (B) or with (C) dsh overexpression. Typically, a small amount of residual Wg activity remains, producing a narrow margin phenotype at the distal anterior margin. Ectopic bristle formation from dsh overexpression is abolished in the absence of Wg activity in third instar (C), although the distal anterior margin may bear somewhat more bristles than in the absence of dsh overexpression. Sibling \( Tb \)s-dsh, \( wg^{\alpha \text{dn}} \) flies showed a reduced panel and expressivity of ectopic bristles, indicating that even heterozygosity for \( wg \) partially suppresses the ectopic bristle phenotype (not shown). The wings have been inflated and flattened, thereby distorting the veins.

**Fig. 3.** N and DI suppress the formation of ectopic bristles in the interior of the wing blade generated by \( Hs \)-dsh. (A) The phenotype of a pupal wing after overexpression of \( dsh \). (B) 5-hour pulse delivered in the last 12 hours of third instar in an \( N \) heterozygote. The view is from the anterior, with the unfused anterior dorsal and ventral surfaces seen. Ectopic bristles are seen throughout the wing blade in most wings of this genotype. (B and C) \( A101 \) staining revealing the SMC pattern in a 2-hour pdf and (B) and (C) 6-hour pdf (C) pupal wing dissected after a 1.5-hour pulses of dsh overexpression during the last 12 hours of third instar. SMCs fill the anterior compartment (compare to Fig. 1, H and I). Although bristles also arise in the posterior, they are not innervated, and their precursors do not express \( A101 \) until later in development. (D) Pupal wing resulting from dsh overexpression (1.5-hour pulse) in a DI heterozygote. The bristle pattern resembles that seen in (A). This view shows the posterior portion of the wing, which is filled with bristles. Some anterior bristles can be seen in the bottom right of this view.
bristle induction.

Physical interaction between Dsh and N. Dsh is the most proximal known component in the Wg signal transduction pathway. Because Dsh is thought to be mainly cytoplasmic (43), and because the N protein is a receptor that resides in the membrane, we tested whether Dsh might interact directly with N to potentiate Wg signal transduction. By using quantitative yeast interaction trap system, we found evidence for a direct physical interaction between Dsh and a fragment of N (Fig. 4) (44). With the use of smaller pieces of Dsh, the interaction was mapped to the NH₂-terminal half of the Dsh protein. Dsh fails to interact with a N fragment bearing the cdc10/ankyrin repeats and adjacent regions. The cdc10/ankyrin repeats or nearby sequences bind Dx and Su(H) (29–32) and appear to be required to mediate N signaling (26–28). Instead, Dsh was shown to interact with the COOH-terminal end of the N cytoplasmic domain. This finding suggests that a physical interaction occurs between Dsh and a domain of N having no previously assigned function.

To corroborate the binding results in a Drosophila assay, we examined the relative colocalization abilities of N and Dsh proteins after coexpressing them in Drosophila Schneider 2 (S2) cells in culture (45). Co-transfected cells were aggregated with cells expressing DI, a membrane-bound ligand for N, to produce a “mutual capping” of N and DI at the point of cellular contact (46). Figure 5A shows colocalization between Dsh and the “capped” N, consistent with binding of Dsh to N. In contrast, we found that deletion of the intracellular domain of N resulted in failure of the Dsh protein to colocalize with the capped, truncated N molecules (Fig. 5B). A requirement for the intracellular domain of N for colocalization of Dsh provides further evidence that Dsh binds the intracellular domain of N. Dsh thus interacts specifically with the cytoplasmic tail of N, causing it to colocalize in S2 cells.

Finally, we examined the interaction between Dsh and the COOH-terminus of N in vivo. We tested the ability of Dsh to bind the COOH-terminus of N by expressing two N derivatives and assaying their effects on ectopic bristle induction (38). First, if Dsh binds to the N COOH-terminus, its activity should be titrated by expression of the N COOH-terminus alone. Figure 6C shows that expression of the N COOH-terminus inhibited Dsh-dependent ectopic bristle induction. Second, if Dsh antagonizes N by binding its COOH-terminus, then deletion of the Dsh binding site from N should produce an activated form of N. Together with Dsh overexpression, expression of a N derivative lacking the Dsh binding site acts as a dominant gain-offunction allele, repressing ectopic bristle induction (Fig. 6D). Therefore, deletion of the Dsh binding site in N allows it to escape suppression by Dsh. Conversely, expression of the Dsh binding site titrates Dsh, blocking its ability to induce ectopic bristles.

The above experiments, taken together, support the proposal that Dsh interacts with the N COOH-terminus. Further, they suggest that these interactions contribute in vivo to the antagonistic roles of Dsh and N in regulation of target gene expression.

Dsh inhibits N function. Wg signaling provides a mandatory inductive signal required to activate proneural genes in the wing, whereas N acts independently to block commitment to the proneural cell fate. The genetic interactions between Dsh and N described above could reflect competition between the two pathways at the level of target gene regulation. However, the evidence for a physical interaction between the two proteins suggests the possibility that an additional mechanism may be
operative inhibition of N signaling by Dsh, or inhibition of Dsh (and Wg) signaling by N. To test whether Dsh overexpression can inhibit N function, we asked whether Dsh can block an activity of N that is independent of Wg, namely, its role in lateral inhibition. The development of the microchaetae on the notum appears not to require Wg, but the density of microchaetae is controlled by the lateral inhibition function of N (47). Dsh overexpression, induced by use of the GAL4 system, produces a marked increase in the density of the microchaetae, demonstrating that Dsh overexpression blocks N activity during lateral inhibition (Fig. 7, A and B) (38). Similarly, during the refinement of the proneural clusters at the wing margin, N function can be inhibited by Dsh. dsh overexpression during this time (120 to 172 hours AEL) results in bristle hyperplasia and loss of normal patterning at the margin, consistent with a failure of lateral inhibition (Fig. 7, C and D) (38). This phenotype is reminiscent of the SMC hyperplasia seen with loss of temperature-sensitive N function during approximately the same time period (34). Thus, dsh overexpression can block N activity in lateral inhibition, supporting the model that Dsh also blocks N function during proneural induction by Wg. In addition, vein formation in the wing is dependent on N, but not on Wg. Overexpression of Dsh in the wing by use of the GAL4 system can disrupt vein formation, producing phenotypes reminiscent of N mutants (39). Disruption of both lateral inhibition and vein development by Dsh overexpression provides evidence that Dsh can antagonize the activity of N.

A model for the roles of Wg, Dsh, and N in cell fate specification. Correct cell fate decisions require the integration of multiple intercellular signals. Inductive signals are produced by cell types, different from the responding cells, and instruct those decisions. In contrast, lateral signaling occurs among a group of equivalent cells to restrict responses to a subset of the group. The mechanisms whereby cells integrate these inputs are largely unknown. The activities of the Wg and N pathways in bristle induction on the wing serve as a model for the integration of information from an inductive and a lateral signaling pathway.

A model for the interaction between N and components of the Wg pathway is presented in Fig. 8. Wg protein is secreted from a localized source that determines the position of the relevant response, such as bristle formation. Wg signaling activates Dsh, which then antagonizes Zw3, thereby derepressing AS-C expression and SMC development and resulting in development of the adult sensilla. Independently, N acts as a suppressor of AS-C. The level at which N acts to suppress AS-C expression is unknown, perhaps working by a general mech-
Although inhibition of N function must occur to induce bristle development, it is not the sole role for Wg signaling in bristle development: in the interior of the wing, simply blocking N activity by making N mutant clones is not sufficient to induce AS-C or to make bristles (34, 51). Rather, Wg must also actively induce AS-C expression, and components of the Wg pathway downstream of Dsh are required for this activity. Dsh may therefore serve as a branch point, both activating the Wg pathway by overcoming ZW3 activity and inhibiting N function by direct binding.

Previous reports that describe the relation between N and Wg signaling show conflicting observations. It has been proposed that N activity synergests with Wg to specify bristles (20, 21), and that N may act as a receptor for Wg (21, 36). In contrast, it has been suggested that Wg can specify AS-C expression at the wing margin in the absence of N, indicating that N is not required for Wg signal transduction (34). These apparently conflicting observations may be reconciled by recent data suggesting that N activity induces Wg expression in imaginal discs during establishment of the D/V boundary (16, 35). The apparent cooperation between Wg and N may therefore reflect this relation. However, a role for N in reception of the Wg signal has not been ruled out.

It is interesting that N, Wg, and Arm are found at adherens junctions, thought to be sites for cell-cell signaling events (52). Furthermore, Dsh contains a disc large homology region (DHR) domain present in several proteins that localize to intercellular junctions (4, 5). This observation suggests that N may interact with Dsh (and perhaps other signaling pathways) by participating in a complex at adherens junctions. Recently, Dsh has been shown to become phosphorylated in response to Wg (42). In addition, a small fraction of the cytoplasmic Dsh pool translocates to the membrane under these conditions. Similarly, a fraction of the total Dsh pool appears to bind N in the colocalization assay. It is not yet known if phosphorylation of Dsh is required for, or is a result of, its interaction with N. In addition, it has been suggested that activated forms of N might bind to the nucleus, where they participate in complexes that directly regulate gene expression (31). It will be important to determine whether Dsh regulates or participates in formation of these complexes. Future molecular models for Dsh function must account for its role as a link between the instructive signaling mediated by Wg and the lateral signaling mediated by N.

REFERENCES AND NOTES

37. Clones of homozygous wild-type mutant cells were generated by sequencing of the FLP recombination (22). Mutant clones were produced by crossing y w::act::GFP (FRT2) females to y w:: act::GFP (FRT2) males and heat-shocking at 37°C for 2 hours during the first instar. GFP is a red-white. Mutant tissue was identified by y, readily apparent in bristles, and 1182, apparent in hair cells. FRT2 and FRT24 are as described [5, 30]. The em mutation is associated with dominant female sterility and has no effect on development of somatic tissues. The timing of clone
induction was determined by collecting white prepupa at timed intervals after heat shock.

38. Wg was overexpressed in the flies of the genotypes pcyGAL4, UASwgr/UASwgr (41), E. L. Wilder and N. Pavilion, Development 121, 477 (1995). Wg was overexpressed as follows. An Eco RI fragment containing the anti-complementary DNA (41) was cloned into the Eco RI site of pCAGGp (91) (G. S. Thummel, A. M. Boulet, H. L. Dickson, Genet. 74, 448 (1988); G. S. Thummel and V. Pirrotta, Chromosoma Information Service 21, 150 (1992)), and the construct, which carries the ren-derived gene, was used for F element-mediated transformation (34, 43). Spraying in Drosophila, A Practical Approach, D. D. Robertson, Ed. (IRL, New York, 1986), pp. 175-188. M. Robertson et al., Genetics 118, 461 (1987). Transformants 789/fsh and 714/fsh, on the other strand, were recombined to make the transgenic flies 789/fsh/714/fsh for chromosome reversion to fsh/fsh. For production of animals with ectopic bristles, Hr+/+ larvae were heat-shocked at 37°C for various times. Timing of the heat shock was predetermined by collecting white prepupa at timed intervals after heat shock. Pupal wings were dissected and mounted in 50% glycerol for examination. Dosage dependence of the bristle phenotype could be demonstrated by varying the duration of the heat shock or the dose of fsh and/or by using different fsh dosages that are associated with varying levels of activity (J. D. Axelrod, unpublished data). Although there is variability in the responses, clear differences between different conditions were observed. This was also observed by use of the GAL4 system (41). UASwgr driven by pcyGAL4 produces ectopic micro- and microchaetae on the scutellum; the rhoGAL4 driver produces extra microchaetae on the notum. To test the dosage dependency of fsh overexpression, we made use of a combination of wg alleles, w^{111}w^{111}/w^{111}w^{111} (2). Larvae of genotype 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111} were grown at 25°C until mid-third instar and then shifted to 30°C, and heat-shocked (on ice) twice for 1.5 hours at 37°C. Pupal wings were inflated in 12% KOH at 65°C and classified from prepupa. In a wild-type background, 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111} produces a phenotype only slightly less robust than the recombinant between 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111}. Larvae of genotype 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111} were grown at 25°C until mid-third instar and then shifted to 39°C, and heat-shocked (on ice) twice for 1.5 hours at 37°C. Pupal wings were inflated in 12% KOH at 65°C and classified from prepupa. In a small wild-type background, 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111} produces a phenotype only slightly less robust than the recombinant between 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111}. Larvae of genotype 789/fsh/714/fsh, w^{111}w^{111}/w^{111}w^{111} were grown at 25°C until mid-third instar and then shifted to 39°C, and heat-shocked (on ice) twice for 1.5 hours at 37°C. Pupal wings were inflated in 12% KOH at 65°C and classified from prepupa. In each case, half the dosages showed the 714/FSH1 staining pattern.

41. A. van Bel, unpublished observations. Wg were monitored by use of the en promoter line A101, which carries a lacZ gene integrated in the normal en gene, whose expression reflects SMC development (F. Huang, C. Camblay-Chaumiere, A. Glynns, Development 111, 1087 (1986); Y. Boulelaine, G. de la Concini, J. A. Campos Ortega, L. Y. Yan, N. Jan, EMBO J. 10, 2975 (1991)). Pupal wing discs were dissected in insect phosphate-buffered saline (PBS: 135 mM NaCl, 7 mM NaH2PO4, 3 mM Na2HPO4, pH 7.2), fixed for 20 min in 3% formaldehyde in insect PBS and stained in X-Gal according to standard methods. Discs were mounted in 50% glycerol and photographed by use of a Nikon episcopic. To examine SMCs in wild-type discs, w^{111}w^{111} females were crossed to A101/TMS, 50 males. To assess the effects of 789/fsh, Hr+/+ females were crossed to A101/TMS, 50 males, and the progeny were heat-shocked as described above. In each case, half the doses showed the A101 staining pattern.

44. J. D. Axelrod, unpublished observations. SMCs were monitored by use of the en promoter line A101, which carries a lacZ gene integrated in the normal en gene, whose expression reflects SMC development (F. Huang, C. Camblay-Chaumiere, A. Glynns, Development 111, 1087 (1986); Y. Boulelaine, G. de la Concini, J. A. Campos Ortega, L. Y. Yan, N. Jan, EMBO J. 10, 2975 (1991)). Pupal wing discs were dissected in insect phosphate-buffered saline (PBS: 135 mM NaCl, 7 mM NaH2PO4, 3 mM Na2HPO4, pH 7.2), fixed for 20 min in 3% formaldehyde in insect PBS and stained in X-Gal according to standard methods. Discs were mounted in 50% glycerol and photographed by use of a Nikon episcopic. To examine SMCs in wild-type discs, w^{111}w^{111} females were crossed to A101/TMS, 50 males. To assess the effects of 789/fsh, Hr+/+ females were crossed to A101/TMS, 50 males, and the progeny were heat-shocked as described above. In each case, half the doses showed the A101 staining pattern.

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