Current Biology 19, 1378–1383, August 25, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.06.042

Report

cis-Inhibition of Notch by Endogenous Delta Biases the Outcome of Lateral Inhibition

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Summary

Lateral inhibition mediated by Delta/Notch (Dl/N) signaling is used throughout development to limit the number of initially equivalent cells that adopt a particular fate [1–3]. Although adjacent cells express both Dl ligand and N receptor, signaling between them ultimately occurs in only one direction. Classically, this has been explained entirely by feedback: activated N can downregulate Dl, amplifying even slight asymmetries in the Dl or N activities of adjacent cells [1–5]. Here, however, we present an example of lateral inhibition in which unidirectional signaling depends instead on Dl's ability to inhibit N within the same cell, a phenomenon known as *cis*-inhibition [6–11]. By genetically manipulating individual R1/R6/R7 photoreceptor precursors in the *Drosophila* eye, we show that loss of Dl-mediated *cis*-inhibition reverses the direction of lateral signaling. Based on our finding that Dl in R1/R6s requires endocytosis to *trans*-activate but not to *cis*-inhibit N, we reexamine previously published data from other examples of lateral inhibition. We conclude that *cis*-inhibition generally influences the direction of Dl/N signaling and should therefore be included in standard models of lateral inhibition.

Results and Discussion

Signaling between R1/R6 and R7 Precursors Is an Example of Biased Lateral Inhibition

Each unit of the fly eye is assembled by reiterative epidermal growth factor (EGF) signaling [12], which simultaneously recruits undifferentiated cells to join the growing cluster of photoreceptor (R) neurons and induces them to transcribe *Delta* (*Dl*) [13]. The R1, R6, and R7 neuron precursors form an equivalence group: those in which Notch (N) is activated adopt the R7 fate, whereas those in which N is not activated adopt the molecularly equivalent R1 or R6 (R1/R6) fate [14, 15]. During normal development, the first two of the precursors to be recruited receive no Dl signal and therefore adopt the R1/R6 fate. They then redundantly use Dl to activate N in the next recruit, which therefore adopts the R7 fate [14, 15]. Because both R1/R6 and R7 precursors coexpress Dl and N [16], we hypothesized that Dl/N signaling among them might be an example of lateral inhibition and that the direction of signaling might simply be biased by the prior expression of Dl in R1/R6s (Figures 1A and 1B; see also Figure S1 available online). If so, then removal of Dl from the R1 and R6 precursors should reverse the direction of signaling. To test this, we used the *GMR-FLP*/mosaic analysis with a repressible cell marker (MARCM) technique to create mosaic adult retinas in which ~11% of R1/R6/R7 precursors were homozygous for a *Dl* null mutation and all other R cells were wild-type [17, 18]. As predicted by our hypothesis, in ommatidia in which both R1 and R6 precursors lacked Dl, the R1 and R6 precursors adopted the R7 fate, and the wild-type R7 precursor adopted the R1/R6 fate, indicating that the direction of signaling was reversed (Figures 1C, 1D, 1F, and 1G; Figure S2). Confirming that N is indeed activated in *Dl* mutant R1/R6 precursors, we found that they expressed the *mδ0.5-lacZ* reporter of N activity [15, 19] and did not adopt the R7 fate if their N pathway was blocked (Figure S3). To confirm that the transformation of *Dl* mutant R1/R6 precursors was caused by their receipt of a Dl signal from the R7 precursor, we examined ommatidia in which all three precursors were homozygous *Dl* mutant. Indeed, *Dl* mutant R1/R6 precursors did not adopt the R7 fate if the corresponding R7 precursor also lacked Dl (Figures 1E and 1H; Figure S2). We therefore conclude that Dl/N signaling between R1/R6 and R7 precursors is an example of lateral inhibition. The classical feedback model predicts that the prior expression of Dl in R1/R6 precursors would downregulate Dl in the R7 precursor, ensuring that the latter cannot signal back and thus biasing the direction of signaling (Figure 1F). We hypothesize that the transformation of *Dl* mutant R1/R6s into R7s was not previously observed because the homozygous clones analyzed included *Dl* mutant R7s [14].

Dl-Mediated *cis*-Inhibition of N in R1/R6s Is Required to Prevent a Reversal in the Direction of Signaling

We noticed that the redundancy of Dl signaling from R1 and R6 exposed a discrepancy between our results and those predicted by the classical feedback model of lateral inhibition. In particular, when only one of the two R1/R6 precursors in an ommatidium is *Dl* mutant and the other is wild-type, the feedback model predicts that both R1/R6 precursors should nevertheless adopt the same fate, because they are both exposed to the same R7 source and therefore level of Dl (Figures 2A and 2B). If Dl in the R7 precursor remains sufficiently downregulated despite the partial reduction in N activation, then both the wild-type and the *Dl* mutant R1/R6 precursor should still adopt the R1/R6 fate (Figure 2A). If, instead, Dl levels in the R7 precursor are sufficiently increased by the partial reduction in N activation, then both R1/R6 precursors should adopt the R7 fate (Figure 2B). However, we found that only the *Dl* mutant R1/R6 precursor adopted the R7 fate, whereas the wild-type precursor remained untransformed, indicating that N was activated in the former but not in the latter (Figures 2C, 2D, 2F, and 2G; Figure S2). Consistent with this interpretation, the fate transformation of the *Dl* mutant R1/R6 precursor depended on Dl from the R7 precursor (Figures 2E and 2H; Figure S2), and only the *Dl* mutant R1/R6 precursor expressed the *mδ0.5-lacZ* reporter of N activity (Figure S3). Because the only difference between the *Dl* mutant and wild-type R1/R6 precursors is their ability to express Dl, we conclude that Dl in R1/R6s autonomously represses N pathway function (Figures 2I and 2J). The only known molecular mechanism for this is *cis*-inhibition: recent work indicates that Dl can bind in *cis* the same region of N that is bound by Dl in *trans*, suggesting that *trans* Dl must outcompete *cis* Dl in order to activate N [11]. Consistent with

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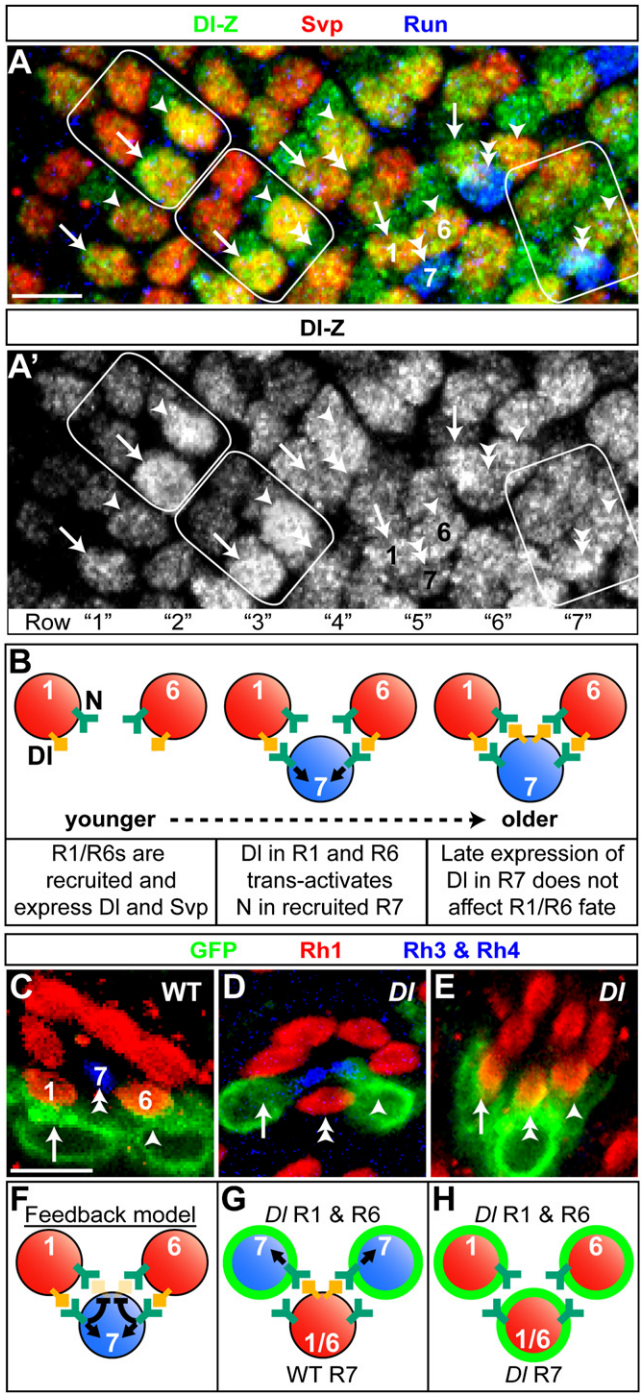

Figure 1. The R1 and R6 Precursors Normally Express Dl First; if R1 and R6 Lack Dl, the Direction of Signaling Is Reversed

(A and A′) A third-instar larval (L3) eye disc heterozygous for a *Dl-lacZ* enhancer trap [32]. During late L3, a wave of differentiation passes from posterior (right) to anterior (left) across the eye disc, resulting in a gradient of ommatidial ages within a single disc: each vertical row of ommatidia is 1.5 hr older than the row to its immediate left. We arbitrarily define the row in which R1/R6 precursors first express *Dl-lacZ* as row "0" (Figure S1). R1 (arrow) and R6 (arrowhead) first express the R1/R6- and R3/R4-specific transcription factor Seven-up (Svp; red) in row "1" (leftmost, unoutlined ommatidium) [25]. R7 precursors (double arrowheads) do not transcribe Dl (green) until row "3" (middle outlined ommatidium) (see Figure S1 for details). R7s ultimately express the R7- and R8-specific transcription factor Runt (Run; blue) [36]. Outlined ommatidia correspond to those in Figure 4A. Scale bar represents 5 μm.

this model, we found that overexpressing full-length wild-type N in R1/R6 precursors could overcome the inhibition by *cis* Dl, causing many R1/R6s to adopt the R7 fate (Figure S4). The simplest explanation for our results is therefore that when one R1/R6 precursor lacks Dl, its N is no longer *cis*-inhibited and can therefore be activated by Dl expressed in R7, whereas N in the nonmutant R1/R6 precursor remains *cis*-inhibited.

Although artificial overexpression of Dl can prevent N activation in many contexts [7–11], the only previous loss-of-function evidence that *cis*-inhibition occurs during normal development remains the example of *Drosophila* wing boundary formation, during which *cis*-inhibition does not influence the direction of signaling but is instead required to keep all signaling off [6]. The possible role of *cis*-inhibition in lateral inhibition has been largely ignored (although see [2, 20, 21]). To test our *cis*-inhibition model further, we wanted to examine the consequence of eliminating N activity specifically from R7 precursors: whereas the feedback model predicts that this would cause a reversal in the direction of signaling, causing R1/R6 precursors to adopt the R7 fate, the *cis*-inhibition model predicts instead that N in R1/R6 precursors would remain *cis*-inhibited, resulting in all three precursors' adopting the R1/R6 fate. For technical reasons, it is not possible to create *N* mutant R7 precursors ([14]; see also Experimental Procedures), but Tomlinson and Struhl [14] used an exclusively repressive form of Su(H) to reduce activity of the N transduction pathway specifically in R7s. Consistent with our *cis*-inhibition model, they found that 10%–20% of such R7s adopted the R1/R6 fate but reported no transformation of the corresponding R1 and R6 precursors into R7s [14]. To confirm and extend this result, we used an alternative strategy, taking advantage of the fact that Dl ligand must be endocytosed in order to

(B) Schematic depicting the timing of Dl expression in wild-type R1/R6/R7 precursors. The three ommatidia depicted correspond to rows "1," "2," and "3" in (A) and (A′).

(C–E) Mosaic adult ommatidia in which a small number of R1/R6/R7 precursors are homozygous for a particular chromosome arm (green; see Experimental Procedures).

(C) An ommatidium in which both the R1 (arrow) and R6 (arrowhead) precursors are homozygous for a wild-type chromosome (*FRT82*): each has a large rhabdomere that remains in the outer part of the ommatidium and expresses the Rh1 rhodopsin (red) [37]. The wild-type R7 precursor (double arrowhead) has a small rhabdomere that extends into the center of the ommatidium and expresses Rh3 or Rh4 (blue) [37]. Scale bar represents 5 μm.

(D) When both the R1 (arrow) and R6 (arrowhead) precursors are homozygous *Dl* mutant, they have small central rhabdomeres that express Rh3 or Rh4 but not Rh1, indicating that they have adopted the R7 fate. The wild-type R7 precursor (double arrowhead) adopts the R1/R6 fate.

(E) If *Dl* is also removed from the R7 precursor (double arrowhead), then *Dl* mutant R1 (arrow) and R6 (arrowhead) precursors no longer adopt the R7 fate and instead become R1/R6s. See Figure S2 for quantification.

(F–H) Schematic representations of how the results in (C)–(E) can be explained by the classical feedback model of lateral inhibition. Precursor identities are indicated by position and black text. The fate each precursor adopts is indicated as in (B) both by color (red represents R1/R6 fate; blue represents R7 fate) and by the corresponding R cell fate number in white. *Dl* mutant cells are outlined in green, and Dl and N are depicted as in (B).

(F) N in the R7 precursor is *trans*-activated by the early expression of Dl in the R1/R6 precursors. According to the feedback model, Dl in the R7 precursor is therefore downregulated, preventing it from later *trans*-activating N in the R1/R6 precursors.

(G) When both R1/R6 precursors lack Dl, N is no longer activated in the R7 precursor. As a consequence, Dl in R7 is not downregulated and thus can activate N in the R1/R6 precursors. The R1/R6 precursors therefore adopt the R7 fate, and the R7 precursor adopts the R1/R6 fate.

(H) When Dl is removed from the R7 precursor, N is no longer activated in the *Dl* mutant R1/R6 precursors: all three precursors adopt the R1/R6 fate.

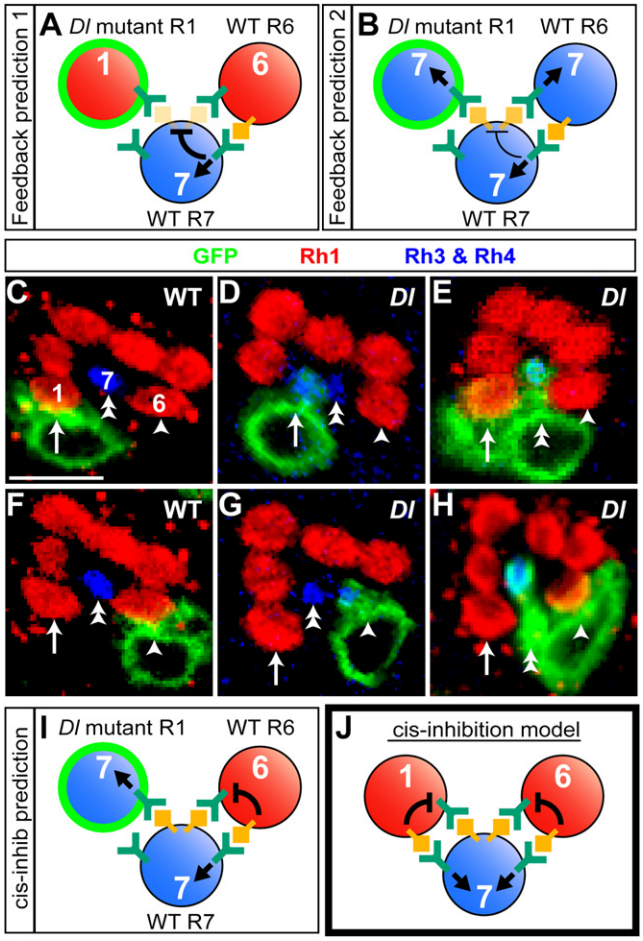

Figure 2. The Phenotype Caused by Loss of Dl from the R1 or R6 Precursor Alone Suggests that Dl *cis*-Inhibits N in R1/R6 Precursors

(A and B) The classical feedback model predicts two possible outcomes of removing Dl from the R1 precursor alone. Previous work has shown that the corresponding wild-type R6 precursor still activates N in the R7 precursor, which therefore adopts the R7 fate [14].

(A) The level of activated N in the R7 precursor may be sufficient to downregulate Dl. Both R1/R6 precursors will therefore adopt the R1/R6 fate.

(B) Alternatively, a reduction in activated N within the R7 precursor may cause an increase in Dl that is sufficient to activate N in R1/R6 precursors. Both R1/R6 precursors will therefore adopt the R7 fate.

(C–H) Mosaic adult ommatidia in which a small number of R1/R6/R7 precursors are homozygous for a particular chromosome arm (green) and are stained for expression of Rh1 (red) and Rh3 or Rh4 (blue). Loss of Dl from the R1 (D; arrow) or R6 (G; arrowhead) precursor alone causes only that precursor to adopt the R7 fate (blue). This fate transformation depends on Dl from the R7 precursor (E and H, respectively). Scale bar in (C) represents 5 μm. See Figure S2 for quantification.

(I and J) Schematic representation of how the results in (C)-(H) are explained by Dl-mediated *cis*-inhibition of N.

(I) Dl from the R7 precursor *trans*-activates N in the *Dl* mutant R1 precursor but does not *trans*-activate N in the wild-type R6 precursor. The only difference between the R1 precursor and the R6 precursor is that the latter expresses Dl. We therefore conclude that Dl inhibits N pathway activation.

(J) Model: Dl in the R1/R6 precursors normally *cis*-inhibits N.

trans-activate N, a process that in R1/R6 cells requires the E3 ubiquitin ligase Neuralized (Neur) [20, 22, 23]. By contrast, a mutant form of the N ligand Serrate that cannot be endocytosed retains the ability to *cis*-inhibit N when artificially overexpressed [24]. We therefore tested whether by removing Neur from both R1 and R6 we could eliminate Dl's ability to

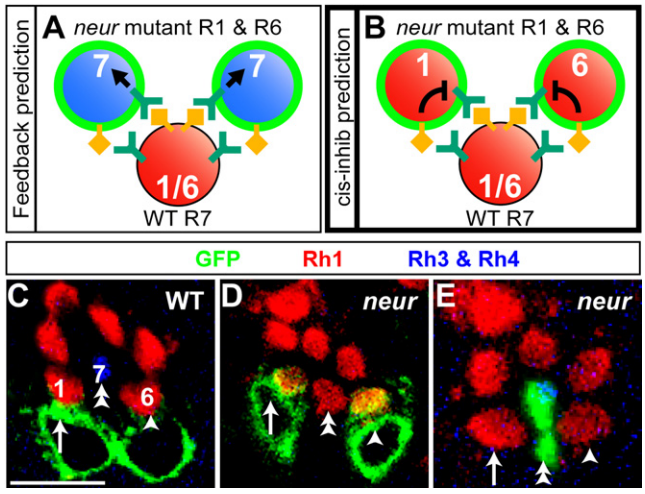

Figure 3. *neur* Mutant R1/R6s Do Not *trans*-Activate N in R7 yet Do Not Transduce the Dl Signal from R7

(A and B) The outcomes predicted by the feedback and *cis*-inhibition models presented in Figure 1F and Figure 2J, respectively.

(A) The feedback model predicts that because Dl in *neur* mutant R1 and R6 precursors cannot *trans*-activate N in R7, Dl in R7 is not downregulated and will *trans*-activate N in the R1/R6 precursors, causing them to adopt the R7 fate.

(B) The *cis*-inhibition model predicts that Dl in *neur* mutant R1/R6 precursors will still *cis*-inhibit N, preventing its *trans*-activation by Dl from the R7 precursor and causing the *neur* mutant R1/R6 precursors to adopt the R1/R6 fate.

(C–E) Mosaic adult ommatidia. Colors and scale bar in (C) are as in Figure 2. See Figure S2 for quantification.

(C) A wild-type ommatidium in which R1 and R6 are homozygous wild-type (*FRT82*).

(D) When both R1 (arrow) and R6 (arrowhead) are *neur* mutant, R7 (double arrowhead) adopts the R1/R6 fate, confirming that *neur* is required for Dl's ability to *trans*-activate N. However, unlike *Dl* mutant R1/R6s, *neur* mutant R1/R6s never adopt the R7 fate, indicating that, as predicted by the *cis*-inhibition model, their N is not activated.

(E) *neur* mutant R7s (double arrowhead) still adopt the R7 fate, confirming that loss of *neur* does not affect N's ability to be *trans*-activated.

trans-activate N in R7 without affecting its hypothesized *cis*-inhibitory activity. The feedback model predicts that *neur* mutant R1 and R6 precursors will adopt the R7 fate for the same reason that *Dl* mutant R1/R6 precursors do so—that is, because Dl in the R7 precursor is no longer downregulated by activated N (Figure 3A). By contrast, the *cis*-inhibition model predicts that Dl in the *neur* mutant R1/R6 precursors will still *cis*-inhibit N, causing all three cells to adopt the R1/R6 fate (Figure 3B). We found that *neur* mutant R1/R6 precursors never adopted the R7 fate, despite failing to activate N in the R7 precursor (Figures 3C–3E; Figure S2). We therefore conclude that Dl *cis*-inhibits N even in *neur* mutant R1/R6s.

cis-Inhibition Maintains the Direction of Signaling Established by Ordered Dl Expression in R1/R6 and R7

We have so far shown that Dl-mediated *cis*-inhibition is required in the R1/R6 precursors to prevent their transduction of a Dl signal from the R7 precursor. Because R7 does not express Dl until approximately 3 hr after the R1/R6s have begun to express the transcription factor Seven-up (Svp), previously shown to be necessary and sufficient to specify the R1/R6 fate (Figure 1A; Figure S1) [17, 25, 26], we hypothesized that *cis*-inhibition prevents a reversal of the R1/R6 precursors' choice of fates. Alternatively, it was possible that loss of Dl

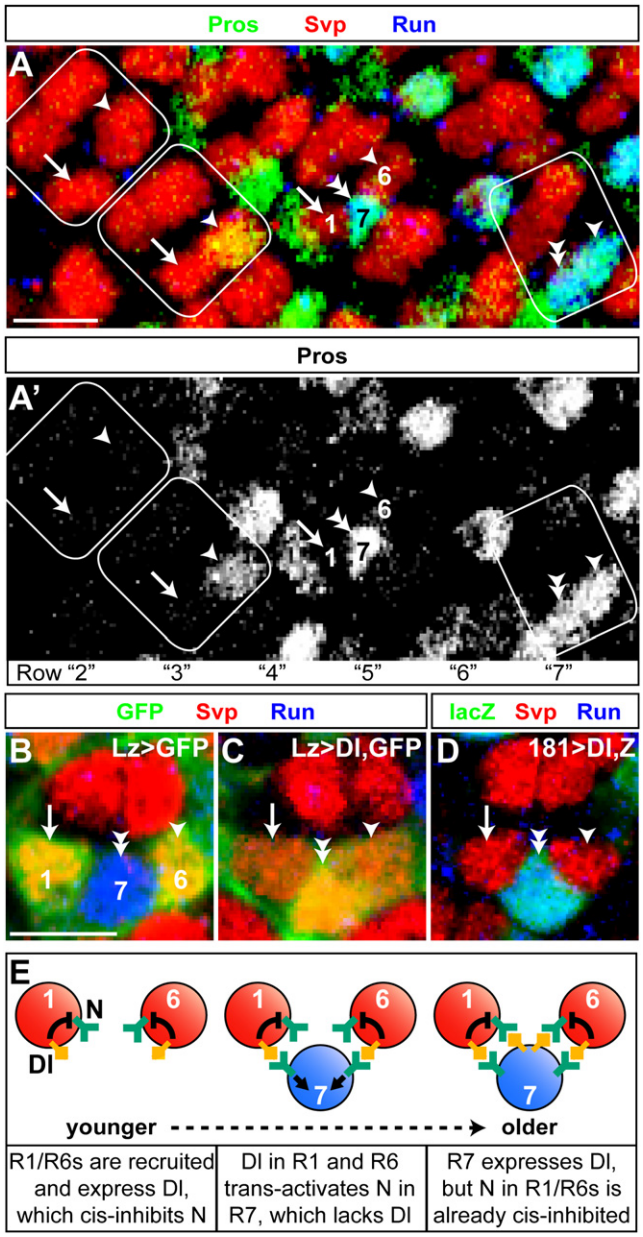

Figure 4. *cis*-Inhibition Maintains the Direction of Signaling Initially Achieved by the Ordered Expression of Dl

(A and A′) An L3 eye disc in which ~11% of R1/R6/R7 precursors are homozygous *Dl* mutant. Because of Gal80 perdurance, homozygous clones are not yet marked (see Experimental Procedures). All R1 and R6 precursors initially express the R1/R6 marker Svp (red), but ultimately 14% (presumably those that are *Dl* mutant; see Figure S5 for details) instead express the R7-specific transcription factor Prospero (Pros; green) as well as Run (blue). The outlined ommatidia correspond to those outlined in Figure 1A. In row "2" (leftmost outlined ommatidium), R1 (arrow) and R6 (arrowhead) express Svp only; at this time, R7 does not yet express *Dl-lacZ* (see Figure 1A; Figure S1). In row "3" (middle outlined ommatidium), a presumably *Dl* mutant R6 (arrowhead) expresses both the R1/R6 marker Svp and the R7 marker Pros; by this time, the R7 has been recruited and first expresses *Dl-lacZ* (see Figure 1A; Figure S1), although its nucleus is not yet apical enough to be visible in this plane. By row "7" (rightmost outlined ommatidium), the presumably *Dl* mutant R6 (arrowhead), like the R7 (double arrowhead), expresses Pros and Run but not Svp (as in Figure 1A, the R1 nucleus is below the plane of view). Scale bar represents 5 μm.

(B–D) L3 ommatidia labeled with antibodies against Svp (red) and Run (blue).

from R1/R6 precursors might delay their differentiation or accelerate the R7 precursor's expression of Dl. To distinguish these possibilities, we examined the time course of *Dl* mutant R1/R6 precursor development. To avoid the complex, pleiotropic effects caused by removing Dl earlier in eye development, we used, as before, *GMR-FLP* to induce mitotic recombination during the final cell division that generates the R1/R6/R7 precursors, resulting in mosaic eye discs in which ~11% of R1/R6/R7s were homozygous *Dl* mutant but all other R cells were heterozygous. As a consequence of this specificity, however, marker proteins are inherited by and perdure in both mutant and nonmutant cells, preventing us from using conventional labeling techniques to distinguish these genotypes in L3 eye discs. Instead, we deduced the time course of *Dl* mutant R1/R6 fate choice as follows. In mosaic eye discs containing *Dl* mutant R1/R6/R7 precursors, we found that all R1/R6 precursors initially expressed Svp (Figure 4A; Figure S5). However, once R7s expressed Dl (row "3" in Figure 1, Figure 4, Figure S1, and Figures S3–S6), ~14% of ommatidia contained R1/R6s that instead expressed the R7-specific transcription factor Prospero (Pros) [27] (Figure 4A; see Figure S5 for an explanation of our quantification). By contrast, in wild-type mosaic discs, all R1/R6 precursors continued to express Svp for approximately 12 hr and did not express Pros (data not shown). We therefore conclude that *Dl* mutant R1 and R6 precursors initially select the R1/R6 fate but that, upon exposure to Dl from R7, their N is activated, and they instead adopt the R7 fate. Consistent with this interpretation, we found that R1/R6 precursors in *Dl* mosaic eye discs first expressed the *mδ0.5-lacZ* reporter of N activity at the same time that the R7 precursors first expressed Dl (row "3," Figure S3). In transitioning from the R1/R6 to the R7 fate, *Dl* mutant R1/R6 precursors did not revert to a common precursor state; instead, they temporarily expressed both Svp and Pros simultaneously (Figures 4A; Figure S5), a combination of transcription factors that is not observed in any wild-type cell in the eye. These results indicate that Dl-mediated *cis*-inhibition prevents the *trans*-differentiation of R1/R6s directly into R7s.

Why does Dl in the R7 precursor not similarly *cis*-inhibit N? One possibility was that R7 expresses Dl too late to prevent activation of N by Dl in R1 and R6. Alternatively, the timing of Dl expression might be unimportant, and instead unknown factors might make N in R7 precursors resistant to or Dl in R7 precursors incapable of *cis*-inhibition. To distinguish these

(B) In *lozenge-Gal4* (*lz-Gal4*), *UAS-GFP* heterozygotes, R1 and R6 express Svp and R7 expresses Run. Expression of GFP (green) initiates approximately simultaneously in progeny of the second mitotic wave (data not shown) [31]. Scale bar represents 5 μm.

(C) In *lz-Gal4, UAS-Dl, UAS-GFP* heterozygotes, many R7 precursors (double arrowhead) express Svp but not Run, indicating that they have adopted the R1/R6 fate.

(D) In *PM181-Gal4, UAS-Dl, UAS-lacZ* heterozygotes, in which expression of β-galactosidase (green) initiates in R7 precursors just after their recruitment (data not shown), R7 precursors never adopt the R1/R6 fate. In this ommatidium, *PM181-Gal4* has been driving expression for approximately 3 hr; older R7s in which *PM181-Gal4* has been expressed for more than 12 hr remain wild-type (data not shown).

(E) Model: R1 and R6 precursors receive the epidermal growth factor (EGF) signal first and begin to differentiate and express Dl. The R7 precursor receives the EGF signal next and is immediately exposed to Dl in R1 and R6 before expressing any Dl of its own; its N is therefore *trans*-activated. By the time R7 expresses Dl, N in R1 and R6 has already been *cis*-inhibited by Dl and so cannot be *trans*-activated. We hypothesize that Dl in R7 *cis*-inhibits N, but too late to prevent commitment to the R7 fate. However, in the interests of clarity, we have left this out of the schematic.

models, we examined whether premature expression of Dl in R7 precursors could *cis*-inhibit N. Because no known promoters drive expression specifically in the R7 precursor prior to recruitment of R1 and R6, we used an insertion of a *Gal4*-containing P element into the *lozenge* (*lz*) locus to drive expression of *UAS-Dl* approximately simultaneously in all cells within the pool from which R1/R6 and R7 precursors are recruited [28] (Figure 4B). We found that this premature Dl expression caused many R7 precursors to become R1/R6s (92 of 254, 36%; Figure 4C) and thus conclude that N in R7s can be *cis*-inhibited. Confirming that premature rather than increased expression of Dl was responsible, we used *PM181-Gal4* to drive expression of *UAS-Dl* specifically in R7s shortly after their recruitment [18]. Indeed, overexpressing Dl in this way did not prevent R7 precursors from adopting the R7 fate (Figure 4D). We conclude that it is the timing of Dl expression that allows Dl-mediated *cis*-inhibition to create unidirectional signaling from R1 and R6 to R7 and propose the following model of R1/R6/R7 specification (Figure 4E). The first two of these cells to be recruited by EGF signaling are somehow prevented from receiving or transducing the Dl signal present in R2–R5 and R8 and so express Svp and adopt the R1/R6 fate. EGF also causes these cells to express Dl, which *cis*-inhibits their N. The next cell to be recruited by EGF is immediately exposed to Dl from R1 and R6 before expressing sufficient Dl to *cis*-inhibit N. N is therefore activated in this third recruit, which therefore fails to express Svp and instead adopts the R7 fate. Although EGF also causes the third recruit to express Dl, the latter cannot *trans*-activate the already *cis*-inhibited N in R1 or R6.

Although the direction of signaling from the R1/R6 to R7 precursors can thus be explained entirely by ordered Dl expression and its consequent *cis*-inhibitory and *trans*-activation effects, it remains possible that other mechanisms including feedback also influence the levels of N activation in R1/R6 and R7 precursors. The antibody mAb323, which recognizes a subset of N targets, has recently been reported to label R1/R6s [29], suggesting either that *cis*-inhibition does not completely prevent activation of N by Dl from R7 or that R1 and R6 precursors can transduce a Dl signal that is present before their N is *cis*-inhibited. One possibility is that R1/R6 precursors can transiently receive the Dl signal expressed earlier by R2–R5 or R8; consistent with this, we noted that a small proportion of *Dl* mutant R1/R6 precursors still adopt the R7 fate even when Dl has been removed from the corresponding R7 precursor (Figure S2). The transcription factor Roughened eye has recently been shown to inhibit transcription of N target genes in R1/R6 precursors and may therefore contribute to protecting R1/R6s from this early source of Dl [30]. We also found that Dl is transcribed at a higher level in R1/R6 precursors than is reached in R7 precursors (Figure S1A), a difference that cannot be explained by their order of recruitment alone. One possibility is that activation of N in R7 does partially downregulate Dl; indeed, we found that ectopically expressing activated N in R1/R6s and R7s caused a modest decrease in Dl levels in all three precursors, although blocking the N pathway in R7s did not increase Dl levels (Figure S6). Factors that affect the levels of Dl's *cis* and *trans* activities, perhaps by regulating endocytosis, would also be predicted to influence the outcome of signaling between R1/R6 and R7 precursors. In summary, many mechanisms may converge to ensure that signaling during lateral inhibition is unidirectional. We have provided the first evidence that ligand-mediated *cis*-inhibition is one such mechanism.

cis-Inhibition by Endogenous Ligand Likely Influences Other Examples of Lateral Inhibition

Although including *cis*-inhibition in a theoretical model of lateral inhibition has been shown to bolster the model's ability to generate unidirectional signaling [21], there has been no previous evidence that *cis*-inhibition normally affects lateral inhibition. Our results suggested that we might detect the influence of *cis*-inhibition on other examples of lateral inhibition by comparing *Dl* and *neur* loss-of-function phenotypes: if *cis*-inhibition does not bias the direction of signaling, these phenotypes should be identical. We have found two such published comparisons. First, in the fly eye, Dl in the R3 precursor is normally upregulated in response to positional information and so activates N in the R4 precursor [19, 31–33]. Loss of *Dl* specifically from the R3 precursor reverses the direction of Dl signaling [31, 32]. By contrast, loss of *neur* specifically from the R3 precursor causes both precursors to become R3s, indicating that N is activated in neither [20, 33]. It was previously proposed that Dl within a *neur* mutant R3 precursor may retain residual activity and so can *trans*-activate N in the R4 precursor enough to downregulate Dl, but not enough to specify the R4 fate [20, 33]. However, given our results, a more parsimonious explanation is that Dl normally *cis*-inhibits N in the R3 precursor. Similarly, presumptive sensory organ precursors (SOPs) in the fly wing use Dl to activate N in surrounding cells and thereby prevent them from also becoming SOPs [1]. Whereas nearly all *Dl* mutant cells that are adjacent to wild-type SOPs become non-SOPs, indicating that their N has been activated (29 of 30 = 97%) [23], a substantial proportion of *neur* mutant cells adjacent to wild-type SOPs instead become SOPs themselves (9 of 33 = 27%) [23], indicating that their N cannot be activated despite being adjacent to Dl signaling cells. Again, the simplest explanation is that the *neur* mutant cells' N remains *cis*-inhibited by Dl. We suggest that *cis*-inhibition of N by endogenous Dl likely plays a general role in regulating the direction of signaling and should be included in standard models of lateral inhibition.

Delta/Serrate/Lag-2 (DSL) signals are used throughout development and adulthood. Our work highlights the importance of protecting cells from inappropriately transducing the DSL signals that inevitably surround them. The R1/R6 fate choice is tenuous: despite having already expressed the R1/R6-specific transcription factor Svp for approximately 3 hr, R1 and R6 remain vulnerable to receiving a Dl signal that directly switches their developmental program. This plasticity may be specific to binary fate decisions determined by lateral DSL signaling or may be a common feature of cell fate choices that are specified by the failure to receive a signal. Abnormally high levels of N activation are associated with a variety of cancers as well as other pathologies [34]. Our results suggest that such activation may in some cases be caused by the loss of DSL ligand.

Experimental Procedures

The R1, R6, and R7 precursors are recruited from a pool of equipotent cells generated by the so-called second mitotic wave (SMW) [12]. We used the *GMR* promoter to express FLP recombinase and thereby induce FRT site-specific recombination specifically during the SMW, resulting in mosaic animals in which ~11% of R1s, R6s, and R7s were homozygous for a given chromosome arm; any combination of one, two, or three of these cells could be homozygous in a given ommatidium [18] (data not shown). Homozygous cells were specifically labeled by the MARCM technique with *act-Gal4* and *UAS-mCD8-GFP*, which labels cell bodies but is excluded from photoreceptor rhabdomeres [17, 35]; Gal80 is specifically expressed in nonmutant cells, where it prevents Gal4-driven expression of GFP [35]. Because

R1/R6/R7 precursors inherit Gal80 protein from their heterozygous parents, homozygous mutant cells do not express GFP until approximately 12 hr after puparium formation (data not shown); they are therefore unmarked in the experiments depicted in Figure 4A, Figures S3A–S3D, and Figure S5. Because *Dl* and *neur* are not transcribed prior to the SMW [13, 33], homozygous mutant R1/R6 and R7 precursors are predicted to lack wild-type protein. However, homozygous *N* mutant R1/R6/R7 precursors created by *GMR-FLP* inherit wild-type N from their heterozygous parents, preventing the use of this technique to remove N from R7. We used the Dl^{RevF10} and $neur^{1}$ null alleles for all analyses presented but found that the Dl^{B2} and $neur^{A101}$ alleles resulted in identical phenotypes (Figure S2). Tissues were dissected, fixed, and stained as described previously [17]. Confocal images were collected on a Leica SP2 microscope and analyzed with Leica or NIH ImageJ (http://rsbweb.nih.gov/ij/) software.

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01319-0.

Acknowledgments

We thank H. Ruohola-Baker, G. Struhl, E. Lai, S. Bray, A. Martinez Arias, D. Ready, S. Britt, P. Gergen, C. Doe, Y. Hiromi, T. Isshiki, N. Baker, the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank for flies and antibodies. We also thank C. Doe, K. Guillemin, J. Eisen, A. Tomlinson, and C. Desplan for discussion and comments on the manuscript. This work was funded by a Burroughs Wellcome Career Development Award to T.G.H. and by an institutional NIH National Research Service Award Genetics Training Grant (5-T32-GM007413) to A.C.M.

Received: March 6, 2009
Revised: May 26, 2009
Accepted: June 18, 2009
Published online: July 23, 2009

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