A Screen for Dominant Modifiers of \(ro^{Dom}\), a Mutation That Disrupts Morphogenetic Furrow Progression in Drosophila, Identifies Groucho and Hairless as Regulators of atonal Expression

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ABSTRACT

\(ro^{Dom}\) is a dominant allele of rough (ro) that results in reduced eye size due to premature arrest in morphogenetic furrow (MF) progression. We found that the \(ro^{Dom}\) stop-furrow phenotype was sensitive to the dosage of genes known to affect retinal differentiation, in particular members of the hedgehog (\(hh\)) signaling cascade. We demonstrate that \(ro^{Dom}\) interferes with Hh’s ability to induce the retina-specific proneural gene atonal (\(ato\)) in the MF and that normal eye size can be restored by providing excess Ato protein. We used \(ro^{Dom}\) as a sensitive genetic background in which to identify mutations that affect \(hh\) signal transduction or regulation of \(ato\) expression. In addition to mutations in several known loci, we recovered multiple alleles of groucho (\(gro\)) and Hairless (\(H\)). Analysis of their phenotypes in somatic clones suggests that both normally act to restrict neuronal cell fate in the retina, although they control different aspects of \(ato\)’s complex expression pattern.

MORPHOGENESIS in higher animals requires the coordination of cell movements or cell shape changes with the implementation of novel programs of gene expression (Smith and Schoenwolf 1998; Leptin 1999; Noselli and Agnès 1999). Retinal differentiation in Drosophila provides a striking example of an intricate pattern of diverse cell fates that is achieved in a short time over a large field of cells with minimum movement or waste of cells and with remarkable accuracy. The precision and rapidity of the process result from a complex network of long-range and short-range signaling events that impose tight temporal and spatial regulation to the differentiation process (Heberlein and Moses 1995; Albagli et al. 1997; Freeman 1997).

Retinal differentiation in Drosophila begins during the third larval instar, when a few cells near the center of the posterior eye imaginal disc margin start clustering and expressing neuron-specific markers (Wolff and Ready 1993). The anterior portion of the disc keeps growing through random cell proliferation, while differentiation sweeps across the disc as a wave, spreading evenly to the lateral margins and eventually reaching the anterior margin over the course of 2 days. At the front of the wave, cells stop dividing and undergo concerted apical constrictions, which creates a narrow indentation, known as the morphogenetic furrow (MF), in the retinal epithelium (Ready et al. 1976; Tomlinson 1985). In the MF, differentiation begins with the expression of the proneural gene atonal (\(ato\)) as a sensitive genetic background in which to identify mutations that affect \(hh\) signal transduction or regulation of \(ato\) expression. In addition to mutations in several known loci, we recovered multiple alleles of groucho (\(gro\)) and Hairless (\(H\)). Analysis of their phenotypes in somatic clones suggests that both normally act to restrict neuronal cell fate in the retina, although they control different aspects of \(ato\)’s complex expression pattern.

Progress of the MF is intimately linked to cross-regulation between \(ato\) and the segment polarity gene hedgehog (\(hh\)). Hh is produced by cells that differentiate, as an indirect consequence of \(ato\) expression, behind the MF (Heberlein et al. 1993; Ma et al. 1993). When Hh signaling is reduced during the third larval instar, using a temperature-sensitive \(hh\) allele, or in somatic clones devoid of the putative Hh receptor Smoothened (Smo; Alcedo et al. 1996), \(ato\) expression is abolished in MF cells (Domínguez and Hafen 1997; Strutt and Mlodzik 1997; Borod and Heberlein 1998). This is accompanied by the premature arrest or severe retardation of MF progression (Heberlein et al. 1993; Ma et al. 1993; Domínguez and Hafen 1997; Strutt and Mlodzik 1997; Borod and Heberlein 1998). The anterior move-
ment of the MF therefore relies on the cells’ transition from a state where they respond to the Hh signal and express ato to a state where they emit the Hh signal as a consequence of differentiating. However, differentiation is not completely abolished within smo mutant clones, indicating that Hh is not the sole activator of ato expression in the MF (Domínguez and Hafen 1997; Strutt and Mlodzik 1997). Rather, Hh may be viewed as a permissive factor that facilitates the progress of retinal differentiation by increasing the rate of Ato accumulation.

Like other proneural genes of the Drosophila peripheral nervous system, ato follows a dynamic transcription pattern. Its expression is first continuous and weak even in the MF, giving rise to an alternating pattern of strongly expressing and less strongly expressing cell clusters (Jarman et al. 1995; Dokucu et al. 1996; Sun et al. 1998). At the posterior edge of the MF, groups of six to eight cells with high Ato protein levels progressively resolve into single, evenly spaced cells that maintain high levels of Ato protein for an additional 6–8 hr and go on to differentiate as R8 photoreceptors (Jarman et al. 1995; Baker et al. 1996). In addition to hh, signals from the Notch (N) and Egr factor receptor (EGFR) pathways have been implicated in the early stages of ato induction (Baker and Yu 1997; Domínguez and Hafen 1997; Domínguez et al. 1998; Spencer et al. 1998; Sun et al. 1998). ato was also shown to activate its own transcription (Sun et al. 1998). While the exact sequence in which these activation mechanisms act has not been fully established, together they account for the rapid accumulation and early patterning of Ato protein in the MF. Behind the MF, downregulation of ato within the intermediate clusters is attributed mainly to lateral inhibition mediated by the receptor Notch (N) and its ligand Delta (Dl; Baker and Zitron 1995), in combination with the adhesion molecule Scabrous (Scb; Lee et al. 1996). Spacing of the R8 precursors may further depend on the patterned activation of the EGFR and/or Ras/Raf signaling pathways (Domínguez et al. 1998; Kumar et al. 1998; Spencer et al. 1998; Greenwood and Struhl 1999). The homeodomain protein Rough (Ro; Tomlinson et al. 1988; Heberlein et al. 1991), an inhibitor of ato transcription expressed at the posterior edge of the MF, ensures the individualization of single ato-expressing cells from an equivalence group including the R8, R2, and R5 photoreceptor precursors (Dokucu et al. 1996).

Given the variety of signals that converge in the MF, a genetic screen based on the disruption of furrow movement should provide insights into many interesting patterning and differentiation processes. Here we report the results of a genetic screen we undertook to identify new genes that promote or prevent progress of the MF. We used a dominant ro allele, robos, that had been isolated in the course of previous investigations of the role of the Rough protein in eye differentiation (Heberlein et al. 1991, 1993). Unlike loss-of-function ro alleles, which cause roughening of the eye surface without affecting eye size (Tomlinson et al. 1988), robos leads to reduced eye size due to premature arrest of MF progression. We found robos to be sensitive to dosage manipulations of several genes involved in eye development. We therefore used it as a sensitive background in which to identify new regulators of MF movement on the basis of their ability to act as dominant modifiers of the stop-furrow phenotype.

Some of the strongest genetic interactions were observed with members of the hh pathway, including hh itself and its antagonist patched (ptc). Our screen also yielded new mutations in genes of the N signaling pathway, such as Dl, grouch (gro), and Hairless (H), as well as uncovered the eyelid (eld) locus (Treisman et al. 1997). Phenotypic characterization of robos shows that the differentiation arrest results primarily from decreased ato expression. We demonstrate that gro and H are normal regulators of ato expression in the MF. gro represses ato expression behind the MF whereas H represses expression anterior to the MF. Their role as suppressors of robos is discussed in the context of their relationship to the N pathway.

**MATERIALS AND METHODS**

**Mutagenesis and mutant recovery:** Males from an isogenic w strain were fed ethyl methanesulfonate (EMS) at a concentration of 25–35 mM in 10% sucrose or exposed to ~4000 rad of X-ray irradiation. They were mated to virgin females of the T(2;3) ro/TM3,St genotype. The strategy to recover mutants is illustrated in Figure 1. The robos translocation acts as a double balancer for second and third chromosomes. Hence the modifiers can be followed easily by their ability to segregate away from the robos-bearing chromosome.

**Fly stocks and culture:** Fly stocks are listed in Table 1. The robos phenotype is more pronounced at 25° than at 18°. The screen and all subsequent crosses were performed at 25° in noncrowded conditions on standard fly medium.

**Induction of somatic clones:** Somatic clones were induced using the yeast Flip recombinase (FLP) and target sequence (FRT) system (Golic and Lindquist 1989; Golic 1991). gro347, H210, and H210 mutations were recombined onto a third chromosome carrying an FRT at position 82B (Xu and Rubin 1993). To obtain marked clones in adults, the following crosses were performed: y, w; hs-FLP122; FRT (82B) P[w+190E] females × w; FRT(82B), H or gro/TM3,St males. Progeny were grown at 25° and submitted to a 1-hr heat shock at 38.5°, once at the end of first larval instar (48 hr after egg laying) and once again at the end of second larval instar (72 hr after egg laying). The presence of mutant clones was inferred from unpigmented patches of retinal tissue in an otherwise normal, w adult eye.

To obtain marked clones in larvae, the following crosses were performed: y, w; hs-FLP122; FRT(82B), arm-lacZ females × w; FRT(82B), H or gro/TM3,St males. The same heat-shock regimen as above was used to induce somatic recombination. The arm-lacZ reporter (Vincent et al. 1994) expresses β-galactosidase ubiquitously in larval tissues. The presence of mutant
clones was inferred from the loss of β-galactosidase activity in patches of eye-disc cells.

The frequency of eye clones was greatly increased when we used a construct that places the FLP recombinase under the control of an eye-specific enhancer element from the eyeless gene, designed by B. Dickson (ey-FLP; Hazelett et al. 1998). Crosses were performed as described above, replacing hs-FLP with ey-FLP, also on the X chromosome. No heat induction was necessary as ey-FLP is expressed constitutively in the eye disc from second instar on.

**Immunohistochemistry and microscopy:** Primary antibodies were kindly provided by the following people and were used at the following dilutions: Rabbit-anti-Atonal (Andrew Jarman; preabsorbed, 1:1000 final); Mouse-anti-Rough (Gerry Rubin; 1:100); Rat-anti-ELAV (Nadean Brown; 1:5); Mouse-anti-Hairy (Larry Zipursky; 1:5).

Dissection, fixation, antibody detection, and β-galactosidase activity staining of eye-imaginal discs were performed as previously described (Kimmel et al. 1990; Treisman et al. 1995). Confocal images were obtained on a Bio-Rad (Hercules, CA)

**TABLE 1**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Reference</th>
<th>Source/thanks to</th>
</tr>
</thead>
<tbody>
<tr>
<td>hh</td>
<td>MOHLER and VANI (1992)</td>
<td>Bloomington</td>
</tr>
<tr>
<td>hh&lt;sup&gt;13&lt;/sup&gt; = hh&lt;sup&gt;11&lt;/sup&gt;</td>
<td>JURGENS et al. (1984)</td>
<td>Bloomington</td>
</tr>
<tr>
<td>ato&lt;sup&gt;y&lt;/sup&gt;</td>
<td>JARMAN et al. (1994)</td>
<td>Y. N. Jan</td>
</tr>
<tr>
<td>ato&lt;sup&gt;y&lt;/sup&gt;</td>
<td>JARMAN et al. (1995)</td>
<td>A. Jarman</td>
</tr>
<tr>
<td>ptc&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PHILLIPS et al. (1990)</td>
<td>D. Kalderon</td>
</tr>
<tr>
<td>HE&lt;sup&gt;31&lt;/sup&gt;</td>
<td>BANG et al. (1995)</td>
<td>J. Posakony</td>
</tr>
<tr>
<td>UAS-shh</td>
<td>CAPDEVILA and GUERRERO (1994)</td>
<td>I. Guerrero</td>
</tr>
<tr>
<td>UAS-ato</td>
<td>JARMAN et al. (1993)</td>
<td>Y. N. Jan</td>
</tr>
<tr>
<td>UAS-ptc (EP(2)0941)</td>
<td>RÖRTH et al. (1998)</td>
<td>BDGP</td>
</tr>
<tr>
<td>GMR-GAL4</td>
<td>FREEMAN (1996)</td>
<td>M. Mlodzik</td>
</tr>
<tr>
<td>h&lt;sup&gt;103&lt;/sup&gt;(h-GAL4)</td>
<td>HUANG and FISCHER-VIZE (1996)</td>
<td>M. Mlodzik</td>
</tr>
<tr>
<td>y, w, hs-FLP122</td>
<td>STRUHL and BASLER (1993)</td>
<td>T. Laverty</td>
</tr>
<tr>
<td>y, w, ey-FLP</td>
<td>HAZELETT et al. (1998)</td>
<td>J. Treisman</td>
</tr>
<tr>
<td>arm-lacZ (on III)</td>
<td>VINCENT et al. (1994)</td>
<td>P. O'Farrell</td>
</tr>
<tr>
<td>FRT(82B), P[ry&lt;sup&gt;+&lt;/sup&gt;, w&lt;sup&gt;+&lt;/sup&gt;90E]</td>
<td>XU and RUBIN (1993)</td>
<td>T. Laverty</td>
</tr>
<tr>
<td>dpp-lacZ (line H1-1)</td>
<td>BLACKMAN et al. (1991)</td>
<td>R. Blackman</td>
</tr>
<tr>
<td>hh-lacZ (line P30)</td>
<td>LEE et al. (1992)</td>
<td>P. Beachy</td>
</tr>
<tr>
<td>Third chromosome deficiency kit</td>
<td></td>
<td>T. Laverty</td>
</tr>
</tbody>
</table>
RESULTS

roDom is a gain-of-function stop-furrow mutation: Flies heterozygous for the roDom mutation have kidney-shaped eyes made of fewer than 100 ommatidia, compared to 700–800 in wild type (Figure 2, A and B). Ommatidia display essentially normal organization and structure (not shown), but are confined to the posterior third of the eye, while the anterior part of the eye is occupied by a few unpatterned pigment cells and by head cuticle. As previously shown (Heberlein et al. 1993), the reduced eye size in roDom results from the premature arrest of retinal differentiation during the third larval instar. roDom mutant discs stained to reveal expression of neuronal markers contain mature photoreceptor clusters all the way to the anterior front of differentiation (Figure 2D), instead of the smooth gradient of maturing clusters observed behind the MF in wild type (Figure
2E). Ommatidial clusters in the posterior disc region therefore finish their maturation, but the induction of new clusters at the front edge is interrupted: this is the hallmark of a stop-furrow mutation.

\( \text{rod}\text{^\text{nos}} \) is an X-ray-induced translocation of the tip of the right arm of chromosome III onto the base of the left arm of chromosome II. One of these breakpoints is close to the \( \text{ro} \) locus (97D5) and molecular mapping revealed a genomic rearrangement 5’ to the \( \text{rough} \) gene, although the coding region appeared intact (U. Heberlein, unpublished observations). Several observations suggest that the stop-furrow phenotype results from a gain-of-function mutation of the \( \text{rough} \) locus. First, a differentiation defect very similar to \( \text{rod}\text{^\text{nos}} \) is observed upon overexpressing the \( \text{ro} \) gene during the third larval instar under the control of a heat-shock promoter (Kimmel et al. 1990). In contrast, mutations that reduce or abolish \( \text{ro} \) function are recessive and do not lead to reduced eye size. Instead, they cause roughening of the eye, due to photoreceptor mis-specification (Tomlinson et al. 1988; Heberlein et al. 1991). Second, the dominant \( \text{rod}\text{^\text{nos}} \) stop-furrow phenotype proved reversible by EMS mutagenesis and all revertants failed to complement loss-of-function \( \text{ro} \) alleles (not shown). Third, removing the wild-type copy of \( \text{ro} \) in a \( \text{rod}\text{^\text{nos}} \) mutant background partially suppressed the reduced eye phenotype (compare Figure 2, H and I), whereas two copies of \( \text{rod}\text{^\text{nos}} \) led to strong enhancement (Figure 2G). Eye-disc antibody staining detected the \( \text{Ro} \) protein in the same cells in wild type and \( \text{rod}\text{^\text{nos}} \), though at slightly elevated levels in the mutant (Figure 2, J–L). On the basis of these genetic and molecular criteria, we believe that the \( \text{rod}\text{^\text{nos}} \) mutation leads to a slight overexpression of \( \text{ro} \).

Loss-of-function mutations in \( \text{hh} \), such as the eye-specific allele \( \text{kh} \), display a stop-furrow phenotype very similar to \( \text{rod}\text{^\text{nos}} \) (Chanut and Heberlein 1997). This suggested that \( \text{rod}\text{^\text{nos}} \) would provide a good genetic background in which to isolate new mutations in the \( \text{hh} \) signaling cascade. Genetic interactions were systematically tested between \( \text{rod}\text{^\text{nos}} \) and known regulators of eye development and furrow movement. Strong interactions were indeed observed with various members of the \( \text{hh} \) signaling pathway (see below). In addition, \( \text{rod}\text{^\text{nos}} \) proved highly sensitive to gene dosage alterations in the \( \text{Notch} \) and \( \text{EGFR} \) signaling pathways (not shown). Interactions with mutations of the cell-cycle regulator \( \text{string} \), members of the \( \text{wingless} \) signaling pathway, and members of the retinal fate specification network were also detected, although they were more modest. No interaction with mutants of the \( \text{dpp} \) signaling cascade was observed.

Because \( \text{rod}\text{^\text{nos}} \) was sensitive to mutations in several pathways regulating eye differentiation, a screen for dominant modifiers of the stop-furrow phenotype was carried out with the aim of isolating novel members of these pathways or novel regulators of furrow progression.

Screening for dominant modifiers of \( \text{rod}\text{^\text{nos}} \) yields new alleles of \text{hedgehog, eyelid, Delta, groucho, and Hairless}: \( \text{rod}\text{^\text{nos}} \) heterozygous females were crossed to wild-type males that had been mutagenized with EMS or X ray (see Materials and Methods). \( F_{1} \) individuals whose eyes were larger or smaller than \( \text{rod}\text{^\text{nos}} \) heterozygotes were crossed to balancer stocks. Interactions with \( \text{rod}\text{^\text{nos}} \) were retested in their progeny, and the modifier mutations allocated to the second or third chromosome (Figure 1). Stocks were established with the appropriate balancer chromosomes and assessed for lethality or visible homozygous phenotypes. Out of 130,000 \( F_{1} \) progeny scored, we recovered 103 modifiers, including 53 enhancers and 50 suppressors (Table 2).

To address the specificity of the modifiers’ effect on \( \text{rod}\text{^\text{nos}} \), we took advantage of a novel mutation recovered in our screen. This mutation, \( \text{rod}\text{^\text{33}} \), displays a dominant stop-furrow phenotype very similar to \( \text{rod}\text{^\text{nos}} \) and \( \text{kh} \), although it maps to a distinct location (K. Woo and U. Heberlein, unpublished results). A large subset of \( \text{rod}\text{^\text{nos}} \) modifiers was found to modify \( \text{rod}\text{^\text{33}} \) as well. These mutations might therefore represent genes with a general role in furrow progression, rather than genes that interact specifically with \( \text{ro} \) or the \( \text{rod}\text{^\text{nos}} \) allele.

Although most modifier stocks carried a lethal mutation, only 15 lethal complementation groups were found, representing a total of 42 independent mutations. This indicates that our mutagenesis was not saturating. Most single hits were not characterized further, unless their interaction with \( \text{rod}\text{^\text{nos}} \) was particularly strong. Complementation groups were mapped by meiotic recombination or by lethal complementation against a third chromosome deficiency collection [Berkeley Drosophila Genome Project (BDGP); Table 1]. Allelism to known loci was tested, and we identified four new alleles of \( \text{Dl} \) and one of \( \text{glass} \) (\( \text{gla} \)) that acted as strong enhancers of \( \text{rod}\text{^\text{nos}} \) and four new alleles of \( \text{H} \), six of \( \text{gro} \), and two of \( \text{hh} \) that acted as strong suppressors. One of the novel loci identified in this screen is \( \text{eyelid} \) (\( \text{eld} \); Treisman et al. 1997), which is allelic to the \( \text{trithorax} \) group gene \( \text{osa} \) and encodes a nuclear protein that affects transcription by participating in a chromatin remodeling complex that also includes \( \text{brahma} \) (Collins et al. 1999; Vázquez et al. 1999). Among the other novel mutations, some modified weakly the wing phenotype of the \( \text{N} \) gain-of-function allele \( \text{Atriptex} \) (\( \text{Ax} \)) and of \( \text{H} \) heterozygotes, suggesting an involvement in the \( \text{N} \) signaling pathway (not shown). For most of them, however, the only detectable interactions were with \( \text{rod}\text{^\text{nos}} \) and \( \text{rod}\text{^\text{33}} \). Examples of strong suppressors of \( \text{rod}\text{^\text{nos}} \) recovered in our screen are shown in Figure 6. They include one \( \text{gro} \) and one \( \text{H} \) allele, mutant \( 519 \), a viable EMS-induced mutation on 3R, and mutant \( 1480 \), a member of a lethal complementation group also mapped to 3R.

**The hh paradox:** Among our strongest suppressors were two new alleles of \( \text{hh} \) (Table 2). The direction of this interaction was perplexing: since \( \text{hh} \) is required for MF progression (Heberlein et al. 1993; Ma et al. 1993),
TABLE 2
Modifiers of roDom

<table>
<thead>
<tr>
<th>Mutation no.</th>
<th>Over roDom</th>
<th>Locus name</th>
<th>Cytological location</th>
<th>Homozygous phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>737, 1582</td>
<td>SSS</td>
<td>hodghehog</td>
<td>94E2</td>
<td>Embryonic lethal, segment polarity, disc patterning</td>
</tr>
<tr>
<td>131*, 347, 781, 942, 1052, 2053</td>
<td>SS</td>
<td>groucho</td>
<td>96F11</td>
<td>Embryonic lethal, disc clones show photoreceptor (PR) hyperplasia (*, viable, recessive rough eye)</td>
</tr>
<tr>
<td>299, 1171, 1997, 3194</td>
<td>SS</td>
<td>Hairless</td>
<td>92E14</td>
<td>Embryonic lethal, dominant bristle loss; adult clones show missing outer PR and additional inner PR</td>
</tr>
<tr>
<td>308, 616, 2438, 1946, 2052, 2791</td>
<td>SS</td>
<td>eyelid = osa</td>
<td>90C7-8</td>
<td>Embryonic lethal, dominant held-out wings. Adult clones lead to scars and loss of retinal differentiation</td>
</tr>
<tr>
<td>519</td>
<td>SSS</td>
<td>?</td>
<td>82E-F</td>
<td>Viable, weak dominant rough eye</td>
</tr>
<tr>
<td>1480, 2104</td>
<td>SS</td>
<td>?</td>
<td>89B12-13</td>
<td>Embryonic lethal, no clonal phenotype</td>
</tr>
<tr>
<td>1993, 2088</td>
<td>S</td>
<td>?</td>
<td>III</td>
<td>Semilethal, recessive rough eyes and small wings</td>
</tr>
<tr>
<td>3023, 877</td>
<td>S</td>
<td>?</td>
<td>III</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>345, 617</td>
<td>wS</td>
<td>?</td>
<td>III, between cu and sr</td>
<td>Recessive lethal, lethal in clones</td>
</tr>
<tr>
<td>923, 1275</td>
<td>wS</td>
<td>?</td>
<td>III</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>2014</td>
<td>wS</td>
<td>?</td>
<td>III</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>51, 1333, 1650, 2821</td>
<td>EE</td>
<td>Delta</td>
<td>92A2</td>
<td>Embryonic lethal, dominant wing-vein deltas, adult clones show aberrant eye morphology</td>
</tr>
<tr>
<td>179</td>
<td>wE</td>
<td>glass</td>
<td>91A1</td>
<td>Viable, recessive rough eye</td>
</tr>
<tr>
<td>1801, 2796</td>
<td>EEE</td>
<td></td>
<td>84B-84D</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>2051, 2376</td>
<td>E</td>
<td>?</td>
<td>III</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>50, 1766</td>
<td>E</td>
<td>?</td>
<td>II, between pr and cn</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>1777, 2586</td>
<td>E</td>
<td>?</td>
<td>72D-73A</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>3222</td>
<td>E</td>
<td>?</td>
<td>73A3-74F</td>
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<tr>
<td>2409</td>
<td>E</td>
<td>?</td>
<td>87C-87D</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>1065, 1483</td>
<td>wE</td>
<td>?</td>
<td>III, between st and cu</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>6 single hits</td>
<td>E, EE</td>
<td>?</td>
<td>II</td>
<td>Recessive lethal</td>
</tr>
<tr>
<td>28 mutants</td>
<td>wE, E</td>
<td>?</td>
<td>III</td>
<td>Recessive lethal</td>
</tr>
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</table>

Only a subset of the 103 modifiers originally recovered is represented in the table, including new alleles of known loci, members of novel lethal complementation groups (more than one hit), and single hits displaying strong interaction with roDom and/or with 2033, an unrelated stop-furrow mutation (K. Woo and U. Heberlein, unpublished results). Novel loci were mapped based on their lethal phenotype. rd (discovered in this screen; Treisman et al. 1997) and 1480 were mapped by lethal complementation against the third chromosome deficiency kit (see MATERIALS AND METHODS). 519 is a viable, strong suppressor of roDom. The suppression was mapped by meiotic recombination using visible markers or by lethal complementation against the third chromosome deficiency kit (see MATERIALS AND METHODS).

we had expected hh mutations to enhance the roDom phenotype. Nevertheless, strong suppression was observed with all hh loss-of-function alleles tested, including our two new alleles (presumed hypomorphs or nulls, on the basis of their recessive lethality; Table 2), the eye-specific hypomorph hh1 and the presumed null hh15 (Figure 3C). Interestingly, loss-of-function mutations in patched (ptc) led to strong enhancement of the stop-furrow phenotype (Figure 3D). ptc encodes an integral transmembrane protein that prevents Smo from transducing the Hh signal (Alcedo and Noll 1997) and has been shown to antagonize hh function in retinal differentiation (Chanut and Heberlein 1995; Ma and Moses 1995; Wehrli and Tomlinson 1995). Its effect on roDom, the reverse of hh’s effect, therefore confirmed that roDom was a reliable sensor of the integrity of the hh signaling cascade, albeit in a counterintuitive fashion. Dosage reduction of smo had no effect (not shown), but several alleles of Cubitus interruptus (Ci), which encodes a transcription factor that modulates Hh signaling (Orencic et al. 1990), acted as strong enhancers (Table 2). As Ci gives rise to several protein products that can act as mediators or repressors of Hh signaling (Aza-Blanc et al. 1997; Hepker et al. 1997), the direction of these interactions is difficult to interpret.

roDom prevents expression of hh target genes in the MF:
To understand better how roDom interferes with furrow progression, we examined the expression of various markers of differentiation and furrow progression in mutant third instar discs. hh transcription was monitored using a lacZ reporter construct inserted at the hh locus (Lee et al. 1992) and was found identical in wild-type and roDom mutant discs (Figure 4, A and B).

Prominent targets of hh signaling in the eye, including
the TGF-β homolog dpp (Heberlein et al. 1993; Ma et al. 1993) and the proneural gene ato (Domínguez and Hafen 1997; Borod and Heberlein 1998), were also examined. Both are transcribed in an overlapping band of cells in the anterior portion of the MF (Figure 4, C and E), and expression of both genes was sharply decreased in roDom discs (Figure 4, D and F; Heberlein et al. 1993). Importantly, roDom did not cause reduced transcription of all genes in the eye-disc anterior portion. For instance, expression of hairy (h), an inhibitor of neural differentiation that slows the anterior progress of differentiation (Brown et al. 1995), remained strong and even appeared to expand ahead of the MF (Figure 4, I and J). Unlike dpp and ato, h is not thought to be a direct target of hh (Greenwood and Struhl 1999). We conclude that roDom affects furrow progression by interfering with Hh signaling and preventing the implementation of furrow-specific cell fates.

**hh signaling in roDom inhibits differentiation:** roDom could affect furrow cells in two ways: it could reduce their ability to transduce the hh signal, or it could induce an aberrant response to hh signaling. To address this question, we tested the effects of excess Hh and excess Ptc on differentiation in roDom.

Excess Hh was provided using a UAS-hh construct (Capdevila et al. 1994) and a GMR-GAL4 construct (Freeman 1996) that drives expression behind the MF, where endogenous Hh is normally produced. While this restores wild-type eye size in hh1 homozygotes (not shown), it resulted in a strong enhancement of the roDom stop-furrow phenotype (Figure 5C). Therefore, in a roDom mutant background, excess Hh signaling can further inhibit differentiation, rather than promote it. Furthermore, the stop-furrow phenotype was substantially suppressed when excess Ptc was provided ahead of the MF, using a UAS-pto line (Rørth et al. 1998) and a GAL4 driver under the control of the hairy promoter (h-GAL4; Huang and Fischer-Vize 1996). Excess Ptc in anterior cells is expected to reduce their ability to respond to Hh as the MF advances. The fact that it can alleviate furrow arrest suggests that in roDom mutants, furrow cells have an abnormal response to Hh signaling. They may activate an inhibitor that interferes with expression of Hh’s transcriptional targets, like dpp and ato. Alternatively, they may have an increased sensitivity to Hh signaling that makes them adopt fates normally encountered further posteriorly, closer to the source of Hh, where neither dpp nor ato are normally expressed.

**roDom is rescued by excess Ato protein:** To determine whether decreased ato expression was sufficient to explain furrow arrest in roDom, we forced expression of a UAS-ato construct (Jarman et al. 1993) anterior to the MF with the h-GAL4 driver. While this had no effect in a wild-type background (not shown), it led to nearly complete rescue of eye size in the roDom background (Figure 5E). Conversely, loss-of-function alleles of ato acted as dominant enhancers of roDom (Figure 3B). In contrast, dpp overexpression had no effect on roDom eye size (not shown). We conclude that the roDom phenotype results primarily from loss of ato expression in and ahead of the MF.

**gro and H affect different aspects of ato expression:** Several alleles of H and gro were recovered as strong suppressors of roDom (Table 2 and Figure 6, A and B). While both genes act in the N signaling pathway, they function at different steps and have opposite effects: H antagonizes signaling by preventing Suppressor of Hairless [Su(H)] from entering the nucleus upon activation of the receptor N (Schweisguth and Posakony 1994). Gro mediates the inhibition of proneural gene expression by acting as a corepressor with members of the Enhancer of split [E(spl)] complex, whose transcription is activated by Su(H) (Oellers et al. 1994; Heitzler et al. 1996). To understand how loss-of-function mutations in both genes may lead to roDom suppression, we analyzed the effect of mutant somatic clones on retinal development. Mutant clones were induced using a heat-induced (hs-FLP) or eye-specific (cyt-FLP) FLP recombinase construct and chromosomes carrying the gro and H mutations and an FRT recombination sequence (Xu and Rubin 1993). Clonal phenotypes were assessed in adults or in third instar eye-antennal discs (see Materials and Methods).

**gro mutant clones led to roughening of the adult eye surface or to scars, and retinal sections showed very disorganized ommatidial arrays (Figure 7A). Omma-
Figure 4.—roDom interferes with expression of hh targets in the MF. (A and B) hh transcription as revealed by β-galactosidase activity staining of third instar discs from wild-type (A) and roDom/+ (B) larvae carrying a lacZ reporter gene inserted at the hh locus. hh is expressed in cells differentiating behind the MF (arrowhead) and in the ocellar region (arrow) at similar levels in roDom/+ and wild type. (C and D) Double staining for neuron-specific nuclear antigen ELAV expression (brown) to reveal differentiation and β-galactosidase activity (blue) to reveal the MF (arrowheads) in wild-type (C) and roDom/+ mutant (D) third instar discs carrying a dpp-lacZ reporter gene. Expression of the dpp-lacZ reporter is sharply reduced in the central portion of the MF in the roDom/+ disc (arrow). As the disc ages, loss of dpp expression affects the whole dorso-ventral span of the MF (not shown). (E–J) Confocal microscopy images of the MF of third instar wild-type (E, G, I) and roDom (F, H, J) discs stained to reveal Ato in green and ELAV (E and F), Ro (G and H), or Hairy (I and J) in red. The anterior, MF-specific band of Ato expression is sharply reduced in the mutant relative to wild type. Ro and Ato maintain complementary expression patterns at the front of differentiation in roDom/+ discs, although expression of both proteins is strongly reduced. Expression of Hairy anterior to the MF is not abolished in roDom/+ mutant discs but appears instead to expand further anteriorly than in wild type.

tidia appeared to have fused, and photoreceptor clusters were commonly found in groups of 20 or more, instead of the normal complement of 8. Rhabdomeres were often deformed and elongated, and clusters of small rhabdomeres, typical of inner photoreceptors R7 or R8, were common.

In third instar eye discs, staining with an antibody directed against the neuronal nuclear protein ELAV (Robinow and White 1991) revealed a greater density of cells differentiating as neurons in gro mutant tissue compared to neighboring wild-type tissue (Figure 7B). While individual clusters were still visible immediately behind the MF, neuronal differentiation seemed continuous in more mature (i.e., posterior) areas of the disc (Figure 7B). Neuronal hyperplasia was restricted to the mutant tissue and therefore appears to be a cell autonomous effect of the gro mutation. Specific antibodies were used to identify the nature of the additional photoreceptors. Boss is specific to R8 cells and appears in wild type as a regular array of single staining cells behind the MF (Figure 7C; Cagan et al. 1992). In contrast, gro mutant clones contained tight clusters of Boss-expressing cells, suggesting that adjacent R8 precursors can coexist in gro mutant tissue. Nevertheless, large gaps persisted between these R8 clusters, indicating that other photoreceptor types must also account for neuronal hyperplasia. Indeed, staining with Bar and Rough antibodies revealed abnormal numbers of R1-6 cells among gro mutant cells differentiating as neurons in gro mutant tissue (not shown).

The existence of multiple Boss-expressing cells in gro mutant tissue suggests a deregulation of ato expression, leading to excess R8 photoreceptor differentiation. This was confirmed by staining with the anti-Ato antibody. gro mutant clones that straddled the MF showed a posterior expansion of the continuous band of Ato protein (Figure 7, D and E). Some refinement of the expression
Gro and H Regulate ato in the Eye

Figure 5.—rd<sup>hos</sup> is rescued by excess ato and enhanced by excess hh. Light microscopy image of the left eye of rd<sup>hos</sup>/+ females carrying various combinations of GAL4 driver and UAS responder transgenes. In all panels, posterior is to the right. (A and B) Left eye of rd<sup>hos</sup>/+ females carrying one copy of the driver construct GMR-GAL4 (A) or hH10-GAL4 (B). The effect of the drivers on rd<sup>hos</sup>/+ eye size is negligible (blurry eye surface results from photographing heads under alcohol). (C) GMR-GAL4 drives UAS-hh expression in differentiating cells behind the MF. Excess hh expression in its normal location reduces rd<sup>hos</sup>/+ eye size. (D and E) hH10-GAL4 drives expression of UAS-ptc (D) and UAS-ato (E) ahead of the MF. Both conditions result in robust increase of the rd<sup>hos</sup>/+ eye size.

The gro clones were also occasionally accompanied by severe overgrowth. This was most obvious in older discs and correlated with the presence of clones that reached the anterior and lateral disc margins (Figure 7, F and G). Overgrowth was not usually associated with ectopic differentiation, as shown by the absence of ectopic ELAV or Ato staining. In contrast, internal clones and clones touching the posterior margin rarely caused overgrowth and were always accompanied by neural hyperplasia.

The effect of H mutations was assessed in similar experiments. In adults, clones of our H<sup>H299</sup> allele led to mild roughening of the retinal surface. Sections revealed mutant ommatidia in regular arrays, but incomplete, most often lacking one or two outer photoreceptors. Ommatidia with two to three inner photoreceptors were also frequent (Figure 8A). Stronger defects were observed with the null allele H<sup>F57</sup> (not shown), suggesting that H<sup>F57</sup> is a hypomorph.

We used H<sup>F51</sup>, which also acts as a strong suppressor of rd<sup>hos</sup> (not shown), to analyze H function in disc clones. Staining for ELAV revealed clusters with reduced numbers of differentiating cells compared with wild-type clusters of similar age (Figure 8, B and C), as expected from the adult sections. Effects on ato expression were complex. In anterior clones, Ato protein was detected further anteriorly than in neighboring wild-type tissue (Figure 8, D and E), suggesting that H normally restricts the anterior span of ato expression. Near the MF, the posterior edge of ato expression was often seen to bulge forward in mutant tissue. This suggests that the refinement of ato expression occurred prematurely in the absence of H protein. Behind the MF, ato-expressing cells were irregularly spaced and sometimes seen in clusters of two or three (Figure 8, D, G, and H). Whether the effect of H mutant tissue on ato expression was cell autonomous or not could not clearly be established. Upregulation of ato in anterior clones seemed confined to the boundaries of the clone (Figure 8, D and E). However, downregulation behind the MF sometimes appeared stronger at the center of large clones than at their edges, suggesting some rescue from the neighboring wild-type tissue (Figure 8F).

Our observations demonstrate a complex effect of H on ato expression during retinal differentiation. H represses ato expression in territories far ahead of the MF, while behind the MF it ensures the timely selection of regularly spaced R8 precursors, presumably by modulating N signaling.

**DISCUSSION**

Genetic screens based on dominant interactions with a gain-of-function mutation represent a powerful and popular means of identifying components of various developmental pathways in flies (Karim et al. 1996; Carrera et al. 1998; Go and Artavanis-Tsakonas 1998; Greaves et al. 1999; Sekelsky et al. 1999; Staehling-Hampton et al. 1999; Rebay et al. 2000). We took advan-
tage of \textit{rd\textsuperscript{dn}}, a dominant stop-furrow mutation that lends itself to both enhancement and suppression, to uncover novel components of retinal differentiation. We screened through 130,000 EMS- and X-ray-induced mutants and recovered 103 mutations that either suppressed or enhanced the \textit{rd\textsuperscript{dn}} reduced eye phenotype. We were interested in recovering dominant modifiers of \textit{rd\textsuperscript{dn}} that were recessive lethal, as they would likely represent genes that had not been identified in previous screens for defects in adult eye morphology. In particular, we expected mutations that affect \textit{hh} signaling, since \textit{Hh} is required for normal furrow movement and \textit{hh} mutations display strong genetic interactions with \textit{rd\textsuperscript{dn}}.

**How does \textit{rd\textsuperscript{dn}} work?** Loss-of-function \textit{ro} mutations cause eye roughness, due to misspecification of photoreceptors R2 and R5, and the formation of ommatidia with more than one R8 photoreceptor (Heberlein \textit{et al.} 1991). Repression of R8 cell fate has been attributed to inhibition of \textit{ato} expression by the Ro homeodomain protein (Dokucu \textit{et al.} 1996). In support of this proposal, Rough and Atonal proteins appear in complementary sets of cells behind the MF, and \textit{ato} expression is expanded behind the MF in \textit{ro} mutants (Kimmel \textit{et al.} 1990; Dokucu \textit{et al.} 1996). Generalized expression of \textit{ro} under a heat-shock promoter (\textit{hs-ro}) leads to loss of \textit{ato} expression in the MF (Dokucu \textit{et al.} 1996) and eventually results in furrow arrest (Kimmel \textit{et al.} 1990). We find that furrow arrest in \textit{rd\textsuperscript{dn}} is also accompanied by loss of \textit{ato} expression in the MF. By analogy to the \textit{hs-ro} phenotype, we propose that \textit{rd\textsuperscript{dn}} leads to excess Ro production, although that excess was not detectable by antibody staining.

\textit{ro} expression at the posterior edge of the MF is under the control of \textit{hh} signaling (Domínguez 1999). The position of \textit{ro}-expressing cells, adjacent to \textit{hh}-expressing cells, suggests that high levels of \textit{hh} signaling are required for \textit{ro} expression. By comparison, \textit{ato}, also a target of \textit{hh} signaling in the MF (Domínguez and Hafen 1997; Borod and Heberlein 1998), is expressed further away from the \textit{hh} source, suggesting a requirement for lower levels of \textit{hh} signaling. We propose that the \textit{rd\textsuperscript{dn}} rearrangement sensitizes the \textit{ro} gene to \textit{hh} signaling, either by removing a negative regulatory cis-element or by bringing in an additional \textit{hh}-responsive enhancer element. The resulting anterior expansion of the \textit{ro} expression domain would prevent \textit{ato} expression and ultimately cause differentiation arrest.

While this model cannot be proven at this point, it provides a simple explanation for the surprising genetic interactions between \textit{rd\textsuperscript{dn}} and \textit{hh}: if the rearranged \textit{ro} gene is more sensitive to Hh, then increasing \textit{hh} gene dosage will cause more Ro production and accelerate the differentiation arrest. On the other hand, reducing Hh signaling, by removing one copy of \textit{hh} or by providing the inhibitor Ptc in excess, will diminish the amount of Ro protein made and restore Ato accumulation. Modifiers recovered in our screen should therefore include, among other things, components of \textit{hh} signaling that affect \textit{ro} or \textit{ato} expression or partners of Ro in the inhibition of \textit{ato} transcription.

Expression of \textit{dpp} is also sharply decreased in \textit{rd\textsuperscript{dn}}. Like \textit{ato}, \textit{dpp} expression could be inhibited by \textit{ro} directly. This may explain its sharp downregulation behind the MF in wild type at the location where \textit{ro} begins to be expressed. Alternatively, its decrease in \textit{rd\textsuperscript{dn}} could be a secondary consequence of decreased \textit{ato} transcription. In support of the latter, \textit{dpp} transcription is sharply reduced in the MF of \textit{ato}\textsuperscript{1} homozygous larvae (Jarman \textit{et al.} 1995). Similarly, Hairy protein levels remain elevated ahead of the MF in \textit{rd\textsuperscript{dn}}, although \textit{h} has been shown to be a target of Dpp signaling (Greenwood and Struhl 1999). The same is true in \textit{ato}\textsuperscript{1} mutants (Jarman \textit{et al.} 1995). This suggests that \textit{h} is under the control of other, as yet unidentified, mechanisms that are not dramatically impaired by the \textit{rd\textsuperscript{dn}} mutation and the accompanying loss of \textit{dpp} transcription. In any case, effects of \textit{rd\textsuperscript{dn}} on \textit{dpp} and \textit{h} expression are unlikely to explain the furrow arrest since the \textit{rd\textsuperscript{dn}} phenotype is not detectably affected by changes of \textit{h} and \textit{dpp} gene dosage.

In contrast, \textit{rd\textsuperscript{dn}} is very sensitive to alterations of \textit{ato}
gro and H Regulate \textit{ato} in the Eye

**Figure 7.** Effect of \textit{gro} somatic mutant clones on retinal differentiation. (A) Tangential section through an adult retina carrying \textit{gro} mutant clones. The blue arrow points to a typical wild-type ommatidium with a single inner photoreceptor (small rhabdomere) surrounded by six outer photoreceptors (large rhabdomeres) and a crown of pigment cells. Mutant clones are marked by the absence of red pigment (see materials and methods). Yellow arrows point to large mutant clones with fused ommatidia that lack pigment cells and contain an excess of disorganized and misshapen inner and outer photoreceptors. (B–G) Third instar discs carrying \textit{gro} mutant clones and stained with antibodies (brown) against the neuronal marker ELAV (B, F), the R8-specific cell surface antigen Boss (C), or the retinal proneural protein Ato (D, E, G). Clones are marked by the absence of the cell-autonomous marker arm-lacZ as evidenced by \(\beta\)-galactosidase activity staining (blue). Red arrowheads indicate the approximate location of the MF. Posterior is to the right. (B) \textit{gro} mutant clones behind the MF fill up with ELAV-expressing cells (white arrows), indicating neuronal hyperplasia. (C) Boss-expressing cells form tight clusters in \textit{gro} mutant clusters behind the MF (white arrows), instead of the evenly spaced single cells seen in neighboring heterozygous tissue (pink arrow) or in the homozygous wild-type twin-spot (blue arrow). (D) In mutant clones that span the MF, furrow-specific Ato expands further posteriorly (white arrow) than in neighboring wild-type or heterozygous tissue. (E) Behind the MF, Ato persists in cell clusters (white arrows), instead of resolving to single cells. (F and G) Mutant clones that reach the anterior and lateral disc margins are associated with severe overgrowth without ectopic differentiation, as evidenced by ELAV (F) or Ato (G) detection. Neuronal hyperplasia is observed in mutant clones located behind the MF (white arrows) or in the ocellar precursor region (red arrows). Black arrows point to epithelial folds indicative of overgrowth.

Gene dosage, as it is enhanced by loss-of-function \textit{ato} alleles and almost completely rescued when high levels of \textit{ato} expression are restored ahead of the MF. The \textit{ro} phenotype therefore appears to result primarily from inhibition of \textit{ato} expression due to excess Ro protein. On the basis of this understanding, we have analyzed the role of two of the strongest suppressors isolated in our screen, new alleles of \textit{gro} and \textit{H}, on \textit{ato} regulation and furrow progression.

**groucho.** \textit{gro} encodes a transcription inhibitor that combines with \textit{b-HLH} genes of the \textit{E(spl)} complex to inhibit expression of proneural genes such as \textit{achaete} and \textit{scute} (Heitzler \textit{et al.} 1996). We find that in \textit{gro} mutant clones, expression of \textit{ato} persists behind the MF longer than in wild-type tissue. This is consistent with a role for Gro in the N signaling events that lead to the refinement of \textit{ato} expression behind the MF.

However, Gro is also known to form inhibitory complexes with other transcription factors of the \textit{b-HLH} class, such as Hairy, or of other classes, such as the c-Rel homolog Dorsal or the homeodomain, segment polarity regulator Engrailed (Paroush \textit{et al.} 1994; Jimenez \textit{et al.} 1997; Fisher and Caudy 1998). Association of Gro with Hairy deserves to be envisaged here, since Hairy has been implicated in inhibition of \textit{ato} as well (Brown \textit{et al.} 1995). We find, however, that \textit{gro} mutant clones expand \textit{ato} expression posterior to the MF, whereas \textit{h} inhibits \textit{ato} expression anterior to the MF (Brown \textit{et al.} 1995). Another intriguing possibility is that Gro associates with Ro to mediate inhibition of \textit{ato} expression behind the MF. Although we cannot completely eliminate this hypothesis, we find it unlikely, because complete loss-of-function phenotypes of \textit{ro} and \textit{gro} are different. While both of them lead to increased \textit{ato} expression and imperfect R8 resolution, this effect is much more extensive and long lasting in \textit{gro} mutant tissue (our data) than in \textit{ro} mutant tissue (Kimmel \textit{et al.} 1990; Heberlein \textit{et al.} 1991; Dokucu \textit{et al.} 1996). In addition, neuronal hyperplasia is not observed in \textit{ro} mutant tissue, which suggests that at least this \textit{gro} function must involve factors other than Ro. On the other hand, removal of \textit{E(spl)} function results in neuronal hyperplasia and excess R8 development very similar to removal of \textit{gro} function (Treisman \textit{et al.} 1997; Ligoxy-
Figure 8.—Effect of H somatic mutant clones on retinal differentiation. (A) Tangential section through an adult retina carrying an Hsoph clone. The clone is marked by the absence of red pigment (see Materials and Methods). Mutant ommatidia are arrayed regularly but contain an abnormal complement of photoreceptors. Some have extra inner photoreceptors and lack outer photoreceptors (green circle); others lack inner photoreceptors (yellow circle). (B–H) Third instar discs carrying H31 clones and stained with antibodies (brown) against the neuronal marker ELAV (B, C, C9) or against Ato (D–H). Clones are marked by the absence of the cell-autonomous clonal marker arm-lacZ as revealed by β-galactosidase activity staining (blue). Red arrowheads indicate the approximate location of the MF. Posterior is to the right. (B, C, C9) Mutant ommatidia contain fewer ELAV-staining cells than wild-type ommatidia of the same stage, appear mis-oriented (C, close-up of area marked in B), and are interspersed with picnotic vesicles (black arrows) indicative of cell death (C9, close-up of another HE31 clone). (D–F) HE31 clones that straddle the MF are associated with accelerated resolution of Ato expression behind the MF (D, blue arrow), expansion of Ato anterior to the MF (D and E, red arrows), or loss of Ato in the MF (F, black arrow). (G and H) Higher magnification views of HE31 mutant clones near the MF, revealing uneven Ato production in the MF and irregular spacing or abnormal clustering of Ato-expressing cells behind the MF.

Therefore, we favor the hypothesis that Gro restricts ato expression by combining with proteins of the E(spl) complex whose expression is induced by N signaling.

Even in the complete absence of gro [or E(spl)] function, some refinement of ato expression still occurs, which indicates that factors independent of N and Gro play important roles in patterning Ato behind the MF. Candidates include Ro (Dokucu et al. 1996), the EGFR inhibitor Argos (Spencer et al. 1998), and Hh (Domínguez 1999). Moreover, outer photoreceptors differentiate in large excess between the R8 precursors and are the main cause of neuronal hyperplasia (Treisman et al. 1997; Ligoxygakis et al. 1998). Neuronal hyperplasia could occur as a direct consequence of the excess of R8 precursors in gro [and E(spl)] mutant tissue, which would, through the normal serial induction process, recruit an excess of neighboring cells into ommatidial clusters. However, differentiation of extra outer photoreceptors was observed with a hypomorphic gro allele in the absence of excess R8 differentiation (Fischer-Vize et al. 1992). The excess of all photoreceptor types observed with our stronger gro allele may therefore reflect an involvement of gro in the restriction of cell fates at each step of ommatidial formation.

gro mutant clones can also induce extensive overgrowth of head capsule and retinal tissues. In the wing, gro clones have been found to cause overgrowth via the induction of ectopic hh expression (de Celis and Ruiz-Gómez 1995). Hh is also a powerful inducer of overgrowth in eye discs, when provided in excess or ectopically (Chanut and Heberlein 1995; Heberlein et al. 1995). However, overgrowth due to ectopic hh expression is accompanied by ectopic and premature photoreceptor differentiation, a phenotype we do not observe in overgrown gro mutant tissue. It is therefore unlikely that gro mutant clones cause ectopic hh expression in the eye. Besides, if gro mutations allowed increased Hh...
production, we would expect enhancement, rather than suppression, of the \( \text{ro}^{\text{pos}} \) phenotype (see above). While scenarios in which a slight increase in cell proliferation allows the MF to progress further in \( \text{ro}^{\text{pos}} \) cannot be eliminated, we find it more likely that \( \text{gro} \) mutations suppress \( \text{ro}^{\text{pos}} \) by allowing Ato protein to persist longer in the MF.

**Hairless:** Hairless inhibits N signaling by preventing Su(H), a transcription factor (Schweisguth and Posakony 1992; Schweisguth 1995), from translocating to the nucleus and activating transcription of N targets such as the \( E(\text{split}) \) complex genes (Bang et al. 1991; Bang and Posakony 1992; Schweisguth and Posakony 1994). In the absence of \( H \), Su(H) is free to enter the nucleus upon activation of N. Su(H) mutant clones lead to expanded Ato expression behind the MF, consistent with a role for Su(H) in the N-mediated lateral inhibition that leads to the refinement of \( ato \) expression (Ligoxygakis et al. 1998). We find \( H \) clones in which the refinement of \( ato \) expression to single cells appears accelerated. This is consistent with a role for \( H \) as an inhibitor of N and Su(H) in lateral inhibition. Surprisingly, however, individual clusters of Ato-expressing cells often persist in \( H \) mutant tissue behind the MF, instead of resolving to single R8 precursors; in adults as well, mutant ommatidia often contain more than one R8. This would suggest that at later stages \( H \) is required to resolve \( ato \) expression to single R8 precursors, a role that is not expected for an inhibitor of the N pathway. Anterior \( H \) mutant clones show precocious \( ato \) expression anterior to the MF. This might explain the patterning defects behind the MF, if precarious and excessive accumulation of Ato protein in the MF interferes with the proper execution of lateral inhibition via N or with downregulation by Ro. In this regard, we note that excess Ato protein, as provided under heat-shock control, was found to perturb the resolution of \( ato \) expression to single R8 precursors (Dokucu et al. 1996).

It has been suggested that early \( ato \) expression, ahead of the MF, is in part the result of an as yet unsuspected “proneural” effect of N signaling (Baker and Yu 1997). The anterior expansion of \( ato \) expression in \( H \) mutant clones is consistent with this model, assuming that \( H \) would act as an inhibitor of N there as well. However, the proneural function of N must not be mediated by Su(H), since removal of Su(H) function did not abolish \( ato \) expression ahead of the MF (Ligoxygakis et al. 1998). Our results may indicate that \( H \) antagonizes the proneural function of N via a mechanism that does not involve Su(H). Alternatively, the role of \( H \) on early \( ato \) expression may be independent of N signaling. Regardless of the exact mechanism, the enhanced expression of \( ato \) ahead of the MF in \( H \) mutants is likely to explain suppression of the \( \text{ro}^{\text{pos}} \) phenotype by counteracting the effect of ectopic Ro on \( ato \) expression in the MF.

Finding that similar levels of suppression can be achieved by loss-of-function mutations in \( H \) and \( gro \), which act in opposite direction in the N pathway, is not unique. A similar situation was encountered in another study where mutations in \( gro \) and \( H \) were both found to enhance the wing and bristle phenotypes associated with loss-of-function mutations in \( EGFR \) (Price et al. 1997). Our observation that mutations in both genes elevate \( ato \) expression in the vicinity of the MF, but at different stages of the differentiation process, helps resolve this paradox. Our results also indicate that the exact timing (or location) of \( ato \) expression might not be crucial to MF progression, provided adequate levels are reached. This conclusion is supported by our finding that Ato supplied anterior to its normal expression domain, in the \( h \) expression domain, restores normal eye size in a \( \text{ro}^{\text{pos}} \) background. Whether proper R8 spacing and ommatidial patterning can be achieved under these conditions remains to be shown.

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