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 oncoprotein 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 temporally separable steps. Between 48 to 96 hours after egg laying (AEL), Wg expression in the ventral compartment is required for correct dorsal/ventral (D/V) patterning (*10*, *11*). Establishment of the D/V 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*, *17*). (iv) Ectopic 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 *wg* mutations in screens for genetic modifiers of *N* (*20*) and vice versa (*21*), and genetic interactions between *N* and *wg* (*20–22*).

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 immature 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 a 938–amino acid intracellular domain bearing 6 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 (Ser), the cytoplasmic protein Deltex (Dx), and the nuclear proteins encoded by *mastermind (mam)*, *Hairless (H)*, the *Enhancer of split complex* [*E(spl)*], and *Suppressor of Hairless* [*Su(H)*] (*23*). Dl and Ser bind to specific EGF repeats in the N extracellular domain (*25*), whereas the intracellular ankyrin repeats are necessary for N signaling activity (*26–28*). The cytoplasmic proteins Dx (*29*, *30*) and Su(H) interact with N at or adjacent to the ankyrin repeats (*31*, *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 *wg* in establishment of D/V pattern in the wing as well as in induction of bristles at the margin (*16*, *34–36*, and references cited above). However, the precise relation between the 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 *wg* 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 D/V compartmentalization (*10*, *11*, *15*), whereas late loss can result in either loss of growth-organizing activity (*16*) or the absence of bristles (*13*,

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14). 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. Pulses of *dsh* overexpression, driven from the *hsp70* promoter (*Hs:dsh*) during late third instar (108 to 120 hours AEL) (*38*), result in the development of supernumerary bristles in the interior of the wing (Fig. 1, D to F). The bristle types (stout, 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. 1G) (*13*, *38*). However, we will argue that the wing provides a semiquantitative 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 wg^{ts} 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 *hsp70* 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. (i) 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

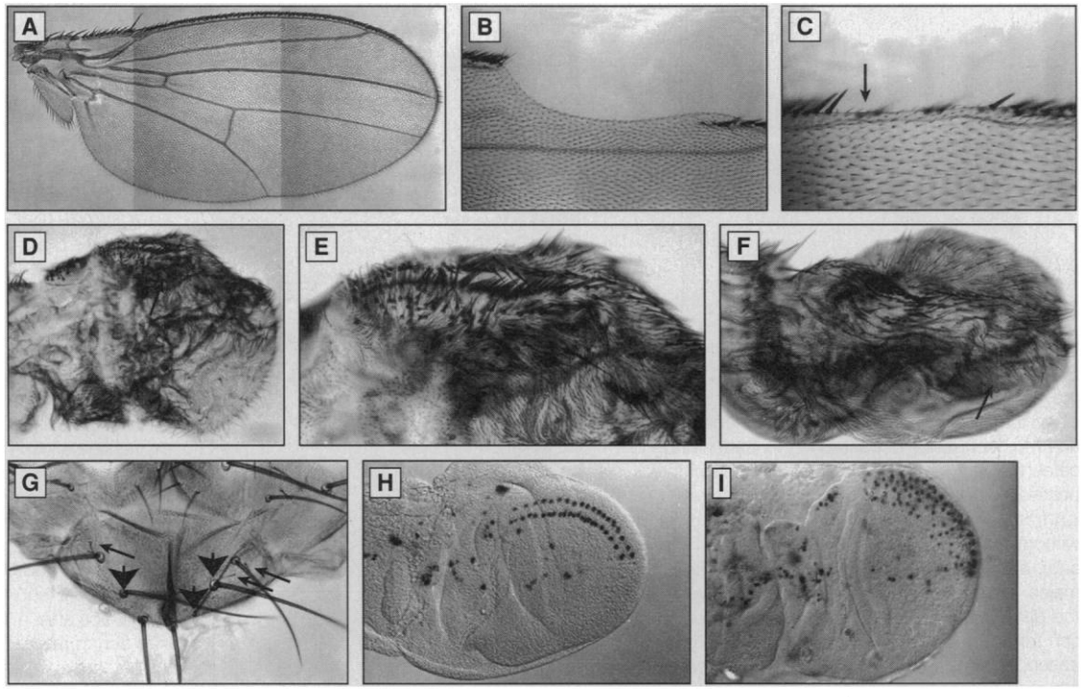

Fig. 1. Effects of altered *dsh* expression on patterning of wing bristles. (**A**) Wild-type wing. (**B**) A large clone of *dsh* mutant cells, induced before 72 hours AEL, is associated with a notch in the wing margin. (**C**) A small clone of *dsh* mutant cells at the wing margin, induced later than 72 hours AEL. The mutant cells, marked with *y* and f^{36a} [producing curved hairs (arrow)], fail to elaborate bristles. (**D** to **F**) Dose dependency of ectopic bristle formation resulting from *dsh* overexpression. Ectopic bristles resulting from (D and E) a 0.75- or (F) 1.5-hour late third instar heat shock of animals carrying one copy of *Hs:dsh*. (E) Detail of the wing shown in (D). Arrows denote the increased extent to which bristles develop in the interior of the wing with increased heat shock. Altering the dose of the *Hs:dsh* transgene has a similar effect on the extent of ectopic bristle development (*38*). (**G**) Ectopic scutellar bristles resulting from overexpression of Dsh from *UASdsh* driven by *ptcGAL4* (*38*). Ectopic macrochaete and microchaete are indicated by large and small filled arrows, respectively. (**H** and **I**) *A101* staining reveals SMCs in wild-type and *Hs:dsh* pupal wings. Discs were dissected from pupae at ~2 hours after puparium formation (apf). In wild-type (H), SMCs develop in two parallel rows along the presumptive margin. At late third instar, only the precursors of the chemoreceptors are visible, and shortly thereafter all SMCs have appeared and undergo divisions between ~2 to 14 hours apf (*50*). (I) Wing disc dissected from a *Hs:dsh*/+ larva marked with *A101*, after heat shock during the last 12 hours of third instar. The two rows of SMCs at the margin are expanded relative to the wild type, thereby producing ectopic SMCs outside the domain of wild-type SMC induction. Additional ectopic SMCs on the opposite side of the disc are out of focus and not clearly visible in this view.

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, marginocentric 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 D/V 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 Dsh and N. 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 marked expansion of the SMC domain is seen (Figs. 3B and 1I). A similar, though less robust, result was obtained when a dominant-negative N construct, $N^{\Delta cdc10}$ (*26*), was expressed together with *Hs:dsh* (*39*). Conversely, duplication of the *N* locus, or expression of an activated N (*Hs:N^{intra}*) (*42*), suppresses the ectopic bristle phenotype (*39*). We conclude that in the interior of the wing blade, N and the Wg pathway exert opposing, 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 Dl is a ligand for N, and both act together in regulation of bristle development on the notum, we tested whether Dl inhibits bristle induction on the wing. Accordingly, ectopic bristles were induced with *Hs:dsh* in a *Dl* heterozygous background. As with *N*, decreasing the dosage of *Dl* 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 *Dl*, the severity of the phenotype was reduced (*39*). Thus, Dl has an activity similar to N in suppressing bristle development. In summary, both N and Dl 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

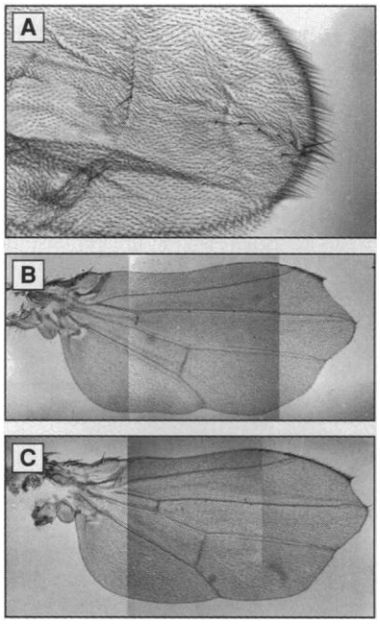

Fig. 2. Wg dependence of ectopic bristles. (**A**) Ectopic bristles induced by expression of Wg in the *ptc* expression domain. *ptc* is expressed along the anterior-posterior boundary of the wing, and the occurrence of bristles in this pattern indicates that ectopic Wg is able to induce ectopic bristles. (**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 *T8hs-dsh, wg^{L114}/Gla* flies showed a reduced penetrance 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.

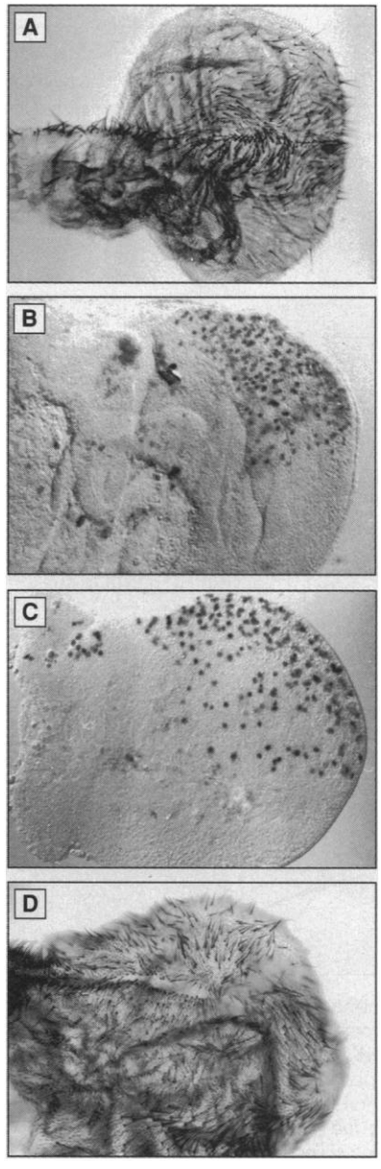

Fig. 3. N and Dl suppress the formation of ectopic bristles in the interior of the wing blade generated by *Hs:dsh*. (**A**) The phenotype of a pupal wing after overexpression of *dsh* (1.5-hour pulse delivered in the last 12 hours of third instar) in an *N* heterozygote. The view is from the anterior, 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 ~2-hour apf (B) and ~6-hour apf (C) pupal wing discs after a 1.5-hour pulse 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* overespression (1.5-hour pulse) in a Dl 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. Using a 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 NH_2-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*). Cotransfected cells were aggregated with cells expressing Dl, a membrane-bound ligand for N, to produce a "mutual capping" of N and Dl 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-of-function 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

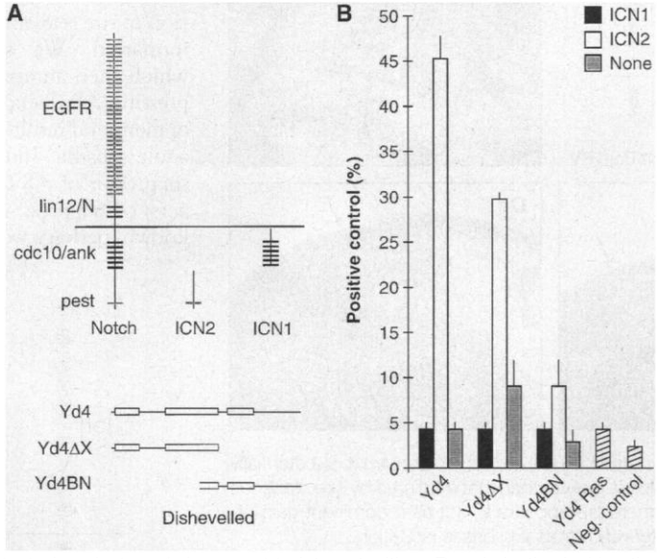

Fig. 4. Dsh binds to N in the yeast interaction trap assay. (**A**) Constructs used to test binding of Dsh and N. N fragments ICN1 and ICN2 were fused in frame to a transcriptional activator domain, and the Dsh fragments were fused in frame to a LexA DNA binding domain (*44*). Boxes in the Dsh protein represent domains conserved among known Dsh proteins from other species. (**B**) Averaged results of four independent assays for interaction of N and Dsh (*44*). Each Dsh construct was tested against ICN1, ICN2, and with no interactor (none). Yd4 was also tested against, and shown not to interact with, a Ras derivative lacking the COOH-terminus that is known to interact with Raf in this assay. A significant interaction was noted only for ICN2 when tested against Yd4 or Yd4ΔX. Note that full-length Dsh (Yd4) and the NH_2-terminus of Dsh (Yd4ΔX) have some intrinsic activity as transcriptional activators. Some of the activity of Yd4ΔX is squelched by ICN2. Error bars, standard deviations.

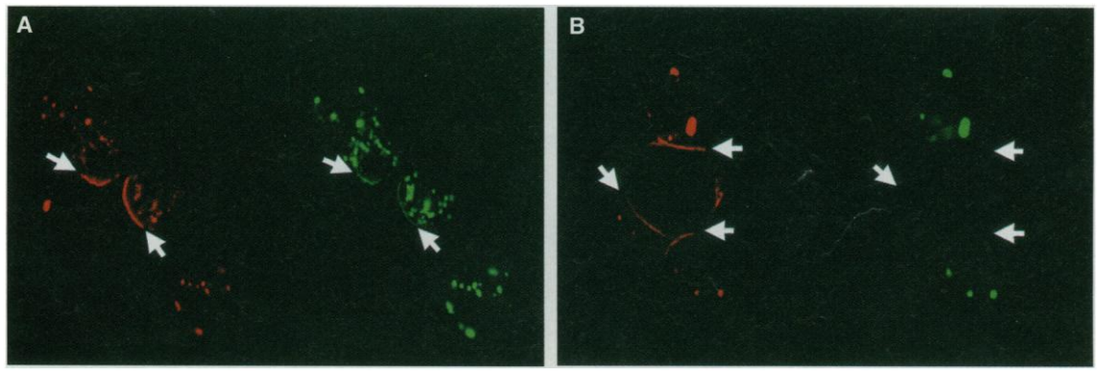

Fig. 5. Colocalization of Dsh and N in transfected Schneider cells. Confocal microscope images of *Drosophila* S2 cells are presented as split images, with Dsh shown in green (right) and N in red (left). Dsh was coexpressed (**A**) with full-length N or (**B**) a truncated form of N that deletes the cytoplasmic domain [pMTECN (*29*)]. After expression, the cells were allowed to aggregate with Dl expressing cells as described (*29*). The subcellular localization of Dsh in relation to the N capping site at the cellular contact between N and Dl is shown (arrows). Colocalization of N and Dsh in the caps depends on the presence of the intracellular domain of N. At least 75% of individual N capping sites showed colocalization with the Dsh protein.

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 microchaete on the notum appears not to require Wg, but the density of microchaete 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 microchaete, 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 122 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-

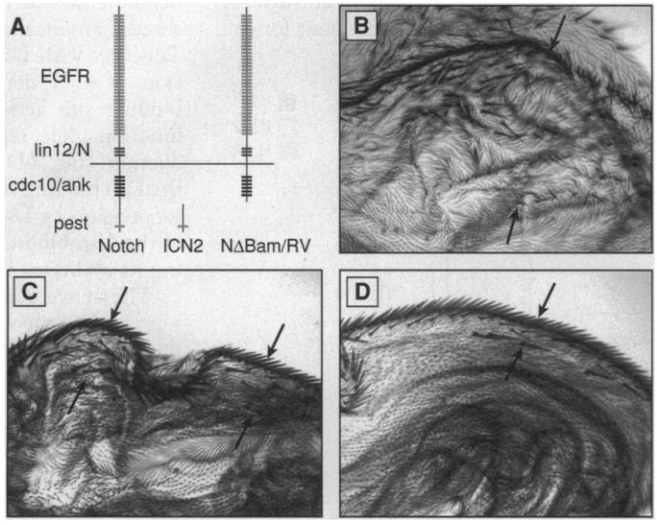

Fig. 6. In vivo function of the Dsh binding site in N. (**A**) Schematic diagram of the structures of *N* derivatives used here. (**B**) Ectopic bristles resulting from Dsh overexpression driven by the heat shock promoter between 7 and 12 hours before puparium formation (bpf) (*38*). (**C**) Simultaneous overexpression of Dsh and $N^{\Delta Bam/RV}$, each from the heat shock promoter between 7 and 12 hours bpf. ICN2 substantially suppresses the ectopic Bristle response, consistent with its ability to bind Dsh. (**D**) Simultaneous overexpression of Dsh and $N^{\Delta Bam/RV}$, each from the heat shock promoter between 7 and 12 hours bpf. The $N^{\Delta Bam/RV}$ construct substantially suppresses the ectopic bristle response. This suppression is greater than that mediated by simultaneous overexpression of wild-type N (not shown). $N^{\Delta Bam/RV}$ therefore appears to act as a dominant gain-of-function allele, escaping repression by Dsh and constitutively repressing bristle induction.

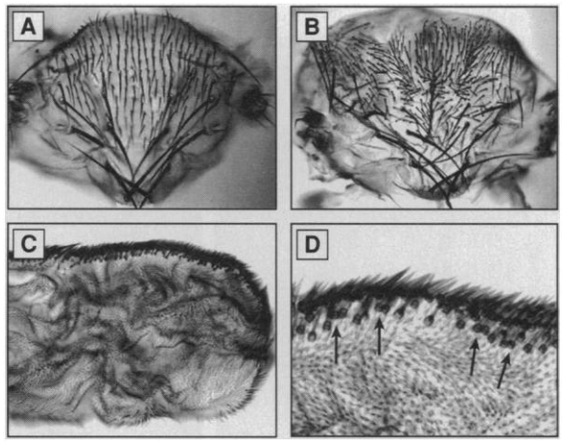

Fig. 7. Overexpression of Dsh disrupts lateral inhibition. To test whether Dsh can block N during lateral inhibition, Dsh was overexpressed during lateral inhibition on the notum and during refinement of the proneural clusters at the wing margin. (**A**) A wild-type notum, showing the normal array of microchaete. (**B**) Notum from an animal in which Dsh was overexpressed from *UASdsh* driven by *rhlGAL4* (*38*). The density of macrochaete is markedly increased, suggesting that N-mediated lateral inhibition is suppressed. (**C**) Wing from a *Hs:dsh* fly after a 1.5-hour heat shock immediately after puparium formation. (**D**) Detail of the anterior margin of a wing similar to that shown in (C). Note the extra bristles clustered around the margin and the loss of normal architecture. Several instances of bristles arising immediately adjacent to one another are indicated [arrows in (C)]. In contrast, the normal margin architecture is retained when the heat shock is performed before puparium formation (see Fig. 1E).

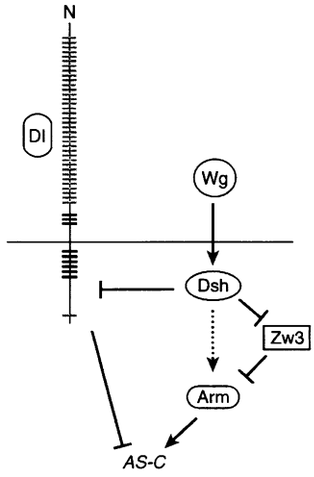

Fig. 8. A model for the roles of Dsh and N in Wg signal transduction. Dsh is proposed to inhibit N function by binding to the COOH-terminal end of the cytoplasmic domain. It may function by stabilizing an inactive conformation of the N protein. The mechanism whereby N blocks *AS-C* transcription is unknown. A cytoplasmic fragment of N has been proposed to complex with Su(H) to regulate transcription of *E(spl)* in a homologous mouse system (*33*), and such a mechanism may function here. However, N may act more directly to inhibit the Wg signaling pathway. Arm localization is regulated by Zw3, and Dsh blocks this activity. Dsh may regulate the activity of the Zw3 kinase directly or may exert a competing effect on its target.

anism that suppresses responses to inductive signals (*23*, *48*, *49*) or perhaps working more directly to affect the function of the Wg pathway. Wg signaling overcomes N-mediated suppression, and we propose that this inhibition of N activity occurs through a direct interaction between Dsh and the COOH-terminal domain of N. Dsh may accomplish this function by disrupting the ability of a N fragment and its associated effectors to act as transcriptional regulatory factors in the nucleus (*33*).

Wg provides an instructive signal that specifies bristle development at the wing margin. As previously summarized, the Wg expression pattern coincides with the bristle pattern, loss of Wg function specifically during the end of third instar results in loss of bristles, and Wg function is required to activate AS-C expression. In addition, we demonstrate that ectopic Wg expression activates ectopic bristle development. Furthermore, components of the Wg signaling pathway are required to generate bristles. Dsh [(*4*, *14*) and in this article] and Arm (*14*, *39*) are needed cell autonomously for bristle induction, whereas *zw3* clones ectopically activate AS-C and produce bristles (*50*). All known components of the Wg signal transduction pathway are therefore active in bristle induction.

N is also involved in bristle development. N appears to play a role in refinement of the proneural region at the wing margin, similar to its role in lateral inhibition during bristle development on the notum. In addition, we describe an earlier role for N during induction of the proneural region: N acts to repress the proneural cell fate change induced by Wg. Wg signaling must therefore overcome this inhibitory activity of N.

The data presented here argue that Wg signaling overcomes N function by a direct interaction. Three independent assays have been presented that indicate that Dsh binds to the COOH-terminal half of the N cytoplasmic domain. An in vivo assay supports the functional relevance of this binding and is consistent with the hypothesis that Dsh antagonizes N by binding to this domain. The Dsh binding domain in N maps distal to the cdc10/ankyrin repeats and therefore defines a previously unrecognized functional element in the N protein.

The physical interaction between Dsh and N suggests that the observed genetic interactions may result from direct contact between these two proteins. Although we would like to order the activities of N and Dsh in a pathway, epistasis analysis is not possible by use of the ectopic bristle assay. However, from the observation that *dsh* overexpression blocks lateral inhibition, and the observation that Dsh binds N, we infer that Dsh acts upstream of N to inhibit its action in bristle induction as well.

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 synergizes 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 discs 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 hyperphosphorylated in response to Wg (*43*). 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 move to the nucleus, where they participate in complexes that directly regulate gene expression (*33*). 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 inductive signaling mediated by Wg and the lateral signaling mediated by N.

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37. Clones of homozygous *dsh* mutant cells were generated by use of the FLP technique (*53*). Marked *dsh* clones were produced by crossing *y w dsh^{v26} f^{36a} FRT$^{9-2}$/FM7* females to *ovo^{D2} FRT$^{9-2}$; FLP^{38}/FLP^{38}* males and heat-shocking at 37°C for 2 hours during third instar. *dsh^{v26}* is a null allele. Mutant tissue was identified by *y*, readily apparent in bristles, and *f^{36a}*, apparent in hair cells. *FRT$^{9-2}$* and *FLP^{38}* are as described (*8*, *53*)]. The *ovo^{D2}* 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 prepupae at timed intervals after heat shock.
38. Wg was overexpressed in flies of the genotype *ptc-GAL4; UASwg^{ts}/UASwg^{ts}* [(*41*); E. L. Wilder and N. Perrimon, *Development* **121**, 477 (1995)]. Dsh was overexpressed as follows: An Eco RI fragment containing the *dsh* complementary DNA (*4*) was cloned into the Eco RI site of pCaSpeR-hs [C. S. Thummel, A. M. Boulet, H. D. Lipshitz, *Gene* **74**, 445 (1988); C. S. Thummel and V. Pirrotta, Drosophila *Information Service* **71**, 150 (1992)], and the construct, which carries the *mini-white$^+$* gene, was used for P-element–mediated transformation [A. Spradling, in Drosophila, *A Practical Approach*, D. B. Roberts, Ed. (IRL, New York, 1986), pp. 175–198; H. M. Robertson *et al.*, *Genetics* **118**, 461 (1988)]. Transformants *T8Hs:dsh* and *T14Hs:dsh*, on the second chromosome, were recombined to make the homozygous viable *rec7 Hs:dsh* chromosome, referred to as *Hs:dsh*. For production of animals with ectopic bristles, *Hs:dsh/+* larvae were heat-shocked at 37°C for various times (see text). Timing of the heat shock was precisely determined by collecting white prepupae at timed intervals after heat shock. Pupal wings were dissected and mounted in 80% glycerol for examination. Dose dependence of the ectopic bristle phenotype could be demonstrated by varying the duration of the heat shock or the dose of *Hs:dsh*, or by using different *Hs:dsh* insertions 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. Dsh was also overexpressed by use of the GAL4 system (*41*). *UASdsh* driven by *ptcGAL4* produces ectopic macro- and microchaete on the scutellum, and the *rhlGAL4* driver produces extra microchaete on the notum. To test the Wg dependence of *dsh* overexpression, we made use of a combination of *wg* alleles, *wg^{IL114}/wg-lacZ*, that is associated with temperature sensitivity (*13*). Larvae of genotype *T8Hs:dsh, wg^{IL114}/wg-lacZ* were grown at 17°C until mid–third instar and then shifted to 29°C, and heat-shocked (or not) twice for 1.5 hours at 37°C. Pupal wings were inflated in 10% KOH at 65°C and dissected from pharate pupae. In a wild-type background, *T8Hs:dsh* produces a phenotype only slightly less robust than the recombinant between *T8Hs:dsh* and *T14Hs:dsh*. *Hs:wg,* inserted on the third chromosome, drives expression of *wg* from the *hsp70* promoter [J. Noordermeer, P. Johnston, F. Rijsewijk, R. Nusse, P. A. Lawrence, *Development* **116**, 711 (1992)] and was coexpressed with *Hs:dsh*. *Hs:dsh* flies were crossed to *N^{83}/Dp (1;Y) w^{+303}*, *Df (3R) Dl^{M2}/TM6C*, or *Dp (3;3) bxd^{110}/TM6C* to generate flies carrying *Hs:dsh* in the backgrounds of *N* or *Dl* deficiencies or duplications. *N^{83}* and *Dp (1;Y) w^{+303}* are a deletion and a duplication of the *N* region, respectively. *Dp (3;3) bxd^{110}* is a duplication, and *Df(3R)Dl^{M2}* is a deletion, of the *Dl* region. The *N* genotypes were identified by gender, and the *Dl* genotypes, by absence of the *Tb* marker, which is present on the *TM6C* chromosome. To test *Hs:dsh* in the background of a dominant-negative or gain-of-function *N* allele, *Hs:dsh* flies were crossed to *Hs:N$^{\Delta cdc10}$* (*27*) and *Hs:N^{Intra}* (*42*), respectively. Pupal wings were dissected and mounted in 80% glycerol for examination. SMCs in flies with *Hs:dsh* and *N* were examined by crossing *Hs:dsh/CyO, en11; A101/+* females to *N^{83}/Dp (1;Y) w^{+303}* males and heat-shocking for 1.5 hours at 37°C during third instar. The appropriate discs were obtained by sorting female larvae, aging as desired, and were dissected and prepared as above. Only discs not staining for *CyO, en11* (an enhancer trap in *wg*) and with the *A101* staining pattern were scored. Overexpression of N subfragments from *Hs:N$^{\Delta Bam/RV}$* (*27*) and *Hs:ICN2* (N amino acids 2109 to 2703) was done simultaneously with *Hs:dsh* in a 1.5-hour pulse during the last 12 hours of third instar to assess their effects on the ectopic bristle response.
39. J. D. Axelrod, unpublished observations.
40. SMCs were monitored by use of the enhancer trap line *A101*, which contains a *lacZ* gene integrated in the *neuralized* gene, whose expression reflects SMC development [F. Huang, C. Dambly-Chaudière, A. Ghysen, *Development* **111**, 1087 (1991); L. Boulianne, A. de la Concha, J. A. Campos-Ortega, L. Y. Jan, Y. N. Jan., *EMBO J.* **10**, 2975 (1991)]. Pupal wing discs were dissected in insect phosphate-buffered saline [PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 (pH 7.0)], fixed for 20 min in 2% formaldehyde in insect PBS, and stained in X-Gal according to standard methods. Discs were mounted in 80% glycerol and photographed by use of Nomarski optics. To examine SMCs in wild-type discs, *y w* females were crossed to *A101/TM3, Sb* males. To assess the effects of *Hs:dsh, Hs:dsh* females were crossed to *A101/TM3, Sb* males, and the progeny were heat shocked as described above. In each case, half the discs showed the *A101* staining pattern.
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44. A yeast interaction trap system was used as described [J. Gyuris, E. Golemis, H. Chertkov, R. Brent, *Cell* **75**, 791 (1993)]. Quantitative *lac-Z* assays were performed [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)]. The bait plasmids expressed either the entire *dsh* coding region, including amino acids 1 to 638 (Yd4), 1 to 393 (YD4dX), or 286 to 638 (Yd4BN). The "prey" plasmids expressed portions of the cytoplasmic domain of N, including amino acids 1027 to 2258 (ICN1, includes cdc10/ankyrin repeats) and amino acids 2109 to 2703 (ICN2). The assay was performed four independent times on independent isolates. Average values are expressed as a percentage of the positive control (an activation domain covalently linked to the DNA-binding domain). The ICN2 fragment has substantial ability to activate transcription (*33*, *39*) and therefore may amplify the apparent strength of the interaction. This activity also precludes the use of ICN2 as a bait.
45. Colocalization of Dsh and N was assayed by cotransfection of expression plasmids into Schneider cells (*29*). Expression constructs placed *N* and *dsh* under the inducible control of the *Drosophila metallothionein* and *hsp70* promoters, respectively. Capping of N was induced by coculture of the transfected cells with cells expressing Delta. To facilitate visualization of Dsh, we inserted two tandem copies of a Myc epitope tag (sequence: EQKLISEEDL) (*54*) between amino acids 393 and 394. The resulting protein is functional in vivo, because, under control of the *dsh* promoter, it can rescue a null mutant (*39*). Dsh was visualized by use of a mouse antibody specific for the Myc epitope, and N was visualized by use of a rat polyclonal antibody. The N expression constructs pTM and pMTECN were as described (*29*).
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54. Abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; I, Ile; K, Lys; L, Leu; Q, Gln; and S, Ser.
55. We thank J. Klingensmith for construction of the *Hs:dsh* plasmid; J. Noordermeer, M. Young, and G. Struhl for fly stocks; R. Finley and R. Brent for plasmids and yeast; and H. Chen, M. Melnick, and B. Noll for technical help. We also thank to A. Manoukian for stimulating discussion and members of the Perrimon lab for critically reviewing the manuscript. We are grateful to A. Manoukian, J. P. Couso, A. Martinez Arias, S. Blair, S. Carroll, and S. Sokol for communicating results before publication. Supported by grants from the National Institutes of Health (J.D.A. and N.P.). N.P. and S. A.-T. are Investigators of the Howard Hughes Medical Institute.

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